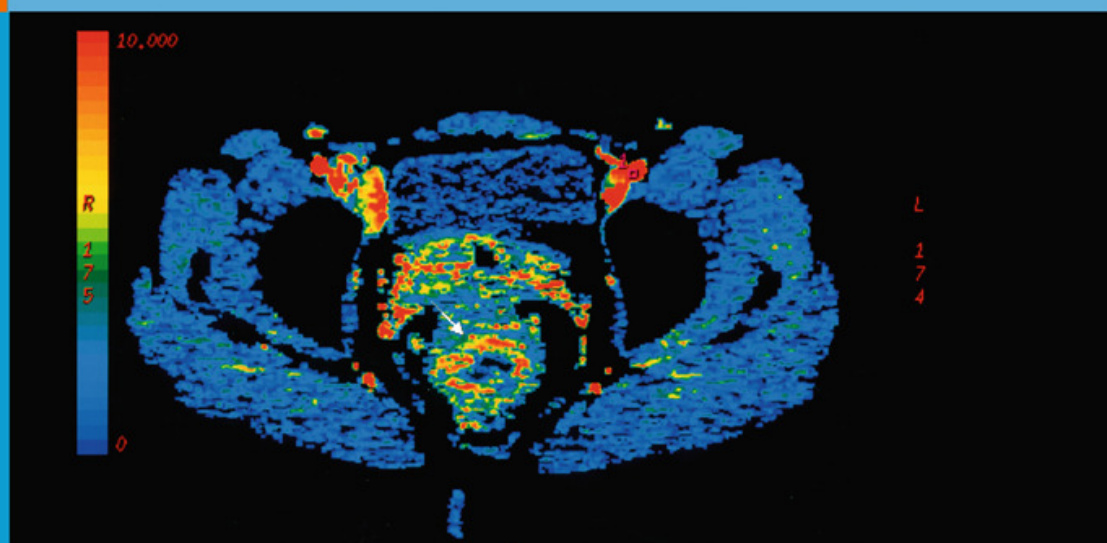


Methods of Cancer Diagnosis, Therapy, and Prognosis

Volume 6

Ovarian Cancer, Renal Cancer, Urogenitary tract Cancer, Urinary Bladder Cancer,
Cervical Uterine Cancer, Skin Cancer, Leukemia, Multiple Myeloma and Sarcoma



M.A. Hayat
Editor



Springer

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Edited by

M.A. Hayat

Department of Biological Sciences,
Kean University, Union, NJ, USA



Springer

Editor

M.A. Hayat

Department of Biological Sciences

Kean University

Union, NJ, USA

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New technology, for better or for worse, will be used, as that is our nature.

Lewis Thomas

You have been given the key that opens the gates of heaven; the same key opens the gates of hell.

Writing at the entrance to a Buddhist temple

Contributors

Laurent Alberti

Centre Léon Bérard, 28, Rue Laennec,
69008 Lyon, France

Juan Luis Alcázar

Department of Obstetrics and
Gynecology, Clínica Universitaria de
Navarra, University of Navarra, Avenida
Pío XII, 36 Pamplona 31008, Spain

Damien Ambrosetti

Service d'Anatomie Pathologique,
Hôpital Pasteur, 30 Avenue de la Voie
Romaine, 06000 Nice, France

Yaser Atlasi

Department of Genetics, Faculty of Basic
Science, Tarbiat Modares University,
P.O. Box: 1411-175, Tehran, Iran

Allyson C. Baker

University of Alabama at Birmingham,
NP 3537, 619 19th Street South,
Birmingham, AL 35249, USA

Surinder K. Batra

Department of Biochemistry and
Molecular Biology, College of Medicine,
Eppley Cancer Institute 7052 Durham
Research Center, University of Nebraska
Medical Center, 985870 Nebraska
Medical Center, Omaha, NE 68198-5870,
USA

Vladimir Bilim

Department of Urology, Yamagata
University Faculty of Medicine, Iida-nishi
2-2-2, Yamagata 990-9585, Japan

Michael J. Birrer

Cell and Cancer Biology, National Cancer
Institute, 37 Convent Drive, Room 1068,
Bethesda, MD 20892, USA

Jean-Yves Bla

INSERM U590, Centre Léon Bérard,
Rue Laennec, 69008 Lyon, France

Malte Böhm

Department of Urology, Otto-von-
Guericke Universität, Leipziger Str. 44,
D-39120 Magdeburg, Germany

Kristin L.M. Boylan

Department of Laboratory Medicine and
Pathology, The University of Minnesota
Medical School, Minneapolis, MN 55455,
USA

Laura Brousseau

Centre Léon Bérard, 28, rue Laennec,
69008 Lyon, France

Fanny Burel-Vandenbos

Service d'Anatomie Pathologique,
Hôpital Pasteur, 30 Avenue de la Voie
Romaine, 06000 Nice, France

Gabriel Caponetti

Department of Pathology, Baystate Medical Center, Tufts School of Medicine, 759 Chestnut Street, Springfield, MA 01109, USA

Nathalie Cardot-Leccia

Service d'Anatomie Pathologique, Hôpital Pasteur, 30 Avenue de la Voie Romaine, 06000 Nice, France

Philippe Cassier

Hôpital Edouard Herriot, Service d'Oncologie Médicale, Pavillon E, 5 Place d'Arsonval, 69003 Lyon, France

Tridib Chakraborty

Division of Biochemistry, Department of Pharmaceutical Technology, Jadavpur University, P.O. Box 17028, Calcutta 700032, West Bengal, India

Malay Chatterjee

Division of Biochemistry, Department of Pharmaceutical Technology, Jadavpur University, P.O. Box 17028, Calcutta 700032, West Bengal, India

David Chhieng

University of Alabama at Birmingham, 619 19th Street South, Birmingham, AL 35249, USA

William A. Cliby

Division of Gynecologic Surgery, Department of Obstetrics and Gynecology, Mayo Clinic, 200 First Street SW, Rochester, MN 55905, USA

Joseph P. Connor

University of Wisconsin-Madison, CSC H4/656, 600 Highland Avenue, Madison, WI 53792, USA

Isabelle Ray Coquard

Centre Léon Bérard, 28, Rue Laennec, 69008 Lyon, France

Dean Daya

Department of Pathology and Nuclear Medicine, Henderson General Hospital, McMaster University, 711 Concession Street, Hamilton, Ontario L8V 1C3, Canada

Anne-Valérie Decouvelaere

Centre Léon Bérard, 28, Rue Laennec, 69008 Lyon, France

Bruce J. Dezube

Department of Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA 02215, USA

Sean C. Dowdy

Division of Gynecologic Surgery, Department of Obstetrics and Gynecology, Mayo Clinic, 200 First Street SW, Rochester, MN 55905, USA

Armelle Dufresne

Hôpital Edouard Herriot, Service d'Oncologie médicale, Pavillon E, 5 Place d'Arsonval, 69003 Lyon, France

Jérôme Fayette

Centre Léon Bérard, 28, Rue Laennec, 69008 Lyon, France

Michael Fiegl

Department of Internal Medicine V, Hemato-Oncology, Innsbruck Medical University, Anichstrasse 35, A-6020 Innsbruck, Austria

Olivier Gheysens

Department of Nuclear Medicine, University Hospital KU Leuven, Herestraat 49, B-3000 Leuven, Belgium

Elfriede Greimel

Department of Obstetrics and Gynecology,
Medical University Graz, Auenbruggerplatz
14, A-8036 Graz, Austria

Perry W. Grigsby

Department of Radiation Oncology,
Mallinckrodt Institute of Radiology,
Washington University School of
Medicine, Box 8224, 4921 Parkview Place,
Lower Level, St. Louis, MO 63110, USA

William E. Grizzle

University of Alabama at Birmingham,
619 19th Street South, Birmingham,
AL 35249, USA

Jennifer A. A. Gubbels

University of Wisconsin-Madison, CSC
H4/656, 600 Highland Avenue, Madison,
WI 53792, USA

Christian Hafner

Department of Dermatology, University
of Regensburg, Franz-Josefstrauss-Allee
11, Regensburg 93042, Germany

Hiroshi Hashimoto

Department of Pathology and Oncology,
School of Medicine, University of
Occupational and Environmental Health,
1-1 Iseigaoka, Yahatanishi-ku, Kitakyushu
807-8555, Japan

Juliette Haudebourg

Service d'Anatomie Pathologique,
Hôpital Pasteur, 30 Avenue de la Voie
Romaine, 06000 Nice, France

Masanori Hisaoka

Department of Pathology and Oncology,
School of Medicine, University of
Occupational and Environmental Health,
1-1 Iseigaoka, Yahatanishi-ku, Kitakyushu
807-8555, Japan

Andrew Horvai

University of California, San Francisco,
1600 Divisadero Drive, B220, San
Francisco, CA 94115, USA

Kazuhiko Ino

Department of Obstetrics and
Gynecology, Nagoya University Graduate
School of Medicine, 65 Tsurumai-cho,
Showa-ku, Nagoya 466-8550, Japan

Toshiyuki Itoi

Department of Urology, Yamagata
University Faculty of Medicine,
Iida-nishi 2-2-2, Yamagata 990-9585,
Japan

Seyed Mehdi Jafarnejad, Department
of Genetics, Faculty of Basic Science,
Tarbiat Modares University, P.O. Box:
1411-175, Tehran, Iran

Hiroaki Kajiyama

Department of Obstetrics and
Gynecology, Nagoya University Graduate
School of Medicine, 65 Tsurumai-cho,
Showa-ku, Nagoya 466-8550, Japan

Shingo Kato

Research Central Hospital for Charged
Particle Therapy, National Institute of
Radiological Sciences, 4-9-1 Anagawe,
Inage-ku, Chiba 263-8555, Japan

Fumitaka Kikkawa

Department of Obstetrics and
Gynecology, Nagoya University Graduate
School of Medicine, 65 Tsurumai-cho,
Showa-ku, Nagoya 466-8550, Japan

Seung Hyup Kim

Department of Radiology, Seoul National
University Bundang Hospital 300,
Gumi-dong, Bundang-Gu, Songnam-si,
Gyeonggi-do, 463-707, Korea

Tobias Klatte

Department of Urology, Otto-von-Guericke Universität, Leipziger Str. 44, D-39120 Magdeburg, Germany

Michael Landthaler

Department of Dermatology, Regensburg University Medical Center, Franz-Josef-Strauss-Allee 11, Regensburg 93053, Germany

Hak Jong Lee

Department of Radiology, Seoul National University Bundang Hospital 300, Gumi-dong, Bundang-Gu, Songnam-si, Gyeonggi-do, 463-707, Korea

Subodh M. Lele

Department of Biochemistry and Molecular Biology, College of Medicine, Eppley Cancer Institute, 7052 Durham Research Center, University of Nebraska Medical Center, 985870 Nebraska Medical Center, Omaha, NE 68198-5870, USA

Jonathan S. Lewin

Department of Radiology, Duke University Medical Center, Duke North-Room 1417, Erwin Road, Durham, NC 27710, USA

Lilie L. Lin

Department of Radiation Oncology, Mallinckrodt Institute of Radiology, Washington University School of Medicine, 4921 Parkview Place, Lower Level, St. Louis, MO 63110, USA

Yair Lotan

Department of Urology, The University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, TX 75390, USA

Ryo Maruyama

Department of Urology, Yamagata University Faculty of Medicine, Iida-nishi 2-2-2, Yamagata 990-9585, Japan

Atsuji Matsuyama

Department of Pathology and Oncology, School of Medicine, University of Occupational and Environmental Health, 1-1 Iseigaoka, Yahatanishi-ku, Kitakyushu 807-8555, Japan

Milena M. Maule

Cancer Epidemiology Unit, CeRMS and CPO-Piemonte, University of Turin, 10126 Torino, Italy

Jiri Mayer

Department of Internal Medicine V, Hemato-Oncology, Masaryk University Hospital, Jihlavska 20, CZ 62500 Brno, Czech Republic

Pierre Méeus

Centre Léon Bérard, 28, Rue Laennec, 69008 Lyon, France

Elmar M. Merkle

Department of Radiology, Duke University Medical Center, Duke North-Room 1417, Erwin Road, Durham, NC 27710, USA

Jean-Francois Michiels

Service d'Anatomie Pathologique, Hôpital Pasteur, 30 Avenue de la Voie Romaine, 06000 Nice, France

Samuel C. Mok

Department of Gynecologic Oncology, M.D. Anderson Cancer Center, T403908, 1515 Holcombe Boulevard, Houston, TX 77030, USA

Felix M. Mottaghy

Department of Nuclear Medicine,
University Hospital KU Leuven,
Herestraat 49, B-3000 Leuven,
Belgium

Seyed Javad Mowla

Department of Genetics, Faculty of Basic
Science, Tarbiat Modares University,
P.O. Box: 1411-175, Tehran, Iran

Akihiro Nawa

Department of Obstetrics and
Gynecology, Nagoya University Graduate
School of Medicine, 65 Tsurumai-cho,
Showa-ku, Nagoya 466-8550, Japan

Rendon C. Nelson

Department of Radiology, Duke
University Medical Center, Duke
North-Room 1417, Erwin Road,
Durham, NC 27710, USA

Torsten O. Nielsen

PGY3 Anatomical Pathology, 1500 JPPN
Vancouver General Hospital, 899 12th
Avenue W, Vancouver, BC V5Z 1M9,
Canada

Tatsuya Ohno

Research Central Hospital for Charged
Particle Therapy, National Institute of
Radiological Sciences, 4-9-1 Anagawe,
Inage-ku, Chiba 263-8555, Japan

Yasemin Ozluk

Department of Pathology, Istanbul
University, Faculty of Medicine, Capa,
Topkapi 34390, Istanbul

Manish S. Patankar

University of Wisconsin-Madison, CSC
H4/656, 600 Highland Avenue, Madison,
WI 53792, USA

Liron Pantanowitz

Department of Pathology, Baystate
Medical Center, Tufts School of
Medicine, 759 Chestnut Street,
Springfield, MA 01109, USA

Louis L. Pisters

Department of Urology, The University
of Texas M.D. Anderson Cancer Center,
1515 Holcombe Boulevard, Houston,
TX 77030, USA

Moorthy P. Ponnusamy

Department of Biochemistry and
Molecular Biology, College of Medicine,
Eppley Cancer Institute, 7052 Durham
Research Center, University of Nebraska
Medical Center, 985870 Nebraska
Medical Center, Omaha, NE 68198-5870,
USA

Francine M. Quan

Division of Hematology/Oncology,
East Carolina University Brody School
of Medicine, 600 Moyle Boulevard,
Greenville, NC 27858, USA

Walter D.Y. Quan

Division of Hematology/Oncology, East
Carolina University Brody School of
Medicine, Brody 3E-127, 600 Moyle
Boulevard, Greenville, NC 27858, USA

Ajay Rana

Division of Biochemistry, Department
of Pharmaceutical Technology, Jadavpur
University, P.O. Box 17028, Calcutta
700032, West Bengal, India

Basabi Rana

Division of Biochemistry, Department
of Pharmaceutical Technology, Jadavpur
University, P.O. Box 17028, Calcutta
700032, West Bengal, India

Dominique Ranchère

Centre Léon Bérard, 28, Rue Laennec,
69008 Lyon, France

Lorenzo Richiardi

Cancer Epidemiology Unit, University of
Turin, Via Santena 7, 10126 Torino, Italy

Alexander Roesch

Department of Dermatology, Regensburg
University Medical Center, Franz-Josef-
Strauss-Allee 11, Regensburg 93053,
Germany

Albert Rübben

Department of Dermatology, University
Hospital RWTH Aachen, Pauwelsstrasse
30, D-52074 Aachen, Germany

Naoki Sasaki

Department of Obstetrics and
Gynecology, National Defense Medical
College, 3-2 Namiki, Tokorozawa,
Saitama 359-8513, Japan

Kiyosumi Shibata

Department of Obstetrics and
Gynecology, Nagoya University Graduate
School of Medicine, 65 Tsurumai-cho,
Showa-ku, Nagoya 466-8550, Japan

Ajay P. Singh

Department of Biochemistry and
Molecular Biology, College of Medicine,
Eppley Cancer Institute, 7052 Durham
Research Center, University of Nebraska
Medical Center, 985870 Nebraska Medical
Center, Omaha, NE 68198-5870, USA

Amy P.N. Skubitz

Department of Laboratory Medicine and
Pathology, The University of Minnesota
Medical School, MMC 609, 420 Delaware
Street, SE, Minneapolis, MN 55455, USA

Keith M. Skubitz

Department of Medicine, The Masonic
Cancer Center, Minneapolis, MN 55455,
USA

Philippe E. Spiess

Department of Urology, The University
of Texas M.D. Anderson Cancer Center,
1515 Holcombe Boulevard, Houston,
TX 77030, USA

Michael P. Stanley

Cell and Cancer Biology, National Cancer
Institute, 37 Convent Drive, Room 1068,
Bethesda, MD 20892, USA

Toru Sugiyama

Department of Obstetrics and
Gynecology, National Defense Medical
College, 3-2 Namiki, Tokorozawa,
Saitama 359-8513, Japan

Marie-Pierre Sunyach

Centre Léon Bérard, 28, Rue Laennec,
69008 Lyon, France

Monalisa Sur

Department of Pathology and Nuclear
Medicine, Henderson General Hospital,
McMaster University, 711 Concession
Street, Hamilton, Ontario L8V 1C3,
Canada

Robert S. Svatek

Department of Urology, The University of
Texas Southwestern Medical Center, 5323
Harry Hines Boulevard, Dallas,
TX 75390, USA

Masashi Takano

Department of Obstetrics and
Gynecology, National Defense Medical
College, 3-2 Namiki, Tokorozawa,
Saitama 359-8513, Japan

Nizar M. Tannir

Department of Urology, The University of Texas M.D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030, USA

Jefferson Terry

PGY3 Anatomical Pathology, 1500 JPPN Vancouver General Hospital, 899 12th Avenue W, Vancouver, BC V5Z 1M9, Canada

Yoshihiko Tomita

Department of Urology, Yamagata University Faculty of Medicine, Iida-nishi 2-2-2, Yamagata 990-9585, Japan

Hiroshi Tsuda

Department of Obstetrics and Gynecology, Osaka City General Hospital, 2-13-22 Miyakojimahondori, Miyakojima, Osaka 5340021, Japan

Thomas Vogt

Department of Dermatology, Regensburg University Medical Center, Franz-Josef-Strauss-Allee 11, Regensburg 93053, Germany

Eiko Yamamoto

Department of Obstetrics and Gynecology, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan

Preface

This volume presents a detailed survey of various methodologies related to diagnosis, therapy, and prognosis of ovarian cancer, renal cancer, urinary bladder cancer, and cervical uterine cancer, while the already published Volumes 1–5 detail similar aspects of breast, lung, prostate, liver, gastrointestinal, colorectal, and biliary tract carcinomas.

It is well established that cancer is the deadliest of human diseases. The following estimated global incidence of seven types of cancers discussed in this volume indicated the seriousness of this malignancy.

Cervical uterine cancer 493,342

Urinary bladder cancer 357,000

Leukemia 300,522

Renal cancer 208,480

Ovarian cancer 204,499

Melanoma of skin 160,177

Multiple Myeloma 85,704

As in the previous five volumes of this series, each chapter is written by distinguished, practicing clinicians/surgeons/pathologists who provide methodologies for diagnosis and treatment of eight types

of cancers. This volume was written by 94 oncologists representing 13 countries. Their practical experience highlights their writings, which should build and further the endeavors of the readers in this important area of disease. The text of each cancer type is divided into subheadings for the convenience of the readers. It is my hope that the current volume will join the preceding volumes of this series for assisting in the more complete understanding of globally relevant cancer syndromes. There exists a tremendous, urgent demand by the public on the scientific community to address cancer prevention, diagnosis, treatment, and hopefully cures.

I am grateful to the contributors for their promptness accepting my suggestions. I respect their dedication and diligent work in sharing their invaluable knowledge with the public through this series. Each chapter provides unique individual, practical knowledge based on the expertise and practical experience of the authors. The chapters contain the most up-to-date practical and theoretical information. I hope that these handbooks will assist the practicing readers in their clinical work.

I am thankful to Dr. Dawood Farahi and Dr. Kristie Reilly for recognizing the importance

of scholarship (research, writing, and publishing) in an institution of higher education and for providing the resources for completing this project. I appreciate receiving expert

help from Myrna Ortiz and Erin McNally in preparing this volume.

M.A. Hayat
March 2009

Introduction

M.A. Hayat

The enormous burden of liver cancer on society becomes clear by considering the fact that approximately 625,000 new cases of this cancer are diagnosed globally each year. Distressingly, the number of deaths is approximately the same at 598,000 per year. Liver cancer, therefore, is the third most common cause of death from cancer. Survival rates for liver cancer are only 3–5% globally. In the United States, 19,160 new cases of liver cancer and 16,780 deaths were reported for 2007. The major risk factors for this cancer include prior infection with hepatitis B and C viruses, with the former more prevalent. Dietary exposure to fungus *Aspergillus fumigatus* (aflatoxins) also contributes to the incidence of liver cancer in many parts of the world. Tobacco use is the most serious preventable cause of cancer, as its use causes cancer of the lung, throat, mouth, liver, pancreas, urinary bladder, stomach, kidney, as well as other types. Alcohol-induced liver injury is another major risk factor for hepatocellular carcinoma (HCC).

In view of these devastating statistics, the urgency of deciphering the molecular mechanism underlying this disease, perfecting reliable diagnostic methods, understanding risk factors, developing effective targeted drugs, improving other treatments, assessing the effectiveness of therapies, and providing improved care for post-treatment patients, becomes apparent. This volume provides up-to-date information on the above-mentioned aspects of liver cancer; specifically, details of the methodologies used are included. The other seven volumes in this series provide similar information on other types of cancers.

This series of handbooks has taken the unique approach of discussing cancer diagnosis, treatment, and prognosis in the same volume. It is pointed out that this vast subject cannot be fully discussed by only one author. This is the primary reason for inviting a large number of oncologists/clinicians/surgeons to write each of the eight volumes of this series of handbooks. Another advantage of involving more than one author is to present different points of view on a specific controversial aspect of cancer. I hope these goals were accomplished in this and other published volumes of this series.

Contents of Volumes 1, 2, 3, 4 and 5

Volume 1

- 1. Breast Cancer: An Introduction**
- 2. Breast Cancer: Computer-Aided Detection**
- 3. Sebaceous Carcinoma of the Breast: Clinicopathologic Features**
- 4. Breast Cancer: Detection by In-Vivo Imaging of Angiogenesis**
- 5. Breast and Prostate Biopsies: Use of Optimized High-Throughput MicroRNA Expression for Diagnosis (Methodology)**
- 6. Familial Breast Cancer: Detection of Prevalent High-Risk Epithelial Lesions**
- 7. Differentiation Between Benign and Malignant Papillary Lesions of Breast: Excisional Biopsy or Stereotactic Vacuum-Assisted Biopsy (Methodology)**
- 8. Multicentric Breast Cancer: Sentinel Node Biopsy as a Diagnostic Tool**
- 9. Breast Cancer Recurrence: Role of Serum Tumor Markers CEA and CA 15-3**
- 10. Breast Cancer Patients Before, During or After Treatment: Circulating Tumor Cells in Peripheral Blood Detected by Multigene Real-Time Reverse Transcriptase-Polymerase Chain Reaction**
- 11. Breast Cancer Patients: Diagnostic Epigenetic Markers in Blood**

- 12. Breast Cancer Patients: Detection of Circulating Cancer Cell-Related mRNA Markers with Membrane Array Method**
- 13. Prediction of Metastasis and Recurrence of Breast Carcinoma: Detection of Survivin-Expressing Circulating Cancer Cells**
- 14. Node-Negative Breast Cancer: Predictive and Prognostic Value of Peripheral Blood Cytokeratin-19 mRNA-Positive Cells**
- 15. Breast and Colon Carcinomas: Detection with Plasma CRIPTO-1**
- 16. Breast Cancer Risk in Women with Abnormal Cytology in Nipple Aspirate Fluid**
- 17. Tissue Microarrays: Construction and Utilization for Biomarker Studies**
- 18. Systematic Validation of Breast Cancer Biomarkers Using Tissue Microarrays: From Construction to Image Analysis**
- 19. Phyllodes Tumors of the Breast: The Role of Immunohistochemistry in Diagnosis**
- 20. Phyllodes Tumor of the Breast: Prognostic Assessment Using Immunohistochemistry**
- 21. Metaplastic Breast Carcinoma: Detection Using Histology and Immunohistochemistry**
- 22. Invasive Breast Cancer: Overexpression of HER-2 Determined by Immunohistochemistry and Multiplex Ligation-Dependent Probe Amplification**
- 23. Operable Breast Cancer: Neoadjuvant Treatment (Methodology)**
- 24. Chemotherapy for Breast Cancer**
- 25. Locally Advanced Breast Cancer: Role of Chemotherapy in Improving Prognosis**
- 26. Relevance of Dose-Intensity for Adjuvant Treatment of Breast Cancer**

- 27. Advanced Breast Cancer: Treatment with Docetaxel/Epirubicin**
- 28. Systemic Therapy for Breast Cancer: Using Toxicity Data to Inform Decisions**
- 29. Chemotherapy for Metastatic Breast Cancer Patients Who Received Adjuvant Anthracyclines (An Overview)**
- 30. Estrogen Receptor-Negative and HER-2/neu-Positive Locally Advanced Breast Carcinoma: Therapy with Paclitaxel and Granulocyte-Colony Stimulating Factor**
- 31. Breast Cancer: Side Effects of Tamoxifen and Anastrozole**
- 32. Breast Cancer: Expression of HER-2 and Epidermal Growth Factor Receptor as Clinical Markers for Response to Targeted Therapy**
- 33. Young Breast Cancer Patients Undergoing Breast-Conserving Therapy: Role of BRCA1 and BRCA2**
- 34. Radiation Therapy for Older Women with Early Breast Cancer**
- 35. Acute Side Effects of Radiotherapy in Breast Cancer Patients: Role of DNA-Repair and Cell Cycle Control Genes**
- 36. ¹⁸F-Fluorodeoxyglucose/Positron Emission Tomography in Primary Breast Cancer: Factors Responsible for False-Negative Results**
- 37. Sentinel Lymph Node Surgery During Prophylactic Mastectomy (Methodology)**
- 38. Breast Conservation Surgery: Methods**
- 39. Lymph Node-Negative Breast Carcinoma: Assessment of HER-2/*neu* Gene Status as Prognostic Value**
- 40. Multifocal or Multicentric Breast Cancer: Understanding Its Impact on Management and Treatment Outcomes**
- 41. Are Breast Cancer Survivors at Risk for Developing Other Cancers?**

- 42. Distant Metastasis in Elderly Patients with Breast Cancer: Prognosis with Nodal Status**
- 43. Concomitant Use of Tamoxifen with Radiotherapy Enhances Subcutaneous Breast Fibrosis in Hypersensitive Patients**
- 44. Malignant Phyllodes Tumor of the Breast: Is Adjuvant Radiotherapy Necessary?**
- 45. Locally Advanced Breast Cancer: Multidrug Resistance**
- 46. Breast Cancer: Diagnosis of Recurrence Using ¹⁸F-Fluorodeoxyglucose-Positron Emission Tomography/Computed Tomography**
- 47. Role of Sentinel Lymph Node Biopsy in Ductal Carcinoma In Situ: Diagnosis and Methodology**
- 48. Breast Conservation Treatment of Early Stage Breast Carcinoma: Risk of Cardiac Mortality**

Volume 2

Part I General Methods and Overviews

- 1. Metabolic Transformations of Malignant Cells: An Overview**
- 2. Detection of Recurrent Cancer by Radiological Imaging**
- 3. Tumor Gene Therapy: Magnetic Resonance Imaging and Magnetic Resonance Spectroscopy**
- 4. Assessment of Gene Transfer: Magnetic Resonance Imaging and Nuclear Medicine Techniques**
- 5. Role of Mutations in *TP53* in Cancer (An Overview)**
- 6. Personalized Medicine for Cancer**
- 7. Radiation Doses to Patients Using Computed Radiography, Direct Digital Radiography and Screen-Film Radiography**

- 8. Cancer Vaccines and Immune Monitoring (An Overview)**
- 9. New Insights into the Role of Infection, Immunity, and Apoptosis in the Genesis of the Cancer Stem Cell**
- 10. Successful Cancer Treatment: Eradication of Cancer Stem Cells**
- 11. Overexposure of Patients to Ionizing Radiation: An Overview**

Part II Lung Cancer

- 12. Lung Carcinoma**
- 13. Extra-Pulmonary Small Cell Cancer: Diagnosis, Treatment, and Prognosis**
- 14. Magnetic Resonance Imaging of the Lung: Automated Segmentation Methods**
- 15. Peripheral Lung Lesions: Diagnosis Using Transcutaneous Contrast-Enhanced Sonography**
- 16. Small Pulmonary Nodules: Detection Using Multidetector-Row Computed Tomography**
- 17. Secondary Primary Cancer Following Chemoradiation for Non-Small-Cell Lung Cancer**
- 18. Advanced Non-Small Cell Lung Cancer: Second-Line Treatment with Docetaxel**
- 19. Non-Small Cell Lung Cancer with Brain Metastases: Platinum-Based Chemotherapy**
- 20. Non-Small Cell Lung Carcinoma: EGFR Gene Mutations and Response to Gefitinib**
- 21. Advanced Non-Small Cell Lung Carcinoma: Acquired Resistance to Gefitinib**
- 22. Prognostic Significance of [^{18}F]-Fluorodeoxyglucose Uptake on Positron Emission Tomography in Patients with Pathological Stage I Lung Adenocarcinoma**

- 23. Non-Small Cell Lung Cancer: Prognosis Using the TNM Staging System**
- 24. Differentiation Between Malignant and Benign Pleural Effusions: Methylation Specific Polymerase Chain Reaction Analysis**
- 25. Pathological Distinction of Pulmonary Large Cell Neuroendocrine Carcinoma from Small-Cell Lung Carcinoma Using Immunohistochemistry**
- 26. Differentiating Between Pleuropulmonary Desmoid Tumors and Solitary Fibrous Tumors: Role of Histology and Immunohistochemistry**
- 27. Non-Small Cell Lung Cancer with Brain Metastasis: Role of Epidermal Growth Factor Receptor Gene Mutation**

Part III Prostate Cancer

- 28. Prostate Carcinoma**
- 29. The Role of Intermediary Metabolism and Molecular Genetics in Prostate Cancer**
- 30. Array-Based Comparative Genomic Hybridization in Prostate Cancer: Research and Clinical Applications**
- 31. Prostate Cancer: Role of Vav3 Overexpression in Development and Progression**
- 32. Prostate Cancer: Detection and Monitoring Using Mitochondrial Mutations as a Biomarker**
- 33. Prognostic Markers in Prostate Carcinoma**
- 34. Prostate Cancer: Detection of Free Tumor-Specific DNA in Blood and Bone Marrow**
- 35. Prostate Carcinoma: Evaluation Using Transrectal Sonography**
- 36. Prostate Cancer: 16β -[^{18}F]Fluoro- 5α -Dihydrotestosterone(FDHT) Whole-Body Positron Emission Tomography**
- 37. Effects of Standard Treatments on the Immune Response to Prostate Cancer**

- 38. Vinorelbine, Doxorubicin, and Prednisone
in Hormone Refractory Prostate Cancer**
- 39. Locally Advanced Prostate Cancer Biochemical Recurrence
After Radiotherapy: Use of Cyclic Androgen Withdrawal
Therapy**

Volume 3

Part I Gastrointestinal Cancers

- 1. Introduction: Gastrointestinal Cancer**
- 2. Metastatic Gastrointestinal Cancer: Safety of Cisplatin
Combined with Continuous 5-FU Versus Bolus 5-FU
and Leucovorin (Methodology)**
- 3. Gastrointestinal Cancer: Endoscopic Submucosal
Dissection (Methodology)**
- 4. Gastrointestinal Epithelial Neoplasms: Endoscopic
Submucosal Dissection (Methodology)**
- 5. Inoperable Abdomino-Pelvic Tumors: Treatment
with Radio-Frequency Ablation and Surgical Debulking**
- 6. Gastrointestinal Neuroendocrine Tumors:
Diagnosis Using Gastrin Receptor Scintigraphy**

Part II Esophageal Cancer

- 7. Distal Esophagus: Evaluation with 18F-FDG PET/CT
Fusion Imaging**
- 8. Endoscopic Ultrasound and Staging of Esophageal Cancer**
- 9. Esophageal Cancer: Role of RNASEN Protein
and microRNA in Prognosis**
- 10. Esophageal Cancer: Initial Staging**

Part III Gastric Cancer

- 11. Automated Disease Classification of Colon and Gastric Histological Samples Based on Digital Microscopy and Advanced Image Analysis**
- 12. Early Gastric Cancer: Prediction of Metachronous Recurrence Using Endoscopic Submucosal Dissection (Methodology)**
- 13. *Helicobacter pylori*-Infected Neoplastic Gastric Epithelium: Expression of MUC2 as a Biomarker**
- 14. Gastric Cancer: Role of Intestinal Metaplasia by Histochemical Detection Using Biopsy Specimens**
- 15. Gastric Cancer: Antitumor Activity of RUNX3**
- 16. Early Gastric Cancer: Laparoscopic Gastrectomy (Methodology)**
- 17. Gastric Cancer: Overexpression of Hypoxia-Inducible Factor 1 as a Prognostic Factor**

Part IV Pancreatic Cancer

- 18. Pancreatic Cancer: Hepatoma-Derived Growth Factor as a Prognostic Factor**
- 19. Pancreatic Cancer: 18F-Fluorodeoxyglucose Positron Emission Tomography as a Prognostic Parameter**
- 20. Imaging and Pathologic Findings of Peculiar Histologic Variants of Pancreatic Endocrine Tumors**
- 21. Periampullary Adenocarcinoma: Diagnosis and Survival After Pancreaticoduodenectomy**
- 22. Unresectable Locally Advanced Pancreatic Cancer: Concurrent Chemotherapy**

Index

Volume 4

Part I Colorectal Cancer

- 1. Introduction: Colorectal Cancer**
- 2. Poorly Differentiated Colorectal Adenocarcinoma: (Methodology)**
- 3. Colorectal Cancer: Immunohistochemical Diagnosis with Heterogenous Nuclear Ribonucleoprotein K**
- 4. Metastases and Recurrence of Colorectal Cancer: Diagnostic Role of Immunoscintigraphy**
- 5. Colorectal Cancer Diagnosis Using DNA Levels in Blood and Stool**
- 6. Colorectal Carcinoma: Identification of MicroRNAs Using Real-Time Polymerase Chain Reaction**
- 7. Colorectal Cancer: Optimization of the Combination of 5-Flouroracil and Irinotecan**
- 8. Detection of Abdominal Abscesses After Colorectal Surgery: Ultrasonography, Computed Tomography, and Gallium Scan**
- 9. Antimetastatic Therapy in Colorectal Cancer: Role of Tumor Cell Matrix Metalloproteinase 9 (Methodology)**
- 10. Endoscopic Resection of Early Colorectal Tumours: Novel Diagnostic and Therapeutic Techniques**
- 11. Role of Stromal Variables in Development and Progression of Colorectal Cancer**
- 12. Quantitative Assessment of Colorectal Cancer Perfusion: Perfusion Computed Tomography and Dynamic Contrast-Enhanced Magnetic Resonance Imaging**
- 13. Colorectal Cancer: Positron Emission Tomography**
- 14. Prognostic Significance of Protein Markers in Colorectal Cancer Stratified by Mismatch Repair Status**
- 15. Colorectal Cancer: Lactate Dehydrogenase (LDH) Activity as a Prognostic Marker**

Part II Colon Cancer

- 16. Detection of Tumor Cells in Lymph Nodes of Colon Cancer Patients Using Real-Time Quantitative Reverse Transcription-Polymerase Chain Reaction**
- 17. Colon Cancer: Laparoscopic Surgery**
- 18. Sentinel Node-Based Immunotherapy of Colon Cancer**

Part III Rectal Cancer

- 19. Rectal Cancer: Preoperative Staging Using Endorectal Ultrasonography (Methodology)**
- 20. Rectal Cancer: Spectral Imaging and Immunohistochemistry of Thymidylate Synthase**
- 21. Cancer of the Rectum: Abdominoperineal and Sphincter-Saving Resections**
- 22. Chemoradiation for Rectal Cancer**
- 23. Resectable Rectal Cancer: Preoperative Short-Course Radiation**
- 24. Preoperative Chemoradiotherapy Allows for Local Control in Rectal Cancer, but Distant Metastases Remain an Unsolved Problem**
- 25. Locally Advanced Rectal Cancer: Combined Chemotherapy During Preoperative Radiation Therapy**

Part IV Colorectal Liver Metastases

- 26. Colorectal Cancer Liver Metastases: Neoadjuvant Therapy with Bevacizumab**
- 27. Colorectal Liver Metastases: Radiofrequency Ablation**

Part V Anal Cancer

- 28. Anal Squamous Cell Carcinomas: Diagnosis Using p63 Immunohistochemistry**
- 29. Anorectal Melanoma: Prediction of Outcome Based on Molecular and Clinicopathologic Features**

Volume 5

Part I Liver Cancer

A. Diagnosis

- 1. Applications of Positron Emission Tomography in Liver Imaging:
An Overview**
- 2. Localized Fibrous Tumor of the Liver: Imaging Features**
- 3. A Radial Magnetic Resonance Imaging Method for Imaging Abdominal
Neoplasms**
- 4. Liver: Helical Computed Tomography and Magnetic Resonance Imaging**

Part II Resectable Liver Cancer

A. Diagnosis

- 5. Selection of Patients for Resection of Hepatic Colorectal Metastases:
18F-Fluorodeoxyglucose/Positron Emission Tomography**

B. Treatment

- 6. Ultrasonography During Liver Surgery**

Part III Unresectable Liver Cancer

A. Treatment

- 7. Intraoperative Magnetic Resonance Imaging for Radiofrequency
Ablation of Hepatic Tumors**
- 8. Surgically Unresectable and Chemotherapy-Refractory Metastatic
Liver Carcinoma: Treatment with Yttrium-90 Microsphere
Followed by Assessment with Positron Emission Tomography**

B. Prognosis

- 9. Unresectable Liver Metastases from Colorectal Cancer:
Methodology and Prognosis with Radiofrequency Ablation**

Part IV Hepatocellular Carcinoma

A. Diagnosis

- 10. Screening with Ultrasonography of Patients at High-Risk for Hepatocellular Carcinoma: Thrombocytopenia as a Valid Surrogate of Cirrhosis**
- 11. Hepatocellular Carcinoma: Contrast-Enhanced Sonography**
- 12. Focal Liver Lesion: Nonlinear Contrast-Enhanced Ultrasound Imaging**
- 13. Hepatocellular Carcinoma: Magnetic Resonance Imaging**
- 14. Expression of Vascular Endothelial Growth Factor in Hepatocellular Carcinoma: Correlation with Radiologic Findings**
- 15. Detection of Small Hepatic Lesions: Superparamagnetic Oxide-Enhanced Diffusion-Weighted T2 FSE Imaging**
- 16. Diagnosis of Hepatocellular Carcinoma: Multidetector-Row Computed Tomography and Magnetic Resonance Imaging**
- 17. Hepatocellular Carcinoma: Effect of Injection Rate/Injection Duration of Contrast Material on Computed Tomography**
- 18. Detection of Combined Hepatocellular and Cholangiocarcinomas: Enhanced Computed Tomography**
- 19. Hepatocellular Carcinoma and Adenomatous Hyperplasia (Dysplastic Nodules): Dynamic Computed Tomography and a Combination of Computed Tomography and Angiography**
- 20. Hepatocellular Cancer in Cirrhotic Patients: Radiological Imaging**

B. Treatment

- 21. Treatment of Hepatocellular Carcinoma with Thalidomide: Assessment with Power Doppler Ultrasound**
- 22. Perfusion Scintigraphy with Integrated Single Photon Emission Computed Tomography/Computed Tomography in the Management of Transarterial Treatment of Hepatic Malignancies**
- 23. Postoperative Interferon Alpha Treatment of Patients with Hepatocellular Carcinoma: Expression of p48 Using Tissue Microarray**

C. Prognosis

- 24. Hepatocellular Carcinoma: Overexpression of Homeoprotein Six 1 as a Marker for Predicting Survival**
- 25. Hepatocellular Carcinoma: KiSS-1 Overexpression as a Prognostic Factor**
- 26. Hepatocellular Carcinoma: Prognosis Using Hepatoma-Derived Growth Factor Immunohistochemistry**
- 27. Hepatitis C Virus-Related Human Hepatocellular Carcinoma: Predictive Markers Using Proteomic Analysis (Methodology)**

Part V Metastases**A. Diagnosis**

- 28. Liver Metastases from Colorectal Cancer: Ultrasound Imaging**
- 29. Preclinical Liver Metastases: Three-Dimensional High-Frequency Ultrasound Imaging**
- 30. Colorectal Liver Metastases: ^{18}F -Fluorodeoxyglucose-Positron Emission Tomography**

Part VI Biliary Cancer**A. Diagnosis**

- 31. Biliary Cystic Tumors: Clinicopathological Features**
- 32. Cholangiocarcinoma: Intraductal Sonography**

B. Prognosis

- 33. Extrahepatic Bile Duct Carcinoma: Role of the p53 Protein Family**
- 34. Extrahepatic Bile Duct Carcinoma: Mucin 4, a Poor Prognostic Factor**

C. Treatment

- 35. Hilar Cholangiocarcinoma: Photodynamic Therapy and Stenting**

Part VII Splenic Cancer

A. Diagnosis

36. Splenic Metastases: Diagnostic Methods

Index

Contents

Contributors	vii
Preface.....	xv
Introduction.....	xvii
Contents of Volumes 1, 2, 3, 4 and 5	xix

Part I Ovarian Cancer

A. Diagnosis

1. Identification of Biomarkers for Clear Cell Ovarian Adenocarcinoma	5
Samuel C. Mok, Michael P. Stanley, Hiroshi Tsuda, and Michael J. Birrer	
Introduction.....	5
Genetic Alterations in Clear Cell Ovarian Cancer.....	6
Clear Cell Ovarian Cancer Has Distinct Transcription Profiles.....	8
Differential Gene Expression in Clear Cell Adenocarcinoma of Different Organs	9
2. Ovarian Carcinoma: Diagnostic Immunohistochemistry of MUCIN4 (MUC4)	13
Moorthy P. Ponnusamy, Ajay P. Singh, Subodh M. Lele, and Surinder K. Batra	
Introduction.....	13
Histopathology of Ovarian Cancer	14
Stages and Prognosis of Ovarian Cancer	14
Biomarkers and Screening of Ovarian Cancer.....	14

Aberrant Mucin Expression in Ovarian Cancer:	
A Novel Class of Biomarkers	15
MUCIN4: Structure and Biology	16
MUCIN4 in Ovarian Cancer	16
Methodology for MUCIN4 Immunohistochemistry	18
Tissue Sectioning	18
Immunolabeling	18
Assessment of MUCIN4 Staining	19
3. Distinguishing Benign from Malignant Complex Adnexal Masses in Ovarian Cancer: Two-Dimensional Power-Doppler Imaging	23
Juan Luis Alcázar	
Introduction	23
Patients and Methods	24
Results	27
Discussion	28
4. Subgroups of Ovarian Carinoma: Identification Using Differential Gene Expression	35
Kristin L. M. Boylan, Keith M. Skubitz, and Amy P. N. Skubitz	
Introduction	35
Ovarian Cancer Heterogeneity	35
Selection of Samples for Gene Microarray Analysis	36
Contamination of Gene Expression Profiles by Other Cells in Tissues	37
Number of Samples to Analyze for Gene Profiling	38
Tissue Processing Protocols	38
Importance of Pathological Quality Control	38
Clinical Correlations	39
Gene Microarray Platforms	39
RNA Isolation for Generating Gene Expression Data	39
Analysis of Gene Microarray Data	40
Need for Secondary Validation of Data	40
Goals for Gene Microarray Analysis	41
Gene Expression Analysis Used to Determine Ovarian Cancer Subgroups	41
Gene Expression Analysis Used to Compare Different Stages or Grades of Ovarian Cancer	43
Gene Expression Profiles Based on Metastasis	46
Correlation of Gene Expression Profiles to Chemotherapeutic Response	48

Correlation of Gene Expression Profiles to Surgical Debulking	51
Correlation of Gene Expression Profiles to Patients' Survival	52
Summary	54

5. Sertoliform Endometrioid Carcinoma of the Ovary:

Diagnosis and Prognosis	59

Monalisa Sur and Dean Daya

Introduction.....	59
Diagnosis.....	59
Clinical Features	59
Gross Findings	60
Microscopic Findings.....	60
Differential Diagnosis	61
Immunohistochemistry	62
Cytokeratins	62
Epithelial Membrane Antigen.....	63
Inhibin	63
Calretinin.....	63
Neural Cell Adhesion Molecule (N-CAM/CD56).....	64
Estrogen and Progesterone Receptors.....	64
Other Makers	64
Prognosis.....	66

B. Prognosis

6. Role of MUC16 (CA125) in the Pathogenesis of Epithelial Ovarian Cancer

71

Jennifer A. A. Gubbels, Joseph P. Connor, and Manish S. Patankar

Introduction.....	71
CA125 and MUC16	71
MUC16 in Epithelial Ovarian Cancer.....	73
Mesothelin and MUC16 Binding: A Model for Metastasis.....	73
Mesothelin.....	73
Mesothelin and MUC16 Binding.....	74
Kinetics of Mesothelin–MUC16 Binding.....	75
Mesothelin Binds to N-Linked Oligosaccharides	
Present on MUC16.....	76
MUC16 Binding to Natural Killer Cells:	
Immunosuppressive Effects	79
A Phenotypic Shift.....	79
NK Cell Differentiation	81
Tumor Cell Layers of Protection	82

7. Clear Cell Carcinoma of the Ovary: Prognosis Using Cytoreductive Surgery	85
Masashi Takano, Naoki Sasaki, and Toru Sugiyama	
Introduction	85
Clinical Characteristics	85
Presentation at Early Stages and Association with Endometriosis	85
Molecular Characteristics	86
Clinical Outcome	87
Resistance to Platinum-Based Chemotherapy	87
Retroperitoneal Involvement.....	87
Prognosis After Cytoreductive Surgery	87
8. Advanced Ovarian Cancer: Prediction of Surgical Outcomes Using Computed Tomography	93
Sean C. Dowdy and William A. Cliby	
Introduction.....	93
Value of Cytoreduction	93
Ability of Computed Tomography to Predict Optimal Cytoreduction.....	96
Other Techniques for Predicting Surgical Outcomes.....	99
Conclusion	101
 Part II Renal Cancer	
A. Treatment	
9. Renal Cell Carcinoma: Follow-Up with Magnetic Resonance Imaging After Percutaneous Radiofrequency Ablation	109
Elmar M. Merkle, Rendon C. Nelson, and Jonathan S. Lewin	
Introduction.....	109
Involution of the Radiofrequency Induced Thermal Ablation Zone.....	110
Magnetic Resonance Signal Characteristics of Radiofrequency Induced Thermal Ablation Zones	110
Residual or Recurrent Tumor.....	112
10. Metastatic Kidney Cancer: Treatment with Infusional Interleukin-2 Plus Famotidine	115
Walter D.Y. Quan, JR and Francine M. Quan	
Introduction.....	115
Patients and Methods	115
Results.....	117
Discussion	117

11. Renal Cell Carcinoma: Preoperative Treatment with Cytokines Followed by Surgery	121
Tobias Klatte and Malte Böhm	
Introduction.....	121
Cytokines for Immunomodulation.....	122
Interleukin-2 (IL-2).....	122
Interferon- α (IFN- α).....	122
Methodological Aspects of Perioperative Immunomonitoring.....	123
Flow Cytometry	124
Enzyme-Linked Immunosorbent Assay.....	126
Perioperative Immunomodulation with Interleukin-2.....	127
Perioperative Immunomodulation with Interferon-Alpha	129
Other Agents	131
Conclusions and Future Directions	132
 12. Metastatic Renal Cell Carcinoma: Use of Bcl-2 and Fas to Predict Responses to Immunotherapy.....	 137
Yoshihiko Tomita, Ryo Maruyama, Toshiyuki Itoi, and Vladimir Bilim	
Introduction.....	137
Apoptotic Machinery and Tumor Cells	138
Fas-Driven Apoptosis and Bcl-2 in Renal Cell Cancer Cells	138
Bcl-2 or Fas and Prognosis of Renal Cell Cancer Patients	139
Absence of Bcl-2 and Fas/CD95/Apo-1 Predicts the Response to Immunotherapy in Metastatic Renal Cell Carcinoma.....	140
Clinical Course of the Patients.....	141
Expression of Bcl-2.....	141
Expression of Fas	142
Detection of Cell Proliferation and Apoptosis.....	142
Conclusion	143
 13. Wilms Tumor: Prognosis Using Microvessel Density	 147
Yasemin Ozluk	
Introduction.....	147
Prognostic Factors in Wilms Tumor	147
Stage I	147
Stage II.....	147
Stage III.....	148
Stage IV	148
Stage V.....	148
Angiogenesis.....	148
Quantification Methods.....	148
Angiogenesis and Wilms Tumor.....	150

Part III Urogenitary Tract Cancer

A. Adrenal

14. Adenomatoid Tumor of the Adrenal Gland: Differential Diagnosis Using Immunohistochemistry	161
Fanny Burel-Vandenbos, Nathalie Cardot-Leccia, Juliette Haudebourg, Damien Ambrosetti, and Jean-Francois Michiels	
Introduction.....	161
General Features	161
Histology and Differential Diagnosis	162
Immunophenotype	163
15. Testicular Cancer: Post-Chemotherapy Retroperitoneal Lymph Node Dissection	167
Philippe E. Spiess, Nizar M. Tannir, and Louis L. Pisters	
Introduction.....	167
Indications for PC-RPLND.....	167
Preoperative Considerations	169
Technical Considerations	170
Treatment-Related Outcomes	173
Potential Complications.....	176
Postoperative Follow-Up.....	176
Conclusions.....	177
16. Survivors of Germ-Cell Testicular Cancer: Increased Risk of Second Primary Tumors	181
Lorenzo Richiardi and Milena M. Maule	
Introduction.....	181
Methods to Investigate Second Primary Cancers.....	181
Cohort Studies.....	181
Nested Case-Control Studies	183
Methodological Limitations.....	184
Second Primary Cancers Among Survivors of Testicular Cancer	185
All Testicular Cancers.....	185
Seminomas and Nonseminomas	186
Chemotherapy and Radiotherapy.....	186

Part IV Urinary Bladder Cancer

Diagnosis

17. Urothelial Bladder Cancer: Screening with Urine-Based Tumor Markers	197
Robert S. Svatek and Yair Lotan	
Rationale	197
Previous Screening Programs	198
Screening in People with Occupational Exposure	198
Hematuria Screening.....	199
Urine-Based Tumor Markers	200
Methodological Aspects of Marker Evaluation	201
Specific Urine-Based Tumor Markers	203
Bladder Tumor Associated Antigen Test	203
Nuclear Matrix Protein-22	203
Urovysion.....	204
ImmunoCyt/uCyt	204
Cost-Effectiveness.....	205
Biases and Pitfalls in Bladder Cancer Screening	206
Future Considerations	207
Conclusions	207
 18. Detection of OCT-4 in Bladder Cancer: Role of Cancer Stem Cell	 211
Seyed Javad Mowla, Seyed Mehdi Jafarnejad, and Yaser Atlasi	
Introduction.....	211
Materials	212
Methods.....	215
Human Clinical Samples.....	215
Total RNA Extraction	215
Analyzing the Quality of Extracted Total RNA	216
Determining the Concentration of Extracted RNA.....	216
Semi-Quantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR).....	216
Agarose Gel Electrophoresis.....	217
Western Blotting	217
Total Protein Extraction	217
Quantification of the Concentration of Extracted Protein.....	217

SDS-PAGE..... 217

 Transfer 217

 Blotting 218

 Stripping and Reprobing the Membrane..... 218

 Immunohistochemistry 219

 Statistical Analyses 219

Results..... 220

 Expression of OCT-4 in Tumor and Non-Tumor Tissues
 of Human Bladder..... 220

 Tissue Distribution and Intracellular Localization of OCT-4
 Protein in Bladder Tumors 221

Discussion 223

Part V Cervical Uterine Cancer

Diagnosis

19. Uterine Cervical Glandular Lesions: Differentiation

Using Immunohistochemistry of Mucins 231

 Allyson C. Baker, William E. Grizzle, and David Chhieng

 Introduction..... 231

 Materials 232

 Solvents, Media, and Solutions..... 232

 Other Materials and Equipment..... 233

 Methods..... 233

 Sectioning of Tissues and Slide Preparation..... 234

 Antigen Retrieval 234

 Delineating Tissue Sections 234

 Inactivation of Endogenous Peroxidase 234

 Blocking Non-specific Binding of Proteins 235

 Primary Antibody Step 235

 Amplification of Primary Antibody 235

 Develop Color with Peroxidase Substrate 235

 Counterstaining 235

 Mounting the Tissue Specimens 236

 Results..... 236

 Discussion 238

20. Uterine Cervical Carcinoma: Preoperative Magnetic

Resonance Imaging Staging 243

 Hak Jong Lee and Seung Hyup Kim

 Introduction..... 243

 Normal Anatomy of Uterine Cervix 243

General Consideration of Uterine Cervical Cancer	244
Magnetic Resonance Imaging Technique for Uterine Cervical Cancer.....	245
Magnetic Resonance Findings of Uterine Cervical Cancer.....	246
Magnetic Resonance Staging of Uterine Cervical Cancer.....	247
Pelvic Computed Tomography Versus Magnetic Resonance	250
Evaluation of Pelvic Lymph Nodes	251

Treatment

21. Cancer Imaging and Intracavitary Brachytherapy for Cervical Cancer.....	257
Shingo Kato and Tatsuya Ohno	
Introduction.....	257
Intracavitary Brachytherapy for Cervical Cancer	258
Applicator Insertion	258
Dose Specification	258
Magnetic Resonance Imaging for Cervical Cancer Brachytherapy.....	259
Image-Based Brachytherapy	260
22. Cervical Cancer: Methods for Assessing the Quality of Life.....	263
Elfriede Greimel	
Introduction.....	263
Concept of Quality of Life.....	263
Selecting Appropriate Quality of Life Measurements	264
First Step: Questions to Be Asked When Selecting a Quality of Life Instrument	264
Second Step: Introducing a Quality of Life Instrument in Clinical Practice.....	265
Psychometric Properties of a Quality of Life Instrument	265
Reliability.....	265
Validity.....	265
Responsiveness to Change	266
Types of Quality of Life Measurements	266
Development and Cross-Cultural Validation of Quality of Life Instruments.....	268
EORTC Modular Approach to Quality of Life Assessment	268
Development of the Cervical Cancer Module (EORTC QLQ-CX24).....	269
Phase I: Generation of QoL Issues.....	269
Phase II: Construction of Items and Translation.....	269
Phase III: Pretesting	269
Phase IV: Testing the Psychometric Properties.....	270

23. Cervical Cancer: Positron Emission Tomography and Positron Emission Tomography/Computed Tomography.....	275
Lilie L. Lin and Perry W. Grigsby	
Introduction.....	275
Background and Staging	275
Directing Therapy	279
Prognosis.....	280
Posttherapy Monitoring	281
24. Endometrial Cancer: Indoleamine 2,3-Dioxygenase as a Prognostic Indicator	285
Kazuhiko Ino, Eiko Yamamoto, Kiyosumi Shibata, Hiroaki Kajiyama, Akihiro Nawa, and Fumitaka Kikkawa	
Introduction.....	285
Materials and Methods.....	287
Antibodies	287
Patients	287
Immunohistochemical Staining	288
Evaluation of Indoleamine 2,3-Dioxygenase Expression	288
Statistical Analysis.....	288
Results.....	289
Immunohistochemical Expression of IDO in Endometrial Cancer Tissues	289
Association of IDO Expression with the Patient Survival	290
Multivariate Analysis of Prognostic Variables in Endometrial Cancer Patients.....	290
Discussion.....	291

Part VI Skin Cancer

Melanoma

25. Neurofibromatosis Type 1-Associated Malignant Melanoma: Molecular Evidence of Inactivation of the <i>NF1</i> Gene	301
Albert Rübben	
Introduction.....	301
Methodology	302
Definition of Cancer Genes.....	302
Identification of Genes Implicated in Oncogenesis	302
Role of <i>NF1</i> Gene Mutations in NF1-Associated Melanoma.....	304
Melanoma Incidence in NF1.....	304
Biologic Role of Neurofibromin in Melanocytes.....	304
Mutations of the <i>NF1</i> Gene in NF1-Associated Malignant Melanoma	305

Inactivation of the <i>NF1</i> Gene in NF1-Associated	
Malignant Melanoma	306
Conclusion	307
26. Malignant Melanoma: Localisation and Characterization	
Using Fluorodeoxyglucose-Positron Emission	
Tomography/Computed Tomography	311
Olivier Gheysens and Felix M. Mottaghy	
Introduction and Clinical Background.....	311
Potential Indications of Fluorodeoxyglucose Positron Emission	
Tomography Imaging in the Management	
of Malignant Melanoma.....	312
Detection of Locoregional Lymph Node Invasion.....	313
Detection of Distant Metastases.....	314
Pitfalls and Additional Value of Integrated PET/CT Imaging	314
Role of FDG-PET in Monitoring Response to Therapy	317
Role of FDG-PET in Patient Management	318
Alternative Tracers for Diagnosing MM and Monitoring	
Therapy Response	318
27. Malignant Melanoma Versus Deep Penetrating Nevus:	
Diagnostic and Prognostic Immunohistochemistry	
of Dipeptidyl Peptidase IV (Methodology)	323
Alexander Roesch, Michael Landthaler, and Thomas Vogt	
Introduction.....	323
The Deep Penetrating Nevus as a Model of Paradoxical	
Melanocytic Invasion	323
Common Melanoma Markers Fail to Separate Between	
Melanocytic Invasion and True Melanocytic Malignancy	324
Immunostaining of Dipeptidyl Peptidase IV Discriminates	
Metastatic Malignant Melanoma from Deep Penetrating	
Nevus – Application of a New Histomorphologic Expression	
Algorithm (Methodology).....	324
Tissue Sample Collection and Immunohistochemistry	325
Immunohistochemical Evaluation.....	325
Discussion and Biologic Background.....	327
28. Nonmelanoma Skin Cancer: Use of EphA1 Receptor	
as a Prognostic Marker.....	333
Christian Hafner	
The Eph/Ephrin Family.....	333
Eph/Ephrin Expression in Adult Human Tissues	334
Eph/Ephrin Expression in Human Skin	334
EphA1 and Nonmelanoma Skin Cancer	336

Part VII Leukemia

29. Pretreated Chronic Lymphocytic Leukemia:

Use of Alemtuzumab..... 343

Michael Fiegl and Jiri Mayer

 Introduction..... 343

 Evolution of Treatments for Chronic Lymphocytic Leukemia 344

 Alemtuzumab as Monotherapy in Pretreated Chronic

 Lymphocytic Leukemia..... 344

 Combination Therapy 350

 Consolidation Therapy with Alemtuzumab 352

Part VIII Multiple Myeloma

30. Immunotherapeutic Strategies, Radiotherapy, and Targeted

Radionuclide Therapy Approaches for the Treatment

of Multiple Myeloma..... 361

Malay Chatterjee, Rangasamy Manivannan, Amalendu Pande,

 Tridib Chakraborty, and Ajay Rana

 Introduction..... 361

 Therapeutic Strategies in Multiple Myeloma 362

 Immunotherapy 362

 Radiotherapy 371

 Targeted Radiotherapy 373

 Conclusion and Perspectives..... 378

Part IX Sarcoma

Diagnosis

31. Low Grade Fibromyxoid Sarcoma: Diagnosis by Detecting

FUS-CREB3L2 Fusion Gene Using Reverse

Transcription–Polymerase Chain Reaction..... 387

Atsuji Matsuyama, Masanori Hisaoka, and Hiroshi Hashimoto

 Introduction..... 387

 Detection of the FUS-CREB3L2 Fusion Transcripts Using

 Formalin-Fixed, Paraffin-Embedded Tumor Tissue 388

 Primers 388

 RNA Extraction..... 389

 RT-PCR 389

 Sequence Analysis 390

Results.....	390
Evaluation of the RT-PCR Results.....	391
32. Synovial Sarcoma: Role of TLE1 as a Diagnostic Immunohistochemical Marker	393
Jefferson Terry and Torsten O. Nielsen	
Introduction.....	393
Materials	395
Methods.....	396
Manual Immunostaining	396
Automated Immunostaining.....	397
Interpretation of TLE Staining.....	398
Results and Discussion	398
33. The Immunohistochemistry of Kaposi's Sarcoma	405
Liron Pantanowitz, Gabriel Caponetti, and Bruce J. Dezube	
Introduction.....	405
Materials	407
Methods.....	408
Interpretation.....	417
Histogenesis	418
Pathogenesis.....	421
HHV8 Infection	421
Angiogenesis.....	422
Chemokines.....	423
Apoptosis	423
Diagnosis.....	424
Therapy	424
Conclusion	425
34. Synovial Sarcoma: Role of Immunohistochemistry and Molecular Genetics in Diagnosis and Prognosis	433
Andrew E. Horvai	
Introduction.....	433
Diagnosis.....	434
Histology	434
Ultrastructure	436
Immunohistochemistry	436
Cytogenetics and Molecular Genetics	438
Prognostic Markers for Synovial Sarcoma	442

Treatment

35. Sarcoma: Treatment with Ecteinascidin-743..... 451
Jérôme Fayette, Philippe Cassier, Laura Brousseau,
Armelle Dufresne, Isabelle Ray Coquard, Laurent Alberti,
Pierre Méeus, Anne-Valérie Decouvelaere, Marie-Pierre Sunyach,
Dominique Ranchère, and Jean-Yves Bla

 Introduction..... 451
 Mechanisms of Action and Resistance 452
 Binding to DNA 452
 Inhibition of Transcription Factors 452
 Inhibition of DNA Repair Machinery 453
 Other Effects 454
 Metabolism and Toxicity of ET-743 454
 Clinical Activity of ET-743 in Patients with Sarcoma..... 456
 ET-743 in Combination with Other Drugs..... 458
 References..... 458

Index..... 461

Part I

Ovarian Cancer

A. Diagnosis

1

Identification of Biomarkers for Clear Cell Ovarian Adenocarcinoma

Samuel C. Mok, Michael P. Stanley, Hiroshi Tsuda, and Michael J. Birrer

INTRODUCTION

Ovarian cancer is the fifth most common form of cancer in women in the United States, accounting for 4% of the total number of cancer cases and 25% of those cases occur in the female genital tract. Because of its low cure rate, it is responsible for 5% of all cancer deaths in women. It was estimated that 12,180 deaths would be caused by ovarian cancer in the year 2006 (Jemal et al. 2006).

Epithelial ovarian tumors are classified as benign, low malignant potential (LMP), or malignant (Serov and Scully 1973) and further distinguished by differences in the histologic type of cell. Benign ovarian tumors are lined by a single, or minimally stratified layer of cells, which are columnar and often ciliated in serous tumors or contain abundant apical cytoplasmic mucin in mucinous tumors. LMP tumors or borderline tumors (BOT) are characterized by atypical epitheliums with cellular proliferation and pleomorphism, but without stromal invasion. Malignant epithelium demonstrates marked atypia, increased mitotic activity, and stromal invasion.

Serous tumors are the most common form of ovarian neoplasm with epithelial cells resembling those of the fallopian tube. They comprise ~ 50–60% of primary epithelial ovarian tumors. Mucinous tumors are cystic tumors with locules lined with mucin-secreting epithelial cells resembling either endocervical or colonic epithelium. They comprise ~ 8–10% of primary epithelial ovarian tumors. Endometrioid and clear cell lesions constitute ~ 10% of epithelial tumors and resemble tumors that originate in the endometrium. Other tumor cell types include Brenner, mixed epithelial type and undifferentiated (Lee et al. 2003).

Among different histological types of ovarian cancers, clear cell ovarian cancer differs from the other histologic types with respect to its clinical characteristics (Russel 1994; Scully et al. 1998). This type of tumor is thought to arise from endometriosis and most of the patients present the disease at early stages (Russel 1994; Scully et al. 1998). Clear cell type ovarian cancer is usually more resistant to systemic chemotherapy compared to other histological types, and has the worst prognosis (Goff et al. 1996; Behbakht et al. 1998).

In fact, in current clinical practice, patients with clear cell type ovarian cancer are treated as those with high-grade neoplasms (Morgan et al. 1996). The molecular pathobiology of clear cell type ovarian cancer remains largely unknown.

GENETIC ALTERATIONS IN CLEAR CELL OVARIAN CANCER

Recent studies demonstrated that 25–75% of clear cell type showed increased DNA copy numbers on 8q11-q13, 8q21-q22, 8q23, 8q24-qter, 17q25-qter, and 20q13-qter, and decreased copy number on 19p by chromosome comparative genomic hybridization (CGH) (Suehiro et al. 2000). However, changes in the DNA copy number on the gene level have not been identified.

Using a 10,816-element cDNA array comparative genomic hybridization (aCGH) microarray platform to identify DNA copy number abnormalities in 30 clear cell ovarian cancer cases and 19 serous cases, Tsuda et al. (2004) identified 12 genes that showed a significant increase in DNA and mRNA copy number, and 5 genes showed a significant decrease in DNA and RNA copy numbers in clear cell tumors compared with those in the serous type. One of the amplified genes was *ABCF2*, which belongs to the ATP-binding cassette gene superfamily and has been shown to amplify in other tumor types (Yasui et al. 2004). Validation studies were performed using real time quantitative PCR and immunohistochemistry. The results showed significantly higher *ABCF2* DNA and mRNA copy numbers and protein levels in clear cell cases compared with those in serous cases. In addition, they also showed that cytoplasmic *ABCF2* expression was

significantly correlated with chemotherapy response despite of the small number of cases. These data suggest that *ABCF2* expression may contribute to the chemoresistant phenotype of clear cell ovarian cancer. However, the role of *ABCF2* in conferring chemoresistance in cancer cells is unclear.

Yasui et al. (2004) reported that *ABCF2* gene is amplified in a chemoresistant cell line (t24/cDDp10), which had chromosome gain at 7q34-36. Besides this study, there are no other reports to date demonstrating the mechanism of *ABCF2* in chemoresistance. *ABCF2* protein is a member of the ABCF transporter superfamily and of the *GCN20* subfamily (Vazquez de Aldana et al. 1995). Like other members of the *ABCF* family, *ABCF2* contains a pair of nucleotide binding domain (NBD) but without any transmembrane domains (Allikmets et al. 1996; Kerr 2004), suggesting its unlikely function as a transporter located on the cell membrane as other members of the ABC family. This is further confirmed by the immunohistochemistry data showing predominantly cytoplasmic localization of the protein.

The functions of many of these twin-NBD proteins remain unknown. Kerr (2004) suggested that a mechanistic similarity exists between eukaryotic members of the *ABCF* family, which are involved in the control of translation initiation and elongation. These proteins may also have functional similarities to prokaryotic ABCF proteins, which have been shown to be involved in translational control, antibiotic resistance, and ribonuclease L inhibition (Kerr 2004). ABCF may induce factors related with chemoresistance. Taken together, *ABCF2* is amplified and overexpressed particularly in clear cell ovarian cancer and may have prognostic values.

Beside CGH analysis, loss of heterozygosity (LOH) rates in multiple chromosome loci were evaluated in clear cell ovarian cancer and compared with those in other histological types. Okada et al. (2002) used microdissected ovarian tumor tissue samples to evaluate LOH patterns in 55 loci on 28 chromosomal arms. They detected LOH primarily on 1p (69%) followed by 19p (45%) and 11q (43%) in clear cell ovarian cancer. In addition, they also found that the incidences of LOH on 5q, 12q, 13q and 17p were significantly lower in clear cell cancer compared with those in the serous type. These findings show that there are differences in LOH distribution patterns among different histological subtypes of epithelial ovarian cancer. In addition, tumor-suppressor genes located on 1p may play an important role in the development of clear cell ovarian cancer (Okada et al. 2002).

Other genetic changes including DNA mutations in specific oncogenes and tumor suppressor genes in clear cell ovarian cancer have been identified. Willner et al. (2007) evaluated alterations in *TP53*, *PIK3CA*, *PTEN*, *CTNNB1* (beta-catenin), *MLH1*, and *BRAF* among 12 clear cell, 26 endometrioid, and 51 serous carcinomas by direct DNA sequencing for mutations. They found *TP53* mutations in 25 (49%) of 51 serous type, and 11 (42%) of 26 endometrioid type, but only in 1 (8.3%) of 12 clear cell type neoplasms. *PIK3CA* mutations were identified in 3 (25%) of 12 clear cell cancer, and 3 (12%) of 26 endometrioid cancer, but not in any of the serous type. *PTEN* and *CTNNB1* mutations were common in endometrioid cancer but could not be identified in the clear cell type. Mutations in *MLH1* and *BRAF* were not common in all types of ovarian

cancers. Besides these mutations, *PIK3CA* amplification could only be identified in high-grade serous type but not in the clear cell type. Based on these findings, they conclude that mutations in *TP53* or in *PTEN/PIK3CA* are alternative pathways in ovarian carcinogenesis. Activation of *PIK3CA* occurs by gene amplification in serous cancer but via somatic mutation of *PIK3CA* or *PTEN* in endometrioid cancer and clear cell cancer.

The lack of genetic alteration in *TP53* in clear cell cancer has been further confirmed by other studies. Using Polymerase Chain Reaction/Single Strand Conformational Polymorphism (PCR-SSCP) and immunohistochemistry. Okada et al. (2002) showed that the incidences of *TP53* mutation and p53 nuclear immunoreactivity differed between clear cell and serous ovarian cancer: 0% and 7% in the former and 64% and 45% in the latter. In addition, Ho et al. (2001) examined p53 alteration in primary or recurrent ovarian clear cell carcinoma obtained from 38 patients. All these tumors were subjected to immunohistochemical and molecular analysis. Genomic DNAs extracted from paraffin blocks of the 38 tumors were subscribed for a nested PCR/SSCP analysis. Tumors showing band shift on SSCP were further prepared for DNA sequencing to determine the site of mutation. The results showed that overexpression of p53 was observed in only one stage III clear cell carcinoma. However, focal positive p53 staining was noted in another five tumors. Of the six tumors showing positive immunohistochemistry, p53 alterations were noted in four tumors. Three tumors revealed a missense point mutation. Another tumor revealed a 12-bp deletion, which might result in a truncated protein. Taken together, mutations in *TP53*

appear to be much less frequent in clear cell adenocarcinoma compared to that in other histologic types of epithelial ovarian cancer, suggesting that p53 alterations may not play an important role in the development of clear cell adenocarcinoma.

CLEAR CELL OVARIAN CANCER HAS DISTINCT TRANSCRIPTION PROFILES

Since the introduction of gene expression profiling more than a decade ago, much information has been gained in regards to identifying dysregulated genes in clear cell ovarian cancer. This technique has provided a global analysis correlation of the transcriptional activity of these tumors with multiple molecular determinates. This technology has been utilized not only to identify genes important to clear cell, but also has been used to identify genes that distinguish clear cell from the other epithelial ovarian histotypes. Profiling analyses have been performed comparing clear cell ovarian tumors to other epithelial ovarian tumor histotypes, and to normal ovarian surface epithelium. Each analysis has provided not only lists of dysregulated genes in clear cell tumors, but also identified genes that can possibly explain why clear cell tumors are more aggressive and chemoresistant than other ovarian cancer histotypes.

Gene expression profiling studies of clear cell ovarian tumors have consistently reported this histotype to be different from other ovarian cancer histotypes. Schwartz et al. (2002) reported the comparison of the expression profiles of clear cell tumors with those of serous, endometrioid, and mucinous tumors. They found clear cells tumors to

have the most distinctive molecular signature among the histotypes. While their analysis included only 7,129 probesets, 73 genes were found to be expressed 2- to 29-fold higher in clear cell tumors when compared to the other histotypes. Examples of these genes that have also subsequently been found to be overexpressed by other investigators include *GPX3* (stress response), *GLRX* (drug resistance), *FXYD2* (transporter), *COL4A2* (extracellular matrix), *ANXA4* (calcium binding), and *TCF2* (cell proliferation).

The overexpression of hepatocyte nuclear factor (HNF) 1beta or *TCF2* in clear cell ovarian cancer was also identified by Tsuchiya et al. (2003) during their comparison of expression profiles between clear cell and non-clear cell ovarian cancer cell lines. They further investigated this gene with real-time quantitative reverse transcriptase PCR and immunoblotting on 83 surgically resected ovarian tumors. *TCF2* was found to be overexpressed in essentially only clear cell tumors. Subsequent knockdown experiments with RNA interference resulted in apoptosis in clear cell cells, suggesting *TCF2* is potentially both a marker and therapeutic target in clear cell ovarian cancer.

A comparison of the gene expression profiles of clear cell and serous tumors by Schaner et al. (2003) demonstrated a gene signature of clear cell tumors, which was distinct from serous ovarian tumors. Genes found to be overexpressed included those involved in drug resistance (*GLRX*, *SLC16A3*), cell-cell adhesion (E-cadherin), and basement membrane component (*NID2*). Interestingly, they found HE4, which has been described as a potential marker of ovarian cancer, to be underexpressed in clear cell tumors. Other genes found to have a lower expression when

compared to serous tumors include the tumor suppressors *WT1* and *GAS1*. Taken together, distinct transcription signatures have been identified in clear cell adenocarcinoma. Further validation and functional studies are warranted to evaluate the roles of these genes in the pathogenesis of clear cell adenocarcinoma.

DIFFERENTIAL GENE EXPRESSION IN CLEAR CELL ADENOCARCINOMA OF DIFFERENT ORGANS

Clear cell adenocarcinomas have been found to develop in different organs such as ovary, uterine corpus, and kidney, and the prognosis of these cancers is usually poor (Abeler et al. 1992; Behbakht et al. 1998; Goff et al. 1996; Yagoda 1990). In the cancer of uterine corpus, clear cell and serous adenocarcinomas have worse prognosis compared to that of the endometrioid type, which constitutes 80% of uterine corpus cancer (Abeler et al. 1992). Clear cell adenocarcinoma of the kidney is thought to be chemoresistant (Bukowski 1997; Yagoda 1990). Clear cell adenocarcinoma of the ovary is morphologically similar to clear cell adenocarcinomas developed from the uterine corpus or the kidney. However, both clear cell adenocarcinomas of the ovary and uterine corpus are Mullerian in origin, while those developed from the kidney are Wolffian duct in origin (Matias-Guiu et al. 1997). Markers that can be used to differentiate these tumors have not been explored.

Zorn et al. (2005) performed a gene expression analysis with an 11,000 element cDNA array of clear cell ovarian adenocarcinomas, with comparisons to normal surface epithelium and to clear cell adenocarcinomas

of endometrial and renal origin. When compared to normal surface epithelium 94 genes were found to be dysregulated in the clear cell ovarian adenocarcinomas. A principal component analysis of clear cell tumors of ovarian, endometrial, and renal origin demonstrated overlap of these expression profiles with no statistically significant differences found.

Recent studies have noted specific expression of hepatocyte nuclear factor (HNF) 1beta (TCF2) in ovarian clear cell adenocarcinoma (Tsuchiya et al. 2003). Osada et al. (2007) examined HNF-1beta expression immunohistochemically in 186 ovarian carcinomas of different histological types and 33 endometrial carcinomas, including 5 clear cell adenocarcinomas. The results showed that the incidence of HNF-1beta immunoreactivity differed significantly between clear cell adenocarcinomas and other histology in both the ovary (100% in the former versus 2% in the latter) and the endometrium (100% in the former versus 0% in the latter). However, the incidence of HNF-1beta immunoreactivity did not show any significant difference between clear cell ovarian and endometrial adenocarcinomas. These data suggest that HNF-1beta would be an excellent marker for distinguishing clear cell adenocarcinomas from other lesions in both the ovary and the endometrium. In addition, HNF-1beta expression seems to be associated with physiopathological cytoplasmic glycogen accumulation in these organs (Osada et al. 2007).

Nishimura et al. (2007) examined 76 clear cell adenocarcinomas of the ovary, 23 clear cell adenocarcinomas of the uterine corpus, and 34 clear cell adenocarcinomas of the kidney to evaluate whether significant differences in ABCF2 protein expression can be identified in clear cell adenocarcinomas

of different organs (Nishimura et al. 2007). The results showed both clear cell adenocarcinomas of the ovary and the uterine corpus showed significantly higher levels of ABCF2 expression, compared with those of the clear cell adenocarcinoma of the kidney. The authors suggested that the differential expression patterns of ABCF2 in clear cell adenocarcinoma of the ovary and the uterine corpus compared with that of the kidney may be explained by the fact that both clear cell adenocarcinoma of the ovary and the uterine corpus are Mullerian in origin, in contrast to clear cell adenocarcinoma of the kidney, which is Wolffian duct in origin.

Taken together, clear cell adenocarcinoma is a distinct entity compared to other histological types of cancer in different organs. In addition, despite the similarities in the histology, clear cell adenocarcinomas of different organs may have different pathogenetic pathways.

In conclusion, clear cell ovarian adenocarcinoma is a relatively rare disease compared to other histological types of ovarian cancer. Multiple genetic changes, which are specific for clear cell adenocarcinoma, have been identified by recent global genetic analyses on large collections of clear cell cancer collected throughout the past few years. These changes may be used as biomarkers for early detection of the disease as well as new therapeutic targets in cancer treatment. To understand the pathogenesis of this histological type of ovarian cancer, further functional studies on the genes involved are warranted.

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2

Ovarian Carcinoma: Diagnostic Immunohistochemistry of MUCIN4 (MUC4)

Moorthy P. Ponnusamy, Ajay P. Singh, Subodh M. Lele, and Surinder K. Batra

INTRODUCTION

Ovarian cancer is the fourth leading cause of cancer deaths among U.S. women and has the highest fatality-to-case ratio of all gynecologic malignancies. It affects > 22,000 women and accounts for > 16,000 deaths every year with a projected 5 year mortality rate exceeding 70% (Jemal et al. 2007). This is, in part, due to the lack of early diagnosis, which makes it one of the most challenging of all cancers to fight. In fact, no observable or defined symptoms of disease are evident until it has metastasized. Therefore, there is a need to develop sensitive and reliable biomarker(s) for early detection, so that the high morbidity and deaths in ovarian cancer patients can be minimized. Current strategies for the detection are based on biochemical markers, such as CA125, and biophysical markers assessed by ultrasound and/or Doppler imaging of the ovaries. The clinical utility of these strategies for early diagnosis, however, is limited due to the lack of specificity and sensitivity.

Mucins have emerged as markers of choice for disease diagnosis and prognosis owing to their aberrant expression in malignant cells and proven functional association of

some mucins with the cancer development. To date, 20 human mucins have been identified and categorized into two classes (secreted/gel forming and membrane-bound) based on their structural characteristics and physiological fates (Hollingsworth and Swanson 2004; Singh et al. 2007). Mucins are produced by secretory epithelial cells for the lubrication and protection of ducts and lumen within the human body. However, mucins are also believed to play an important role in the pathogenesis of benign and malignant diseases of secretory epithelial cells (Hollingsworth and Swanson 2004). An aberrant expression of mucins has been reported in a variety of carcinomas. MUC4, which belongs to the membrane-bound mucin family, is aberrantly expressed in several types of carcinomas (Singh et al. 2007; Carraway et al. 2002; Chauhan et al. 2006). It is a multifunctional protein that is implicated in numerous cellular functions including cell adhesion, motility, signal transduction, tissue regeneration and differentiation, and tumor growth and metastasis. The diagnostic significance of MUC4 for ovarian carcinoma was recently evaluated in our laboratory by using immunohistochemical

analysis of archival specimens (Chauhan et al. 2006). It was demonstrated that MUC4 could be a potential candidate marker for early diagnosis of epithelial ovarian carcinoma and can be utilized in combination with MUC16 to achieve greater sensitivity for the detection of late-stage tumors signifying the clinical applicability of MUC4 immunohistochemistry.

HISTOPATHOLOGY OF OVARIAN CANCER

The ovaries contain three main types of cells, germ cells, stromal cells, and epithelial cells that give rise to germ cell, stromal and epithelial ovarian tumors, respectively. Epithelial ovarian cancer (EOC) is the most commonly observed ovarian cancer type and consists of various histological subtypes. Approximately 90% of all ovarian cancers are epithelial, i.e., derived from relatively pluripotent cells of the celomic epithelium or “modified mesothelium”. These cells originate from the primitive mesoderm and can undergo metaplasia. Approximately 10% to 20% of epithelial ovarian neoplasms are borderline or low malignant potential tumors, which are characterized by high degree of cellular proliferation in the absence of stromal invasion. Of the invasive epithelial ovarian cancers, ~ 75% to 80% are serous, 10% are mucinous, and 10% are endometrioid. Less common cell types include clear cell, transitional (Brenner), small cell, and undifferentiated carcinomas. Many of the histological subtypes represent the epithelial features of the lower genital tract, e.g., papillary serous tumors have an appearance resembling the glandular epithelium lining the fallopian tube, mucinous tumors contain cells resembling endocervical glands, and

endometrioid tumors contain cells resembling the endometrium. Non-epithelial types of ovarian cancer include sex cord-stromal tumors (6% of ovarian cancers) and germ cell tumors (3%) (Breedlove and Busenhardt 2005; Escudero 1999; Jemal et al., 2007; Hightower et al. 1994).

STAGES AND PROGNOSIS OF OVARIAN CANCER

One of the most significant prognostic factors is the stage of disease at the time of diagnosis. A study based on the National Survey of Ovarian Cancer (NSOC), with long-term survival data on > 5,000 patients reported 5-year survival for patients with stage Ia, Ib, and Ic disease of 92%, 85%, and 82%, respectively. The 5-year survival was 67% in stage IIa disease, 56% in stage IIb, and 51% in stage IIc. NSOC showed a 39% 5-year survival for stage IIIa disease, whereas it was 26% for stage IIb, 17% for stage IIc, and 12% for stage IV (Nguyen et al. 1993). A study based on the National Cancer Institute’s Surveillance, Epidemiology, and End Results (SEER) data, reflected improvements in 5-year survival rates for ovarian cancers as follows: stage I (93%), stage II (70%), stage III (37%), and stage IV (25%) (Ozols, 1990; Nguyen et al. 1993; Escudero 1999; Jemal et al., 2007).

BIOMARKERS AND SCREENING OF OVARIAN CANCER

The prognostic importance of tumor stage at diagnosis and the fact that most tumors are not symptomatic until in advanced stage has motivated efforts to develop

screening programs for ovarian cancer. Unfortunately, the value of screening programs for epithelial ovarian cancer with pelvic examinations, tumor markers, and ultrasonography has not been clearly established by prospective studies for either the general population or high risk groups. Routine annual pelvic examination has been disappointing for the early detection of ovarian cancer. Transvaginal ultrasonography has demonstrated encouraging sensitivity for the detection of early ovarian cancer, but specificity continues to be limited (Van et al., 1990). Cancer antigen-125 (CA-125), is a protein that is found at levels in most ovarian cancer cells that are elevated compared to normal cells. CA-125 is present at the surface of cells and is also released in the blood stream. Population based studies found that CA-125 measurements with reference value of 30 U/mL still missed almost 30% of stage I tumors (Woolas et al. 1993). The screening group underwent three annual CA-125 measurements, with pelvic ultrasound if the CA-125 was 30 U/mL or higher, and gynecologic evaluation if the ovarian volume was 8.8 mL or greater on ultrasound.

The past 3 years have seen significant rise in the research efforts devoted to the detection of early ovarian cancer, although none of the methods identified thus far will probably be sufficient to result in the accurate and reliable screening test that is needed. It is likely that a combination of tests will be necessary to ensure sufficient sensitivity and specificity. Although progress in the development of methods for early detection of ovarian cancer has historically been very slow, the recent advances in technology and the associated enthusiasm for investigation in this field have ensured that the development of a

reliable sensitive screening test for ovarian cancer is one step closer (Breedlove and Busenhardt 2005).

ABERRANT MUCIN EXPRESSION IN OVARIAN CANCER: A NOVEL CLASS OF BIOMARKERS

Mucins are expressed by epithelial cells lining the gastrointestinal, urogenital tracks and glandular organs. In epithelial cancers, the metastatic process has been associated with alterations in cell surface and cell-associated glycoprotein expression (Hakomori 1989; Giuntoli et al. 1998). Specifically mucin glycoproteins have been implicated in the pathogenesis of epithelial cell malignancies (Hilkens et al. 1992; Bhavanandan 1991). Aberrantly glycosylated mucins are overexpressed by tumors and secreted into the circulation of cancer patients. These oligosaccharides present on mucins and/or mucin glycoproteins can be detected by antibodies in patients' sera and serve as tumor markers. Mucin expression from ovarian cancer tissues has also been related to tumor histology, stage, and patient survival (Rump et al. 2004). CA125, a serum marker, used routinely in gynecologic practice to monitor patients with ovarian cancer is in fact a mucin glycoprotein 16 (MUC16) (Rump et al. 2004). If serial CA-125 testing results double beyond normal parameters of the Skate algorithm, then this suggests progression of the cancer (Yin et al. 2002; Rump et al. 2004; McLemore and Aouizerat 2005). The identity of MUC16 as CA125 was based on (1) the isolation of peptides from purified CA125 that were contained in the deduced amino acid

sequence of MUC16 and (2) a precise correlation between *MUC16* mRNA expression, established by Northern blotting and CA125 expression, determined by serological analysis, in a panel of cancer cell lines. It is a membrane-anchored glycoprotein and has highly O-glycosylated repeats that are the landmark of the mucin family. CA125 is upregulated in most ovarian cancer cells as compared to the normal cells (Rump et al. 2004). Further CA-125 tests for stage II, III, and IV ovarian cancer patients during chemotherapy have been used to determine the activity of the cancer and the status of chemotherapy on the cancer (McLemore and Aouizerat 2005). MUC1 and MUC4 are other membrane-bound mucins, which are also aberrantly expressed in ovarian carcinoma and are being explored as potential diagnostic markers for epithelial ovarian cancer (Chauhan et al. 2006).

MUCIN4: Structure and Biology

MUC4 is a member of the membrane-bound mucin family that consists of two subunits: MUC4 α (an extracellular mucin – type glycoprotein subunit) and a transmembrane subunit MUC4 β (membrane-anchored subunit with three EGF-like domains and a short cytoplasmic tail). Depending on the size of central tandem-repeat domain, molecular weight of the nascent MUC4 protein may range from 550 to 930 kDa (Nollet et al. 1998; Moniaux et al. 1999). Due to similarities in structural organization, MUC4 is believed to be the homologue of the rat sialomucin complex (SMC, rat Muc4).

MUC4/SMC is also a heterodimeric glycoprotein composed of the O – glycosylated mucin subunit ASGP-1, and N-glycosylated transmembrane ASGP-2, which contains

two epidermal growth factor (EGF) domains in the juxtamembrane region. SMC has been well studied and has been shown to facilitate tumor development/progression by multiple mechanisms (Carraway et al. 2002). The SMC acts as a ligand for the receptor tyrosine kinase ErbB2/HER2/neu via one of its two EGF like domains, and induces its phosphorylation (Carraway et al. 2002). Over-expression of SMC is associated with the suppression of both cell adhesion and immune killing of tumor cells by altering cell surface properties and promoting tumor growth in vitro/vivo via suppression of tumor cell apoptosis by altering intracellular signaling to favor cell survival (Komatsu et al. 2001).

In our recent studies, we have observed important functions of MUC4 in determining the malignant phenotype of cancer cells (Singh et al. 2004; Chaturvedi et al. 2007). MUC4 was shown to potentiate tumorigenicity by enhancing the cell proliferation and suppressing the apoptosis. Additionally, silencing of MUC4 expression was associated with decreased cell motility and invasion. Interestingly, we also identified an important role of MUC4 in modulating the expression of receptor tyrosine kinase, HER2. MUC4 colocalizes and physically interacts with the HER2 in pancreatic and ovarian cancer cells (Chaturvedi et al. 2008; Ponnusamy et al. 2008); however, the mechanism of MUC4-mediated HER2 regulation is not yet established. MUC4 overexpression is also associated with metastatic capacity of tumor cells and altering the tumor cell extra cellular matrix interaction (Singh et al. 2004; Chaturvedi et al. 2007).

MUCIN4 in Ovarian Cancer

MUC4 mucin frequently displays an altered expression in multiple malignancies.

Over-expression of MUC4 mRNA has also been reported in ovarian cancer (Giuntoli et al. 1998; Lopez-Ferrer et al. 2001). Studies from our laboratory have revealed aberrant expression of MUC4 mucin in > 90% of different histological subtypes and grades of ovarian tumors with very low or undetectable expression in the normal ovary (Figure 2.1) (Chauhan et al. 2006). MUC4 exhibited the highest percentage of reactivity in early and late stage tumors as compared to MUC1 and MUC16 (CA125), although its expression decreases in late

stage cases. A combined panel of MUC4 with MUC16 detected 100% of the late-stage tumor cases without compromising the specificity. The expression of MUC4, however, did not significantly correlate with the survival of the ovarian cancer patient, while a significant correlation of MUC16 with poor prognosis was observed. The profiles of mucin gene expression in different histological types revealed the highest sensitivity of MUC4 for mucinous, endometrioid and clear cell carcinomas; however, the size was limited, and thus

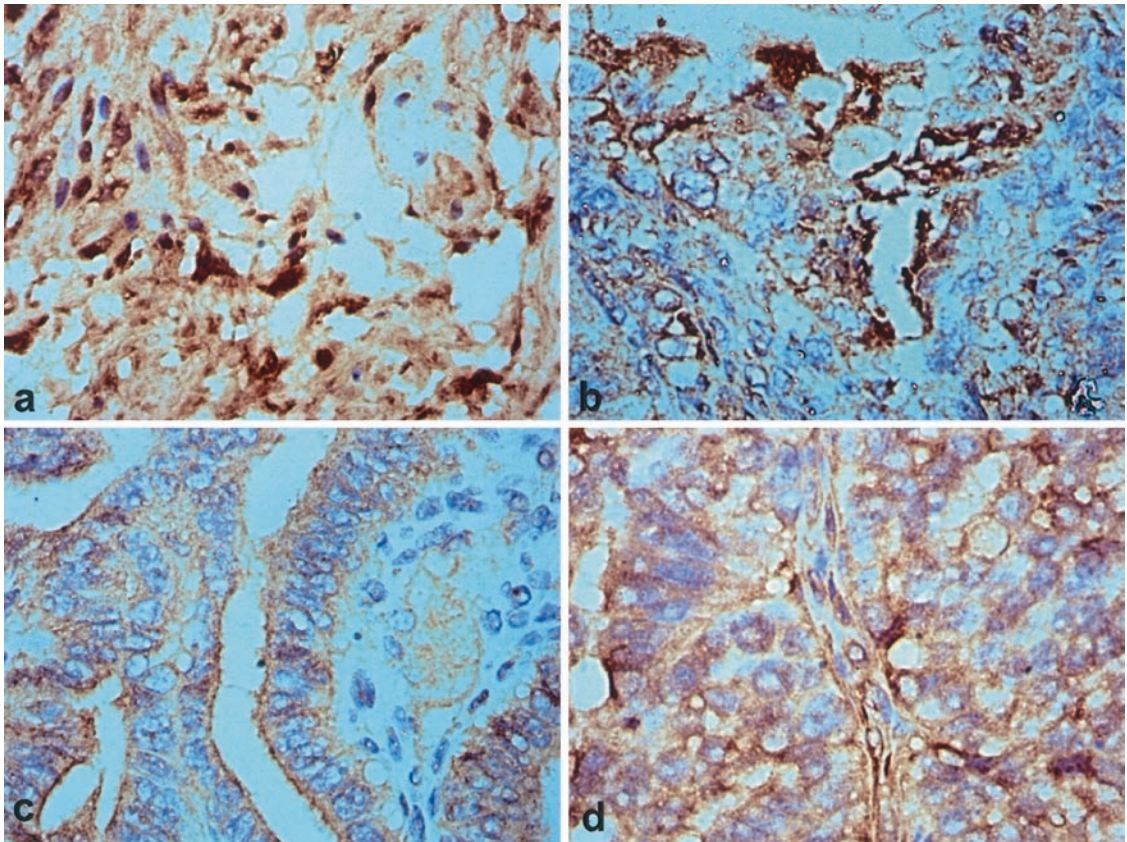


FIGURE 2.1. The expression analysis of MUC4 in ovarian tumor tissues by immunohistochemistry. Tissue sections, cut from the paraffin-embedded blocks, were immunostained with anti-MUC4 MAb after non-specific blocking with serum. Anti-MUC4 immunostaining revealed very weak or negative staining in normal ovarian epithelial cells. The expression of MUC4 in four major histological types of epithelial ovarian tumors is illustrated: (a) Serous; (b) mucinous; (c) endometrioid; (d) clear cell. Original magnifications – 400×

could not determine any significant correlation. These observations suggest an important role of MUC4 in the pathogenesis of ovarian cancer. Furthermore, we found that MUC4 plays a major role in ovarian cancer cell motility, in part, by altering actin-arrangement and potentiating HER2 downstream signaling in human ovarian cancer cells (Ponnusamy et al. 2008).

METHODOLOGY FOR MUCIN4 IMMUNOHISTOCHEMISTRY

Immunohistochemistry is a research technique employed to localize the antigens in-situ in tissue sections by using specific reactive antibodies (Hayat 2004–2006). Because immunohistochemistry involves specific antigen–antibody reaction, it has an apparent advantage in diagnosis by detecting specific marker(s) such as mucins in cancer. Therefore, immunohistochemistry has become crucial and is being widely used in many medical research laboratories as well as clinical diagnostics. Tissue preparation is the cornerstone of immunohistochemistry. To ensure the preservation of tissue architecture and cell morphology, prompt and adequate fixation is essential. However, inappropriate or prolonged fixation may significantly diminish the antibody binding capability. Nonetheless, the development of antigen retrieval techniques has greatly enhanced the use of formalin as routine fixative for immunohistochemistry (Hayat 2002).

Tissue Sectioning

For immunohistochemistry, the first step is the tissue preparation and sectioning. After excision, tissue sample can either be frozen in liquid nitrogen or preserved in

formalin (10%). Formalin-fixed tissues are later embedded in paraffin for sectioning. Paraffin sections (4–5 μ m thick) are prepared by microtome and mounted on the slides. Since its introduction, paraffin has remained the most widely used embedding medium for diagnostic histopathology in routine histological laboratories. Paraffin sections produce satisfactory results for the demonstration of the majority of tissue antigens with the use of antigen retrieval techniques. Frozen sections are first cut into small pieces and embedded in OCT compound (Sakura Fine Technical Co., Tokyo, Japan) for cryosectioning. Sections (4–5 μ m thick) are cut on a LEICA CM 1850 cryostat and mounted on super-frost positively-charged glass slides.

Immunolabeling

MUC4 cell surface antigens survive routine fixation and paraffin-embedding. The paraffin-embedded sections are first deparaffinized using EZ-De Wax (Bio Genex, San Ramon, CA) for 30 min and hydrated using graded alcohols (95%, 70%, 50%, and 30%) for 5 min each. Heat induced antigen retrieval is performed in citrate buffer (pH-6.0) by heating slides in a microwave oven at 700 W for 15 min. The frozen sections do not need antigen-retrieval but need to be fixed before further processing. Sections are washed thrice with phosphate buffered saline (PBS), fixed in chilled methanol and kept at -20°C for at least 10 min prior to immunolabeling. Sections (from both frozen and paraffin-embedded tissues) are washed three times with phosphate buffer saline and incubated with vectastain normal horse serum (vector ABC kit, Vector laboratories, Burlingame, CA) for 30 min at room temperature to

block non-specific binding. Endogenous peroxidase activity is quenched by incubating sections in 3% H_2O_2 in PBS for 20 min. Sections are then incubated with 1:2000 dilution of anti-MUC4 mouse mono-clonal antibody (8G7) for 60 min at room temperature and washed with PBS-T containing 0.05% Tween 20 (3×5 min). To confirm the specificity of the IHC staining, one set of the slides is incubated with PBS without any primary antibody (no antibody control). Slides are washed 3–5 min with PBS containing 0.05% Tween-20 (PBS-T), then the sections are incubated with biotinylated-secondary antibody for 30 min, and then slides are washed again for 3–5 min with PBS-T before incubation with ABC solution (Vector Laboratories) at room temperature. The reaction color is developed by treating the tissue sections with 3,3-diaminobenzidine (DAB) substrate (DAB substrate kit, Vector Laboratories). A reddish-brown precipitate indicates positive immunoreactivity. The slides are washed with water, counter-stained with hematoxylin, dehydrated with alcohol grades (30%, 50%, 70%, and 95% for 5 min each) and mounted with Vectamount permanent mounting media (Vector Laboratories).

Assessment of MUCIN4 Staining

Stained sections are observed under an optical microscope. The intensity of immunoreactivity is graded on a 0 to 3 scale (0 for no staining, + 1 for weak immunoreactivity, + 2 for moderate immunoreactivity, and + 3 for strong immunoreactivity). The percentage of cells that showed positive immunoreactivity within the normal epithelial/cancerous region of the section was scored as follows: 1 for 0–25%; 2 for 26–50%; 3 for 51–75%; and 4 for 76–100%. The values of the staining intensity and the percent of immu-

noreactive cells were multiplied to obtain a composite score ranging from 0 to 12. The obtained values were subjected to a suitable statistical analysis to determine the clinical relevance.

In conclusion, an aberrant expression of MUC4 in multiple malignancies and its association with the disease progression is now well established. These observations can be of immediate significance for diagnostic and/or disease management purposes and for developing MUC4-based effective therapies after precise examination of its mechanism of action. MUC4 staining has successfully been demonstrated in both formalin-fixed paraffin-embedded and frozen tissue sections of ovarian cancer using a tandem-repeat peptide-specific monoclonal antibody (Chauhan et al. 2006). This monoclonal antibody (from clone 8G7), generated in our laboratory has been well characterized and used effectively in previous studies for similar applications (Swartz et al. 2002; Park et al. 2003; Jhala et al. 2006). Considering the heterogeneous nature of disease and multiple disease subtypes, we propose that immunostaining for other ovarian cancer-associated antigens (such as MUC16) in combination with MUC4 should be evaluated to enhance the levels of sensitivity and specificity.

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3

Distinguishing Benign from Malignant Complex Adnexal Masses in Ovarian Cancer: Two-Dimensional Power-Doppler Imaging

Juan Luis Alcázar

INTRODUCTION

Differentiating benign from malignant adnexal masses represents one of the most challenging problems in gynecological practice. It has been estimated that 5–10% of US women with a suspected adnexal mass will undergo surgery, but only 13–21% of these patients will have a mass that proves to be malignant (NIH Consensus Conference 1995). Accurate surgical staging and cytoreductive surgeries have proved to be among the main prognostic factors in ovarian cancer (Farias-Eisner et al. 1994). For this reason patients with questionable adnexal masses should be referred for primary surgery to specialized centers for gynecologic oncology with experienced surgeons and adequate resources. On the other hand, benign tumors may be treated by minimally invasive surgery (Guerriero et al. 2005) or expectant management may even be an option (Alcázar et al. 2005a), and it is well known that most functional ovarian cysts will resolve spontaneously (Alcázar et al. 1997a,b). Therefore, accurate diagnosis is essential in order to establish the optimal management for these patients.

Clinical impression, serum tumoral markers, and ultrasound are the most employed diagnostic methods for differentiating benign from malignant adnexal masses in current practice (Curtin 1994). Clinical impression has a limited value, especially in small tumors and obese patients (Padilla et al. 2000). CA-125 is the most widely used serum marker for discriminating ovarian tumors. However, this serum marker is elevated in only about half of patients with early stage ovarian cancer, and its false-positive rate is considerably high in premenopausal women (Gadducci et al. 1992).

Currently, ultrasound is the diagnostic tool which is the most commonly used in the clinical setting. Ultrasound correlates images with gross pathologic features of ovarian tumors. This correlation has been found to be high, especially when transvaginal ultrasound is employed. B-mode gray-scale ultrasound has been shown to have an acceptable sensitivity, ~ 90%, either by subjective examiner impression or applying scoring systems (Timmerman et al. 1999; Alcázar et al. 2003). There are several sonographic features, such as the presence of thick wall, thick septations, papillary projections, solid nodules, and ascites associated

with a higher probability of malignancy. Multivariate analyses have demonstrated that the most predictive features for malignancy are papillary projections and solid nodules (Alcázar et al. 2001a; Schelling et al. 2000). However, the false-positive rate is ~ 25% because many benign tumors may exhibit questionable or even suspicious appearance (Timmerman et al. 1999).

Pulsed and color Doppler allow the assessment of tumor vascularization. This technique was introduced in an attempt to improve the diagnostic performance of gray-scale ultrasound. Although initial studies were encouraging (Kawai et al. 1992; Alcázar et al. 1996), subsequent studies challenged these results, showing a great overlap of pulsed Doppler indexes between benign and malignant tumors, making this technique non-reproducible and clinically unreliable (Tekay and Jouppila 1996). Multivariate analyses showed that blood flow location within the tumor was the most predictive parameter for distinguishing benign from malignant ovarian tumors using color Doppler (Alcázar et al. 2001a; Schelling et al. 2000). Notwithstanding, a meta-analysis showed that the addition of color Doppler to gray-scale ultrasound would increase the specificity of this technique (Kinkel et al. 2000). The problem centers on the integration of both examinations to yield reproducible and clinically relevant results.

A decade ago, a variation of conventional color Doppler imaging (CDI) termed power-Doppler (2D-PD), was introduced in clinical practice. This technique is based on amplitude shift rather than on frequency shift. It has some advantages, such as higher sensitivity for flow detection, over CDI (Rubin et al. 1994).

In the present chapter we will present our experience on the use of 2D-PD for

distinguishing benign from malignant complex masses.

PATIENTS AND METHODS

From January 2002 to December 2005, 409 women diagnosed as having an adnexal mass were evaluated and treated at our institution. Patients' mean age was 43 years, ranging from 14 to 84 years. Two hundred and ninety-four (72%) were premenopausal and 115 (28%) were postmenopausal. Menopausal status was defined as > 1 year of absence of menses in patients > 45 years old. Hysterectomized patients > 50 years old were considered as postmenopausal.

Two hundred and ninety-eight (73%) were asymptomatic, whereas 111 presented with some complaints such as abdominal or pelvic pain ($n = 59$), menstrual disorders ($n = 13$) or abdominal swelling ($n = 39$). Forty-seven (11.5%) women had bilateral masses.

All women underwent physical examination, serum CA-125 level determination, and transvaginal ultrasound as diagnostic work-up.

Staff specialists in obstetrics and gynecology, three of whom specialized in gynecology oncology, performed physical examination in all cases. Findings were stated as "inconclusive", when no reliable information could be obtained, "non-suspicious", in the presence of a < 8 cm maximum diameter adnexal mass, mobile at examination, of cystic or solid consistency but regular contours and no evidence of ascites, or "suspicious", in the presence of at least one of the following: fixed and/or irregular adnexal mass regardless the size, a size > 8 cm, evidence of ascites.

In all cases, on the same day of physical and ultrasound examination, blood samples

were collected to measure CA-125 plasma concentration. CA-125 measurements were performed using an enzyme-immunoassay with a monoclonal antibody (Cobas-Core CA-125 II, Laboratories Roche, Basel, Switzerland). The sensitivity was < UI/mL. The intra- inter-assay coefficients of variations were < 5.3% and < 7.5%, respectively. A CA-125 level ≥ 35 UI/mL was considered as suspicious of malignancy. Examiners and sonographer were unaware of CA-125 results.

Transvaginal power-Doppler ultrasound was performed using a Voluson 730 (GE Medical Systems, Milwaukee, USA) with a 5–7.5 MHz endovaginal probe and color, power and pulsed Doppler as well as 3D ultrasound capabilities. Transabdominal ultrasound (3.5–5 MHz) was also performed in large tumors. Ultrasound examination was performed in all cases by the author using a predetermined scanning protocol. Initially a thorough gray-scale morphologic evaluation was performed. First, tumor

volume was calculated according to the prolate ellipsoid formula ($A \times B \times C \times 0.5233$, expressed in cm^3). Morphological evaluation was performed analyzing the following parameters: bilaterality, wall thickness (thin < 3 mm, thick ≥ 3 mm), septations (not present, thin < 3 mm, thick ≥ 3 mm), papillary projections (not present, thin < 3 mm length, thick ≥ 3 mm length), solid areas (not present, presence of any solid area $\geq 1 \times 1$ cm in internal wall surface or septum) and echogenicity (cystic-anechoic, homogeneous content, heterogeneous content or solid). The presence of ascites or free fluid in the pouch of Douglas ($> 25 \text{ cm}^3$) and acoustic shadowing were also recorded.

On B-mode ultrasonography a complex mass was defined in the presence of thick papillary projections, solid areas or mostly solid echogenicity (Figure 3.1). Masses in which the echo features were highly characteristic of a given pathology such as endometrioma (Alcázar et al. 1997b), mature



FIGURE 3.1. Transvaginal ultrasound of a complex adnexal mass. This mass shows mostly solid appearance on B-mode ultrasound

teratoma (Caspi et al. 1996), hemorrhagic cyst (Okai et al. 1994), simple cyst (Castillo et al. 2004), hydrosalpinx (Guerriero et al. 2000) and cystadenofibroma (Alcázar et al. 2001b) were considered as benign. Any multiloculated or uniloculated complex or solid mass which shows echo texture not suggestive of benign histology was categorized as questionable.

After B-mode evaluation was done, 2D Power-Doppler gate was activated to assess tumor vascularization. Power Doppler settings were set to achieve maximum sensitivity to detect low velocity flow without noise (frequency: 5 MHz, Power Doppler gain: 0.8 (range: 1–30), dynamic range: 20–40 dB, edge: 1, persistence: 2, color map: 1, gate: 2, filter: 3, PRF: 0.6 kHz). If blood flow was detected, it was stated as “peripheral” (color signals in tumor wall or periphery of a solid tumor) or “central” (blood flow detected in septa, papillary projections, solid areas or central part of a solid tumor) (Figure 3.2). Pulsed Doppler

was used to interrogate color spots identified to obtain a flow velocity waveform and to confirm the arterial nature of the vessel. Spectral pulsed Doppler analysis was completed, but the data were not used in this study. A malignancy was suspected when blood flow was detected within a papillary projection, solid area or central area of solid tumors.

All patients underwent surgery and definitive histological diagnosis was obtained. Tumors were classified according to WHO criteria. Primary ovarian carcinomas were surgically staged according to FIGO criteria. Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and accuracy were calculated for 2D-PD, physical examination and CA-125, and compared using the McNemar test. The Kolmogorov–Smirnov test was used to assess normal distribution of continuous data. Continuous data were compared using the one-way ANOVA or U Mann–Whitney test, according to their distribution. Categorical

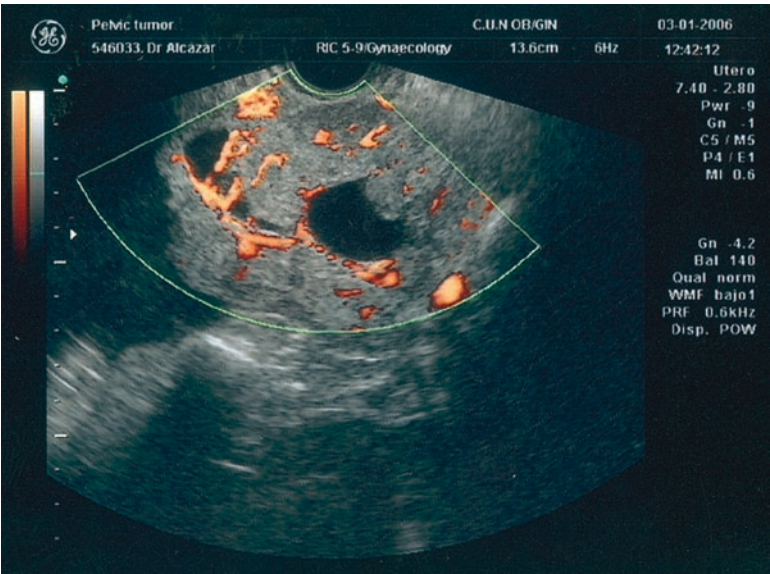


FIGURE 3.2. Transvaginal 2D-PD ultrasound of the mass as seen in Fig. 1. High vascularization within solid portions of the tumor are clearly seen, making this adnexal mass suspicious for malignancy

variables were compared using the chi-square test for dichotomous variables.

RESULTS

Three hundred and thirty-six (74%) tumors were proved to be benign and 117 (26%) were malignant. Malignant tumors were significantly more frequent in postmenopausal women (60%) than in premenopausal women (19%) ($p < 0.0001$). Physical examination was suspicious in 76% of malignant tumors and 6% of benign tumors ($p < 0.0001$). Malignant tumors were bilateral in 28% of the cases as compared with 7% of benign lesions ($p < 0.0001$). Median serum CA-125 was significantly higher in malignant tumors (250.15 UI/mL, range: 1.7–3,1494 UI/mL) as compared with benign tumors (21.9 UI/mL, range: 2.10–1,100 UI/mL) ($p < 0.0001$).

Figure 3.3 shows the results of ultrasound examination and histologic data. On B-mode

gray-scale ultrasound, 201 tumors (44%) showed a complex or suspicious appearance. In this latter group, 2-D Power Doppler showed central blood flow in 130 lesions, whereas 71 did show peripheral blood flow ($n = 45$) or no flow was detected ($n = 26$). Out of the 130 adnexal masses with complex gray-scale appearance and central blood flow, 113 were proven to be malignant and 17 were benign.

Out of the 252 tumors with non suspicious B-mode findings, 251 were benign and one malignant (one primary ovarian cancer stage IIIc – positive lymph nodes – in a multiloculated cyst without solid areas or papillary projections, with a tumor volume of 408.6 cm³).

Out of the 71 adnexal masses with complex B-mode appearance but non-suspicious 2D power Doppler findings, 68 were benign and 3 were malignant. These cases were one primary ovarian adenocarcinoma stage Ic, in a unilocular cyst with a papillary projection without detectable flow, one primary

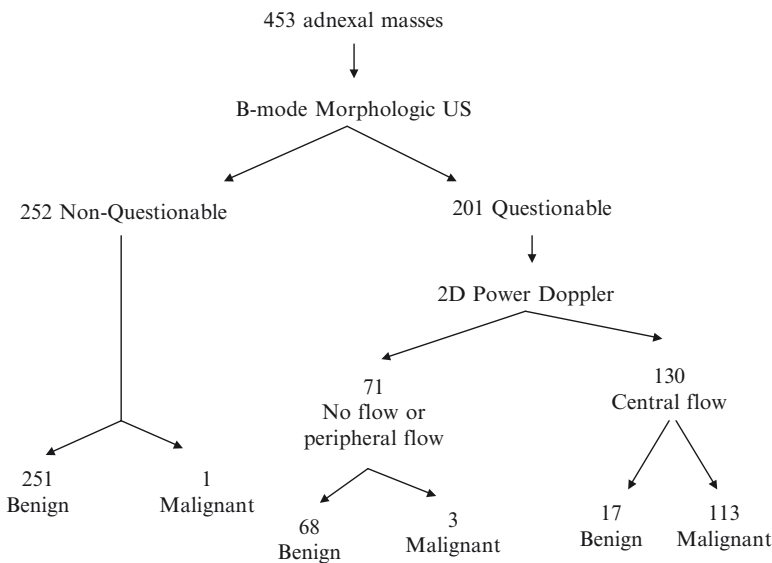


FIGURE 3.3. Chart flow diagram depicting the findings on B-mode ultrasound, 2D-PD and final histological diagnosis in this series of 453 adnexal masses

ovarian cancer stage IIIb in a solid tumor without blood flow, and a metastatic tumor to the ovary from the appendix in a solid lesion without blood flow.

Overall, sensitivity, specificity, positive predictive value, negative predictive value, and accuracy for 2D power-Doppler ultrasound was 96.6%, 94.9%, 86.9%, 98.8% and 95.4%, respectively. These figures were similar in premenopause (94.7%, 94.9%, 73.5%, 99.2% and 94.9%, respectively) and postmenopause (96.4%, 94.9%, 94.7%, 96.6% and 95.2%, respectively). 2D-PD showed a higher diagnostic performance (sensitivity 96.6%, specificity 94.9%, positive predictive value 86.9%, negative predictive value 98.8%, and accuracy 95.4%) than physical examination (sensitivity 76.1%, specificity 93.8%, positive predictive value 80.9%, negative predictive value 91.8%, and accuracy 89.2%) and CA-125 using a cut-off > 35 UI/mL (sensitivity 86.4%, specificity 68.3%, positive predictive value 61.3%, negative predictive value 89.6%, and accuracy 74.9%).

DISCUSSION

In this chapter we have presented our experience in the use of 2D Power Doppler imaging for distinguishing between benign and malignant complex adnexal masses.

To date, few studies have evaluated the role of 2D power Doppler imaging for distinguishing the nature of adnexal masses. Tailor et al. (1998) were the first to address this issue. These authors used 2D-PD in 67 women with known adnexal masses. Their aim was to determine whether examination with 2D-PD was better, in terms of sensitivity and specificity, than conventional color Doppler. They found no differences

between both methods. However, they used 2D-PD, as conventional color Doppler, to identify blood vessels within the tumor for further pulsed Doppler analysis using the pulsatility index (PI) and the time-averaged maximum velocity (TAMXV). Therefore, their criteria for discriminating between benign and malignant tumors were based ultimately on PI and TAMXV values, and the controversial value of these Doppler parameters is well known (Tekay and Jouppila 1996).

Almost simultaneously, Guerriero et al. (1998) published a paper assessing the role of 2D-PD as a secondary test in diagnosing adnexal malignancies in persistent masses. These authors proposed a different approach based on B-mode gray scale ultrasound as the first test to be used and then 2D-PD as a secondary test in the complex or questionable adnexal masses. They evaluated 240 women diagnosed as having persistent adnexal masses using this sequential approach, considering a tumor as malignant when a complex B-mode appearance and arterial blood flow was visualized in an echogenic portion of a mass. These authors found that B-mode ultrasound showed 100% sensitivity in detecting adnexal malignancies, and the addition of 2D-PD increased the specificity of B-mode ultrasound (83% to 92%). Pulsed Doppler analysis was not helpful.

However, the question that which remained to be answered was: was 2D-PD better than conventional color Doppler using this approach? Therefore, a comparative study between both techniques was undertaken in two European University Hospitals. In this study, 328 adnexal tumors were evaluated in one institution by conventional color Doppler and 328 adnexal tumors in the other institution by

2D-PD (Guerriero et al. 2001). Prevalence of ovarian cancer was similar in both centers (21.3% and 21.6%, respectively). The scanning protocol was that proposed by Guerriero et al. (1998). The specificity of both techniques was similar (95% and 93%, respectively). However, 2D-PD was more sensitive than conventional color Doppler (100% versus 87%, $p < 0.05$). This could be explained by the fact that 2D-PD is more sensitive to low-velocity blood flow. Again, a great overlap of pulsed Doppler parameters was found between benign and malignant tumors in both institutions. Subsequently, a multicenter European study on 826 complex adnexal masses confirmed these results, demonstrating that the use of 2D-PD increases the specificity of B-mode ultrasound without decreasing significantly sensitivity, both in premenopausal and postmenopausal women (Guerriero et al. 2002).

Our present data also confirmed these results. In our series, only one case of ovarian cancer was missed after B-mode examination. The addition of 2D-PD correctly identified most benign and malignant complex adnexal masses. Only three ovarian cancers were misclassified as benign after 2D-PD examination, but most benign complex adnexal tumors were correctly identified. However, in spite of these highly encouraging results some questions are still open and some factors should be kept in mind when using 2D-PD. First, one must consider reproducibility. To date, no study about reproducibility on the use of 2D-PD for discriminating benign from malignant adnexal masses has been published. The approach proposed is based on a subjective impression of the examiner, and in order to be generalized, the technique needs to be reproducible. A rough estimation of

reproducibility is similar results obtained in the different studies reported. However, some studies are being conducted which specifically address this question, and we await their results.

A study by a French group assessed the performance of a power Doppler vascularity index in adnexal masses (Marret et al. 2005). These authors developed a power Doppler vascular index, termed “PDI” using software to quantify colored pixels within a given region of interest. It is a ratio between colored pixels and gray-scale pixels. This was used in an attempt to quantify objectively the amount of color in a given area. This was completed over a selected two-dimensional image. Their results showed that this method was reproducible. However, their diagnostic performance in terms of sensitivity (100%) and specificity (97.4%) was similar to our results and to those previously published (Guerriero et al. 1998, 2001, 2002). The main disadvantage of the method proposed by Marret et al. (2005) is that it is more time consuming than the method proposed by Guerriero et al. (1998, 2001, 2002), it requires storage and off-line analysis of the data, software support, and computer analysis.

On the other hand, 2D-PD machine settings have not been standardized thus far. We recommend using those proposed in Material and Methods section, which are intended for a maximum sensitivity for low velocity blood flow, although we have to bear in mind that these settings may change from one machine to another. A third question to solve is the false negative cases in 2D-PD. Most of these cases will show no flow on 2D-PD assessment. This might be explained by the fact that some tumors may exhibit large areas of necrosis with no vessels within them. A possible

solution for this problem might be the use of contrast-enhanced power Doppler ultrasound. Fundamentals of contrast enhanced ultrasound are discussed in another chapter of this volume.

Orden et al. (2000) reported that the use of contrast-enhanced ultrasonography increased the number of vessels detected by power-Doppler both in benign and malignant tumors, but this increase was much more evident in malignancies. A subsequent study from this group analyzed the kinetics of US contrast agent in adnexal tumors and showed that after microbubble contrast agent injection, malignant and benign adnexal lesions behave differently in degree, onset, and duration of Doppler US enhancement (Orden et al. 2003). Malignant tumors exhibited an earlier onset, higher degree, and longer Doppler US enhancement. However, overall diagnostic performance was similar to that reported by non-enhanced ultrasound studies. Furthermore, reproducibility was rather low (coefficient of variation > 10% for most parameters analyzed). More recently, additional studies have confirmed these results (Marret et al. 2004; Testa et al. 2005a).

One question of paramount importance is whether this diagnostic two-step approach for discriminating benign from malignant adnexal masses could help clinicians select the best management option for a given patient. To date only one prospective study has been conducted and published. Guerriero et al. (2005) applied this approach in 453 adnexal tumors, classifying the tumors in four categories according to gray-scale and Power-Doppler ultrasound: very low risk, low risk, high risk and very high risk for malignancy. Surgical approach was selected according to the risk established. Very high-risk tumors were submitted to laparotomy, high-risk were submitted to

laparoscopy or laparotomy, and low-risk and very low-risk tumors were treated by laparoscopy. No tumor submitted to laparoscopy was proved to be malignant. All 95 malignant tumors were correctly identified and treated by laparotomy with the exception of four cases treated by laparoscopy, because they were thought to be borderline tumors.

Future perspectives are based on the use of three-dimensional Power-Doppler ultrasound (3D-PD). The introduction of 3D ultrasound has opened a new and fascinating way for performing ultrasound and also it is a formidable tool for researching. This technique overcomes some limitations of conventional 2D ultrasound, allowing a more detailed assessment of morphologic features of the object studied, with no restriction on the number and orientation of the scanning planes. Furthermore, this technique allows a novel assessment of tumor vascularity by depicting the vascular network architecture or by calculating 3D-derived vascular indexes (Alcázar 2005).

Kurjak et al. (2000) analyzed a series of 120 ovarian lesions by 3D-PD. They described the vascular network architecture using a three-dimensionally reconstructed image. They based their diagnostic criteria for malignancy suspicion on vessel architecture as depicted by 3D, such as branching pattern, vessel caliber, and presence of microaneurysms or vascular lakes. This was based on the chaos theory (Breyer and Kurjak 1995), which established that vascular architecture of a vascular network of newly formed vessels in malignant tumors is built following a chaotic distribution but not in a predetermined fashion. In this study, they found that 3D-PD was more sensitive than conventional color Doppler imaging (100% versus 90.9% respectively) with

similar specificity. A subsequent study from the same group found similar results (Kurjak et al. 2001). In this study the use of contrast agent to 3D-PD added little to diagnostic performance. However, this approach is based on the subjective impression of the examiner regarding how the vascular tree looks. We think that the reproducibility of this approach would be low or at least difficult to achieve because it is ultimately based on a subjective analysis of a 3D reconstructed image.

On the other hand, no comparison with 2D-PD was completed in these studies. We performed a prospective study comparing 2D-PD (using an identical approach to that proposed in this chapter) and 3D-PD (using the approach proposed by Kurjak et al. 2001). Two different examiners, blinded to each other, evaluated 69 complex adnexal masses, one examiner by 3D-PD and the other by 2D-PD. We did not find statistical differences between both techniques in terms of sensitivity (97.8% versus 97.8%) nor specificity (93.6% versus 89.9%). Therefore, we concluded that 3D-PD vascular tree assessment was not better than a simpler approach of blood vessel location with 2D-PD (Alcázar and Castillo 2005).

We have explored the role of 3D-PD in evaluating complex adnexal masses with detectable blood flow within papillary projections or solid areas. These lesions should be categorized as “very highly suspicious for malignancy”. However, a considerable number of benign lesions, ~ 10–15%, may have this appearance; for example, cystadenofibromas, fibromas, granulosa cell tumor, Brenner tumor and tubo-ovarian abscesses. It is virtually impossible to distinguish these benign lesions from those true malignant tumors using 2D-PD in the absence of other findings, such as ascites.

3D-PD sonography provides a new manner of assessing tumor vascularization by calculating 3D power-Doppler derived vascular indexes from those vascularized areas within the tumor. We have termed this approach 3D-PD vascular sampling (Alcázar et al. 2005b). In our study we evaluated 49 vascularized complex adnexal tumors using this approach. Forty tumors proved to be malignant and nine were benign. We found that 3D-PD derived vascular indexes in malignant tumors were significantly higher than those in benign tumors. No differences could be found in pulsed Doppler indexes.

Using a similar approach, an Italian group found similar results to ours in solid pelvic tumors (Testa et al. 2005b). An interesting fact is that this approach is an objective one and showed good intra- and inter-observer reproducibility in both studies.

In conclusion, in view of the data presented in this chapter and those reported in the literature, 2D-PD ultrasound, when used as a second step technique after B-mode evaluation of adnexal masses, has proven to be a very useful tool for discriminating benign and malignant adnexal tumors. It is a simple technique, easy to learn and perform, available in most US diagnostic laboratories and relatively cheap, compared with other imaging techniques such as CT scan, MRI and PET scan. The role of 3D-PD needs to be further evaluated in future prospective studies.

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4

Subgroups of Ovarian Carinoma: Identification Using Differential Gene Expression

Kristin L. M. Boylan, Keith M. Skubitz, and Amy P. N. Skubitz

INTRODUCTION

During the process of cellular transformation from normal to cancerous, numerous genes may become deregulated. By developing high-throughput screening techniques to simultaneously analyze most or all of the cellular transcriptome, it has become possible to generate a signature pattern of gene expression that may be correlated with clinically significant parameters. These techniques allow the quantification of thousands of different mRNA levels simultaneously in many samples. During the past decade, many high-throughput techniques have been developed for this purpose, including: gene microarray analysis, serial analysis of gene expression, high density cDNA hybridization filter, and suppression subtractive hybridization. Furthermore, additional techniques have been developed to analyze DNA copy number variations (e.g., array comparative genomic hybridization), genetic alterations (e.g., microsatellite analysis or single nucleotide polymorphism analysis), and micro RNA profiling. However, gene microarray analysis remains the technique of choice for the majority of studies in ovarian Cancer. Res.earch (Le Page et al. [2006a](#)).

This review will focus on the methodology and results of our group and others, who have reported the identification of genes differentially expressed in sets of serous ovarian carcinomas and normal ovary samples compared with sets of different normal and diseased tissues using gene microarrays. We will review the application of gene expression profiles in the classification and diagnosis of ovarian cancer. In addition, we will review recent studies that have found distinctive gene expression patterns that may be clinically useful in the near future to predict response to chemotherapy and/or survival.

OVARIAN CANCER HETEROGENEITY

Ovarian cancer is an important cause of morbidity and mortality, accounting for ~ 3% of all cancers in women in the United States. The most common ovarian malignancies are surface epithelial tumors (carcinomas), with serous carcinomas being the most common subtype. Although the designation “surface epithelial” tumors of the ovary imply an origin from the specialized epithelial cells

on the ovarian surface, the cell of origin of these tumors is controversial.

Ovarian cancer comprises a heterogeneous group of tumors with different biological behavior. Some of this heterogeneity can be identified by light microscopy, including the general classification into different surface epithelial tumor types: serous, endometrioid, mucinous, and clear cell type. Within these categories, the tumors are further subclassified into benign; borderline or low malignant potential, i.e., tumors that usually follow a benign course (BL-OVCA); or invasive high-grade tumors (H-OVCA) that have a much worse prognosis. However, even the group of ovarian cancer classified as high grade exhibits important clinical heterogeneity. This heterogeneity is manifested clinically by different rates of growth, metastasis, response to chemotherapy, and survival. A better understanding of this heterogeneity is important to study therapy against this disease. Because serous cancers are the most prevalent type of ovarian cancer, this review will focus on the heterogeneity of serous ovarian cancers.

SELECTION OF SAMPLES FOR GENE MICROARRAY ANALYSIS

A wide variety of ovarian cancer samples have been analyzed using gene microarrays. Solid tumors resected from the ovary of patients with ovarian cancer are the most common type of tissue sample used. In addition, ovarian cancer tissue samples may also be resected from abdominal organs or distant sites from patients with Stage III or IV disease. These samples are used to compare the gene expression profile of the tumor at secondary sites to that of the primary tumor within the ovary. Alternatively,

ovarian cancer cells may be isolated from patients' ascites fluid or pleural fluid, because these cells represent a "purer" population of ovarian cancer cells. Gene expression studies using ovarian carcinoma cells that have spread to the pleural cavity may also provide insight into a subgroup of cancer cells that express a highly aggressive phenotype (Schaner et al. 2005).

Although controversial, it is widely accepted that epithelial ovarian carcinomas arise from the thin layer of epithelial cells surrounding the ovary. Not surprisingly, it is difficult to obtain sufficient quantities of normal ovarian surface epithelial (NOSE) cells for further analysis as a "normal" control in gene expression experiments. Some studies have used portions of "normal" ovaries as the control tissue. These ovaries may have been obtained from women who had a bilateral salpingo-oophorectomy due to abnormal bleeding, an unrelated disease (e.g., endometriosis, fibroids, or another type of cancer), or even a family history of ovarian cancer. In these cases, the surface epithelial cells represent a very low percentage of the total normal ovary cells that are included in the microarray analysis. Some studies have used benign ovarian epithelial tumors (Ismail et al. 2000) as the "normal" control tissue for NOSE cells. Other groups have circumvented this problem by enriching the NOSE cells by creating short-term NOSE cell cultures (Hough et al. 2000; Ismail et al. 2000). Because primary cells are frequently difficult to maintain in culture for extended periods of time and are slow to proliferate, other groups have immortalized NOSE cells with SV40 large T-antigen (Jazaeri et al. 2002) or telomerase (Zorn et al. 2003).

The use of cell lines in gene microarray experiments has its advantages and disadvantages. One advantage of using cell lines

is that they are a pure population of cells, not a mixture of cancer cells, endothelial cells, and fibroblasts, as is the case in intact tissues. Secondly, the cells are healthy, in that there are no necrotic or fibrotic areas to avoid, as is the case in some tumors. A third advantage is that cell lines are easy to obtain (commercially or from collaborators) and they can be grown in unlimited numbers for a large yield of RNA for gene expression experiments. However, when using cells that have been grown in tissue culture for extended periods of time, it is important to keep in mind that the cells may experience phenotypic drift. Over time, the expression levels of some genes may increase (or decrease), and may not reflect the original expression levels, thus altering gene expression results. For example, Santin et al. (2004) showed that genes over-expressed in established ovarian serous papillary carcinoma (OSPC) cell lines had little correlation with those over-expressed in short term cultures of primary OSPC. In contrast, Bignotti et al. (2006) compared the gene expression profiles of paired snap frozen tumor tissues and primary tumor cells grown in cultures for 2 weeks and found only 0.35% of the genes were differentially expressed between the tumor tissue and the short-term cultures. Many of the differentially expressed genes were genes whose expression is associated with nonepithelial (i.e., stromal cells or infiltrating lymphocytes) cells. In the case of immortalized cells, it is possible that the transformation process itself may alter the gene expression profile. Zorn et al. (2003) have recently shown that the development and maintenance of NOSE cell lines alter the gene expression pattern when compared to whole normal ovaries or brushings taken from the surface of fresh normal ovaries.

CONTAMINATION OF GENE EXPRESSION PROFILES BY OTHER CELLS IN TISSUES

When solid tissues are used in gene microarray expression analysis studies, the relative contributions of tumor cells, stromal or infiltrating immune cells, or other elements to the gene expression data are not always clear. For example, in one of our studies, we noted over-expression of immunoglobulin genes in two subgroups of H-OVCA (Skubitz et al. 2006). We also found immunoglobulin genes and interferon-inducible genes overexpressed in H-OVCA compared with BL-OVCA. Gilks et al. (2005) also observed an increased expression of immunoglobulin genes in serous ovarian cancer compared with borderline tumors. In other ovarian cancer classification studies, this was attributed to lymphocytes infiltrating the tumor (Schaner et al. 2003; Gilks et al. 2005). The presence of intratumoral T-cells has been reported to correlate with improved clinical outcome in advanced ovarian carcinoma. Zhang et al. (2003) found the presence of intratumoral T-cells was associated with increased expression of interferon-gamma, interleukin-2, and lymphocyte-attracting cytokines in the tumors. The lack of knowledge of the cellular origin of the gene expression detected does not diminish the potential utility of whole tissue, such as biopsy specimens, in gene expression analysis.

In order to obtain a more homogeneous population of cells for gene microarray experiments, new techniques have been developed to isolate the cells of interest from tissue blocks by microdissection or laser capture microdissection. For example, ovarian cancer cells can be isolated from a solid tumor, while NOSE cells can be isolated from the surface of a normal ovary. If the yield of RNA from

the isolated cells is too low, then the RNA may need to be amplified.

NUMBER OF SAMPLES TO ANALYZE FOR GENE PROFILING

When performing gene microarray experiments, not only does the quality of tissue samples matter, but so does the number of samples used. If too few samples are analyzed, it is not possible to get a true profile of that type of tumor. Thus, by analyzing a large number of tissues, a more accurate picture of ovarian carcinoma gene profiles can be obtained. Furthermore, if one wants to define a gene as “specific” to a particular type of cancer (e.g., for use as a biomarker), then it is also important to analyze samples (normal, diseased, and cancerous) from other types of tissues. For example, in one of our recent studies (Skubitz et al. 2006), we performed gene microarray analysis with tissues from 29 ovarian cancer samples (21 H-OVCA and 8 BL-OVCA) and 512 samples from 17 different types of nonmalignant tissues. By using such a large number of tissues, it was possible to identify genes that were differentially expressed between the ovarian cancer, normal ovary, and other types of tissues. More importantly, it also allowed us to identify heterogeneity within the H-OVCA sample set by Eisen clustering and principle component analysis (PCA).

TISSUE PROCESSING PROTOCOLS

All tissue samples should be rapidly processed from the operating room following a standard operating procedure for procurement.

It is very important that tissue samples undergo stringent quality control measures in order to preserve the integrity of the RNA before use in gene microarray experiments. Initially, tumor and normal samples are identified by the pathologist, who will retain a sufficient amount of tissue in order to make a diagnosis. The pathologist will then dissect the tumor or tissue/organ of interest, and the tissue should be snap frozen in liquid nitrogen within 30 min of resection from the patient. Tissue sections of each sample may be prepared before freezing, and examined by light microscopy after H&E staining to confirm the pathologic nature of the sample. Diagnoses should be determined by the surgical pathologist at the time of surgery, and confirmed by a second pathologist experienced in the field of ovarian cancer.

IMPORTANCE OF PATHOLOGICAL QUALITY CONTROL

Slides of the ovarian tumors from which samples have been obtained should be reviewed by a single pathologist at one sitting in a blinded manner. All slides available from primary tumors and implants/metastases should be reviewed, without knowledge of the original diagnosis or the clinical characteristics of the patients. Based on this review, tumors should be assigned a histologic type (serous, endometrioid, mucinous, or clear cell) and, when appropriate, a secondary pattern. Classification into borderline tumors and carcinomas should be made based on the identification of destructive stromal invasion (but not microinvasion) within the primary ovarian tumor.

In our studies (Skubitz et al. 2006), all tumors were assigned a grade using three different grading systems currently in use: the universal grading system for ovarian epithelial carcinoma, the FIGO grading system, and the MD Anderson system. In the universal grading system for ovarian epithelial carcinoma, tumors are assigned scores based on the predominant architecture, nuclear grade, and mitotic count. In the FIGO system, grade 1 is used for tumors with < 5% solid tumor growth pattern, grade 2 for 5–50% solid growth pattern, and grade 3 for > 50% solid growth pattern. The two-tiered MD Anderson grade is assigned as low or high based primarily on nuclear grade.

CLINICAL CORRELATIONS

Many of the gene microarray studies published recently have a “translational” appeal, in that they not only have multiple sets of high quality tissue (one set for testing and a second set for validation), but also have clinical data for each patient. The best clinical data sets are comprised of demographic information (age, sex, race, ethnicity, height, weight, etc.), health history (diseases, etc.), smoking status, alcohol use, family history (cancer, other diseases), chemotherapy, radiation therapy, response to therapy, follow-up data, and survival. In studies of ovarian cancer, it is also desirable to document the number of pregnancies, number of live births, date of last menstrual cycle, menopausal status, and use of oral contraceptives. Thus, once the gene expression data have been generated for the patients’ tissues, it is possible to statistically correlate the data with the clinical parameters.

GENE MICROARRAY PLATFORMS

A variety of microarray platforms are currently commercially available from companies such as Affymetrix, Agilent Technologies, the GE Healthcare division of General Electric, Research Genetics, Clontech, and Swegen. In addition, several institutions, such as the National Cancer Institute and Stanford University, have developed their own microarrays. The vast majority of gene microarray studies in the field of ovarian cancer have used Affymetrix GeneChips®, in particular: Affy U95, U95A, U95Av2, HuFL, U133, U133A, and U133A Plus 2.0. The GeneChip® U_133 is the current series of microarrays made by Affymetrix; it contains oligodeoxynucleotides corresponding to ~ 40,000 genes and expression sequence tags (ESTs). The following methodology for RNA isolation and preparation is focused on the techniques used for this product.

RNA ISOLATION FOR GENERATING GENE EXPRESSION DATA

When tissues have been properly processed, a standard operating procedure should be followed for the isolation of RNA and subsequent gene microarray analysis, as we have previously described (Skubitz et al. 2006). Briefly, RNA is isolated by homogenization of frozen tissue in extraction buffer under RNase-free conditions. Kits are commercially available from many manufacturers, with easy to follow protocols. RNA quantity is determined spectrophotometrically, and the quality assessed on agarose gels, or

with an Agilent Bioanalyzer to confirm the presence of non-degraded RNA. Tissue samples should not be used if the RNA yield is low or RNA degradation is evident. When using Affymetrix GeneChips®, biotinylated cRNAs are prepared using standard Affymetrix protocols. RNA is converted to first strand cDNA followed by second strand synthesis. Double-stranded cDNA is used as the template for in vitro transcription using biotinylated ribonucleotides to generate biotin-labeled cRNA. Biotinylated cRNA is fragmented for target preparation and then hybridized on the Affymetrix GeneChip®. Following hybridization, the microarrays are washed and stained using an automated fluidics system. The microarrays are then digitally scanned and images of the average probe intensities are visually monitored for any irregularities in the microarrays. Samples should be rehybridized when images appear flawed in any way. The integrity of the RNA sample can be further monitored by examining the relative expression of a probe from the 3' end of beta-actin compared with the expression of a probe from the 5' end of the same gene. In addition, internal controls can be added to each Affymetrix microarray, and samples with "flawed" data should not be analyzed.

ANALYSIS OF GENE MICROARRAY DATA

A variety of computational methods and algorithms are available to analyze gene microarray data. In the common fold-change analysis, the geometric means of the expression intensities of the relevant gene fragments are computed, and the ratios reported as the fold change (up or down).

Confidence intervals and p-values on the fold change are calculated using a two-sided Welch modified two-sample t-test. A variety of software is both freely and commercially available to analyze gene microarray data, including Principle Component Analysis (PCA) and Eisen clustering software. Also for "supervised" analysis of microarray data, Stanford University Labs has developed Significance Analysis of Microarrays (SAM) (<http://www-stat.stanford.edu/~tibs/SAM/>) and Prediction Analysis of Microarrays (PAM) (<http://www.stat.stanford.edu/~tibs/PAM/Rdist/index.html>). Gene expression patterns may also be analyzed by multidimensional scaling or pattern identification, which would include heuristic algorithms or neural networks. The validity of the data can be enhanced by including multiple regions of each gene as targets on the array, improving image acquisition via analyzing scanner records, and enlisting the aid of a biostatistician for data processing (e.g., normalization, background subtraction, and standardization).

NEED FOR SECONDARY VALIDATION OF DATA

Once gene microarray data have been generated and analyzed, it is very important to validate the findings. First, the experiments should be repeated using replicates of each RNA sample and a completely different set of RNA samples in order to ensure reproducibility and eliminate "noise" in the data. A key step in determining the diagnostic potential of gene expression profiling is to compare the gene expression of a variety of tumors derived from many different organs. Due to the high

costs incurred in performing gene microarray experiments on some platforms, it may become prohibitively expensive to replicate and validate all of the data using large numbers of samples from a wide variety of tissues. However, the RNA expression levels should be validated by semi-quantitative reverse transcription PCR (RT-PCR), real time RT-PCR, Northern blots, ribonuclease protection assays, or in situ hybridization using tissues or cell lines. In order to determine whether the RNA expression levels correlate with the protein expression levels, further validation experiments can be conducted, such as immunohistochemistry, immunocytochemistry, Western immunoblotting, immunoprecipitation, flow cytometry, or mass spectrometry. For example, in one of our studies, we used > 300 other tissues in order to determine the specificity of the upregulated genes to ovarian carcinoma, and we verified our findings by immunohistochemistry using 45 ovarian tissues (Hibbs et al. 2004). Others have rapidly validated the expression of their proteins of interest by immunohistochemically staining tissue microarrays, a high throughput platform comprised of tens-to-hundreds of formalin-fixed paraffin embedded tissue biopsies on one slide (Le Page et al. 2006a).

GOALS FOR GENE MICROARRAY ANALYSIS

Since 1999, large-scale gene expression analyses have been performed to identify differentially expressed genes in ovarian carcinoma (Hibbs et al. 2004; Le Page et al. 2004). In the remainder of this review, we will focus on several of the specific aims that researchers have set out to accomplish by performing gene microarray analysis.

One aim has been to identify specific and sensitive tumor markers for the diagnosis of early-stage ovarian cancer as well as for the recurrence of the cancer. Researchers envision utilizing some of these novel tumor markers as targets for improved therapy and/or treatment of ovarian cancer. Some gene microarray studies have compared borderline tumors to highly invasive tumors, while other studies have focused on examining tumor grade and metastatic potential. Many of these studies have found subgroups of patients and heterogeneity within the sample sets; providing quantification of the observation that not all patients with serous ovarian cancer are the same. Furthermore, researchers have correlated gene expression data to the ovarian cancer patients' response to chemotherapy and survival, as well as the effect of optimal vs. suboptimal surgical cytoreduction. The goal of these latter studies is to develop a subset of genes that is shared by ovarian cancer patients in each category (e.g., responders to therapy, long-term survivors, etc.) which can be used to predict response to therapy, recurrence of cancer, and/or survival. Finally, a long-term goal shared by many of these studies is to develop an individualized treatment regimen for each patient with ovarian cancer, based on gene microarray profiling.

GENE EXPRESSION ANALYSIS USED TO DETERMINE OVARIAN CANCER SUBGROUPS

In this first section, we will review studies that have used gene microarray expression analysis to identify subgroups of ovarian cancer. For example, in a recent study we sought to identify subgroups of papillary

serous ovarian cancer based on gene expression profiles (Skubitz et al. 2006). In this analysis, we quantified gene expression levels in H-OVCA, BL-OVCA, and normal tissues using the Affymetrix GeneChip® U_133 microarray. In a set of 21 ovarian cancer samples originally classified as H-OVCA, two major subsets were identified on the basis of gene expression using fold-change analysis, PCA, and Eisen clustering. To gain insight into the biologic differences that might be reflected by the subsets, we tested a variety of functional gene sets, including 14 different metabolic pathways and a gene set that we had previously published as being able to discern two distinct subgroups of conventional renal cell carcinoma. The same two major subgroups of H-OVCA samples were routinely observed, H-OVCA-A and H-OVCA-B. In the PCA, five H-OVCA-A samples routinely clustered with eight BL-OVCA samples, and may represent less aggressive disease. These two H-OVCA subsets were then analyzed together and separately to search for genes uniquely expressed in each set compared with 512 individual tissue samples from 17 sets of non-malignant tissues. Our study demonstrated that gene expression patterns can be used to identify subsets of H-OVCA directly, without searching for differences based on clinical correlates. This approach also allowed for the identification of several potential subsets that could be obscured by searching for patterns that discriminate between two predefined groups determined by a particular clinical outcome. Such gene expression profiles may be useful in subclassifying ovarian cancer, characterizing ovarian cancer, and identifying potential targets for therapy.

Three additional studies revealed the presence of subgroups among ovarian tumors

based on differential gene expression. Welsh et al. (2001) compared 27 serous papillary tumors and ovarian cancer cell lines, to normal ovaries and other normal tissues using the Affymetrix HuFL gene chip. Using unsupervised hierarchical clustering, they found evidence for tumor subgroups. One subgroup of ovarian cancer tumors clustered with the normal ovary samples. These tumors were mostly well differentiated tumors, and this subgroup of normal and tumor samples was characterized by high expression of a group of ribosomal genes, suggesting a high metabolic rate. Another subgroup of tumors clustered with the ovarian cancer cell lines, and was characterized by the expression of genes associated with cell cycle regulation and cell proliferation (CDC28 protein kinases 1 and 2, CDC25B and CDC20). These tumors were poorly differentiated, consistent with a more aggressive phenotype.

In a similar study, Matei et al. (2002) identified ovarian cancer subgroups in a comparison of primary cultures of normal ovarian epithelial cells and serous epithelial ovarian cancer. Again, one subgroup of tumors clustered with the normal ovary tissues, expressing genes that may be important for normal ovary differentiation. Other tumor subgroups were associated with high expression of genes involved in cell proliferation (CDC2, cyclin A2, cyclin B1, CDC28 protein kinase, and CDC20), extracellular matrix proteins and cell adhesion molecules (biglycan, integrin β 1 like protein, and lumican) or proteasome-related proteins.

Bild et al. (2006) analyzed the gene expression data of > 100 advanced stage (III or IV) ovarian cancer tissues using the Affymetrix Hu133A GeneChip array. Using a gene signature for oncogenic pathways

(comprised of Myc, Ras, E2F3, Src, and beta-catenin) that was derived from breast cancer samples, they found that the ovarian cancer samples clustered into two major subgroups. One subgroup exhibited elevated co-deregulation of beta-catenin and Src; these patients had a very poor survival compared to the other subgroup that exhibited diminished coderegulation of beta-catenin and Src. They concluded that, “the ability to predict the deregulation of various oncogenic pathways through gene expression analysis offers an opportunity to identify new therapeutic options for patients by providing a potential basis for guiding the use of pathway specific drugs. The major value of this approach may be the capacity to direct combinations of therapies – multiple drugs that target multiple pathways – based on information that specifies the activation state of the pathways.”

GENE EXPRESSION ANALYSIS USED TO COMPARE DIFFERENT STAGES OR GRADES OF OVARIAN CANCER

In this section, we will review studies that have used gene expression microarray analysis to compare different stages or grades of ovarian cancer. In an early study, Tapper et al. (2001) examined differences in gene expression between advanced and poorly differentiated and localized, highly differentiated serous adenocarcinomas, and benign adenoma. Genes that were overexpressed in the advanced tumors compared to the localized tumors included genes involved in cell adhesion and invasion (collagens COL1, COL3A1, and COL6A1;

fibronectin, biglycan, and semaphorin1), oncogenes and tumor suppressors (MET, CFOS, STAT1), and developmental regulators (NOTCH1, JAG2, and SMO). Genes that were expressed more in the well differentiated tumors (grade I) included genes that play a role in apoptosis (BCL2, TRAP3, ICE, BFL1, and caspases 7 and 8), cytokines and growth factors (IL1B, CD27 ligand) and DNA damage response and repair genes (ATM, HMLH1).

Warrenfeltz et al. (2004) compared gene expression between benign adenomas, borderline tumors of low malignant potential, and invasive adenocarcinomas using the Affymetrix U95A GeneChip®. Unsupervised hierarchical clustering showed segregation of the samples into groups according to their histopathological designation (benign, borderline or invasive), with one exception. The top ten genes most highly correlated with each group (i.e., showed high expression in that group and not the other groups) were identified. Interestingly, expression of many of the genes that correlated with either the benign or invasive tumors was at an intermediate level for the borderline tumors. When the gene expression data for the different groups of tumors were compared by ANOVA, 163 genes were found to be present at statistically significant different levels. Again, 40 genes were expressed in the borderline tumors at intermediate levels between the expression levels of the benign and invasive tumors. These data are consistent with the idea that borderline tumors represent a transitional state between benign adenomas and malignant adenocarcinomas. Functional analysis of gene expression associated with tumorigenesis by gene ontology found that genes with functions associated with cell proliferation and DNA metabolism were highly

expressed in the cancer samples. In contrast, genes with functions in cell adhesion, regulation of cell growth, calcium transport and insulin-like growth factor (IGF) signaling were expressed at low levels in the cancer samples.

Meinhold-Heerlein et al. (2005) reported differences in gene expression patterns between noninvasive BL-OVCA tumors and H-OVCA using the Affymetrix U_95A microarray set. They observed similar gene expression profiles of BL tumors compared with well differentiated grade 1 tumors. Similarly, when we examined the gene expression patterns between BL-OVCA and H-OVCA using Affymetrix HU_133 GeneChips®, we reported ~ 300 gene fragments to be differentially expressed by > threefold between the BL-OVCA and the H-OVCA sample sets (Skubitz et al. 2006). These studies are compatible with the hypothesis that borderline and low grade tumors develop by a different mechanism than do higher grade tumors, rather than high-grade tumors evolving from borderline/grade 1 tumors by subsequent mutations (Hauptmann and Dietel 2001). When we performed PCA using a set of probes from the U_133 microarray set, corresponding to 25 of the 40 genes described by Meinhold-Heerlein et al. (2005) from the U_95A gene set, our H-OVCA-A samples grouped with the BL-OVCA samples, distinct from the H-OVCA-B samples. Similar to previous studies (Tapper et al. 2001; Warrenfeltz et al. 2004; Meinhold-Heerlein et al. 2005), who reported that high grade tumors were characterized by the expression of genes associated with the cell cycle, and by expression of STAT1, STAT3, JAK and downstream Jak/Stat signaling targets, we also found that many genes overexpressed in H-OVCA-B compared with BL-OVCA

were associated with cell division/cell cycle, including: CDC2, CDCA1, CDC20, CDC3A, CDCA7, cyclin B2, cyclin E1, and STAT1. These findings are supported by Ouellet et al. (2005) who also compared BL-OVCA with invasive OVCA using the HuGene FL microarray chips and found CKS1B, cyclin E1, and KRT19 differentially expressed between these subgroups.

In another study, Gilks et al. (2005) performed gene microarray expression analysis on 10 BL-OVCA samples and 13 serous carcinomas using the Stanford University cDNA chips. They found considerably more genes over-expressed by the BL-OVCA samples compared with the H-OVCA samples, many of which we also identified in our study (Skubitz et al. 2006).

Bonome et al. (2005) reported that their BL-OVCA samples formed a distinct cluster from their late-stage high-grade ovarian tumors; this latter group was separated into two distinct subgroups, similar to the H-OVCA samples in our study (Skubitz et al. 2006). The genes that they found to be most different between the two groups of samples were genes linked to cell cycle progression, which were upregulated in the H-OVCA samples compared with the BL-OVCA samples. Our study corroborated their findings, as we also noted that CDC2, cyclin B2, cyclin E1, CDC20, RFC4, and PTTG were expressed at increased levels in H-OVCA samples compared to BL-OVCA samples, whereas CDKN1A was upregulated in the BL-OVCA samples (Skubitz et al. 2006).

Another recent study also examined the differences in gene expression between benign, borderline, and malignant tumors. Biade et al. (2006) used cDNA microarrays containing 7,000 elements to analyze gene expression from 120 tumors of varying histologic subtypes and grades. Similar to

Warrenfeltz et al. (2004), they found by unsupervised hierarchical clustering that the tumors segregated into three groups: one containing primarily benign and borderline tumors, a second containing mostly high grade malignant tumors, and a third containing borderline and moderate grade malignant tumors. Genes that were upregulated in the mostly benign group included: collagens COL1A2, COL6A1, and COL6A3, IGFBP5, connective tissue growth factor (CTFG), and monoamine oxidase. Some of the genes upregulated in the mostly malignant group were: K-cadherin, E-cadherin, STK4, and CD24. Although different histological subtypes were analyzed in these experiments, clustering did not clearly distinguish between them. Using PAM analysis they identified a 25 gene predictor that best characterized the gene expression differences between the three classes. Most of the genes in the predictor set were upregulated in the benign and borderline compared to the malignant tumors, with only three genes (PAX8, SLC23A1, and OPCML) upregulated in the malignant tumors. Using a combination of genes identified in the supervised and unsupervised analyses, Biade et al. (2006) validated the gene expression of 21 candidate genes in an independent set of tumors by quantitative RT-PCR. When the results of the gene expression by qRT-PCR were viewed by hierarchical clustering, two groups were identified; one group was composed of the benign and most of the borderline tumors, the other consisted of the malignant tumors and some of the borderline tumors. The authors' interpretation of these results was that borderline tumors may be more variable in their gene expression profiles than either the benign or malignant tumor groups, and, similar to what was shown

by Warrenfeltz et al. (2004), may represent an intermediate group of tumors. However, in contrast to Warrenfeltz et al. (2004), no gene set uniquely characterized the borderline tumors, and the clustering of some of the borderline tumors with the malignant tumors suggests that some borderline tumors may have a more malignant phenotype, capable of progression to an invasive tumor.

In a similar study, Le Page et al. (2006b) analyzed the gene expression of primary cultures derived from 54 epithelial ovarian cancer tissues, including borderline tumors of low malignant potential (LMP), invasive solid tumors, and tumor cells derived from ascites. They identified 126 genes to be differentially expressed between the primary cultures of the tumor tissues and the primary cultures of NOSE. Cluster analysis using these 126 genes identified three tumor subgroups: one subgroup consisted of NOSE, the LMP tumors, and three grade 2 solid tumors (considered the "low grade" tumor group); the second subgroup contained the remaining solid tumors of all histological types; and the third subgroup was composed of samples derived from ascites. Using these three subgroups, the tumor samples were individually compared to NOSE to identify genes that might distinguish the three classes. Not surprisingly, they found the most differences in gene expression between NOSE and ascites cells (270 genes), including 16 genes that also differentiated between solid, invasive tumors and NOSE. Ten unique genes were identified that differentiated the LMP group from NOSE. Using 18 of the 26 genes that discriminated between the LMP tumors, solid invasive tumors and ascites and NOSE as a predictor gene set, they reclassified their 65 samples, correctly classifying 60/65 samples. To further test their model, they used publicly available gene

expression data from the HuFL genechip. Although they were correctly able to classify samples as normal or cancer with ~ 90% accuracy, they did not attempt to distinguish LMP samples. Indeed, the gene expression profiles of the LMP were quite similar to the NOSE samples; however, the gene expression differences between LMP and solid, invasive tumors were not determined.

To explore the genes involved in tumor differentiation, Jazaeri et al. (2003) looked at gene expression in stage III serous papillary carcinomas of differing grades. While unsupervised analysis revealed no clustering of the tumors based on grade, 99 genes with significantly different expression between grade I and grade III tumors were identified. Surprisingly, several of the genes overexpressed in the poorly differentiated tumors (grade III) mapped to chromosome 20q13, a region that is frequently amplified in ovarian and other types of cancers. A second distinguishing feature of the poorly differentiated tumors was the aberrant expression of genes with functions related to centrosome replication and mitosis (cyclin E1, cyclin B1, STK15, NEK2, BUB1, and CSE1L). Based on this data, they proposed a model where over-expression of STK15 or low copy number gain of 20q13 results in transformation. Subsequent disruption in centrosome replication and cell cycle control leads to chromosomal instability and aneuploidy, both characteristics of poorly differentiated tumors.

GENE EXPRESSION PROFILES BASED ON METASTASIS

In this third section, we will review manuscripts that define gene expression profiles of metastatic tumors of ovarian cancer.

In one of our studies, we sought to improve upon earlier studies by comparing the gene expression of ovarian carcinoma tissue samples to > 300 other tissue samples using Affymetrix HU_95 gene chips (Hibbs et al. 2004). By examining a large number of other types of tissues, it was possible to identify genes relatively specific to ovarian carcinoma, without relying entirely upon the gene expression profile of normal ovary tissues. Seven genes that were overexpressed in ovarian carcinoma tissues were selected for further analysis: bone morphogenetic protein-7 (BMP7), the $\beta 8$ integrin subunit, claudin-4, cellular retinoic acid binding protein-1 (CRABP1), collagen type IX $\alpha 2$ (COLIX $\alpha 2$), forkhead box J1 (FOXJ1), and S100A1. In order to verify the corresponding protein expression of these seven genes, immunohistochemical staining was performed using normal ovaries, ovarian carcinoma tissues, and ovarian carcinoma tumors metastatic to the omentum. The design of our study had several advantages in identifying potential ovarian carcinoma tumor markers compared to many of the earlier ovarian cancer gene expression studies. First, a relatively large number of ovarian tissues were utilized for the microarray analyses (50 normal ovaries, 20 serous papillary ovarian tumors, and 17 ovarian tumors metastatic to the omentum). Secondly, protein expression was verified by immunohistochemistry using a relatively large number of ovarian tissue samples (15 normal ovaries, 15 ovarian carcinoma tumors, and 15 ovarian carcinomas metastatic to the omentum). A third advantage of this study was that gene microarray analysis was conducted using 321 tissue samples from 24 other sites in order to determine the specificity of the genes to the ovarian carcinoma tissues.

More recently, we searched for genes that are uniquely expressed in ovarian carcinoma tissues compared with a variety of normal tissues, using Affymetrix HU_133 gene chips (Skubitz et al. 2006); these genes may prove to be the most promising biomarkers and potential targets of therapy. We analyzed the expression of ~ 40,000 genes/ESTs in search of genes overexpressed in H-OVCA compared with 17 different normal tissue types comprising 512 tissue samples. Our results using the newer Affymetrix U_133 microarray chips confirmed and extended the findings of our earlier report in which we used the Affymetrix U_95 microarray set (Hibbs et al. 2004). In particular, CAPS, FOXJ1, KLK10, CCNA1, TMPRSS3, KLK6, SPON1, CDKN2A, MSLN, PRAME, PRSS21, SIAHBP1, and UBE2H were found to be selectively over-expressed in ovarian cancer compared with normal tissues. Interestingly, many of the same genes were found to be over-expressed in BL-OVCA and H-OVCA compared with normal tissues, including: CCNA1, TMPRSS3, FGF18, KLK6, and SPON1, suggesting that these genes are specific to ovarian carcinoma irrespective of stage or grade.

Relatively few studies have directly compared gene expression profiles of primary vs. metastatic ovarian tumors. In our initial gene expression study (Hibbs et al. 2004), we looked at gene expression in 20 serous papillary carcinomas and 17 omental metastases compared to 50 normal ovary samples and 24 other normal and cancer tissue sets. While we observed that overall, the ovarian carcinoma samples were more similar to each other than to the normal ovaries, several genes were found to be differentially expressed by more than tenfold between the ovary tumors and the omentum. Similarly, Adib et al. (2004) found

comparatively few genes differentially expressed in their study of six paired primary and metastatic ovarian serous adenocarcinomas compared to macrodissected epithelium from normal ovaries; all differentially expressed genes were found to be upregulated in the metastatic tumors compared to the primary tissues. Many of the differentially expressed genes were markers of inflammation (serum amyloid A1) or leukocyte infiltration (immunoglobulin lambda), or reflected the high number of adipocytes in the omentum (adipsin, fatty acid binding protein 4, and lipoprotein lipase). Additionally, they found that a number of genes previously identified as predictive of metastasis in other solid tumors were over-expressed in both the primary and metastatic ovarian tumors. Both of these studies support the idea that some type of metastatic gene signature in primary tumors could be a useful predictor of metastasis.

In the studies by Schaner et al. (2005), gene expression patterns from 38 effusions (28 peritoneal and 10 pleural) were compared to that of primary tumors, including 8 paired primary tumors and malignant effusions from the same patient. Using unsupervised cluster analysis, they found a significant amount of heterogeneity among the tumor samples. Four of the primary tumors clustered together, but apart from their effusions; the other four primary samples were clustered with their effusions, but apart from other patients. Using supervised analysis to identify gene expression patterns characteristic of each tumor location, they found few differences between the peritoneal and pleural effusions. However, the expression of genes associated with epithelial cells (such as claudin 7, keratin 7, and keratin 19) was higher in the effusions, while genes associated with stroma

(such as collagens COL1A1, COL5A2, and COL6A2 and SPARC) were more highly expressed in primary tumors.

In a relatively large scale study, Lancaster et al. (2006) analyzed 47 primary and metastatic serous ovarian carcinoma samples from 20 patients using the Affymetrix U95A array. They identified 56 genes that were differentially expressed between tumor samples derived from the ovary compared to the omentum. One third of these genes was associated with metastasis, or had functions involving cell motility, migration, or the cytoskeleton. Ten of the 56 genes were associated with the p53 tumor suppressor pathway, which has been shown to be important in ovarian cancer progression. Additionally, three of the genes identified as upregulated in the omental metastases (immunoglobulin lambda, adipose most abundant transcript 1, and fatty acid binding protein 4) were also shown to be overexpressed in the omental metastasis by Adib et al. (2004).

Finally, a recent analysis by Bignotti et al. (2007) examined gene expression profiles in 14 primary ovarian tumors and 17 metastatic tumors (unpaired) using the Affymetrix U133A chip. In contrast to previous studies, unsupervised hierarchical clustering separated the two sample sets based on differences in gene expression. They identified 156 genes that were differentially expressed between primary and metastatic tumors, including a number of genes predictive of invasion and metastasis such as uPA, MMP2, MMP11, thrombospondins 1 and 2, and CXCL12. A significant proportion of the gene signature associated with the metastatic tumors appeared to be derived from non-epithelial components of the tumor, including different types of collagens (COL1A1, COL5A1, COL5A2,

COL8A1, and COL11A1) usually associated with fibroblasts, and actin gamma 2 (a smooth muscle marker). Interestingly, Lancaster et al. (2006) also found actin gamma 2 expression to be elevated in the omental metastases compared to the primary ovary tumors. However, Schaner et al. (2005) found that several collagen genes (COL1A2, COL3A1, COL5A2, COL6A1, COL6A2, and COL6A3) were more highly expressed in the primary tumors.

CORRELATION OF GENE EXPRESSION PROFILES TO CHEMOTHERAPEUTIC RESPONSE

Despite an initial positive response to therapy, the majority of ovarian cancer patients will ultimately relapse, developing resistance to first-line chemotherapy agents. Several approaches have been used to identify genes involved in resistance to chemotherapeutic drugs, including gene microarray analysis of ovarian cancer cell lines and patients' tissue samples.

Ovarian cancer cell lines that show varied sensitivity to platinum-based chemotherapy drugs have been used in a number of studies examining gene expression differences by cDNA microarray and proteomic profiling methods. Cheng et al. (2006) used paired ovarian cancer cell lines and their chemoresistant sublines to identify pathways associated with resistance to cisplatin. They identified 26 cDNA elements that were differentially expressed in four out of six pairs of cell lines, representing 22 genes and 2 ESTs. Among the genes upregulated in the cisplatin-resistant cell lines were: annexin A1, apolipoprotein E, claudin 4, tissue inhibitor of metalloproteinase

1 (TIMP1), and the oncogene Jun. A particular strength of this study was the use of multiple replicates for each cell line, from independently isolated RNA samples.

Roberts et al. (2005) evaluated the gene expression of 14 ovarian cancer cell lines relative to their sensitivity to four platinum containing drugs: cisplatin, carboplatin, oxaliplatin, and AMD473. These cell lines were independently isolated from patients who had been treated or untreated with platinum-based drugs, and displayed a wide range of sensitivity to chemotherapeutic drugs. Interestingly, one of the top ten genes associated with oxaliplatin resistance was MAL, the gene for the T-cell differentiation protein that has also been shown to be upregulated in patients with short survival (Berchuck et al. 2005) and in ovarian tumors compared to NOSE (Lancaster et al. 2004). They also showed that increased STAT1 expression was associated with decreased sensitivity to both cisplatin and AMD473. They validated the expression of STAT1 RNA and Stat1 protein. Further, they showed that transfection of STAT1 into cell lines with low levels of Stat1 expression increased resistance to cisplatin and AMD473, but not oxaliplatin. They also used a pharmacologic inhibitor of Jak/Stat signaling on cell lines with the highest expression of Stat1 protein, and showed an increase in sensitivity to AMD437 and cisplatin, but not to oxaliplatin. Interestingly, the one cell line pair isolated from a patient before and after developing resistance to cisplatin had significantly increased expression of STAT1 and other interferon-inducible genes in the drug resistant cell line.

Using a combined proteomics and gene expression approach, Stewart et al. (2006) compared cisplatin sensitive and resistant

lines of the ovarian cancer cell line IGOV-1. They used the quantitative proteomics technique, ICAT, to identify and quantify 1117 proteins, 121 of which were differentially expressed between the cisplatin sensitive and resistant cells. Many of the proteins identified had been previously associated with gene expression in cancer cells. Claudin 4, CDC42 binding protein kinase β , mitogen activated protein kinase, and spectrin β were overexpressed in chemoresistant cells. Integrin $\alpha 5$ and annexin IV were over-expressed in cisplatin sensitive cells. Additionally, they correlated the proteomic data with gene expression data generated by MPSS (massively parallel signature sequencing), a technique which generates millions of sequence tags from different cDNA libraries, that then are counted and compared. From the MPSS data, they found opposing mRNA expression levels compared to protein for more than half of the transcripts examined, suggesting that the posttranscriptional regulation of protein expression is an important factor in tumor cell function.

A number of studies have used gene expression from primary tumors to predict response to standard platinum-based chemotherapy. Surprisingly, only a minimal overlap in the gene signatures is observed, although common features of resistance to chemotherapy include an increase in expression of anti-apoptotic genes and a decrease in expression of proapoptotic genes (Peters et al. 2005; Spentzos et al. 2005; Bachvarov et al. 2006), as well as deregulation of genes involved in signal transduction, cell cycle control, cell proliferation, and cell adhesion (Hartmann et al. 2005; Helleman et al. 2006; Newton et al. 2006). Additionally, Bachvarov et al. (2006) identified alterations

in the expression of genes involved in membrane transport molecules, lipid metabolism, and inflammation/immune response in resistant vs. sensitive tumors, suggesting additional mechanisms for resistance to chemotherapy.

Perhaps some of the disparity in gene expression between studies relates to experimental details such as microarray platform (cDNA vs. oligonucleotide, and the number of transcripts evaluated), or definition of chemosensitivity. For example, Spentzos et al. (2005) identified a 93 gene Chemotherapy Response Profile (CRP) based on results of second-look laparoscopy, indicating a pathologic complete response. This criterion is more stringent than clinical criteria (CA125 levels) used in other studies. Previously, they described a prognostic gene signature based on survival (Spentzos et al. 2004). Interestingly, they observed no overlap between the two signatures, and suggested that the different endpoints used could have resulted in profiles describing different tumor characteristics, such as tumor aggressiveness and response to second-line treatments, compared to complete response to first-line therapy.

Several studies included analysis of gene expression profiles in tumor cells after exposure to chemotherapy that could identify genes involved in acquired resistance. Jazaeri et al. (2005) examined 24 resistant tumors, 21 sensitive tumors, and 15 tumor samples harvested after chemotherapeutic treatment. They found significant differences in gene expression between the primary tumors and tumors obtained post-chemotherapy, even between primary tumors classified as resistant to chemotherapy; suggesting that intrinsic and acquired resistance represent different pathways. Interestingly, genes for

extracellular matrix related proteins were significantly over-expressed in the post-chemotherapy tumors. In addition, the post-chemotherapy tumors had higher expression levels of anti-proliferative genes, supporting the previously stated idea that resistance to chemotherapy is related to decreased tumor proliferation and apoptosis. In a similar analysis, L'Esperance et al. (2006) examined six paired tumor samples taken before and after chemotherapy. They also found altered expression of genes that function in proliferation in post-chemotherapy samples; expression of genes that positively regulate proliferation was decreased, while negative regulators of proliferation were over-expressed. In an in vitro investigation, Peters et al. (2005) used primary cultures of tumors from six patients (three resistant and three sensitive to carboplatin) to do a temporal analysis of gene expression in response to drug treatment. Again, the results indicated a significant proportion of the differentially expressed genes were involved in apoptosis, cell adhesion, or proliferation.

Bernardini et al. (2005) examined the gene expression profiles of 22 serous epithelial tumors and identified 123 genes with significantly different expression levels between responders and nonresponders to carboplatin and taxol. Using the 10 tumors with the most extreme differences in response to therapy (based on changes in CA125 expression), they identified 22 genes that predicted resistance to chemotherapy. Three of the 15 genes for which functional annotation was available encoded subtypes of β -tubulin, supporting the hypothesis that alterations in expression of tubulin subtypes are associated with resistance to taxane. Interestingly, they also identified five genes (GAPD, HMGB1, HMGB2, HSC70,

and GRP58) with reduced expression in chemoresistant tumors, which may play a role in chemosensitivity. These genes encode proteins that form a nuclear complex that binds to DNA in response to conformation changing chemotherapy agents, such as cisplatin, and may serve as sensor proteins that promote apoptosis in response to DNA damage. Cells deficient in one component of the complex have increased resistance to chemotherapy, suggesting a novel class of genes responsible for drug resistance in ovarian cancer. To examine the possibility that the OVCA subgroups that we identified in our PCA analysis reflect differences in response to chemotherapy, we compared gene expression from the Affymetrix U_133 microarray data using a gene set (29 gene fragments) corresponding to the 14 known genes from this predictive set. PCA and Eisen clustering using this gene set yielded two major groups from our OVCA samples; separating our BL-OVCA and H-OVCA-A samples from our H-OVCA-B samples (Skubitz et al. 2006); suggesting that response to chemotherapy may be one component of the gene expression differences in our OVCA subgroups.

In a recent comprehensive analysis, Dressman et al. (2007) examined gene expression in a training set of 83 serous tumors (59 complete responders, 24 incomplete responders), and identified a 100 gene set signature profile that was able to distinguish resistance to platinum therapy with 78% accuracy in a test set of 36 tumors. Analysis of the gene ontology for the highest weighted genes in the platinum prediction response model showed enrichment for genes involved in cell growth and proliferation. Additionally, they used gene signatures indicative of activated oncogenic signaling pathways based on

previous work (Bild et al. 2006) to identify activation of Src, Myc or Rb/E2F pathways in chemoresistant tumors, and showed that patients with activated Src had the worst prognosis. In parallel, a panel of ovarian cancer cell lines was similarly evaluated for deregulated oncogenic signaling and then tested for cell proliferation using drugs targeting different pathways. The results of this analysis showed a relationship between the prediction of pathway deregulation based on gene expression and sensitivity to pathway-targeting drugs.

Similarly, Potti et al. (2006) identified gene expression signatures that predict sensitivity to non-platinum based chemotherapeutic agents such as adriamycin and topotecan, both of which are used in salvage therapy for ovarian cancer. Additionally, they were able to link the resistance to particular chemotherapeutic agents to the expression of different oncogenic pathways. Using these predictors on a panel of ovarian cancer cell lines, they found that those cell lines which were predicted to be topotecan-resistant had a high likelihood of Src pathway deregulation. The topotecan-resistant cell lines were also more likely to show sensitivity to SU6656, a drug which inhibits the Src pathway.

CORRELATION OF GENE EXPRESSION PROFILES TO SURGICAL DEBULKING

An important prognostic factor in advanced ovarian cancer is the amount of residual tumor following primary surgery. Berchuck et al. (2004) used gene expression profiling to determine whether the outcome of debulking surgery is due to some underlying biologic features of the tumor and not

due solely to the cytoreduction. In this study, 44 advanced serous tumors (19 with optimal and 25 with suboptimal debulking) were analyzed. Of the optimally debulked patients, 14 survived more than 7 years, compared with only 9/25 suboptimally debulked patients. Using complex statistical modeling, a 32 gene prediction model for debulking status was developed. Leave one out validation for this model achieved 72.7% accuracy. Two of the genes that were over-expressed in the suboptimally debulked tumors were RARB (retinoic acid receptor B) and P2X6 (p53 inducible protein). RARB may induce tumor differentiation and decrease chemosensitivity, contributing to poor outcome in these tumors. In contrast, two genes related to the fibroblast growth factor receptor (FGFR3 and FGFR1 oncogene partner) and genes for two MAP kinase family members (MAP2K4 and MAP3K) were more highly expressed in optimally debulked tumors. MAP2K4 has been shown to be downregulated in ovarian cancer relative to NOSE and may be a metastasis suppressor. These data suggest that the more favorable outcome of optimally debulked tumors is at least partly due to the biological properties of the tumor.

CORRELATION OF GENE EXPRESSION PROFILES TO PATIENTS' SURVIVAL

Microarray analysis of gene expression has recently been used in ovarian carcinoma to discern genes whose expression is associated with overall survival and may have prognostic significance. Lancaster et al. (2004) compared the gene expression of 31 advanced stage serous cancers to

normal ovarian surface epithelium, using the Affymetrix Human GeneFL array. The expression of genes involved in oncogenesis and cell proliferation was increased in tumors, including the gene for the T-cell differentiation protein MAL, which has been shown in other studies to be associated with short term survival and resistance to chemotherapy (Berchuck et al. 2005; Roberts et al. 2005). Additionally, tumors from patients with survival less than 2 years or more than 7 years were compared. When based on the overall gene expression, the samples did not segregate by survival. However, clusters of genes that were associated with patient survival were observed, including a number of genes encoding immune system functions (for example, cytokine receptors IL2R and IL4R, chemokine ligands CCL4 and CCL5, and T cell receptors TCRA and TCRB); and interferon pathway genes IFI30 and ISGF3/STAT1.

Collins et al. (2004) used cDNA microarrays to evaluate the expression of 2382 genes with cancer-related properties in 20 patients with serous epithelial ovarian cancer with defined clinical outcome. They identified 92 genes that were differentially expressed in ovarian cancer compared to normal ovaries. Comparing tumors from patients with recurrent disease to those from patients with no evidence of disease, 11 genes were significantly over-expressed and three genes were found to be over-expressed in patients who had died. Additionally, they compared the genes differentially expressed in tumors, to gene expression in normal adult tissues by analyzing the distribution of cDNAs in the NCBI-EST database present in different adult tissues (Digi-Northern). From this analysis, several genes with somewhat

tissue-restricted expression were identified, and validated by immunohistochemistry. Five of the differentially expressed genes were evaluated with tissue microarrays (TMA) containing 93 independent epithelial ovarian cancer (EOC) samples. While the results of the TMA analysis mirrored the gene expression results, no significant association with survival could be made based on the five markers tested.

In an evaluation of 68 patients with EOC (of which 62 cases were serous), Spentzos et al. (2004) randomly selected half of the patients as a training set. From the training set, tumors from 14 patients with the most extreme survival (< 2 years and > 5 years) were used for the development of a 115 gene class predictor, termed the Ovarian Cancer Prognostic Profile (OCP). The OCP was associated with survival in an independent 34 sample validation set, with a median overall survival of 30 months and not yet reached (after a median of 47 months follow-up) for patients with unfavorable and favorable profiles, respectively. Although patients in the favorable group were more likely to achieve remission after first-line chemotherapy, exclusion of the patients who did not respond to chemotherapy still yielded a significant difference in overall survival between patients in the favorable and unfavorable groups. This suggests that the OCP is at least partly independent of response to treatment, and may identify other biological features of the tumors, such as proliferation or metastatic potential. Some of the genes with increased expression in the unfavorable OCP include PAI1 (plasminogen activator inhibitor 1), VEGFC, and thrombospondin-2, which have previously been shown to be associated with tumor aggressiveness and poor outcome in EOC or endome-

trial cancer. Additionally, overexpression of the estrogen receptor binding site antigen 9 gene, and other estrogen pathway genes in the favorable group, suggests that estrogen-responsiveness may be associated with improved survival. In order to determine whether the OCP gene set would subdivide our H-OVCA tissue samples, we generated the set of 115 Affymetrix U_95 probes described by Spentzos et al. (2004), and analyzed 19 H-OVCA samples and 5 BL-OVCA samples for which we had data from the Affymetrix U_95 microarray set (Hibbs et al. 2004). Interestingly, the OCP gene set, in general, separated our 24 OVCA samples into two groups that were similar to, but not identical to, the subgroups that we had defined (Skubitz et al. 2006).

Berchuck et al. (2005) analyzed the gene expression of 65 serous ovarian carcinomas [30 short term survivors (< 3 years) and 24 long term survivors (> 7 years)] to develop a predictor of survival using two different statistical methods (classification and regression trees, and linear discriminant analysis). The predictor was validated using leave one out cross-validation, and by using early stage (11 stage I/II) cancers as a test set. The top five genes that distinguished short-term from long-term survivors (MAL, APMCF1, NUDT4, PKP4, and SSR1) all had increased expression in the long-term survivors, with the exception of the T-cell differentiation gene, MAL which had higher expression in the short-term survivors. In all of the models identified, the early-stage tumors were classified as long-term survivors. The association between early-stage tumors and advanced stage tumors with more favorable outcomes may reflect a similar underlying biology that is representative of less aggressive disease. They

also tested their predictive model using an independent set of ovarian tumors that had been previously reported by Spentzos et al. (2004) using a different microarray platform. In the independent data set, 68 tumors were clustered into two major groups which were significantly different with respect to outcome. A number of genes identified in their linear discriminant analysis were again found to be over-expressed in tumors from long-term survivors in the independent data set. We generated a set of 26 Affymetrix U_133A probes described by Berchuck et al. (2005) as including the top 5 discriminating genes, and those probes appearing in 15 or more of their leave-one-out models discriminating between short-term and long-term survival, and analyzed our BL-OVCA and H-OVCA samples. By PCA and Eisen clustering, this gene set also tended to group our BL-OVCA and H-OVCA-A samples together, but there was some overlap with our H-OVCA-B sample set (Skubitz et al. 2006).

Finally, Partheen et al. (2006) examined gene expression in a set of 54 stage III serous papillary carcinomas, including 20 patients with survival of more than 5 years. The strength of this study lies in the relatively large number of tumor samples with a homogenous stage and histological type. Hierarchical clustering identified a subgroup of 12 survivors with similar gene expression. When tumors from the subgroup of 12 survivors were compared to the remaining tumors, 204 genes were identified with significantly different expression levels. Of these, TACC1, MUC5B and PRAME were identified as candidates for tumor markers. TACC1 and MUC5B were both over-expressed in the survivor subgroup of tumors, while PRAME was expressed at lower levels in this group. PRAME was

previously shown to be over-expressed in ovarian cancer compared to normal ovaries (Hibbs et al. 2004; Skubitz et al. 2006).

SUMMARY

Ovarian carcinoma is variable in its clinical behavior, and gene expression is thought to underlie these differences. Previously, the classification of ovarian carcinoma has been determined by light microscopy of H&E stained tissues, in which recognizable characteristics are identified in the tumors. Classification of tumors by gene expression patterns has the potential to provide additional useful information that is free of observer bias and variability, and may aid in tumor classification and diagnosis. Based on the many publications that have appeared since 1999 using gene microarray technology with ovarian cancer samples, it is clear that gene expression profiles are highly useful in the sub-classification of ovarian cancer. Gene expression profiles distinguish subsets within a group of ovarian cancer tumors that may reflect different biological behavior. These studies support the use of gene expression patterns as a complementary set of data that may augment the use of light microscopy to help classify ovarian carcinoma. Differences in gene expression profiles in different ovarian carcinoma cases may yield clues to their pathogenesis and may be useful in diagnosis, pre-operative tumor assessment, and studies of the basic biology of ovarian carcinoma. In fact, the results derived from gene microarray analyses have already led to the discovery of many promising new biomarkers that are in various stages of validation using sera samples for the diagnosis of ovarian cancer (Le Page et al.

2006a; Meinhold-Heerlein et al. 2007). Some of these genes may also indicate possible new targets to which anti-tumor therapy could be directed. Interestingly, although many of the studies reviewed herein focused on identifying genes that could discriminate between short-term and long-term survival, predict a good or poor response to chemotherapy, or distinguish between patients with a favorable vs. unfavorable overall survival following chemotherapy, the genes selected to comprise the prognostic gene sets were not the same. By increasing the number of samples used in gene microarray experiments, the clinically and biologically relevant subgroups of ovarian cancer should become more clearly distinct. Consequently, in the near future it should be possible to develop an individualized treatment regimen for patients with ovarian carcinoma based on gene microarray profiling.

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5

Sertoliform Endometrioid Carcinoma of the Ovary: Diagnosis and Prognosis

Monalisa Sur and Dean Daya

INTRODUCTION

Sertoliform endometrioid carcinoma (SEC) of the ovary is an uncommon variant of endometrioid carcinoma of the ovary resembling sex cord-stromal tumor of pure Sertoli and Sertoli-Leydig cell type (SLCT). This entity was first described by Young et al. (1982). Since then only a few cases of this entity have been reported (Roth et al. 1982; Guerrieri et al. 1998; Remadi et al. 1995). SEC occurs in peri- and post menopausal women with an age range of 41–89 years (mean, 60 years). Abdominal enlargement secondary to a unilateral ovarian mass is the most common presentation. Up to 10% cases show bilaterality (Young et al. 1982). The patients very rarely present with androgenic symptoms such as virulization and, to date estrogenic manifestations have been reported in one case in a series of 13 cases (Young et al. 1982). Adequate sampling, a careful search for areas of conventional endometrioid carcinoma and immunohistochemical stains is helpful in the evaluation of SEC from true ovarian sex cord stromal tumors. It is important to recognize this variant of endometrioid carcinoma and especially differentiate it from a sex

cord stromal tumor. SEC as it behaves, is a low grade malignancy and displays a good prognosis if confined to the ovary.

DIAGNOSIS

Clinical Features

Despite histologic similarities between pure Sertoli and SLCT, there are important clinical differences between SEC and sex cord stromal tumors of the ovary. SEC presents in perimenopausal and postmenopausal women with age range of 40–90 years. In contrast, sex cord stromal tumors present in the younger age group with average age of 24–38 years with only 8% patients above 50 years of age (Young and Scully 1984).

Clinically, up to 50% of sex cord stromal tumors exhibit endocrine manifestations of androgen overproduction (Kurman 2002), whilst patients with SEC may occasionally present with virulising signs that may lead to an incorrect clinical diagnosis (Ordi et al. 1999). This hormonal activity is attributed to the presence of luteinized stromal cells present in some of the tumors (Young et al. 1982). Patients with SEC usually present with a unilateral pelvic or

In one series, ascites was present in two patients, uterine prolapse in one patient, and abdominal pain with enlargement in three patients (Young et al. 1982).

Gross Findings

Microscopic Findings

FIGURE 5.1. **(a)** Sertoliform endometrioid carcinoma showing anastomosing small hollow tubular glands with a solid component of epithelial cells in a fibromatous background resembling Sertoli-Leydig cell tumor. ($\times 100$). H&E. **(b)** Conventional glandular and papillary areas of sertoliform endometrioid carcinoma with low nuclear grade. ($\times 100$). H&E

cords with a stratified cell pattern. Solid anastomosing cords combine with regions demonstrating small tubular structures in a fibromatous stroma which may sometimes demonstrate leutinized cells. The two most common patterns leading to confusion of SEC with well differentiated SLCT are: (1) small hollow tubular glands or solid anastomosing tubular structures separated by varying amounts of fibromatous stroma (Figure 5.2a) and (2) presence of intraglandular proliferations of cells creating solid tubular patterns (Figure 5.2b) (Young et al. 1982).

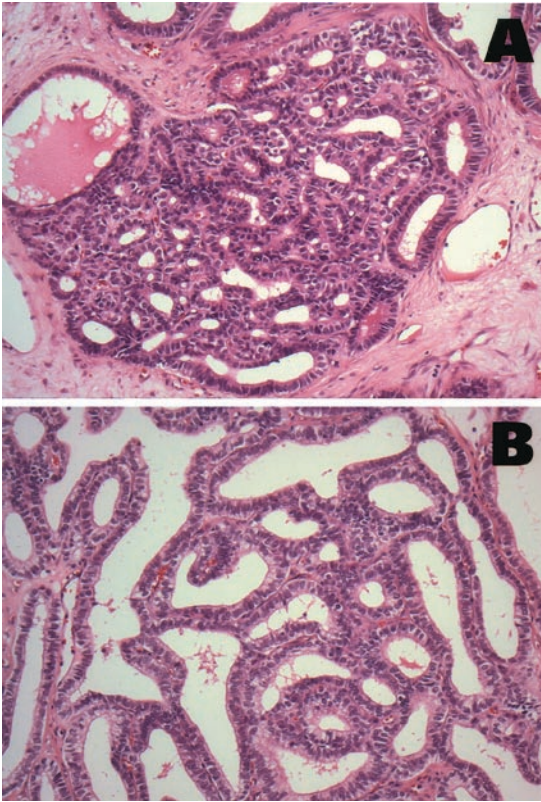


FIGURE 5.2. (a) Sertoli-Leydig cell tumor showing tubular and solid areas intimately admixed mimicking sertoliiform endometrioid adenocarcinoma. ($\times 100$).H&E. (b) Sertoli-Leydig cell tumor showing predominant pseudoglandular pattern mimicking areas of conventional endometrioid adenocarcinoma. ($\times 100$). H&E

The tubular glands are lined by cuboidal to low columnar epithelial cells and may contain PAS positive, diastase resistant eosinophilic secretions in the lumen. Cytologic atypia is mild to moderate in the sex cord like areas. Mitotic activity can vary from 1/10 high power fields to 10/10 high power fields. Areas of squamous differentiation are rare but may be observed (Ordi et al. 1999). Transitional patterns are also noted in occasional cases (Young et al. 1982). Areas of endometrioid adenofibroma may be present, ranging from benign appearing endometrial glands in a fibrotic

stroma with minimal to absent epithelial stratification to foci with atypical proliferative changes (Young et al. 1982; Roth et al. 1982). In the case series reported by Young et al. (1982) two cases demonstrated discrete islands of cells with uniform round nuclei and scant cytoplasm creating a low power resemblance to a granulosa cell tumor. Nuclear grooves, that are a feature of granulosa cell tumor, were not identified in these cases of SEC. In the series described by Ordi et al. (1999) two cases had extensive calcification with two cases showing cells with optically clear cytoplasm and two cases with cells showing prominent eosinophilic cytoplasm.

Based on the Silverberg (2000) histopathologic grading for ovarian carcinoma, most SEC would qualify as Grade 1 and rarely as Grade 2 based on predominant architectural pattern, cytologic atypia, and mitotic figures/10 high power fields. According to the FIGO grading system based on architectural criteria proposed for endometrial cancers, some areas in SEC would qualify for Grade 3 based on predominance of a solid growth pattern ($> 50\%$). However, adjacent well differentiated endometrioid carcinoma areas would still make it a Grade 1 or 2 (Benedet et al. 2000). Similarly, as per the three tier nuclear grading system of Christopherson et al. (1983) SEC is graded as either Grade 1 or 2.

DIFFERENTIAL DIAGNOSIS

There are many ovarian tumors that can mimic endometrioid adenocarcinoma due to the glandular, tubular, and pseudo-tubular patterns, and these have been well summarized by Clement and Young (2000) (Tables 5.1 and 5.2). There are two histologic features

TABLE 5.1. Ovarian tumors that have an endometrioid-like glandular pattern

Endometrioid carcinoma
Mucin-poor mucinous adenocarcinoma
Endometrioid-like yolk sac tumor
Sertoli-Leydig cell tumor
Endometrioid carcinoma from uterus or fallopian tube
Metastatic adenocarcinoma from the gastro-intestinal tract, pancreas, biliary tree, lung or breast

TABLE 5.2. Ovarian tumors that may have a tubular or pseudotubular pattern

Endometrioid adenocarcinoma
Sex cord tumor with annular tubules
Sertoli and Sertoli-Leydig cell tumors
Carcinoid tumor
Metastatic adenocarcinomas from other organ sites

which help to differentiate SEC from sex cord stromal tumors, particularly Sertoli and Sertoli-Leydig cell tumors (SLCT): (1) areas of conventional endometrioid carcinoma and (2) presence of mucin at the apical borders of the tumor cells. Other features that may favor the diagnosis of SEC include squamous differentiation, well developed cilia, presence of endometriosis, or a concomitant adenocarcinoma in the endometrium (Ordi et al. 1999). The epithelium of SEC is often pseudostratified, while the tubular elements of SLCT often form a single layer (Young et al. 1982). The nuclei of SEC lack the paired cell or antipodal arrangement that can occur in SLCT (Ordi et al. 1999).

Although ovarian endometrioid adenocarcinomas morphologically can mimic SLCT and granulosa cell tumors, mimicry of an endometrioid neoplasm by a sex cord-stromal tumor is also a diagnostic pitfall. Ovarian Sertoli-Leydig cell tumors containing a predominant

component of tubules with a pseudo-endometrioid appearance (pseudo-endometrioid Sertoli-Leydig cell tumor) can be misdiagnosed as an endometrioid adenocarcinoma (McCluggage and Young 2007). In the series by McCluggage and Young (2005, 2007), nine cases of SLCT had areas containing hollow, dilated tubules resembling endometrioid glands. In a few of these cases, the psuedoendometrioid tubules were embedded in a fibrous stroma, reminiscent of an endometrioid adenofibroma or borderline malignancy. The proportion of the tumor made up of pseudoendometrioid tubules ranged from 10% to > 90%. When widespread, their presence resulted in confusion with a borderline endometrioid adenofibroma or a well differentiated endometrioid adenocarcinoma. However, extensive sampling revealed areas with typical sertoli tubules and one or more of the characteristic patterns of SLCT as well as Leydig cells. Thus, the presence of more typical Sertoli cell elements and Leydig cells, an absence of squamous elements, endometriosis or associated adenofibroma many conversely assist in differentiating SLCT from SEC.

IMMUNOHISTOCHEMISTRY

Various immunohistochemical stains have been reported to aid in the diagnosis of these unusual tumors.

Cytokeratins

Endometrioid carcinoma shows diffuse cytoplasmic staining for cytokeratins (CKs) as opposed to focal, punctuate or dot-like and paranuclear staining in sex cord stromal tumors (Aguirre et al. 1989; Viale et al. 1988; Costa et al. 1997). SEC shows positive

staining for CAM5.2, AE1/AE3, CK7, and negative staining for CK20. Broad spectrum CKs, including AE1/AE3, are of no value in distinguishing between an endometrioid and a sex cord-stromal neoplasm because, in addition to expected positivity in epithelial tumors, sex cord-stromal neoplasms may be positive (Costa et al. 1995; Czernobilsky et al. 1985). CK7 may have more value in differentiating these neoplasms as SEC is positive for CK7 but SLT, including tumors with a predominance of pseudoendometrioid tubules, are negative for CK7 (McCluggage and Young 2007). In the distinction between a primary ovarian endometrioid adenocarcinoma and a metastatic colorectal adenocarcinoma with an endometrioid appearance, differential CK staining is very useful, with the former usually being diffusely CK7 positive and CK20 negative, whereas the latter generally exhibits diffuse CK20 reactivity and is CK7 negative (McCluggage et al. 2007).

Epithelial Membrane Antigen

Epithelial membrane antigen (EMA) is very useful stain in differentiating SEC from sex cord stromal tumors because it is almost never expressed in the latter (Guerrieri et al. 1998; Aguirre et al. 1989; Costa et al. 1997). EMA reactivity is usually negative in SLT in the Sertoli cell areas, Leydig cell component, and areas showing pseudoendometrioid type tubules, which can be positive for broad spectrum CKs (Costa et al. 1995). Focal immunoreactivity is seen (50% of a small series) in ovarian granulosa cell tumors (McCluggage and Young 2005). However, a definitive diagnosis of sex cord stromal tumor should not be based on the absence of EMA staining alone, in the view of the paucity of positive staining with EMA in some of the SEC cases.

Inhibin

Inhibin, a glycoprotein hormone that suppresses the synthesis and release of pituitary follicle stimulating hormone, is produced by ovarian granulosa cells and testicular Sertoli cells and is a useful marker to differentiate carcinomas from sex cord stromal tumors. With the exception of rare clear cell carcinomas, all epithelial ovarian neoplasms, including SEC have been reported to be negative for inhibin as opposed to sex cord stromal tumors which are positive (Guerrieri et al. 1998). Most sex cord – stromal tumors show focal to diffuse cytoplasmic reactivity with alpha inhibin, although fibromas, poorly differentiated Sertoli-Leydig and sarcomatoid granulosa cell tumors can sometimes be negative (Yao et al. 2003; Costa et al. 1997; Deavers et al. 2003). Since ovarian sex cord- stromal tumors may be confused morphologically with endometrioid neoplasms such as SEC, inhibin along with other sex cord – stromal markers such as calretinin may be useful in the primary diagnosis of SLCT. These two antibodies are also used in confirmation of a metastatic SLCT which may occur at a much later date (Flemming et al. 1995).

Calretinin

Calretinin is a 29 kDa calcium binding protein, best known for its role in the diagnosis of mesothelioma. Calretinin is also found in most ovarian sex cord stromal tumors (Cao et al. 2001; Movahedi-Lankarani and Kurman 2002). In comparison to inhibin, calretinin is a slightly more sensitive, but a less specific marker for ovarian sex cord stromal tumors. Calretinin has a staining pattern similar to inhibin, although it is reported to show more immunoreactivity

in ovarian fibromas when compared to inhibin. Although ovarian adenocarcinomas are more immunoreactive with calretinin when compared to inhibin, there is no staining for calretinin seen in SEC (Cathro and Stoler 2005). In difficult cases, Deavers et al. (2003) recommended that both inhibin and calretinin be used as part of the diagnostic panel.

Neural Cell Adhesion Molecule (N-CAM/CD56)

CD56 is a widely used neuroendocrine marker with a high sensitivity for neuroendocrine tumors and is commonly used as part of a panel to distinguish between a neuroendocrine tumor and other tumors in the differential diagnosis (Kontogianni et al. 2005; Lantuejoul et al. 1998). This marker also shows positive immunoreactivity in ovarian sex cord stromal tumors, but is negative in ovarian endometrioid neoplasms including SEC (McCluggage et al. 2007). In a large series of 85 cases of sex cord-stromal tumors, these authors reported focal to diffuse membranous staining for CD56 in > 50% of the tumor cells in 84 cases. In this series, the sex cord-stromal tumors which were positive for CD56, included adult and juvenile granulosa cell tumors, Sertoli and SLCT, sclerosing stromal tumor, sex cord-stromal tumor with annular tubules, steroid cell tumor, and fibroma. CD56 positivity is almost universal in ovarian sex cord-stromal tumors of all the major histologic subtypes but is of no value in differentiating a sex cord-stromal from a neuroendocrine neoplasm. The diagnostic utility of CD56 comes into play especially in cases which may morphologically resemble SEC, but are negative for alpha inhibin and/or

calretinin. Therefore, this antibody can be used to differentiate sex cord-stromal tumors which are negative for other sex cord-stromal tumor markers from SEC, mimicking Sertoli and SLCT.

Estrogen and Progesterone Receptors

In the series reported by Ordi et al. (1999), 80% of the SECs were positive for estrogen receptor (ER) and 90% for progesterone receptor (PR), a frequency that is higher than the frequency observed in other series of ovarian endometrioid carcinomas (Slotman et al. 1990; Fukuda et al. 1998). However, ER and PR receptor immunostaining is highly variable and does not appear to have diagnostic utility. Furthermore, some sex cord stromal tumors mainly granulosa cell tumors have been reported to express ER (Mulvany and Riley 1997). Expression of these receptors appears to correlate with tumor grade (Fukuda et al. 1998) and prognosis (Slotman et al. 1990).

Other Makers

SEC is negative for CD99 (MIC2 gene product), neuroendocrine markers (synaptophysin, NSE and chromogranin), actin, and desmin. Ovarian sex cord-stromal tumors may show varying degrees of positivity for CD99 (69%), synaptophysin (50%), NSE (50%), chromogranin (29%), actin (22%), and desmin (20%) (Loo et al. 1995; Oliva et al. 2005). These markers are, however, neither specific nor sensitive in making a diagnosis of SLCT. A103 is a melanocyte-associated monoclonal antibody that recognizes the Melan-A/MART-1 antigen in melanomas. The Melan-A/MART-1 antigen is also expressed in Leydig cells, adrenal tissue, and steroid-secreting tumors. A103

is relatively less sensitive than inhibin for recognizing sex cord- stromal tumors, but does not appear to be expressed by ovarian surface epithelial tumors (Yao et al. 2003). It may have some use in identifying lipid-containing cells in sex cord tumors but is not superior as a diagnostic immunohistochemical tool when compared to inhibin or calretinin.

When confronted with a difficult case of ovarian SEC, the recommended immunohistochemical panel for diagnosis should include epithelial markers: AE1/AE3, CAM5.2, EMA, CK7, CK20, inhibin, calre-tinin, and CD56. The immunotyping that applies to endometrioid neoplasms including SEC is positive staining for AE1/AE3, CAM5.2, CK7, and EMA (Figure 5.3) and negative staining for CK20, inhibin, calretinin, and CD56. SLCT in contrast, are negative for EMA (Figure 5.4a) and CK7 and generally positive for CD56, inhibin (Figure 5.4b) and calretinin; immunoreactivity with inhibin and calretinin is generally stronger and more diffuse in the Leydig than the Sertoli

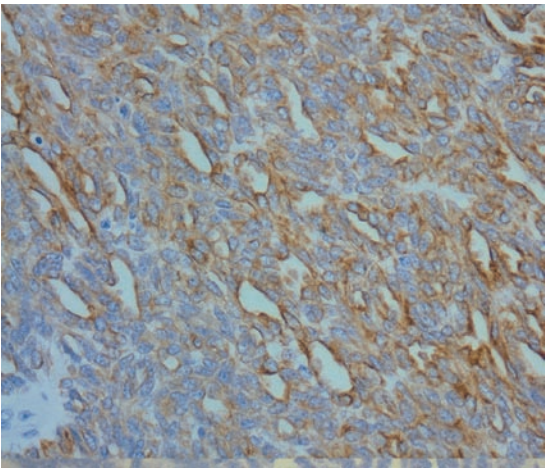


FIGURE 5.3. Sertoliform endometrioid carcinoma positive for EMA (× 200)

cell component (McCluggage and Young 2005). These findings are summarized in Table 5.3.

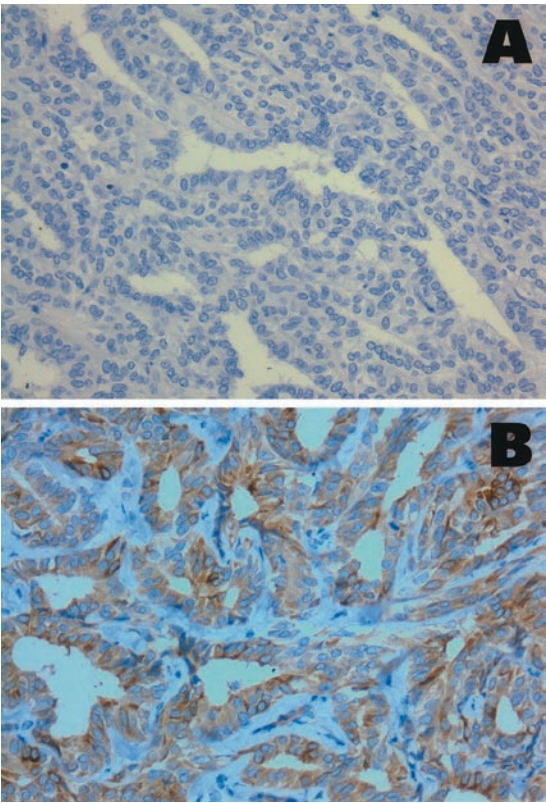


FIGURE 5.4. (a) Sertoli-Leydig cell tumor negative for EMA (× 200). (b) Sertoli-Leydig cell tumor positive for inhibin (× 200)

TABLE 5.3. Typical immunohistochemical reaction patterns in Sertoliform Endometrioid Carcinoma (SEC) and Sertoli and Sertoli-Leydig cell Tumor (SLCT)

Immunostains	SEC	SLCT
CAM5.2	Diffuse +	– /focal +
AE1/AE3	Diffuse +	focal +
EMA	+ / –	–
CK7	+	–
CK20	–	–
Alpha inhibin	–	+
Calretinin	–	+
CD56	–	+
CD99	–	+ / –

+ positive; – negative.

PROGNOSIS

The prognosis of SEC is generally good if limited to the ovary (FIGO Stages). According to the various grading systems, SEC qualifies as a low to intermediate grade tumor. The clinical prognosis tends to follow that of the adjacent conventional endometrioid carcinoma areas seen in SEC even when the sertoliform areas predominate (Roth et al. 1982; Ordi et al. 1999). In the series by Ordi et al. (1999), all tumors were grade 1 or 2, based on the three tier nuclear grading system by Christopherson et al. (1983). In the same series, 10 out of 13 (79%) tumors were FIGO Stage I in sharp contrast with most series of endometrioid adenocarcinomas of the ovary which were diagnosed at a more advanced stage (Brescia et al. 1989; Martin-Jimenez et al. 1994).

In the series of 145 patients with endometrioid carcinoma of the ovary from the M.D. Anderson Cancer Center, 38 patients (26.2%) had FIGO stage I disease, 28 (19.3%) stage II, 60 (41.4%) stage III, and 11 (7.6%) stage IV; 8 patients (5.5%) were unstaged. Grade 2 or 3 histology was seen in 119 patients (82.1%). In addition to surgical therapy, 128 patients underwent chemotherapy, including single-agent therapy, non-cisplatin combination therapy, and cisplatin-based therapy. No statistically significant improvement in median survival was noted among these three chemotherapy groups for FIGO stages II, III, and IV ($P = 0.22$). A significant improvement in median survival was noted for those patients who achieved a complete clinical response, regardless of the type of chemotherapy.

In the series by Ordi et al. (1999), 6 patients with Stage I and 1 patient with Stage II tumor had no evidence of disease 10–120 months (mean, 57 months) after initial treatment.

Two cases with disseminated disease (FIGO Stage III) at initial surgery died of disease, one of whom had a component of serous carcinoma which adversely affected the overall prognosis in this patient. Although limited by the rarity of this variant and small number of cases in literature, the prognosis of FIGO low stage SEC appears to be excellent (Benedet et al. 2000). When compared with Stage I high grade conventional endometrioid carcinoma, Stage I SEC has a good prognosis. (Brescia et al. 1989; Czernobilsky et al. 1970).

In summary, SEC should be considered as a well differentiated low grade endometrioid carcinoma with relatively good prognosis if limited to the ovary. Due to morphological similarities, this entity may be confused with sex cord stromal tumors particularly Sertoli and Sertoli-Leydig cell tumors. Clinical history, age of presentation, extensive sampling for areas of conventional endometrioid carcinoma, and assessment of immunoprofile are all crucial factors in making a definitive diagnosis.

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B. Prognosis

6

Role of MUC16 (CA125) in the Pathogenesis of Epithelial Ovarian Cancer

Jennifer A. A. Gubbels, Joseph P. Connor, and Manish S. Patankar

INTRODUCTION

Epithelial ovarian cancer, known formidably as the “silent killer”, is the most deadly of gynecological cancers in the United States and is the fifth leading cause of cancer related death in women (Jemal et al. 2006). More than 25,000 new cases of epithelial ovarian cancer (EOC) are reported each year, responsible for 14,000 to 15,000 deaths annually in the United States. The deep anatomic location of the ovaries and vague symptoms of the disease make early diagnosis difficult, and there is no accurate diagnostic marker or screening test. Thus, most patients are diagnosed at a late stage, which accounts for the high mortality rate of this disease. Ninety percent of EOC cases could be cured with surgery and currently available chemotherapy if diagnosed at stage 1; however, only 19% of cases are diagnosed at this stage (Jemal et al. 2006). The current 5 year survival rate for all cases is only 45% (Jemal et al. 2006).

CA125 and MUC16

Currently, the tumor marker CA125 is best used for monitoring the progression of known disease, but not for diagnostic

purposes. Its use in early diagnosis of EOC is hampered by its lack of specificity and sensitivity. Its lack of specificity is clear in that women with endometriosis, other cancers (gynecologic, gastrointestinal, and breast), or liver cirrhosis can also present with very high levels of serum CA125, as can women in their first trimester of pregnancy (Bast et al. 1981). Although serum CA125 is elevated in 80% of stage III–IV ovarian cancers, it is only abnormal in approximately 50% of stage I disease, making it an unreliable marker of low sensitivity for early detection of EOC. Complicating matters further, not all patients with EOC have high levels of serum CA125. For the purposes of this chapter, however, we will discuss data that will lead to future therapies for those patients who do present with considerable amounts of CA125. Although a serum level of > 35 U/mL is considered abnormal (McLemore and Aouizerat 2005), in our experience, women with advanced stages of ovarian cancer typically present with serum levels in the hundreds to thousands of U/mL. The peritoneal fluid (PF) values can range from 20,000 to 100,000 U/mL, although levels as high as 700,000 U/mL are not uncommon.

CA125 was discovered in high levels in women with EOC by Bast et al. (1981), and was later shown (Lloyd et al. 1997; Yin et al. 2002) to be an epitope present in the tandem repeat domain of the large molecular weight mucin, MUC16 (O’Brien et al. 2001). This mucin is secreted in vast amounts by ovarian tumor cells, and is also expressed on the surface of these cells; however, its biological function is largely unknown. Mucins are large, multi-functional glycoproteins that typically express a great number of O-linked and N-linked oligosaccharide chains (Hatstrup and Gendler 2008). MUC16 is a type 1 transmembrane protein, with its carboxy terminus and amino terminus on opposite sides of the cell membrane (Figure 6.1). The carboxy terminus includes both an intra-cellular cytoplasmic tail and a transmembrane region. The cytoplasmic tail contains a region for tyrosine phosphorylation and a potential proteolytic cleavage site (Hatstrup and Gendler 2008). MUC16 is found in

both a soluble and a cell-surface bound form; however, the exact mechanism of secretion is unknown. It is hypothesized that it is dependent on cytoplasmic phosphorylation followed by proteolytic cleavage (Hatstrup and Gendler 2008). The MUC16 molecule consists of three separate domains: (1) the amino terminal domain, (2) the Variable Number Tandem Repeat (VNTR) domain (a hallmark of mucins), and (3) a carboxy terminal domain which includes a transmembrane anchor with a short cytoplasmic tail (Hatstrup and Gendler 2008) (Figure 6.1). The amino terminal domain is rich in serine and threonine, making it capable of being very heavily O-glycosylated. There are no CA125 epitopes in this domain, however, the tandem repeat domain, which is just downstream of the amino terminal domain, contains many CA125 epitopes. Each of the repeat domains, consisting of 156 amino acids, is repeated 9–60 times. The epitopes for the anti-CA125 antibodies,

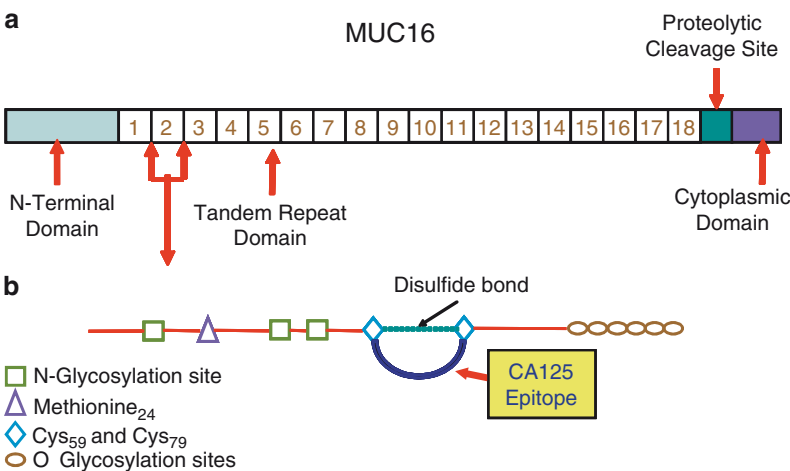


FIGURE 6.1. A model of the MUC16 molecule

M11 and OC125, are located within each of these repeats. MUC16 also contains 16 SEA (sea urchin, enterokinase, agrin) modules, in contrast to other mucins which have only a single SEA domain. This implies that MUC16 may have evolved separately from other mucins (Hattrup and Gendler 2008).

MUC16 in Epithelial Ovarian Cancer

Some known functions of mucins are the protection and lubrication of the surfaces of epithelial tissues lining the ducts and lumens of the body, and postulated functions include epithelial cell renewal and differentiation, cell signaling, and cell adhesion. Altered cell behavior, such as that found in cancer, can cause a disruption in mucin homeostasis and function, rendering pathological consequences (Hattrup and Gendler 2008).

MUC16 on the surface of ovarian tumor cells is shed into the PF and accumulates in the peritoneal cavity of these patients. MUC16 then makes its way through the lymphatic system to the blood, where we have found it binds to a specific subset of natural killer (NK) cells (Belisle et al. 2007). Although the role of this mucin in EOC is unknown, the massive amounts of this protein in patients with advanced EOC suggest that it may be involved in the progression of this disease. Our data suggests that MUC16 may be vital in contributing to the metastasis of ovarian tumors through its binding to mesothelin, a protein present on mesothelial cells that line the peritoneal cavity and peritoneal organs. In addition, it may also have immunosuppressive properties which function to inhibit the

tumor killing abilities of NK cells. These two properties of MUC16 as a metastasis promoter and immunosuppressive agent make this molecule a tempting target for therapies directed against EOC.

MESOTHELIN AND MUC16 BINDING: A MODEL FOR METASTASIS

The most common site for ovarian cancer metastasis is the wall of the peritoneal cavity. Tumor cells expressing high levels of MUC16 are sloughed off of the primary tumor and are then carried by the clockwise flow of the PF to other areas of the peritoneal cavity (Tan et al. 2006). These floating cells are then able to attach to the layer of mesothelin-expressing mesothelial cells that line the peritoneal cavity and peritoneal organs. After this initial binding, ovarian tumor cells invade the layer of mesothelial cells and secrete factors to promote angiogenesis. Successful metastasis is then dependent upon the formation of a blood supply to bring nourishment to the invading tumor cells to promote their proliferation (Tan et al. 2006).

Mesothelin

Research from several groups (including our own) has led to the understanding of how MUC16-mesothelin binding can lead to ovarian cancer metastasis. The cDNA for mesothelin was isolated in 1996 (Hassan et al. 2004). This data showed that the monoclonal antibody Mab K1 recognized mesothelin as a 40-kDa glycoprotein present on the surface of mesothelial

cells, mesotheliomas, and ovarian cancers. Mesothelin is a glycosylphosphatidylinositol-linked protein that originates as a 69 kDa polypeptide. This polypeptide is then cleaved to yield a 32 kDa soluble protein called megakaryocyte potentiating factor (MPF), and a 40 kDa cell membrane bound protein called mesothelin (Hassan et al. 2004). This cleavage process is not completely understood. The normal function of cell membrane bound mesothelin is unknown, and mesothelin knock-out mice have no known abnormalities (Hassan et al. 2004). Mesothelin is overexpressed by the tumor cells found in ovarian cancer, pancreatic cancer, mesotheliomas, and some squamous cell carcinomas (Hassan et al. 2004).

Serum levels of mesothelin are elevated in patients with mesothelioma and ovarian cancer. These serum levels decreased rapidly and were undetectable 7 days after surgical cytoreduction in patients with peritoneal mesothelioma, suggesting that serum mesothelin may be a useful test to monitor treatment response in mesothelin-expressing cancers (Hassan et al. 2006). Another study by Yen et al. (2006) correlated mesothelin immunoreactivity to clinicopathologic features in ovarian serous carcinoma using immunohistochemistry. They found that mesothelin expression correlated with prolonged survival in patients with high-grade ovarian serous carcinoma (Yen et al. 2006). According to our hypothesis as to how EOC metastasizes, high levels of soluble mesothelin would bind to MUC16 on the surface of ovarian cancer tumor cells, preventing them from binding to mesothelin expressed on the mesothelial cells that line the peritoneal cavity. This would then contribute to prolonged survival.

Mesothelin and MUC16 Binding

There have been several studies that have contributed to our understanding of mesothelin and MUC16 binding. A study by Rump et al. (2004) showed that mesothelin binds to OVCAR-3 (a human ovarian cancer cell line that over expresses MUC16) cells. OVCAR-3 cells were also shown to bind to a monolayer of mesothelin-expressing LO cells (murine endothelial-like cell line). Anti-mesothelin antibody concentrations of 22.5 mg/L completely inhibited the binding between the two cells (Rump et al. 2004). This was the first observation that showed that these two proteins do indeed bind. Another group showed that aggregates of cultured ovarian tumor cells, or spheroids, can bind and invade live mesothelial cell layers, further establishing the ability of ovarian tumor cells to attach to the mesothelium (Burleson et al. 2004). After 7 days of incubation, the ovarian tumor cells had proliferated by 200-fold, while the mesothelial cells receded (Burleson et al. 2004). Another study by the same group demonstrated this phenomenon using spheroids from patient PF. Spheroids from the PF of seven ovarian cancer patients were plated on a live human mesothelial cell layer, and again, these cells adhered to and disaggregated on the monolayer within 7 days (Burleson et al. 2006).

Other molecules, such as β integrins and CD44 (a major receptor for the extracellular matrix protein hyaluronic acid), have also been shown to be important in the aggregation and binding of ovarian tumor cells. Davidson et al. (2003) have shown using immunohistochemistry on ovarian cancer tumor cell effusions that β 1 integrin subunits were expressed in 96% of these patients. Another group has investigated

the function of $\beta 1$ integrins in the binding of NIH:OVCAR5 (an ovarian cancer cell line) to extracellular matrix (ECM) proteins ibronectin, laminin, and type IV collagen. When adding NIH:OVCAR5 cells to glass chamber slides coated with the different ECM proteins in the presence of anti- $\beta 1$ integrin antibodies, the number of spheroids attached to the slide decreased ($p < 0.001$) for each ECM protein compared to the control (Casey et al. 2001). Other researchers have demonstrated that CD44H (an isoform of CD44 that preferentially binds to hyaluronate) partially mediates binding of hyaluronate to mesothelial cells. CD44 expressing ovarian cancer cell lines labeled with chromium were plated on mesothelial cell layers in the presence of anti-CD44 antibodies, which inhibited binding $44\% \pm 7\%$ compared to controls where no antibody was present (Cannistra et al. 1993). These authors stated that because the inhibition of binding was not complete, other molecules may be involved in the attachment process. These studies motivated us to look more closely at the MUC16-mesothelin interaction as an additional mechanism that contributes to the attachment of tumor cells to the peritoneal cavity.

Kinetics of Mesothelin–MUC16 Binding

We have completed extensive studies on the molecular characteristics of the mesothelin–MUC16 interaction in collaboration with Dr. Ira Pastan (Gubbels et al. 2006). Dr. Pastan's group has provided us with a mesothelin chimera construct which consists of the mesothelin molecule with a rabbit Fc tag (henceforth called meso-Fc). In order to show the specificity of mesothelin to MUC16, we added meso-Fc to OVCAR-3 cells (MUC16 positive)

and OVCAR-3 derived sublines (some of which are MUC16 negative). Meso-Fc bound only to the MUC16 expressing cells. To determine the binding kinetics of mesothelin to MUC16, increasing amounts of meso-Fc were added to OVCAR-3 cells, which were then measured for bound meso-Fc. Meso-Fc bound with very high affinity to the MUC16 on the OVCAR-3 cells with an apparent K_d of 5–10 nM. In a time course flow cytometry assay, maximum interaction occurred within 5 min of incubation of the recombinant mesothelin with the OVCAR-3 cells and significant binding was observed even after 10 s. These assays demonstrate that mesothelin binds tightly and rapidly to MUC16. In addition, cells that express mesothelin form more hereotypic doublets to cells that express MUC16 than any combination without either of these molecules present on the cell surface.

High concentrations of soluble MUC16 are present in the peritoneal fluid of EOC patients (Harlozinska et al. 1997). Therefore, the proposal that this interaction facilitates peritoneal metastasis of ovarian tumors seems counter-intuitive since the soluble MUC16 may inhibit mesothelin–MUC16 binding. However, our data suggests that soluble MUC16 has a lower affinity for mesothelin compared to cell-surface MUC16. This may occur via a modification of the soluble MUC16 molecule after proteolytic cleavage from the surface of the ovarian tumor cell. According to our observations, mesothelin binds to N-linked oligosaccharides present on MUC16, therefore, another possibility may be that glycosidases within the peritoneal fluid clip the N-linked oligosaccharides of MUC16, rendering mesothelin unable to bind to MUC16.

Mesothelin Binds to N-Linked Oligosaccharides Present on MUC16

N-linked oligosaccharides present on MUC16 are important for mesothelin binding. We have shown that by selectively removing the MUC16 N-linked glycans using PNGase F, the binding of meso-Fc to MUC16 is abrogated. In addition, the lectins WGA and EPHA, which bind to N-linked glycans, were able to inhibit the binding of meso-Fc to MUC16 in Western blot overlay assays.

The proteolytic processing that occurs when the MUC16 is shed from the surface of the tumor cells or subsequent digestion of the N-linked glycans by glycosidases in the PF may alter the soluble MUC16 and make it a less effective ligand for mesothelin. The lower affinity of mesothelin for soluble MUC16 also makes it less likely that the mucin can act as a cross-linking agent by attaching to mesothelin expressed on the mesothelial and the ovarian tumor cells (which overexpress both MUC16 and mesothelin).

We have completed several experiments to demonstrate the lower affinity of soluble MUC16 to mesothelin. Firstly, A431 cells transfected with mesothelin (A431 Meso+) bind only small amounts of soluble MUC16. To determine the effect of soluble MUC16 on the binding between MUC16 on OVCAR-3 cells and meso-Fc, a fivefold molar excess of soluble MUC16 was added to the OVCAR-3/meso-Fc mixture. Soluble MUC16 was unable to inhibit the binding between cell-surface MUC16 and meso-Fc (data not shown). We have also shown that A431 Meso+ cells form doublets with OVCAR-3 cells even in the presence of patient PF, which contains high levels of soluble MUC16 (Figure 6.2).

Under investigation in our laboratory is the possibility that mesothelin may act as a lectin to bind a subset of N-linkages on MUC16. If true, the inhibition of the mesothelin–MUC16 binding by lectins WGA and E-PHA (lectins with many binding sites for N-linked glycans) suggests that polylactosamine or bisecting type N-linked glycans may be the potential ligands for mesothelin (Cummings and Kornfeld 1982; Gallagher et al. 1985; Ivatt et al. 1986; Merkle and Cummings 1987). The weak inhibition of mesothelin binding to OVCAR-3 cells by ovomucoid and obo-transferrin (glycoproteins isolated from chicken eggs that contain high amounts of N-linked oligosaccharides) supports the involvement of the bisecting type N-glycans.

Not only must there be N-linked glycans present for mesothelin to bind to MUC16, but they must be presented appropriately. This is demonstrated by our data that meso-Fc has no affinity for human erythrocytes, which have many binding sites for WGA and E-PHA. We propose that precise positioning and/or expression of appropriate glycans in high density is required for mesothelin to bind to candidate glycoprotein partners. MUC16, as a mucin, is extensively glycosylated. These glycans are likely to be presented in a repeating array on the tandem repeat domain of MUC16, making it a very high affinity mesothelin ligand.

Studies involving recombinant forms of MUC16 are now being undertaken in our laboratory to determine the precise binding site of mesothelin to the mucin. Glycomic analysis of MUC16 fragments will be completed to obtain a clearer picture of the carbohydrates on the molecule, and more specifically, where the N-linked

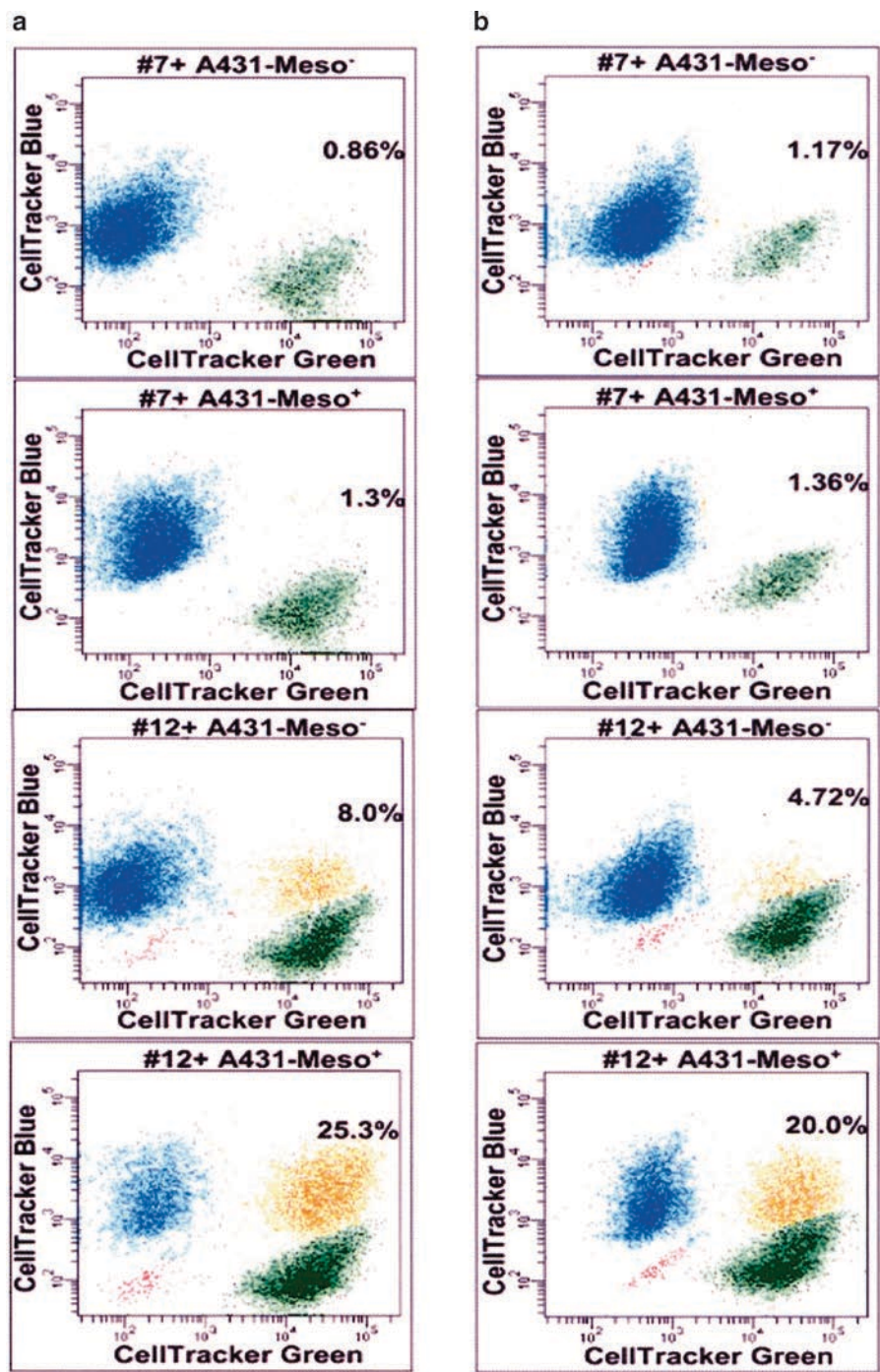


FIGURE 6.2. MUC16 expressing ovarian tumor cells form conjugates with mesothelin positive cells. (A) CellTracker green labeled sublines #12 and #7 (green) were coincubated with either A431-Meso+ or A431-Meso- cells (blue) in PBS containing 1% BSA and heterotypic doublet formation (orange) was analyzed by flow cytometry. The percentages of all live cells that form heterotypic doublets are shown for each plot. Cell debris is in red. (B) Heterotypic doublet formation between the sublines #12 and #7 and the A431 cells in the presence of ascites from patient#15 is shown, same color scheme as (A)

oligosaccharides that contain the mesothelin binding site on MUC16 are located. It should be noted, however, that mesothelin does not carry any of the classical carbohydrate recognition domains that are found in a majority of the mammalian lectins. Thus, if mesothelin directly binds to oligosaccharide ligands, it may do so via an as yet unidentified carbohydrate binding peptide epitope.

As we have mentioned previously, binding of the tumor cells to the mesothelium via the mesothelin–MUC16 interaction may provide a necessary first step for metastasis, somewhat akin to the process of neutrophil migration and the role of selectins in wound healing. However, this interaction in itself may not be strong enough to attach the tumor cell to the mesothelial cell layer. This initial binding may instead lead to recruitment of strong adhesive events mediated by CD44, β -1 integrins, and other cell adhesion molecules (Cannistra et al. 1993; Cannistra et al. 1995; Strobel and Cannistra 1999). It remains to be demonstrated if simultaneous utilization of antibodies directed against mesothelin, CD44 and β -1 integrins will substantially inhibit binding of ovarian tumor cells to the mesothelium. Mesothelin, like MUC16, is overexpressed by the ovarian tumor cells (Bast et al. 2005). The expression of both of these molecules on the tumor cells could facilitate the formation of spheroids, or clumps of tumor cells. This phenomena could increase recruitment of tumor cells to the metastatic site. Increase in tumor load, then, could be contributed not only to the uncontrolled proliferation of tumor cells at the metastatic site, but also to the increase in binding of additional tumor cells that have been sloughed off into the PF from primary or secondary tumor sites. Our data has confirmed other groups' find-

ings (Burleson et al. 2004, 2006; Casey et al. 2001) that ovarian cancer cells form multicellular spheroids in PF. We have shown that ovarian tumor cells that express MUC16 form more homotypic doublets than those without MUC16.

Therapies to block the mesothelin–MUC16 interaction with antibodies could be beneficial to patients, and studies have been undertaken to determine if this interaction can be inhibited using this technique. Bergan and coworkers used a novel yeast-expression system to produce secreted biobodies (yeast secreted recombinant antibodies) and generated anti-mesothelin biobodies that inhibited CA125/mesothelin-dependent cell attachment. These experiments suggest that mesothelin and MUC16 binding is a protein–protein interaction; however, as previously described, our laboratory has data to suggest that it is a protein–carbohydrate interaction. More studies need to be undertaken to fully understand the molecular basis of this binding in order to target specific therapies that will be the most beneficial for patients with ovarian cancer.

We propose that MUC16-mesothelin binding may be crucial in the initial binding of the two cell types, with integrins and other factors becoming more involved as the tumor cell attaches more firmly to the mesothelial cell layer. Blocking the MUC16-mesothelin interaction has been thought to be hampered by the fact that there is soluble MUC16 within the peritoneal fluid; however, our laboratory has shown that soluble MUC16 has less affinity for mesothelin compared to cell-surface MUC16. The apparent structural differences between soluble and cell-surface MUC16 are unknown, but is a question that our lab is currently pursuing. Therapies that would specifically target the cell-

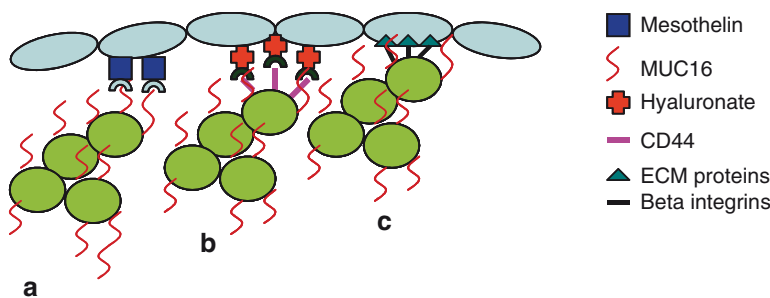


FIGURE 6.3. Model hypothesis for ovarian cancer metastasis to the peritoneal cavity. (A) Spheroids (clumps of tumor cells) initially bind to the peritoneal cavity via the mesothelin-MUC16 interaction. (B) CD44-hyaluronate interactions tighten the binding. (C) Beta-integrins binding to ECM proteins further enhances binding between tumor cells and the peritoneal cavity

surface MUC16 molecule may be most effective in preventing or slowing down metastasis.

A model showing the role of the mesothelin–MUC16 interaction in the peritoneal metastasis and ovarian tumor aggregate is shown in Figure 6.3. The combination of CD44 and hyaluronate, integrins, and mesothelin–MUC16 interactions may all contribute to the binding of tumor cells to the peritoneal cavity. Therefore, a therapy to prevent metastasis may be most successful if targeted towards all of these mechanisms.

MUC16 BINDING TO NATURAL KILLER CELLS: IMMUNOSUPPRESSIVE EFFECTS

In addition to facilitating metastasis through binding to mesothelin, MUC16 may have a role in suppressing the activation of immune cells within the peritoneal cavity. Natural killer (NK) cells are an important arm of the innate immune system, and function mainly to kill tumors and viruses. Elimination of target cells is accomplished either by ADCC (antibody-

dependent cellular cytotoxicity) or through activation of the Fc receptor, CD16. NK cells make up 5–10% of the cells in the peripheral blood (PB) circulation, as well as 4–10% of the PF in ovarian cancer patients (De Leonardis et al. 1993); however, there is minimal invasion of NK cells into the tumor mass (Zhang et al. 2003). Studies of NK cells from the PF of EOC patients have shown decreased overall cytotoxicity as well as decreased cytotoxicity in response to IL-2 stimulation (Lai et al. 1996). In addition, increased numbers of peritoneal fluid NK (PFNK) cells have been associated with worse outcomes in EOC patients (Dong et al. 2006). These studies motivated us to take a closer look at the phenotype of the PFKN in order to understand the cause for decreased cytotoxicity.

A Phenotypic Shift

There are two main types of NK cells based upon their expression of CD56 and CD16: $CD56^{\text{bright}}CD16^{\text{dim}}$ ($CD56^{\text{bright}}$) and $CD56^{\text{dim}}CD16^{\text{bright}}$ ($CD56^{\text{dim}}$) (Nagler et al. 1989). The $CD56^{\text{dim}}$ subset is highly cytotoxic and has a large number of intracellular granules. The $CD56^{\text{bright}}$ subset, however, is less cytotoxic and its

main function is to produce cytokines (Nagler et al. 1989). Normally, the ratio of these subsets of NK cells in the PB is 90% CD56^{dim}, and 10% CD56^{bright} (Nagler et al. 1989). We have analyzed the PF and PB of EOC patients for these subsets of NK cells, as well as the PB of healthy donors. We have observed that in the PFNK of patients with ovarian cancer, the ratio shifts

from 90:10 CD56^{dim}/CD56^{bright} to 60:40 CD56^{dim}/CD56^{bright} (Figure 6.4). We also analyzed these cells for MUC16 binding, and found that MUC16 binds preferentially to the CD56^{dim} subset in the PF (Figure 6.5). There could be several reasons for this shift in phenotype. By conducting proliferation and apoptosis assays, we have proven that this shift is not due to excessive

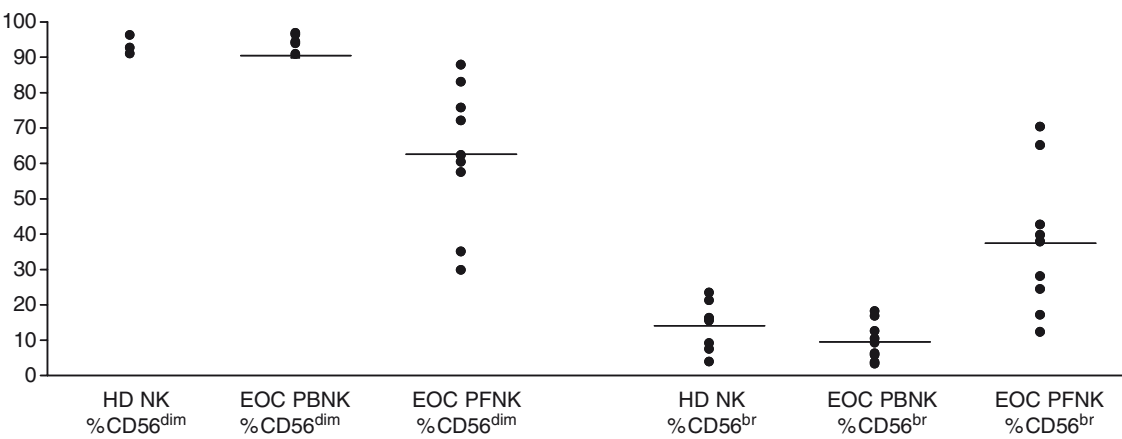


FIGURE 6.4. Distribution of CD56^{dim} and CD56^{bright} NK subsets in the PB and PF of EOC patients. The numbers of CD56^{dim} and CD56^{bright} as a percentage of total NK cells are shown for all eight HD (healthy donor) PBNK (peripheral blood NK cells) and PFNK (peritoneal fluid NK cells) samples. The data point for each HD or EOC sample represents a mean of eight independent experiments

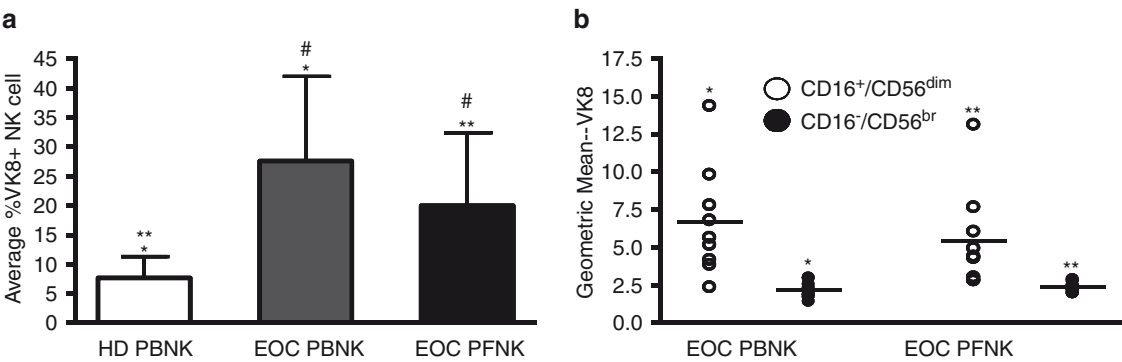


FIGURE 6.5. Restricted binding of MUC16 to NK cell subsets. (A) MUC16 is present on a significant population of the PB and PF NK cells. Data shown is the average from seven HD and nine EOC patients. (B) Distribution of MUC16 on the CD56^{dim} and CD56^{bright} NK cell subsets from the PB and PF of nine EOC patients

proliferation of the CD56^{bright} population, nor excessive death of the CD56^{dim} population (data not shown). Another reason for the change in phenotypic ratios could be the selective accumulation of the CD56^{bright} cells from the secondary lymphoid tissues. Because accumulation of PF is in large part due to the clogging of lymphatic ducts by tumor cells, and CD56^{bright} cells are found mainly in the secondary lymphoid tissues, this may be a cause for the increased numbers of this subset in the PFNK. Yet another possibility is that the CD56^{bright} cells are selectively trafficked into the peritoneal cavity. The CD16^{dim}/CD56^{bright} NK cells express CD62L (L-selectin) that likely helps in their localization to the lymph nodes (Frey et al. 1998). It remains to be demonstrated if the CD16⁻/CD56^{bright} PFNK also express CD62L.

What factors within the PF could be causing this shift? It has been shown previously that PFNK of ovarian patients have a decrease in CD16 expression (Lai et al. 1996). We have recently published the observation that NK cells incubated with MUC16 have decreased cytotoxicity and also have decreased CD16 expression (Patankar et al. 2005). The PF contains an abundant amount of MUC16, and we found MUC16 bound to the CD56^{dim} subset of NK cells. Therefore, MUC16 may be one molecule involved in the down-regulation of NK cells within the PF. To further test this hypothesis, we incubated healthy donor NK cells with PF from EOC patients for 72 h. The results were the same as NK cells from EOC patients: we found that the CD56^{bright} population had increased to 40%, while the CD56^{dim} population had decreased to 60%. In addition, MUC16 was found bound to the CD56^{dim} population.

NK Cell Differentiation

As we consider reasons for this change in NK cell phenotype, the pathway of NK cell differentiation from hematopoietic progenitors becomes important to understand. Development of the NK cells from the progenitors occurs in five distinct stages – defined by the relative expression of CD34, CD117, CD94, and CD56 (Freud and Caligiuri 2006). CD34⁺/CD38⁻ hematopoietic precursor cells, on their pathway towards terminal differentiation to NK cells, transition from pro-NK (Stage 1; CD122⁻/CD34⁺/CD117⁻/CD94⁻/CD16⁻) to pre-NK (Stage 2; CD122⁺/CD34⁺/CD117⁺/CD94⁻/CD16⁻) and to immature NK cells (stage 3; CD34⁻/CD117⁺/CD94⁻/CD16⁻) 24, 25, 26. The immature NK cells initially differentiate into Stage 4 when the phenotype is defined as CD34⁻/CD117^{+/-}/CD94⁺/CD16⁻/CD56^{br}. Cells with this phenotype are primarily found in the secondary lymphoid tissues and only in minor numbers in the PB. The final stage of differentiation from stage 4 cells into stage 5 cells results in the development of CD34⁻/CD117⁻/CD94^{+/-}/CD16⁺/CD56^{dim} NK cells that are abundant in the PB. It is thought that this step is irreversible; however, we suggest that perhaps factors within the PF can cause them to revert back from the CD56^{dim} (stage 5) to the CD56^{bright} (stage 4) phenotype. This could account for the phenotypic shift we see in NK cell populations in the PF of EOC patients.

At this time it is not clear if the CD56^{bright} and CD56^{dim} populations we have identified in the PFNK and in the HD PBNK exposed to PF are equivalent to those found in the PBNK of healthy donors. More involved studies to analyze the expression of other receptors expressed on

these subsets will be needed to determine if these populations are similar in phenotype. Our lab is now dedicated to the task of determining the receptor for MUC16 on NK cells.

Tumor Cell Layers of Protection

There may be several layers that contribute to tumor cells being protected against NK cell defenses. MUC16 may be one, by not only binding to NK cells and possibly causing them to change to a noncytotoxic phenotype, but also by steric hinderance. NK cells must be able to make physical contact with their target cell in order to form an immunological synapse. This synapse is deemed activating when activating NK cell receptors such as LFA-1 and CD2 are polarized towards the cell–cell interface (Davis et al. 1999). The large molecular weight and heavily glycosylated structure of MUC16 may interfere with the immunological synapse formation process, adding another physical layer of protection to the ovarian cancer tumor cells.

The expression of MHC class I can also be a factor in ovarian tumor cell protection from both T and NK cells. NK cells lyse cells that have low or absent amounts of MHC class I molecules and T cells recognize tumor antigens as small peptides presented by a particular MHC class I molecule. Tumor cells generally downregulate MHC class I to avoid T cell attack, however, this would make them susceptible to NK cell lysis. Yet tumors still grow and proliferate in many hosts, despite the lack of MHC molecules on the tumor cell surface. The contribution of MHC class I to NK cell lysis protection in tumor cells is still a mystery that needs to be solved. NK cell cytolytic responses are

controlled by inhibitory or activating KIR receptors, which bind to MHC class I ligands (Moretta et al. 1996). Other non-KIR receptors also exert influences over the actions of NK cells. An NK cell's actions are ultimately decided by the information input generated by the number and strength of inhibitory or activating signals that are engaged (Moretta and Moretta 2004). Therefore, as an NK cell binds to an ovarian tumor cell, the expression of MHC class I on that tumor cell may then also affect NK cell cytotoxicity.

In conclusion, epithelial ovarian cancer is a multi-faceted disease that may engage many layers of protection against immunological attack, especially by NK cells. MUC16 may be one molecule involved in this protection, and it may also have a role in facilitating metastasis by binding to the peritoneal cavity protein, mesothelin. Although the MUC16 molecule is difficult to study because of its large molecular weight and carbohydrate composition, its role in the pathogenesis of EOC is important to understand in the context of both future diagnosis procedures and therapies. New diagnostic tools directed against MUC16 specifically found in EOC patients may someday be available for screening, making the CA125 test more specific. Therapies that inhibit the binding of MUC16 to mesothelin or prevent the downregulation of NK cells could also greatly advance the treatment and survival of women with EOC.

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Clear Cell Carcinoma of the Ovary: Prognosis Using Cytoreductive Surgery

Masashi Takano, Naoki Sasaki, and Toru Sugiyama

INTRODUCTION

Clear cell carcinoma of the ovary was initially termed as “mesonephroma ovarii” by Schiller (1939), as the tumor resembled renal cell carcinoma and was believed to originate from mesonephric structure. Approximately, three decades ago, it was strictly defined by the World Health Organization classification of ovarian tumors (Serov et al. 1973) as lesions characterized by clear cells growing in solid/tubular or glandular patterns as well as hobnail cells. Recently, many publications have identified the distinctive clinical behavior of clear cell carcinoma; resistance to platinum-based chemotherapy (Omura et al. 1991) and poor prognosis in comparison with other histologic subtypes (Sugiyama et al. 2000; Pectasides et al. 2006). The distinguished characteristics of clear cell carcinoma are described in this chapter: clinical and molecular characteristics of the tumor and prognosis after comprehensive cytoreductive surgery.

CLINICAL CHARACTERISTICS

Presentation at Early Stages and Association with Endometriosis

Clear cell carcinoma of the ovary frequently appears during early stages, and proportion of stage I/II tumors ranges from 59% to 71% (Heintz et al. 2006; Takano et al. 2006a). Among these tumors, 30% of clear cell carcinoma is upstaged to stage III disease due to retroperitoneal metastasis (Takano et al. 2006a), leading to high frequency of no measurable lesion after the primary debulking surgery. On the other hand, patients with serous cystadenocarcinoma often present at advanced-staged tumors and harbor measurable disease after initial operation. The lack of measurable disease causes difficulty in the evaluation of effective anti-cancer drugs. The distribution of histological subtypes is summarized in Table 7.1, indicating high frequency of stage I/II tumors in clear cell carcinoma of the ovary. The cooperative study analyzing the histological frequency

TABLE 7.1. Distribution by stage and histologic subtypes (Heintz et al. 2006)

Histology	No. of patients	Stage I (%)	Stage II (%)	Stage III (%)	Stage IV (%)
Serous	3085	14.1	7.3	62.6	15.0
Clear cell	494	55.3	10.8	27.7	6.2

of ovarian cancer revealed the distinctive characteristics of clear cell and mucinous carcinoma of the ovary in comparison with serous adenocarcinoma.

The association with endometriosis and neoplasm is often reported in clear cell carcinoma and endometrioid adenocarcinoma. Atypical endometriosis is considered to be a precancerous lesion, and endometriosis frequently develops into epithelial ovarian cancer including clear cell carcinoma (Kobayashi et al. 2007). According to their report, the ovarian cancer risk was elevated significantly among patients with ovarian endometrioma (relative risk = 12.4; 95% CI, 7.9–17.3). The risk significantly increased with increasing age at ovarian endometrioma diagnosis, especially over the age of 50, suggesting that malignant change of endometriosis occurs near menopause stage.

Sekizawa et al. (2004) reported that K-ras mutation is one of the triggers of malignant change of endometriosis. p53 and PTEN mutations are also frequently observed in ovarian cancers, suggesting that they are carcinogenesis-related genetic changes (Sato et al. 2000). Genetic alterations, including these changes, would contribute to the carcinogenesis in ovarian endometrioma.

MOLECULAR
CHARACTERISTICS

Considering molecular characteristics as well as clinical behavior, it may be hypothesized that clear cell carcinoma belongs to

a different entity from other histological subtypes of ovarian tumors. There are many publications describing molecular markers highly expressed in clear cell carcinoma compared with other histology, supporting this hypothesis. Mutation in p53 is much less frequent in clear cell carcinoma than in other histologic types of epithelial ovarian cancers, suggesting that there is another carcinogenesis mechanism in the development of clear cell carcinoma (Ho et al. 2001). Wilms tumor suppressor 1 gene (WT1) and WT1-antisense promoter were significantly methylated in clear cell carcinoma compared with serous adenocarcinoma (Kaneuchi et al. 2005). Multi-drug resistance protein 3 (MRP3), a well-known resistance marker to anticancer drug, is also highly expressed in clear cell carcinoma (Ohishi et al. 2002). Also, hepatocyte nuclear factor 1beta (HNF-1beta) is highly expressed, and has anti-apoptotic effects in clear cell carcinoma (Tsuchiya et al. 2003). Hirasawa et al. (2003) reported that 17q21–q24 gain and consequent overexpression of two potential targets, PPM1D and APPBP2, are associated with malignant phenotypes of clear cell carcinoma, and could be used as a predictor for prognosis. Tsuda et al. (2005) documented significantly higher ABCF2 DNA and mRNA copy number and protein levels in clear cell tumors compared with those in serous tumors. Moreover, Ki-67 labeling index was significantly lower in clear cell carcinoma than in serous tumors (Itamochi et al. 2002a), and doubling time for clear cell carcinoma

cells was significantly longer than that for serous adenocarcinoma cells (Itamochi et al. 2002b). These genetic background combined with cell growth activity could be correlated with the distinct behavior of clear cell carcinoma. Suppression of the genes as shown above or acceleration of cell cycle might be a useful strategy for the future treatment of clear cell carcinoma of the ovary.

CLINICAL OUTCOME

Resistance to Platinum-Based Chemotherapy

During the last ten years, many publications of relatively small samples have identified that clear cell carcinoma showed resistance to platinum-based chemotherapy, and the response rate was lower in clear cell tumors compared with serous tumors (Omura et al. 1991; Sugiyama et al. 2000). After establishment of paclitaxel and carboplatin as “gold standard” regimens for epithelial ovarian cancer, they have been widely used for all histological subtypes of ovarian tumors (McGuire et al. 1996). But response in measurable cases of clear cell carcinoma treated with paclitaxel and carboplatin was relatively low, ranging from 18% to 56% (Enomoto et al. 2003; Ho et al. 2004; Pectasides et al. 2006; Utsunomiya et al. 2006; Takano et al. 2006b). Although there is no larger phase II study confirming these results, clear cell carcinoma clearly showed resistance to paclitaxel and platinum as well as conventional platinum-based chemotherapy.

Retroperitoneal Involvement

In stage I ovarian cancer of all histologic subtypes, the incidence of positive lymph

nodes was reported to range from 5.1% to 20% (Sakuragi et al. 2000; Cass et al. 2001; Morice et al. 2003). Takeshima et al. (2005) reported that serous tumor had a higher incidence of lymph node metastasis than nonserous tumors. A study of a large number of clear cell carcinomas revealed lymph node metastasis was observed in 9.1% of stage Ia tumors, 7.1% of stage Ic tumors, and 10.8% of pT2 tumors (Takano et al. 2006a). Approximately, 10% of clinical stage I/II tumors were upstaged as stage IIIc based on lymph node status.

The impact of retroperitoneal lymph node status on prognosis in early-staged ovarian cancer patients is still controversial. Some reports showed a positive relationship: survival rates with node positive disease were significantly lower in clinical stage I and II disease (Sakuragi et al. 2000; Kanazawa et al. 1999; Negishi et al. 2004). In contrast, another report showed that the prognoses for clinical stage I/II patients with or without lymph node metastasis were similar (Onda et al. 1998). In clinical stage I clear cell carcinoma patients, lymph node status was identified as a strong prognostic factor, suggesting that it is essential to accurately evaluate the lymph node status through complete surgical staging procedures (Takano et al. 2006a).

Prognosis After Cytoreductive Surgery

From a large retrospective cooperative study, five-year progression-free survival and overall survival was 84% and 88% in stage I, 57% and 70% in stage II, 25% and 33% in stage III and 0% and 0% in stage IV, respectively (Takano et al. 2006a).

During the last three decades, differences have not been reported in survival between clear cell carcinoma patients and

serous adenocarcinoma cases. However, some reports have shown worse survival in patients with clear cell carcinoma. Kennedy et al. (1989) reported stage I/II clear cell carcinoma patients had a survival similar to patients with non-clear cell type carcinomas, but stage Ic cases experienced a poor survival. Behbakht et al. (1998) indicated that stage I clear cell carcinoma had a higher recurrence rate, but the survival was similar to that of other histological subtypes.

The status of peritoneal cytology in stage I ovarian cancer is also controversial. One recent report analyzing prognosis of early-staged ovarian cancer including only 25 clear cell carcinoma cases (26.6%) in 94 carcinomas showed no statistical significant difference between stages Ic preoperative versus intraoperative rupture (Leitao et al. 2004). But another report including higher ratio of clear cell carcinoma patients identified that stage Ic intraoperative rupture patients showed significantly poorer survival than stage Ia patients (Mizuno et al. 2006). The same tendency was observed in clear cell carcinoma specific study: tumor progression was observed in 11% of stage Ic intraoperative rupture tumors and 3% of stage Ia tumors (Takano et al. 2006a). These results implied the importance to remove the tumor mass without intraoperative rupture and implantation of tumor cells, especially in clear cell carcinoma patients. Progression-free survival of the patients with stage Ic (ascites/malignant washing) and Ic (ovarian surface) was significantly worse than that of stage Ic (capsule ruptured) ($p = 0.04$) (Figure 7.1; Takano et al. 2006a). Multiple regression survival analysis for stage Ic patients with clear cell carcinoma revealed that positive

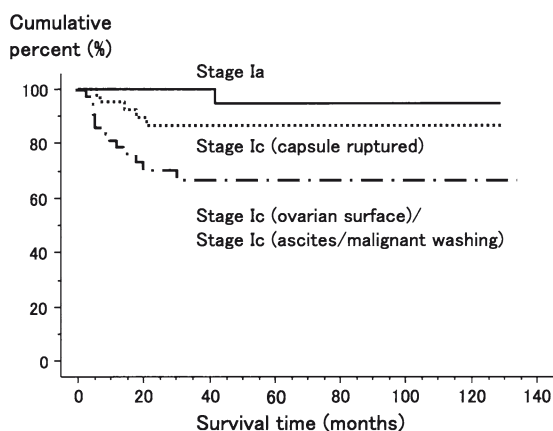


FIGURE 7.1. Progression-free survival of patients with FIGO stage I patients (Takano et al. 2006a)

peritoneal cytology was the only independent prognostic factor ($p = 0.03$; relative risk, 3.40; 95% CI, 1.14–10.18). These results implied that positive peritoneal cytology meant microscopic implantation of clear cell carcinoma cells which potentially harbored resistant clones for anti-cancer agent drugs, and these diagnoses would lead to early relapse of the disease despite postoperative chemotherapy.

In advanced ovarian tumors, it is well known that optimal surgery achieving residual tumor diameter less than 1 cm improved survival of the patients. Since 1986, the Gynecologic Oncology Group (GOG) has used the definition, “less than 1 cm”, in GOG studies (Omura et al. 1991). In our retrospective analysis (Figure 7.2; Takano et al. 2006a), the patients with complete resection showed a significantly higher survival rate compared with those with residual tumor > 1 cm ($p < 0.01$) and those with tumor < 1 cm ($p = 0.04$). There is no significant prognostic difference between the patients with the tumor diameter < 1 cm and those with the tumor diameter > 1 cm ($p = 0.40$). Median progression-free survival duration was 39 months

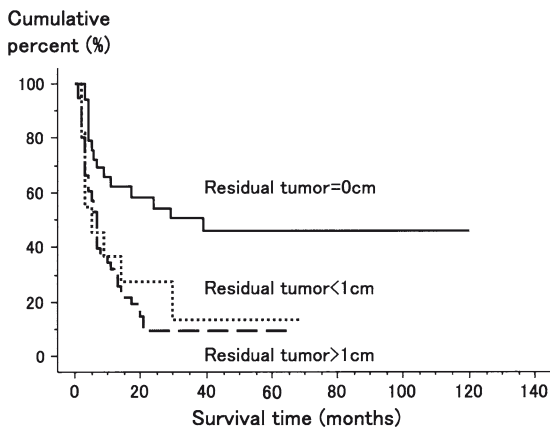


FIGURE 7.2. Progression-free survival of stage III, IV patients according to the residual tumor diameter (Takano et al. 2006a)

in the patients with no residual tumor, 7 months in those with the tumor diameter < 1 cm, and 5 months in those with residual tumor diameter > 1 cm, respectively. Multiple regression analysis in stage III and IV patients confirmed these results that only residual tumor diameter was an independent prognostic factor in stage III and IV patients ($p = 0.02$). From these results, it is suggested that cytoreductive surgery achieving no residual tumor could only improve the prognosis of advanced clear cell carcinoma of the ovary: “optimal” cytoreduction in clear cell carcinoma might be defined as “no residual tumor” in the near future.

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8

Advanced Ovarian Cancer: Prediction of Surgical Outcomes Using Computed Tomography

Sean C. Dowdy and William A. Cliby

INTRODUCTION

The characterization of adnexal masses has been facilitated remarkably by the use of pelvic ultrasound during the last decade. Its ease of use, accessibility, relatively low cost, and the recent introduction of color and duplex scanning has made this modality invaluable. Please see other chapters in this volume for a discussion of the use of ultrasound for differentiating benign from malignant ovarian masses. For patients with advanced ovarian cancer and a known pelvic mass which can be palpated on bimanual exam, however, ultrasonography is rarely helpful other than to confirm the presence of ascites. While magnetic resonance imaging (MRI) provides precise information regarding the location of disease and invasion of tissue planes, such detailed anatomic relationships are generally not clinically relevant for treatment planning in patients with advanced ovarian carcinoma, particularly given the associated costs. This is in contradistinction to cervical cancer, in which MRI has been shown to be sensitive for the detection of parametrial involvement, a parameter which significantly alters the

plan of treatment if present (Yu et al. [1998](#)). ^{18}F fluorodeoxyglucose-positron emission tomography (FDG-PET) is highly sensitive for cancerous lesions and may ultimately prove useful for the detection of recurrences, but the cost/benefit ratio is poor for the preoperative evaluation of primary ovarian cancer. Computed tomography (CT) offers advantages over other techniques: relatively low cost, fast scan times, wide availability, and evaluation of the entire abdominal cavity. Furthermore, the use of intravenous and oral contrast improves visualization of retroperitoneal anatomy (Figure [8.1](#)). For these reasons, CT has become a common diagnostic procedure to assess the extent of disease and plan surgical interventions in patients with advanced ovarian cancer. This chapter addresses the use of CT imaging in this cohort of patients and its potential to predict surgical outcome.

VALUE OF CYTOREDUCTION

To understand how preoperative evaluation with CT could benefit patients with ovarian cancer, it is first necessary to understand

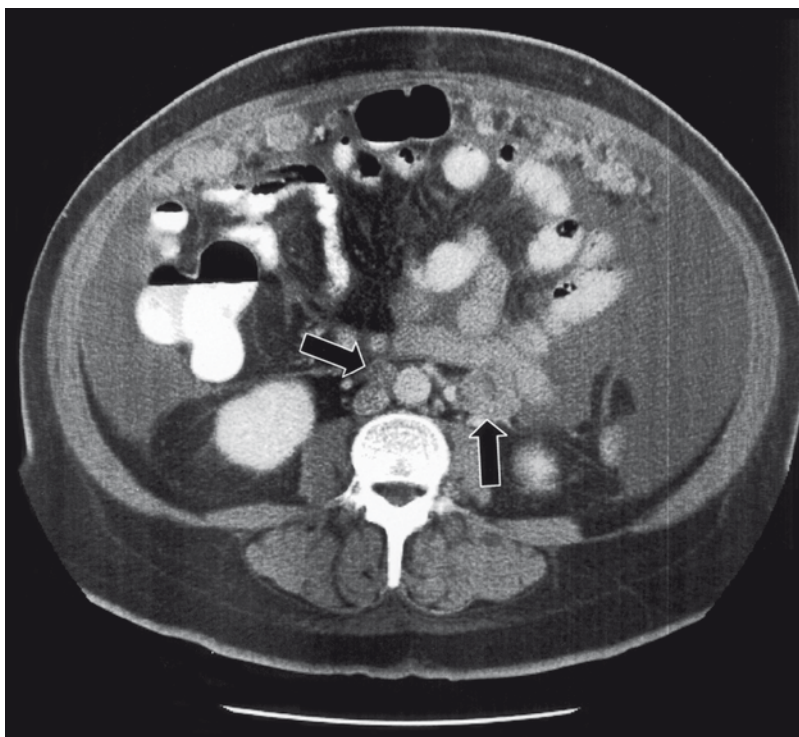


FIGURE 8.1. Example of para-aortic lymphadenopathy, indicated by *arrows*

the value of what gynecologic oncologists refer to as “optimal cytoreduction.” Although never investigated in a randomized trial, the value of optimal cytoreduction (resection of all disease > 1 cm in diameter) in patients with ovarian cancer has been repeatedly demonstrated during the past several decades (Griffiths et al. 1979; Heintz et al. 1986; Eisenkop et al. 1998). Bristow et al. (2000) performed a meta-analysis investigating survival associated with maximal cytoreduction in 81 published patient cohorts totaling 8,000 patients. Comparing cohorts with a high proportion of optimal cytoreduction ($> 75\%$) to cohorts with a rate $< 25\%$, there was a 50% increase in median survival (33.9 vs. 22.7 months). The reason for this observation is likely to be multifactorial, but often attributed to the presumption that adjuvant therapy

is more effective for diminished tumor volumes. Large tumors tend to harbor a high proportion of cells in the G0 phase of the cell cycle. These cells are nondividing and essentially resistant to chemotherapy. Cytoreduction increases the vulnerability of the remaining cells to cytotoxic therapy by increasing their growth fraction. A large proportion of bulky tumor masses are poorly vascularized. Resection of these areas removes tumor that would be expected to receive low concentrations of chemotherapy. Furthermore, it has been suggested that each cycle of chemotherapy reduces cell numbers by a constant proportion, the so called “fractional cell kill hypothesis” (Skipper 1978). In this model, the lower the number of cells exposed to chemotherapy, the lower the number of treatments needed for remission.

This may also reduce acquired chemoresistance, as there are fewer cells being exposed to chemotherapy for prolonged periods (Goldie and Coldman 1979).

Improved response rates to chemotherapy have been documented for patients with an optimal cytoreduction, as illustrated by a comparison of two investigations performed by the Gynecologic Oncology Group (McGuire et al. 1996; Ozols et al. 2003). In the first trial, a complete pathologic response was documented by second look laparotomy after treatment with cisplatin and paclitaxel. For this suboptimally cytoreduced cohort, the complete pathological response rate was 26%. In contrast, the second investigation documented a complete pathologic response rate of 49% for patients optimally cytoreduced. This response rate was also determined by second look laparotomy after treatment with paclitaxel and either carboplatin or cisplatin. The difference in median survival between cohorts was 15 months (38 and 53 months, respectively).

Despite the abundance of data supporting cytoreduction, in the absence of randomized trials some authors have proposed that the improvement in survival seen in patients with optimal cytoreductions is related to the biology of the tumor rather than the surgery itself. In other words, patients with extensive disease that cannot be optimally resected are proposed to have a biologically aggressive cancer with corresponding poor outcomes. In contrast, patients with advanced, but low volume disease that can be easily resected are supposed to have a less aggressive biology and a better prognosis. This philosophy has caused many surgeons to question the value of performing radical procedures in order to achieve an optimal cytoreduction.

Many authors have attempted to delineate cause from effect in this setting. In a retrospective review, Aletti et al. (2006) analyzed 194 consecutive patients with stage IIIC ovarian cancer at the Mayo Clinic. Consistent with prior investigations, the volume of residual disease predicted survival in the entire cohort and within the subset with carcinomatosis. If survival were to be reflective of the tumor biology only, those patients rendered optimal with relatively minimal surgical effort would be expected to have better survival than those who required extensive surgery to achieve an optimal cytoreduction. To explore this hypothesis, the survival of two groups of patients who underwent an optimal cytoreduction was compared. The radical surgery group underwent procedures including bowel resection, splenectomy, hepatic resection, and diaphragmatic resection, while the other cohort did not. Despite the disparate radicality of the procedures needed to remove all tumor > 1 cm, 5 year disease specific survival was nearly identical, 46% vs. 47% ($p = 0.80$). Thus, regardless of the extent of surgery required, optimal cytoreduction appeared to abrogate the effects of extensive tumor burden as a poor prognosticator. It is particularly important to recognize that while grade, stage, and performance status are all correlated with survival, the only predictor of survival under the surgeon's control is the extent of cytoreduction.

While the mass of literature supports the concept that optimal cytoreduction improves survival, there is equal evidence that suboptimal cytoreduction influences survival no more than biopsy alone. Hoskins et al. (1994) reanalyzed 294 stage III patients with a suboptimal cytoreduction from a randomized trial performed by the Gynecologic Oncology Group to

determine the effect of residual disease diameter on survival. Patients were classified according to residual disease into seven groups with corresponding relative risks of dying from ovarian cancer: residual disease < 2 cm, relative risk 1.00; 2 to 2.9 cm, relative risk 1.90; 3 to 3.9 cm, relative risk 1.91; 4 to 5.9 cm, relative risk 1.74; 6 to 7.9 cm, relative risk 1.85; 8 to 9.9 cm, relative risk 2.16; ≥ 10 cm, relative risk 1.82. While survival differences between those with < 2 cm residual disease and those with ≥ 2 cm residual disease was significant ($p < 0.01$), there was no significant difference in the risk of dying between groups with residual disease ≥ 2 cm. This demonstrates that cytoreductive surgery has a negligible effect on survival unless the largest diameter of residual disease measures < 2 cm.

Taken together these concepts suggest that women with ovarian cancer may benefit if we were able to determine preoperatively whether or not their tumors could be optimally resected. Those women with resectable tumors would undergo surgery that has been shown to improve survival. In contrast, those with disease too advanced for optimal resection could be spared a fruitless and morbid procedure and go on to receive neoadjuvant chemotherapy, either palliatively, or with the intent of later performing an interval cytoreduction in the event of adequate response to chemotherapy (van der Burg et al. 1995; Schwartz et al. 1999).

Ability of Computed Tomography to Predict Optimal Cytoreduction

In an attempt to identify patients most likely to benefit from cytoreduction and exclude patients less likely to benefit,

several authors have investigated the ability of CT to correctly classify patients preoperatively. In total, six manuscripts have investigated this topic (Nelson et al. 1993; Meyer et al. 1995; Bristow et al. 2000; Dowdy et al. 2004; Qayyum et al. 2005; Axtell et al. 2007). However, some were of limited size, used an antiquated definition of optimal cytoreduction (< 2 cm), or included stage I and II patients in their analysis.

The first well-designed investigation to use CT for predicting surgical outcomes in patients with ovarian cancer was performed by Bristow et al. (2000). During a 2-year period, 41 patients with stage III or IV ovarian cancer from the Johns Hopkins Medical Institutions and from the Massachusetts General Hospital were analyzed retrospectively. All were evaluated preoperatively with CT and subsequently underwent surgical cytoreduction. The use of oral and IV contrast was used in all subjects with some receiving rectal contrast if the colon or rectum was not adequately visualized. Twenty patients (49%) were optimally cytoreduced (< 1 cm). Preoperative CT scans were evaluated using 25 radiographic features to determine the extent of disease. Radiologists were blinded as to whether the patients underwent an optimal cytoreduction. A Predictive Index score was then calculated for each patient based on 14 of the 25 parameters examined. These 14 radiographic features were chosen based on the following criteria: specificity $\geq 75\%$, PPV $\geq 50\%$, and NPV $\geq 50\%$. These features included the following: peritoneal thickening; peritoneal implants ≥ 2 cm; small bowel mesentery disease ≥ 2 cm; large bowel mesentery disease ≥ 2 cm; omental tumor extension to the stomach, spleen, or lesser sac; tumor

extension to the pelvic sidewall, parametria, or hydroureter; large volume ascites (on all sections); performance status ≥ 2 ; suprarenal paraaortic lymph nodes ≥ 1 cm; diaphragm or lung base disease ≥ 2 cm or confluent plaque; inguinal canal disease or lymph nodes ≥ 2 cm; liver lesion ≥ 2 cm on surface, or parenchymal lesion of any size; porta hepatis or gallbladder fossa disease ≥ 1 cm; infrarenal paraaortic lymph nodes ≥ 2 cm. Nine parameters were assigned a Predictive Index value of 2 points based on their accuracy of $\geq 60\%$; the remaining five were assigned a value of 1. Applying this model to the same cohort of patients and using a Predictive Index value cutoff of 4, no patients would have been unnecessarily explored while 15% would have been inappropriately unexplored. Thus, while all patients explored would have undergone an optimal cytoreduction, 15% of the patients who would have received optimal cytoreduction would not have undergone laparotomy. This preliminary investigation suggested that preoperative CT evaluation may be useful in predicting which patients with advanced ovarian cancer undergo optimal cytoreduction.

In a follow-up to the investigation performed by Bristow et al. (2000), Dowdy et al. (2004) analyzed the records from 321 patients who underwent primary cytoreductive procedures for ovarian cancer in a 5-year period. 87 patients had stage III/IV disease in addition to a CT scan of the abdomen and pelvis with oral and IV contrast. Optimal cytoreduction was achieved in 71%. CT scans were retrospectively evaluated by two radiologists for 17 criteria including the 14 found predictive in the study by Bristow et al. (2000). The three additional parameters were bowel encasement, omental caking, and disease

near the root of the mesentery. The most predictive parameter was diffuse peritoneal thickening, defined in this study as ≥ 4 mm involving at least two of the following five areas: lateral colic gutters, lateral conal fascia, anterior abdominal wall, diaphragm, and pelvic peritoneal reflections (Figure 8.2). In a separate prospective analysis of 43 patients within the same investigation (20 previously read as harboring diffuse peritoneal thickening) this definition was shown to be highly reproducible (agreement in 93% of cases). Other significant predictors on univariate analysis were large volume ascites (present on two-thirds of CT images), bowel encasement, omental extension to the spleen or pancreas, and diaphragm or lung tumors > 1 cm (Figure 8.3). Using multivariate analysis, however, only diffuse peritoneal thickening was found to be an independent predictor of optimal cytoreduction. Using the five most significant parameters as determined by univariate analysis, the most useful model included only diffuse peritoneal thickening and large volume ascites. If both of these parameters were present, the optimal cytoreduction rate was only 32% versus 71% for the entire cohort. Conversely, if both predictors were absent, 82% of patients were optimally resected. The presence of these two factors predicted 52% of suboptimal cytoreductive surgeries. While these two parameters appeared somewhat useful for predicting surgical outcomes, the authors were cautious given that the findings differed to such a degree from Bristow et al. (2000). Radical procedures were performed in 52% of patients in this series and the authors concluded that the results of this investigation could not necessarily be applied to a less aggressive surgical practice.

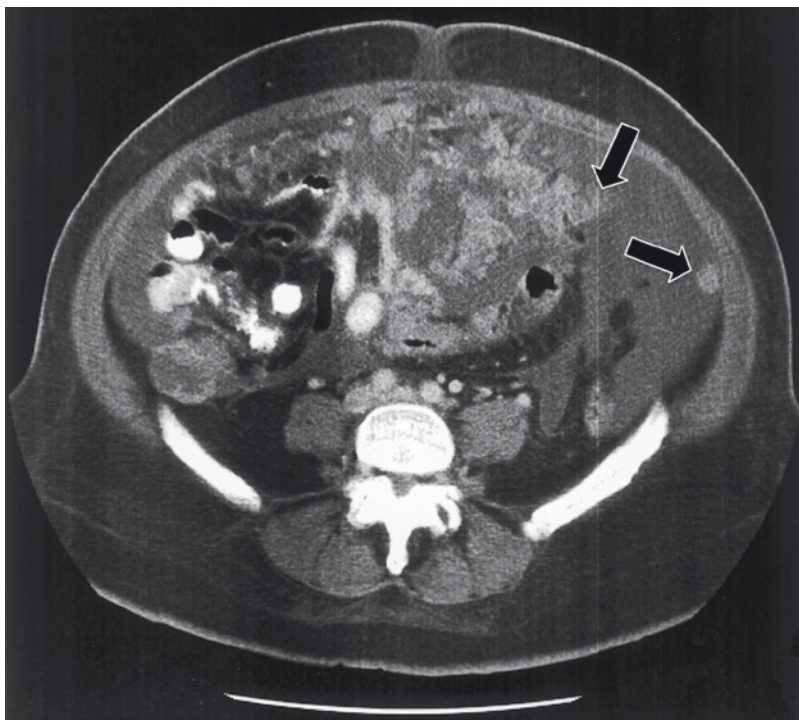


FIGURE 8.2. Diffuse peritoneal thickening, outlined by extensive ascites

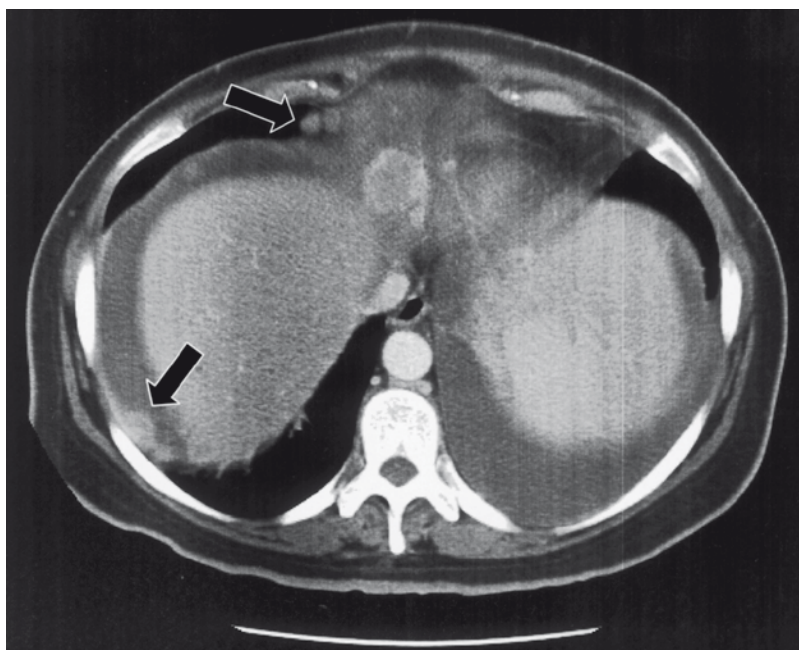


FIGURE 8.3. Diaphragmatic metastases in a patient with advanced ovarian cancer

Discrepancies between the two investigations discussed above left many unanswered questions and the role of CT for defining patients with optimally resectable disease was unclear. In an attempt to explore this further, a multi-institutional reciprocal validation study was performed (Axtell et al. 2007). Data obtained from the investigations by Bristow et al. (2000) and Dowdy et al. (2004) as well as 65 additional patients from four institutions affiliated with the University of California at Los Angeles was included. Data from UCLA was first used to create yet another model to predict surgical outcome. In this group of patients, diaphragm disease > 2 cm and large bowel mesentery disease were significant predictors on multivariate analysis. Next, each of the three independent models was applied to data sets from the other two institutions as a cross-validation. While the UCLA model had an accuracy rate of 77% when applied to their own patient cohort, this rate dropped to 34% and 64% when applied to the Johns Hopkins and Mayo Clinic cohorts, respectively. The accuracy rate of the Johns Hopkins model dropped from 93% to 74% when applied to the UCLA cohort. Similarly, the Mayo Clinic model had an accuracy rate of 79%, but dropped to 48% when applied to the UCLA cohort.

Taken together, these data demonstrate that resectability is a relative term. Cogent prognosticators of suboptimal cytoreduction appear to vary between groups of surgeons with differing surgical techniques and philosophies. Even within single institutions, varying optimal cytoreduction rates between individual surgeons suggest that pertinent parameters to predict surgical outcome will also vary. Ultimately, some individualization is necessary, and

preoperative CT predictors should be used with caution when deciding between surgical cytoreduction and neoadjuvant chemotherapy.

OTHER TECHNIQUES FOR PREDICTING SURGICAL OUTCOMES

Elevated levels of specific proteins have been associated with carcinomas of some organ systems. For example, the correlation between carcinoembryonic antigen (CEA) and colon carcinoma is well known. In patients with advanced ovarian cancer, serum CA 125 is often elevated. However, the specific value varies widely, with some patients having only minimal elevations in the range of 100–200 units/mL, while others may have values > 10,000. This observation has led to speculation that the CA 125 value may be reflective of tumor burden and therefore may be correlated with surgical outcome. Chi et al. (2000) from Memorial Sloan-Kettering Cancer Center performed a retrospective review of 100 consecutive patients with stage III ovarian cancer. Using a receiver operator curve they identified a CA 125 level of 500 units/mL as having the highest predictive value of optimal cytoreduction. For patients with CA 125 values < 500, 73% were optimally cytoreduced whereas only 22% had an optimal cytoreduction if their preoperative CA 125 level was > 500. This simple serum test had a positive predictive value of 78% and a negative predictive value of 73%, not significantly different from results obtained using computed tomography.

In another series of 112 patients with stage III or IV disease, CA 125 > 500 units/mL had a PPV of 74% and NPV of

52% for prediction of optimal cytoreduction (Cooper et al. 2002). However, these findings have not been duplicated at other institutions. For example, CA 125 level was not sufficiently predictive of outcome to be used in the model proposed by Bristow et al. (2000). In the Mayo Clinic series of 87 patients the optimal cytoreduction rate was 71% in patients with a CA 125 level > 500 units/mL, and 69% in those patients with a value < 500 units/mL (Dowdy et al. 2004). Similar findings have been described in other investigations (Memarzadeh et al. 2003; Axtell et al. 2007). The source of this discrepancy is unclear, but may in part be due to differences in overall optimal cytoreduction rates, 45% in the Chi et al. (2004) series and 71% in the Mayo Clinic series. Regardless, while the CA 125 level may be partially reflective of tumor burden and stage, it is clearly not reflective of the location or extent of spread of the tumor. The authors recently optimally debulked a 45 pound ovarian cancer in a patient with a CA 125 of 23,000. This patient had no peritoneal disease, benign lymph nodes, and was thus rendered optimal after a simple hysterectomy and bilateral salpingo-oophorectomy. In other instances, patients with mucinous ovarian cancers may have only minimal elevations in CA 125, but harbor diffuse peritoneal seeding that proves unresectable. Furthermore, CA 125 and any modifications of this test are obviously not reflective of the philosophy of the attending surgeon regarding radical surgery and will be unlikely to predict resectability.

Several more sophisticated imaging techniques, including MRI and PET, have also been investigated in this context (see other chapters in “General Imaging

Applications” for further discussion of these modalities in oncology). In a prospective investigation of MRI, 34 patients with suspected recurrent ovarian carcinoma were studied (Forstner et al. 1995a,b). Magnetic resonance imaging was successful in finding recurrent cancer in 70% of patients. However, accuracy for lesions < 2 cm was only 35%, and there was very poor sensitivity for prediction of implants on the mesentery or peritoneum. This finding is particularly disappointing given that most patients who undergo suboptimal cytoreduction do so because of diffuse peritoneal disease measuring < 2 cm in diameter. The low accuracy of MRI for detecting disease within the small bowel, mesentery, and lesser sac has been confirmed by other investigators (Ricke et al. 2003). In a prospective evaluation using CT in 91 patients and MRI in 46, these two imaging techniques were equally effective in predicting surgical outcome ($p = 1.0$) (Qayyum et al. 2005). This finding has been reproduced by others (Semelka et al. 1993; Buist et al. 1994). Additional reports have demonstrated that the staging accuracy of MRI is no better than 75% (Stevens et al. 1991; Forstner et al. 1995a,b). Despite assertions by some of the above authors that MRI may help identify patients with unresectable tumor, no study has shown it to offer any real advantage over CT despite the significant increase in cost.

At present, indications for the use of PET are still in the process of development. Although no studies have investigated this technique to predict surgical outcomes in patients with ovarian cancer, data collected in other settings provide some indication of its potential. This technique was performed prior to laparotomy

in a series of 40 patients with suspected ovarian cancer (Schroder et al. 1999). Malignancy was found in 30 patients and 1 false positive test was noted in a patient with a tubo-ovarian abscess. Furthermore, two borderline tumors and a low grade ovarian cancer were missed by PET. Although this investigation was not designed to predict surgical outcome, they did find that PET was not particularly useful for detecting peritoneal carcinomatosis, a common reason for suboptimal cytoreduction. Although specificity was 100% for carcinomatosis in this group of 14 primary ovarian tumors, sensitivity was 71% and accuracy 85%. As is the case with MRI, the accuracy of PET will need to be improved significantly in order to justify the significant costs. It will also be necessary to demonstrate an associated reduction in operative morbidity with the use of these techniques, yet there must be a preservation of overall rates of optimal cytoreduction. This would ensure that patients who would have been optimally resected are not declined surgery.

Additional technologies are developing rapidly and may ultimately give the surgeon an understanding of relative tumor burden as well as detailed information regarding the distribution of spread that may prove clinically useful. Particularly valuable would be a reliable indicator for diffuse serosal involvement of the small bowel, a finding that generally results in suboptimal cytoreduction. Promising research continues with carrier proteins and fluorescent dyes which may improve upon current techniques. The folate receptor has proven useful to selectively tag cancerous cells with iron oxide or silica nanoparticles which can then be imaged with high sensitivity (Choi et al. 2004;

Santra et al. 2005). Further discussion of molecular imaging can be found in another chapter in this volume.

CONCLUSION

During the past several decades, multiple investigations have demonstrated that the volume of tumor remaining after cytoreduction is the only prognostic factor in the control of the surgeon. Conversely, suboptimal cytoreduction has been shown to offer no more benefit to the patient than deferring surgery altogether. Surgical debulking is a potentially morbid procedure and techniques are needed to predict preoperatively which patients are not resectable. While several models have been proposed using specific CT parameters to predict surgical outcome, none have been validated in independent patient cohorts. Given the wide disparity in optimal cytoreduction rates, surgeon philosophy, and radicality present between, and even within institutions, CT is unlikely to reliably predict surgical outcome in patients with ovarian cancer to an extent that surgery could be reliably deferred. Magnetic resonance imaging, PET, and serum CA 125 are no more accurate than CT in this context, but it is hoped that emerging technologies will improve upon our current capabilities.

It is important to recognize that while no preoperative evaluation to date has proven particularly useful for predicting surgical outcome, it does not belie their usefulness for preoperative planning. There is no question that many of these modalities are obligatory for proper surgical planning. In our practice, we rarely utilize MRI preoperatively, but nearly universally perform CT.

This allows the surgeon to counsel the patient as to the likelihood that more extensive procedures would be performed, including colon resection or splenectomy. Furthermore, visualization of parenchymal liver metastases allows the opportunity for preoperative consultation with a hepatobiliary surgeon. Developing technologies may soon allow us to make more informed decisions regarding which patients stand the most to gain from initial laparotomy and cytoreduction, reducing the number of patients who suffer unnecessary morbidity.

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Part II

Renal Cancer

A. Treatment

9

Renal Cell Carcinoma: Follow-Up with Magnetic Resonance Imaging After Percutaneous Radiofrequency Ablation

Elmar M. Merkle, Rendon C. Nelson, and Jonathan S. Lewin

INTRODUCTION

The estimated number of new cases of renal cell cancer (RCC) in the United States in 2005 was 22,490 for men and 13,670 for women (Jemal et al. 2005). This equates to greater than a 30% increase in the incidence of RCC over the last 10 years and a greater than 100% increase in the incidence of RCC since 1950 (Boring et al. 1994; Zagoria 2003). Most of this increase has occurred because of the diagnosis of small, localized tumors detected incidentally in patients imaged for other reasons (Chow et al. 1999; Zagoria 2003).

While radical nephrectomy has long been considered the standard treatment for localized RCC, nephron-sparing surgery has grown in popularity (Licht and Novick 1993). Segmental resection is particularly valuable in patients who have undergone a prior nephrectomy or have a contralateral non-functioning renal unit. Other minimally invasive treatment modalities, such as laser ablation and radiofrequency thermal ablation, are increasingly chosen for patients who are either not surgical candidates because of their comorbidities or who refuse surgery. During the past

several years, substantial experience has been gained by numerous research groups in the radiofrequency thermal ablation of patients with RCC (Gervais et al. 2000, 2003; Lui et al. 2003; Mayo-Smith et al. 2003; Raj et al. 2003; Roy-Choudhury et al. 2003; Lewin et al. 2004).

The post-procedure surveillance protocol usually consists of a dedicated contrast-enhanced computed tomography (CT) scan of the kidney performed 4–6 weeks post-ablation and then at 3 and 6 months. Further surveillance scans are usually performed every 6 to 12 months (Gervais et al. 2003). Unfortunately, areas of thermal ablation are poorly visualized on precontrast CT imaging on surveillance scans, and the diagnosis of residual or recurrent tumor is mainly based on contrast enhancement characteristics (Goldberg et al., 2003; Su et al. 2003). While CT as the primary imaging modality is justified because of cost and availability, a substantial number of these patients cannot be exposed to iodine-containing contrast agents due to preexisting allergies or impaired renal function (creatinine levels greater than 2.0 mg/dL) (Gervais et al. 2003). These patients are usually referred

for contrast-enhanced magnetic resonance (MR) scans of the kidneys (Gervais et al. 2003). The subject of this chapter is to describe the MR appearance of renal cell cancer within the first 6 months following radiofrequency thermal ablation.

INVOLUTION OF THE RADIOFREQUENCY INDUCED THERMAL ABLATION ZONE

After an average initial increase in size of ~ 10% on bi-dimensional measurements within the first 2 weeks after ablation, involution of the radiofrequency thermal ablation zones is observed during 6-month follow-up imaging by an average of ~ 30% (Merkle et al. 2005). This involution is most likely caused by the elimination of coagulation necrosis by macrophages and other components of the human immune system and follows the same pattern seen in animal studies of the liver, pancreas, and kidneys (Merkle et al. 1999a,b,c; Crowley et al. 2001).

MAGNETIC RESONANCE SIGNAL CHARACTERISTICS OF RADIOFREQUENCY INDUCED THERMAL ABLATION ZONES

Radiofrequency thermal ablation encompasses a spectrum of tissue damage processes, including deactivation of enzymes, cell membrane rupture, alteration of tissue structure, protein denaturation and aggregation, and vasoconstriction and intravascular coagulation (Graham et al. 1999).

These effects manifest during different temperature elevations and heating durations, and only a subset of these effects are observable as obvious signal alterations in MRI. In addition, various tissue types demonstrate a varied response in terms of MR signal characteristics post-thermal treatment. Graham et al. (1999) have shown that various tissue types can be classified into four groups: fat; fibrous/glandular tissue (such as muscle, liver, or kidney); blood; and neural tissue.

Fat predominantly consists of triglycerides which undergo *reversible* effects during thermal ablation process (Graham et al. 1999). Reversibility explains lack of permanent signal alterations on post-thermal treatment MR imaging in perirenal fatty tissue. In short, thermal ablation zones extending into perirenal fat appear bright on T1- and T2-weighted images (Figure 9.1). In contrast, fibrous/glandular tissue, such as renal parenchyma, demonstrates *irreversible* effects, including denaturation and shrinkage of proteins such as collagen, and increased hydrophobic interactions resulting in the extrusion of water. These irreversible effects most likely cause shortening of the T2 relaxation time after thermal ablation, which ultimately leads to the uniform hypo-intense appearance of ablation zones on T2-weighted imaging (Figure 5.1). This hypo-intense appearance on T2-weighted MR images in humans coincides with experimental data acquired in animal models after renal thermal ablation (Merkle et al. 1999a, b, c; Aschoff et al. 2001); this hypo-intense appearance on T2-weighted MR images also resembles the same pattern seen in the human liver and brain.

On the other hand, the appearance of the thermal ablation zones on unenhanced

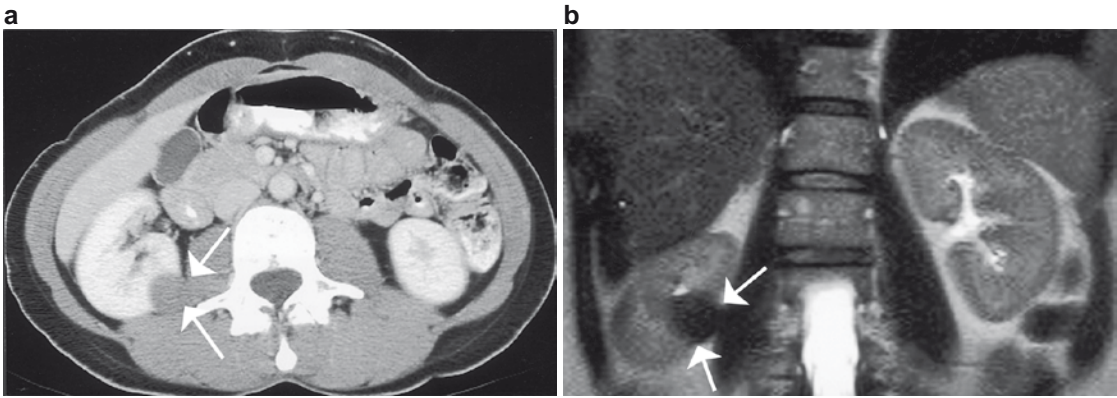


FIGURE 9.1. Forty-six-year-old female with right-sided renal cell cancer. **(a)** Contrast enhanced axial CT acquired before percutaneous radiofrequency ablation shows a solid lesion (*arrows*) within the medial-lower pole of the right kidney that represents a renal cell carcinoma. **(b)** Coronal single shot T2-weighted fast spin echo image acquired 6 months after percutaneous radiofrequency thermal ablation shows a hypo-intense area (*arrows*) within the medial-lower pole of the right kidney that represents the area of thermal ablation

T1-weighted imaging shows a higher degree of variability than the uniform hypo-intense appearance on T2-weighted imaging. While a hypo-intense, iso-intense or hyper-intense appearance of the thermal ablation zone compared to the uninvolved renal cortex is equally common on immediate post-procedure low-field 0.2 Tesla unenhanced T1-weighted spin echo imaging, in the majority of cases, thermal ablation zones appear hyper-intense compared to the uninvolved renal cortex on further follow-up imaging using gradient echo T1-weighted sequences in high-field 1.5 Tesla MR systems (Merkle et al. 2005).

Reduction of the T1 relaxation time during thermal ablation correlates with the degree of tissue vascularity (Graham et al. 1999). Thus, radiofrequency thermal ablation zones appear slightly hyperintense on unenhanced T1-weighted gradient echo imaging of the liver and kidney. Renal radiofrequency thermal ablation zones should appear slightly brighter than hepatic radiofrequency thermal ablation

zones as kidney is more vascular than the liver (Hwang et al. 2004). However, increased vascularity does not explain why renal radiofrequency thermal ablation zones demonstrate a variable appearance on unenhanced T1-weighted imaging, ranging from hypo-intense to markedly hyper-intense compared to uninvolved renal cortex, a finding usually seen to a lesser degree, or not at all, following radiofrequency thermal ablation of focal hepatic lesions.

Procedure-related hemorrhage within the thermal ablation zone is the most likely explanation for this apparent discrepancy. Reasons for this hemorrhage are probably twofold. First, the purely arterial blood supply of the kidneys compared to the mainly portal venous hepatic blood supply to hepatic parenchyma increases the risk of bleeding during renal biopsies. Second, RCC in general is a more hypervascular tumor compared to hypovascular colorectal metastases to the liver. Blood itself exhibits an abrupt decrease in longitudinal T1

and transverse T2 relaxation times at temperatures greater than 60°C, resulting in its hyperintense appearance on T1-weighted imaging (Graham et al. 1999). Intra- or peritumoral hemorrhage may also explain why the hyperintense appearance of renal radiofrequency thermal ablation zones is more often appreciated on gradient echo high-field imaging than on spin echo low-field imaging. On post-contrast T1-weighted imaging, no significant enhancement is observed within the radiofrequency thermal ablation zone (Fig. 1). However, rim enhancement is noted on all post-contrast post-ablation scans. Rim enhancement resolves gradually over time and is barely detectable at the 3-month examination.

RESIDUAL OR RECURRENT TUMOR

The major reason for surveillance imaging post-renal radiofrequency thermal ablation is the early detection of residual or recurrent tumor. While post-contrast magnetic resonance imaging findings are quite similar to the findings seen on CT imaging, the radiofrequency thermal ablation zone is also very well depicted on precontrast T2-weighted imaging. This additional information can be very helpful as residual renal cancer is best seen on both fast spin echo T2-weighted and contrast-enhanced T1-weighted images (Merkle et al. 2005).

In summary, radiofrequency thermal ablation zones in the kidneys follow the same pattern as radiofrequency thermal ablation zones in the liver in terms of the temporal evolution of their size. After an initial increase in size within the first 2 weeks, gradual involution occurs in the course of follow-up imaging. While signal

intensity characteristics of the liver on T2-weighted imaging are the same as those in the liver after radiofrequency thermal treatment (both appear hypo-intense), the appearance of the renal radiofrequency thermal ablation zone is different on T1-weighted images, where the thermal ablation zones are brighter than those in the liver. This is best appreciated on gradient echo images and most likely reflects hemorrhages within the thermal ablation zone. The high vascularity of RCC and the purely arterial blood supply to the kidneys (compared to the mainly portal venous blood supply to hepatic parenchyma) may explain this finding.

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10

Metastatic Kidney Cancer: Treatment with Infusional Interleukin-2 Plus Famotidine

Walter D.Y. Quan, JR and Francine M. Quan

INTRODUCTION

The administration of high dose continuous infusion Interleukin-2 is able to elicit cytotoxicity of cancer cells by lymphocytes, predominantly CD56 positive natural killer cells. These Lymphokine Activated Killer cells (LAK) are able to lyse natural killer cell-resistant tumor cells in vitro and renal cancer cells in vivo (Ellis et al. 1988; McMannis et al. 1988; Weil-Hillman et al. 1989; Horton et al. 1990; Dillman et al. 1993). Moderate to high-dose infusional Interleukin-2 (9–18 MIU/m²/24 h × 72–120 h) yields tumor response rates of up to 26% (Foon et al. 1992; Dillman et al. 1993). Importantly, complete responses have been seen. Numerous trials have sought to increase the response rate for patients with kidney cancer. These have included devising regimens with other immunologic agents and/or cultured effector cells (Sosman et al. 1988; Kradin et al. 1989; Dillman et al. 1991; Figlin et al. 1999). None of these approaches appear to improve response rates compared to Interleukin-2 alone.

The antihistamine famotidine is an agent which may augment the antitumor ability

of lymphocytes. Tsunoda et al. (1992) described that in the presence of famotidine, lymphocytes displayed significantly enhanced uptake of radiolabelled Interleukin-2, resulting in higher tumor cell cytotoxicity by LAK and other tumor infiltrating lymphocytes. The dose of famotidine required for this effect corresponds to a clinically achievable dose. Other investigators have described greater infiltration of cancers in patients treated with famotidine preoperatively (Parshad et al. 2002). For these reasons, the combination of high-dose infusional Interleukin-2 with famotidine has been explored in patients with metastatic kidney cancer (Quan et al. 2004, 2006). With this regimen, we have seen activity in this disease including patients who are now disease-free.

PATIENTS AND METHODS

Twenty-one patients with measurable evidence of histologically-confirmed metastatic kidney cancer have been treated. Patients felt to be candidates for this therapy met previously published eligibility criteria (Quan et al. 2006).

Eastern Cooperative Oncology Group (ECOG) performance status ≤ 1 , estimated survival of at least 3 months, white blood cell count $\geq 3,500/\text{mm}^3$, platelets $> 100,000/\text{mm}^3$, hemoglobin $> 9.0 \text{ gm/dL}$, and serum creatinine $< 2.0 \text{ mg/dL}$. Patients were excluded for autoimmune diseases such as inflammatory arthritis which could potentially be exacerbated by Interleukin-2, any medical illness requiring corticosteroids or other immunosuppressive agents such as methotrexate, current untreated brain metastases, and history of significant cardiovascular disease including myocardial infarction, congestive heart failure, primary cardiac arrhythmias, angina pectoris, or cerebrovascular accident. Informed consent was obtained from all patients prior to study. Prior to treatment on this regimen, all patients had to have undergone low-level cardiac stress test and/or cardiac evaluation to exclude occult atherosclerotic heart disease.

All patients underwent complete history and physical examination, complete blood count with differential (CBC), hepatic profile, serum creatinine, chest x-ray or chest computed tomography (CT) scan, tumor measurements, and evaluation of performance status. Blood studies including CBC, liver enzymes, electrolytes, and creatinine were obtained during the course of the inpatient IL-2 therapy. Radiographic studies to evaluate for response were done after every 2 cycles. Standard response criteria for biologic therapy were utilized (Dillman et al. 1997a,b). Partial responses were measured from the date that response was determined by tumor measurements.

Four patients received Interleukin-2 $9 \text{ MIU/m}^2/\text{day}$ in 500 cc 5% dextrose stabilized with 0.1% albumin by continuous infusion for 72 h; 17 patients received Interleukin-2

$18 \text{ MIU/m}^2/\text{day}$ in 500 cc 5% dextrose stabilized with 0.1% albumin by continuous infusion for 72 h. All patients received famotidine 20 mg intravenously twice per day during the continuous IL-2 infusions.

All patients received their treatment from oncology trained nurses on either an oncology floor or a Stem Cell Transplant Unit. Treatment cycles were repeated every 3 weeks for 4 cycles, then every 3–4 weeks for 2 cycles, then every 4–6 weeks in the absence of disease progression or intolerable toxicity. Patients were premedicated with ondansetron 32 mg intravenously per day in divided doses, meperidine 25 mg IV when needed for rigors, acetaminophen 650 mg orally every 6 h, and ibuprofen 400 mg four times per day orally if needed. Patients were examined daily to assess their tolerance of therapy. Electrolytes were replaced intravenously on a daily basis. For systolic blood pressures $< 90 \text{ mmHg}$, patients received dopamine starting at 5 mcg/kg/min and titrated to keep systolic blood pressure $> 90 \text{ mmHg}$. Patients experiencing serum creatinine $\geq 2.1 \text{ mg/dL}$ or urine output $< 250 \text{ cc/12 h}$ were treated with dopamine 1 mcg/kg/min for renal perfusion. No IL-2 doses reductions were permitted during a cycle. Patients experiencing grade III toxicity unresponsive to standard measures had their infusions stopped. Once infusions were stopped for toxicity, they were not restarted during that cycle.

Patient characteristics are listed in Table 10.1 and include male predominance, median age 60, good Eastern Cooperative Oncology Group performance status (presence of only mild symptoms related to cancer), and just under half of the patients had undergone no prior systemic treatment for their metastatic disease.

TABLE 10.1. Patient characteristics

Male/female	15/6
Median age (range)	60 (29–75)
Performance status (median)	1
Most common metastatic sites	
Lung	14
Lymph node	9
Bone	7
Liver	5
Prior therapy for metastases	
None	10
Interleukin-2	9
Interferon	4
Other immunotherapy	3
Chemotherapy	5
Hormonal	1
Radiation	4
Surgery	3

RESULTS

A total of 89 cycles were administered. The median number of cycles received was 3 (range: 1–10). The most common toxicities were hypophosphatemia (85%), hypomagnesemia (85%), increased creatinine (76%), fever (71%), rigors (67%), nausea/emesis (62%), hyponatremia (57%), pulmonary edema (57%), and metabolic acidosis (52%). Sixty-four percent of the scheduled infusions were completed. The most common reasons for the cessation of IL-2 administration were hypotension (11), pulmonary edema (7), and elevated creatinine (6). Overall, while patients experienced substantial toxicity requiring supportive care, this toxicity was rapidly reversible. Patients were typically able to be discharged from the hospital on the morning after having completed their Interleukin-2 infusions. Seven patients experienced pulmonary edema as seen on physical examination by two different examiners or by chest x-ray. All episodes

of pulmonary edema were reversible with intravenous furosemide and no patients required endotracheal intubation.

Eight responses occurred for a total response rate of 38% (95% Confidence Interval: 21–59%). One patient had a complete response of his bone metastases. Seven other patients had responses in sites including lungs, lymph nodes, liver, and bones. The median duration of all responses was 7 months. All major responses occurred in patients treated at the 18 MIU/m² dose level. An additional patient treated on the 9 MIU/m² dose level had a decrease in pancreatic and lung metastatic sites which did not meet the criteria for a major response and was not included as a responder. This minor response was ongoing at 4+ months. Three patients, the complete responder and two other patients who have undergone surgical resection of residual disease, one of the original kidney primary and the second, a solitary abdominal soft tissue site, are currently free of disease at a median of 42+ months.

Four of the 10 previously untreated patients have experienced a major clinical response. Four of 11 patients who had progressive disease on other therapies (including 9 who had had previous therapy with low-dose Interleukin-2) prior to receiving treatment on this regimen have achieved a partial response. The median survival of all 21 patients treated with this regimen, including four patients recently enrolled, has not been reached at 6+ months. Of note, five patients are alive at >36+ months.

DISCUSSION

High-dose Interleukin-2 may be given by either infusional dose of ≥ 18 MIU/m²/day or in the older bolus (600,000 IU/kg

every 8 h) schedule, yielding typical response rates of ~15% (Dillman et al. 1993; Fisher et al. 2000). Weiss et al. (1992), in the only large randomized study comparing infusional with bolus IL-2, found no statistically significant difference in either total or complete response rates (both groups also received ex vivo activated LAK cells).

Type-2 histamine antagonists were previously described as having the ability to augment lymphocyte killing of cancer cells. Cimetidine has been shown to enhance natural killer cell activity (Flodgren and Sjogren 1985; Kikuchi et al. 1985; Allen et al. 1987). Famotidine increases the tumor infiltration by lymphocytes (Parshad et al. 2002). Tsunoda et al. (1992) described increased in-vitro LAK activity with famotidine. This has been attributed to increased uptake of IL-2 by the IL-2 receptor when exposed to famotidine. Such an action is of value because the IL-2 receptor needed to induce LAK activity has relatively low affinity for the cytokine, necessitating either high IL-2 serum concentrations or continuous exposure.

To our knowledge, no other clinical studies have purposefully examined the combination of intravenous famotidine with high-dose infusional Interleukin-2. A review of prior kidney cancer trials in which various oral antihistamines were given to prevent gastrointestinal toxicity related to concurrent continuous infusion Interleukin-2 revealed seemingly less activity with a total response rate of 22% (95% Confidence Interval: 17–28%) (Walker et al. 2005). It should be noted that the nausea/emesis commonly caused by Interleukin-2 therapy has no bearing on famotidine levels achievable with intravenous famotidine, an advantage of

the current study which may account, at least in part, for the higher response rate.

Progressive disease on prior therapy with low-dose, such as subcutaneously administered, Interleukin-2 did not obviate the activity of our regimen. This may be because, theoretically, high Interleukin-2 serum levels appear necessary to bind to the relatively insensitive IL-2 receptor on lymphocytes to generate LAK activity (Domzig et al. 1983; Yang and Rosenberg, 1997). Such levels are not felt to be achievable with outpatient IL-2 dosing thereby limiting consistent generation of LAK cells. Because our regimen uses high dose continuous infusion Interleukin-2, the failure of prior therapy to generate LAK would have no bearing on the activity of this treatment.

Our experience with this regimen suggests that while toxicity is substantial, no toxicity beyond what is commonly experienced with Interleukin-2 therapy has been identified, and most importantly, these side effects are manageable on either an oncology floor or stem cell transplant/intermediate care unit. There have been no treatment-related deaths, no patients have required Intensive Care Unit admission nor has endotracheal intubation been required in any of the patients treated.

Of note, this IL-2 regimen is tolerated by older patients. Two responses in patients at least age 70 have been seen, one in liver, the other in lungs. We believe that the combination of famotidine with Interleukin-2 can be used in older patients with excellent performance status (Quan et al. 2005).

In conclusion, the combination of infusional Interleukin-2 and famotidine is active in patients with metastatic kidney cancer. While toxicity from this regimen is

substantial, it is reversible and can be managed safely without intensive care support by physicians experienced in the use of Interleukin-2 therapy.

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11

Renal Cell Carcinoma: Preoperative Treatment with Cytokines Followed by Surgery

Tobias Klatte and Malte Böhm

INTRODUCTION

Surgery and immunotherapy are mainstays of the treatment of renal cell carcinoma. Disease confined to the kidney can be cured by surgery alone. However, up to 19% of patients with histopathologically organ-confined disease (i.e., tumor stage pT1-2N0M0 or ROBSON I) die from tumor recurrence in the first 5 years after tumor nephrectomy (Lam et al. 2007). Some reasons and histopathological considerations were reviewed by the authors (Böhm et al., 2002, 2003). Even if one considers the methodological hazards of using different systems of tumor classification (ROBSON versus UICC-TNM classification, modifications of the TNM classification in 1987, 1997, and 2002) it appears that more patients die from recurrent disease after surgically complete resection than can be expected from the frequency of undetected micrometastases at the time of operation. For this and other reasons the suggestion by (Robson et al. 1969) that the renal artery be ligated first and then the specimen be resected and removed *en bloc* is still valued in many surgical departments.

Surgery, including renal surgery is associated with complex perioperative immunodysfunction (Böhm et al. 2001). This involves both cellular and humoral components of the immune system. It was noted that less surgical trauma is associated with less perioperative immunodysfunction and hence better outcome (Whitson et al. 2007), but this association has been challenged (Landman et al. 2004).

Renal cell carcinomas metastasize primarily via the blood circulation into lung, bone, liver, and brain. Hematogenous dissemination of tumor cells is not uncommon in patients with renal cell carcinoma. In 15 of 20 patients with renal cell carcinoma bearing a mutation in the *VHL* gene, this mutation could be detected in the peripheral blood stream (Ashida et al. 2000), suggesting the presence of circulating tumor cells at the time of diagnosis. A similar finding was reported in 13 of 28 patients with organ-confined renal cell carcinoma in whom MN/CA9-mRNA (MaTu N-component/Carbonic Anhydrase isoenzyme 9) was detected in peripheral blood using a reverse transcriptase-polymerase chain reaction

(RT-PCR) assay (McKiernan et al. 1999). After selection of cytokeratin positive cells (i.e., carcinoma cells) from peripheral blood using magnetic cell sorting (MACS), circulating tumor cells were detected in 19 of 59 patients with clear cell renal cell carcinoma (Bilkenroth et al. 2001). Using a RT-PCR assay on buffy coat preparations of peripheral blood cadherin-6 mRNA was found in 15 of 43 patients with renal cell carcinoma without clinically detectable metastases (Shimazui et al. 2004). In a recent study of 41 patients with nonmetastatic renal cell carcinoma, preoperative detection of circulating tumor cells using a RT-PCR assay of the tumor-specific *CA9* gene was associated with worse prognosis (Gilbert et al. 2006). The 5-year disease-free survival rates were 88.1% for patients without and 39.5% for patients with detectable expression of this gene.

Only few of these circulating tumor cells seem to escape elimination by the immune system, and can adhere to and migrate through the vessel wall, invade, grow, and metastasize. An impaired immune defense in the early postoperative phase could, therefore, facilitate this process and dissemination of the tumor. This led to the approach to modulate the immune system shortly before surgery in order to mitigate or counteract perioperative immunodysfunction. Here, relevant clinical studies are compiled. Only those studies are included in this chapter that have been used in a perioperative setting. Adjuvant and neoadjuvant immunotherapies are dealt with in another chapter. Emphasis is placed upon the most common immuno-modulating substances used in patients with renal cell carcinoma: Interleukin-2 and interferon-alpha.

CYTOKINES FOR IMMUNOMODULATION

Interleukin-2 (IL-2)

IL-2 is an established modulator of the immune system, possibly the most effective in a clinical setting. The gene is located on human chromosome 4q. The cDNA codes for a 153 amino acids precursor protein, of which the first 20 amino acids are a signal peptide. The mature 15.5 kDa glykoprotein protects T cells from glucocorticoid induced apoptosis and increases the production of immunoglobulins. In vivo T cells, in particular activated T helper (CD4+) type 1 cells, are the main source of IL-2. The main effect of IL-2 is the activation of a variety of cells, such as T helper cells, cytotoxic T cells, B cells, macrophages, natural killer (NK) cells, and lymphokine activated killer (LAK) precursor cells, of the immune system. Acting as an autocrine growth factor, IL-2 effects the clonal expansion of antigen-activated immune cells; thus, stimulating both cellular and humoral immunity. For these reasons, recombinant IL-2 plays a key role in immunotherapeutic and combined immunotherapeutic/cytotoxic treatment modalities of various malignancies including renal cell carcinomas. In newer approaches using dendritic cells or cancer vaccines IL-2 is used as a costimulating agent.

Interferon- α (IFN- α)

This term includes several structurally related proteins. The gene cluster comprises 13 genes on the terminal short arm of chromosome 9 (region 9p21-pter). The gene product has 165 or 166 amino acids, of which IFN- α 2a, IFN- α 2b, and IFN- α con1 are main pharmaceutical products.

The main source of IFN- α are mononuclear leukocytes, but virtually all cells have the ability to synthesize IFN- α . It inhibits viral replication and enhances expression of class I MHC (major histocompatibility complex) molecules on virus infected cells. In the therapy of renal cancer, IFN- α is used because of a multitude of effects that are, however, only partly understood. It has the ability to activate natural killer cells and macrophages, to stimulate T helper type 1 (Th1) cells, to induce the synthesis of various cytokines and to inhibit cell replication. It also induces terminal differentiation of dendritic cells and inhibits angiogenesis. A direct anti-proliferating effect on kidney cancer cell lines has been described.

METHODOLOGICAL ASPECTS OF PERIOPERATIVE IMMUNOMONITORING

From a methodological point of view, there is still wide disagreement on the appropriate ("surrogate") parameters that can validly assess the function of the human immune system. Three basic discriminations can be made: (1) In vitro tests using cell lines and cultured cells. They feature usually well controlled and reproducible testing conditions, but the parameters measured are artificial and many interactions functional in vivo are not assessable. Conclusions with regard to the human immune system can, therefore, be drawn only with caution. (2) Animal experiments. In vivo interactions of the multitude of factors that constitute the function of the immune system are more easily accessible than with in vitro tests. However, the human immune system

has evolved late during evolution and inter-species variations are vast even between mammals. This makes the translation of results from animal experiments to the human setting not much easier than from in vitro tests. Together with the considerable organizational, ethical, and financial efforts, this may be a reason why animal tests have not gained widespread popularity among researchers of the immune system. (3) Human setting. Modern medicine offers a number of accesses to the human immune system, that do not harm the patient and take advantage of the extensive up-to-date monitoring of patients, in particular oncological patients and those undergoing surgery. Partly due to growing national and international co-operation and collaboration between doctors and researchers surgical, anesthetic and associated procedures are becoming more and more standardized, documented and controlled. This makes it feasible to conduct immunological studies on patients who undergo standard surgical procedures. This setting provides access to the peripheral and tumor supplying blood vessels at various times and, as tissue is removed from most oncological patients during the procedure, also access to relevant tumor and adjacent nontumor tissue. This allows the combination of immunological, functional, and histopathological data in various body compartments. Histopathology can be refined by specific techniques such as immunohistochemistry and laser microdissection in order to combine functional and morphological findings. For these and other reasons the statement appears safe that the setting of patients undergoing surgery offers many advantages for studies on perioperative immunodysfunction and immunomodulation.

From various immunomonitoring arrays that are currently available, the focus below is on flow cytometry (FCM) and enzyme-linked immuno sorbent assay (ELISA), which have been shown to be reliable and clinically applicable methods for the monitoring of immunomodulating therapy with IL-2 and IFN- α . As a rule, immune parameters should be chosen according to the administered immunomodulating agent. In the following two paragraphs we present our preferred methods and discuss some reasons for them.

Flow Cytometry

Flow cytometry (FCM) is a technique used in basic and clinical research to study immune parameters of patients treated with immunotherapy, for monitoring hematologic malignancies, HIV infection, and other diseases. For FCM, cells or their components are stained with fluorescent conjugated monoclonal antibodies and/or fluorescent reagents. After activation with a laser, the emitted fluorescence from each cell is collected by a series of photo-multipliers. The electrical events are collected and subsequently analyzed by a computer that assigns a fluorescence intensity signal in flow cytometry standard data files.

Fluorescence-activated cell-sorting (FACS) is a specialized type of flow cytometry. The acronym FACS is trademarked and owned by Becton Dickinson (BD), but it is used within the scientific community as a general term. FACS allows sorting of cells and thus collection of various specified cell types from a mixture of cells into different containers based upon the specific light scattering and fluorescent characteristics of each cell. Thus, it provides both quantification

and separation of individual cells and possesses the ability to perform multi-marker analyses on a single cell. For FACS analyses, we collected patient's venous blood (100 μ L, EDTA) and stained with monoclonal antibodies conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE), or R-phycoerythrin cyanin 5.1 (PC5). After incubation with the antibody (20 min), erythrocytes were lysed by incubation with a lysing solution for 15 min. After fixation, the leukocyte subsets were determined by flow cytometry using a FACS Calibur with CellQuest Pro software. A typical histogram of an analysis is shown in Figure 11.1.

In our studies, we measured several immune parameters at various time intervals. Peripheral venous blood was collected before (day-7) and during (day-6) the administration of IL-2 or IFN- α 2a, one day before (day-1) and immediately after the operation (day 0), and on the first (day 1), third (day 3), fifth (day 5) and tenth (day 10) postoperative day. The markers included T-cell markers CD3, CD4, and CD8, B-cell marker CD19, monocyte marker CD14,

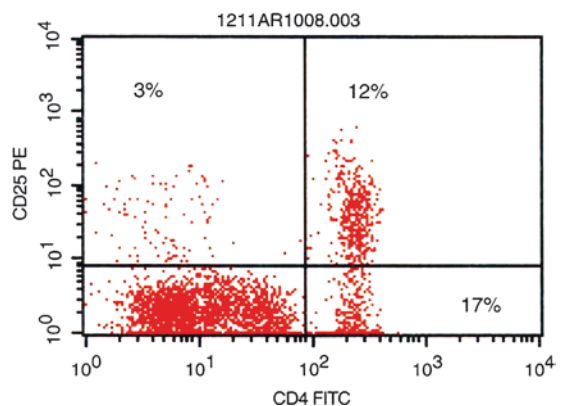


FIGURE 11.1. FACS-histogram for CD4+ and CD25+ cells. The proportion of CD4+CD25+ cells was 12% in this analysis, while 17% were CD4+CD25- and 3% CD4-CD25+

NK (natural killer) cell markers CD16, CD56, and CD57, activation markers CD6, CD25, CD28, CD69, and HLA-DR, and progenitor cell marker CD34.

We processed whole blood samples within a few hours after blood withdrawal. It is safe to process fixed cells within a short period of time because of decreasing marker-associated fluorescence and an increased forward and side scatter, which are used for identification of cells and for exclusion of debris and dead cells. A recent study by (Stewart et al. 2007) showed that fixation causes a significant decrease in both forward and side scatter after 48 h. The decrease in marker-associated fluorescence was up to 39% for CD16 on neutrophil leukocytes 96 h after fixation.

Markers remain stable using different FACS machines (Böhm et al. 2001, 2002; Klatte et al. 2006) indicating that the methodology is mature and stable as is required for (routine) clinical application. We conducted a pilot study in 65 blood samples which were stained with two different antibodies against CD3 from different manufac-

turers conjugated with FITC and PC5, respectively. Correlation analysis of the absolute number of cells showed a mean correlation coefficient of 0.99, indicating a highly significant and reproducible methodology, irrespective of the used conjugation. Furthermore, this significant correlation was present on all assessed days. Figure 11.2 shows the respective curves and correlation coefficients. At this point it is safe to say that FACS analyses need not be carried out in duplicate in such a setting.

For our analyses, we used absolute rather than relative numbers. The authors believe that this approach is generally more accurate than relative values. Relative values are commonly used (Landman et al. 2004), but they poorly reflect the true, absolute changes in the blood stream.

Materials

1. Monoclonal antibodies directed against CD3, CD4, and CD8 (T-cell markers), CD19 (B-cell marker), CD14 (monocyte marker) CD16, CD56, and CD5 (NK cell markers), CD6, CD25, CD28,

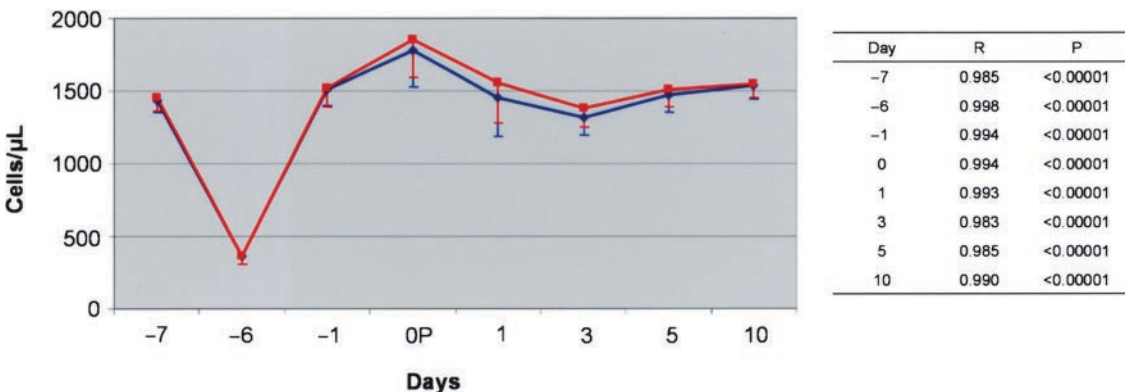


FIGURE 11.2. Correlation analysis of absolute numbers of CD3+ cells, processed at the same time with a differently conjugated antibody. Mean correlation coefficient (R) was 0.99. Each correlation was highly statistically significant ($p < 0.00001$)

CD69, and HLA-DR (activation markers), and CD34 (progenitor cell marker), conjugated with FITC (Fluorescein-Isothiocyanate), phycoerythrin, or PC5 (R-phycoerythrin-cyanin 5.1, Immunotech, Marseille, France)

2. BD-FACS-Lysing-Solution (Becton Dickinson, Heidelberg, Germany)
3. BD-Cell-Wash-Solution (Becton Dickinson, Heidelberg, Germany)
4. BD-CellFIX-Solution (Becton Dickinson, Heidelberg, Germany)

Methods

1. Incubate whole blood (100 μ L, EDTA) for 20 min with the monoclonal antibody directed against the respective CD marker. The amount of antibody that has to be added varies (see manufacturer's instructions).
2. Lyse erythrocytes by incubation with 2,000 μ L FACS lysing solution for 15 min. Centrifuge at 1,600 rpm for 5 min and suck off lysed erythrocytes.
3. Wash cells by adding 2,000 μ L BD-Cell-Wash-Solution, centrifuge at 1,600 rpm for 5 min and suck off supernatant.
4. Add 400 μ L BD-CellFIX-Solution.
5. Store samples at +4°C until analysis with flow cytometer.

We used absolute rather than relative values. For this reason, an additional differential blood count of the whole blood sample was performed (Coulter Electronics, Krefeld, Germany). From the relative values indicated by flow cytometric analysis, absolute numbers of the respective cellular parameter were calculated with the formula: absolute value of the cellular parameter [cells/ μ L] = absolute value of lymphocytes/monocytes [cells/ μ L] $\times$ relative value of the cellular parameter [%].

Enzyme-Linked Immunosorbent Assay

Enzyme-linked immuno sorbent assay (ELISA) is a technique used to detect the presence of an antibody or an antigen in a sample. The sample with an unknown amount of antigen is immobilized on a solid support by adsorption to the surface or by an antigen-specific antibody. The detection antibody is then added, which forms an antigen-antibody-complex. The detection antibody can be linked to an enzyme or can be visualized through a secondary enzyme-conjugated antibody. Between each step, the plate is washed to remove nonspecifically bound elements. An enzymatic substrate is finally added, resulting in the production of a visible signal, whose intensity correlates with the quantity of the antigen. Samples should be processed according to manufacturer's instructions. Each sample should be assayed in triplicate in order to validate reliability and stability of the method; the mean is then calculated and used as final value. All samples from one patient are assayed simultaneously. Optical density is determined on a microtitre-plate reader (e.g., Benchmark Plus Microplate Reader, Tacoma, WA).

Materials and Methods

Sample preparation:

1. Use citrate-supplemented tubes for blood collection.
2. Processed samples for plasma within 1 h after blood withdrawal with two-step centrifugation (10 min at 3,000 rpm, 6 min at 4,000 rpm).
3. Prepare plasma aliquots and store at -80°C until analysis.

For clinical purposes, it is recommended to use ELISA kits provided by various manufacturers. The requirement of serum

or plasma samples depends upon the antigen or antibody of interest. Other than for FACS analyses, we recommend at this point that each ELISA analysis is carried out in duplicate or triplicate. Further, if more than one sample from one patient has to be investigated, all samples of the same patient should be studied with the same assay (Klatte et al. 2007a).

PERIOPERATIVE IMMUNOMODULATION WITH INTERLEUKIN-2

In an early study, 12 patients who underwent open resection of colorectal carcinomas received perioperative low dose IL-2 (1.8 Mio IU/m² twice daily s.c. 1 day before until the fifth postoperative day, total dose 18 Mio IU/m²). An initial decrease of circulating T cells (CD3), in particular T helper cells (CD4), and activation marker CD25 was observed followed by an increase of these parameters on day 7 (Nichols et al. 1992). A similar study reproduced these results (Nichols et al. 1993). The authors reported relative values which hampers comparisons with other studies. The time course of the alterations of the markers CD3, CD4 and CD25 indicates that perioperative immunomodulation should begin earlier before the operation in order to achieve the maximum effect of IL-2 already in the critical early postoperative period.

In a study on nine patients who received a rather low dose of IL-2 (9 Mio IU b.d. s.c. for 3 days starting 4 days before surgery, total dose 54 Mio IU) before they underwent bowel resection for colorectal cancer (Deehan et al. 1995), the percentage of CD25 positive cells and soluble

IL-2 receptor were increased compared with controls. IL-6 peak levels on the first postoperative day were higher in the IL-2 pretreated group. However, seven out of nine patients (versus three of nine controls) received blood transfusions, suggesting that pretreated patients suffered from a higher degree of surgical trauma in this small study. Possibly, immunomodulation was initiated too shortly before the operation to achieve an optimal immune response.

Brivio et al. (1996) reported on two series of a total of 37 patients who received the same IL-2 pretreatment scheme (total dose 54 Mio IU) before they underwent open bowel resection for colorectal cancer. The authors found elevated lymphocyte counts and elevated CD3, CD16, and CD25 levels from the third postoperative day compared with controls, which is consistent with earlier studies (Nichols et al. 1992). The authors also found increased tumor infiltration with eosinophils and a better prognosis of IL-2 pretreated patients. Ten patients with metastatic renal cell carcinoma also received this IL-2 pretreatment scheme (total dose 54 Mio IU) before undergoing tumor nephrectomy (Scardino et al. 1997). All patients and controls received adjuvant IL-2 therapy. Perioperative lymphopenia was neutralized and postoperative complications were reduced in pretreated patients. In a study on twenty NSCLC patients (Masotti et al. 1998) undergoing lobectomy or pneumonectomy who also received the IL-2 application scheme mentioned above (total dose 54 Mio IU) an effect on CD3, CD4, CD8, and CD16 positive cells was seen comparable to other studies (Nichols et al. 1992). Here prolonged survival was reported for the IL-2 treated patients.

In a study on 63 patients (Böhm et al. 2002) who underwent tumor nephrectomy, 26 patients received 4 doses of 10 Mio IU/m² IL-2 twice daily s.c. 7 days before the operation (total dose of 80 Mio IU/m², group *IL-2*); 37 patients received no IL-2 (*TU*). 20 patients who underwent open renal surgery for non-malignant reasons (*NT*), 24 patients whose kidneys were treated with extracorporeal shock wave lithotripsy without anaesthesia (*ESWL*), and 39 healthy probands who received no treatment (*C*) were used as controls. Parameters of cellular and humoral immunity (differential blood count, T-cell markers CD2, CD3, CD4, and CD8, B-cell markers CD19 and CD20, monocyte markers CD13 and CD14, NK-cell marker CD16, activation markers CD25, CD26, CD69 and HLA-DR, as well as the cytokines IL-1 receptor antagonist, IL-2, soluble IL-2 receptor, IL-6, and IL-10) were measured in peripheral venous blood before administration of IL-2, 1 day before and immediately after the operation, and on the first, third, fifth, and tenth postoperative day. In order to assess the effect of anaesthesia, additional blood was drawn from a subset of 18 patients before and after the induction of anaesthesia, but before a skin incision was made.

In an attempt to discriminate whether the changes originate within the tumor bearing kidney or are a phenomenon of redistribution between various body compartments, in a subset of patients ($n = 19$) renal venous blood and a sample from the general blood pool (peripheral or central) were collected at the same time during operation. As expected, little alterations were seen in the *ESWL* group. After open surgery (*TU* and *NT*) leukocytes and granulocytes were elevated until the third postoperative day, but T cell and activation

markers were decreased. The postoperative decrease of CD25 (i.e., membrane bound IL-2 receptor) positive cells was reflected by an increase of soluble IL-2 receptor. Cytokines IL-10 and IL-6 with immunosuppressive properties were elevated postoperatively. These alterations were more marked in carcinoma patients (*TU* vs. *NT*) and remained significant when matched patients with a similar surgical trauma were compared. All patients who received IL-2 suffered from IL-2 related toxicity WHO grade I-II, one patient suffered from toxicity WHO grade III. The alterations of T cell and activation markers were less accentuated in IL-2 pretreated patients. Monocytes and IL-10 and IL-6 levels were lower in pretreated patients. A significant effect of general anaesthesia was not discernible. Renal venous measurements corroborate our previous finding (Böhm et al. 2001) that IL-6 is present in the tumor bearing kidney in higher concentrations than in the general venous blood pool. It may be concluded that T cell mediated immunity and activation of the immune system is impaired and that blood levels of cytokines with immunosuppressive properties are elevated after tumor nephrectomy. Pretreatment with IL-2 can modulate these perioperative alterations.

The time course of the perioperative alterations of T cellular markers CD3 and CD4 and of activation marker CD25 – first described in a clinical setting by Nichols et al. (1992) and reproduced by many others (Klatte et al. 2006) – indicates that IL-2 administration should be initiated at least about a week before surgery in order to allow the immune system enough time to respond. A dose decrement toward the operation can both mitigate counter-regulation (Klatte et al. 2006) that includes

Agent	Study arm	-7	-6	-5	-4	-3	-2	-1	OP
IL-2	IL-2 (Mio.IE/m ²)	2x10	2x10	1x3	1x3	1x3	1x3	1x3	-
	Control	No immunomodulation							
IFN-a	IFN (Mio. IE)	2x9	2x9	1x3	1x3	1x3	1x3	1x3	-
	Control	No immunomodulation							

FIGURE 11.3. Regimen of perioperative immunomodulation with IL-2 (*upper line*), or IFN- α (*lower line*)

IL-2 and its receptor and monocytes and TGF β among others, and avoid cumulating toxicity that would threaten the surgical procedure. A combination with other immunomodulators such as IFN- α is conceivable in order to maximize anti-tumoral effect and minimize toxicity, a principle being used in combination cytotoxic therapy.

Recently, long-term outcome following pretreatment with IL-2 has been reported (Klatte et al. 2006). Of 116 patients enrolled into a prospective phase II trial, 58 received preoperative IL-2 (IL-2 group), 58 did not (control group). Patient and tumor characteristics were balanced between the groups. Median follow-up was 40 months. The regimen of IL-2 administration is shown in Figure 11.3. The 1- and 5-years tumor-specific survival rates for the IL-2 group vs. control group were 98% vs. 81%, and 86% vs. 73%, respectively ($p = 0.04$, Figure 11.4). Furthermore, in multivariate analysis, preoperative administration of IL-2 was retained as an independent prognostic factor of tumor-specific survival. However, this study was not randomized and larger, randomized trials are needed to confirm the superiority of preoperative IL-2 versus the standard approach consisting of nephrectomy alone. A concise synopsis of perioperative immunomodulation in patients with IL-2 in patients has been compiled (Böhm et al. 2003).

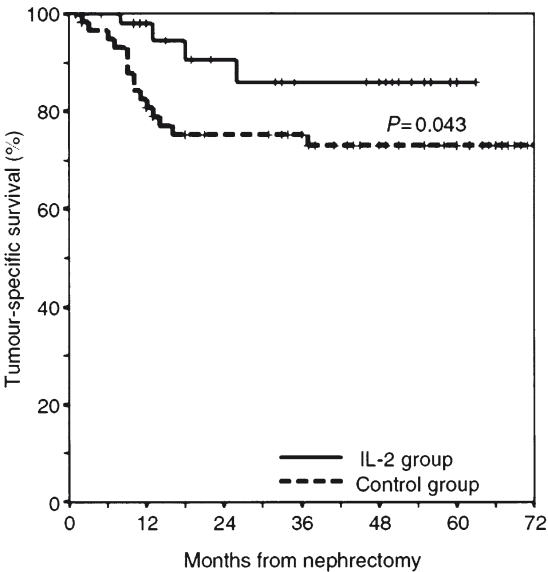


FIGURE 11.4. Kaplan-Meier survival estimates of patients treated with IL-2 (—) and without preoperative IL-2 (---). Tumor-specific survival was significantly longer ($p = 0.04$) for patients who have been pretreated with IL-2 (adapted from Klatte et al. 2006)

PERIOPERATIVE IMMUNOMODULATION WITH INTERFERON-ALPHA

Fewer data than on interleukin-2 are available on perioperative immunomodulation with IFN- α . The available evidence is partly conflicting, and no effects on prognosis have been published. In an early prospective randomized study 15 patients who underwent surgery for gastric or

colorectal cancer received low dose IFN- α (2 Mio. IE/day) from 1 day before surgery until the sixth postoperative day (Sedman et al. 1988), NK cell cytotoxicity, assessed with an in vitro assay against cell line K562, was elevated on days 1 to 5 after the operation, but the postoperative decrease of IL-2 production was not altered. Toxicity of the regimen was low as expected. No effect of blood transfusions on NK cell toxicity was demonstrated. Prior to tumor nephrectomy 12 patients were treated with 3 Mio. IU/day im. IFN- α (Fujioka et al. 1994), and tumor infiltrating lymphocytes were retrieved from the nephrectomy specimen and cultured in an IL-2 enriched medium. The percentage of activated (HLA-DR+) cells and of T suppressor cells (CD8+CD11+) was elevated in pretreated patients but cytotoxicity against cell lines K562 or Daudi, and against allogenic or autologous renal cell carcinoma cells in vitro was unaltered.

In an uncontrolled study (Houvenaeghel et al. 1997), 23 patients with advanced carcinomas received a two week dose increment of IFN- α (2 to 12 Mio IU/day s.c.) beginning 3 days before surgery. Cytotoxicity of peripheral lymphocytes against cell lines K562 or Daudi, and other parameters were unchanged at four different time points, irrelevant of the IFN- α dose. Toxicity (fever) up to grade 3 was observed, which was expected from the high doses administered. However, no controls make it difficult to assess the effects of IFN- α in this study. The Fas/Fas-Ligand system is functional in the apoptosis of tumor cells. Pretreatment with 5 Mio. IE/day IFN- α for 2 weeks (Sejima and Miyagawa 1999) was associated with an increased portion of Fas positive and apoptotic cells in ten patients. However,

most patients in both the treatment and the control group showed an expression of the Fas ligand. The authors concluded that the tumor cells evade or are protected against Fas mediated apoptosis.

Nichols et al. (1993) administered a combination of IFN- α (2 Mio IU/day s.c.) and low dose interleukin-2 (1.8 Mio. IE/m² twice daily s.c.) to 17 patients who underwent open resection of colorectal carcinoma beginning a day before surgery until the fifth postoperative day. In a similar fashion as in an earlier study by the same group using interleukin-2 only (Nichols et al. 1992) T cells (CD3), in particular T helper cells (CD4), and IL-2 serum levels decreased initially. However, only relative values are presented which hampers the comparison with other studies.

Recently, we reported (Klatte et al. 2008) the results of a phase II trial on peri-operative immunomodulation therapy with IFN- α in renal cell carcinoma. The study design (Fig. 3) was similar to a study that showed a survival benefit for patients who underwent perioperative immunomodulation with IL-2 (Klatte et al. 2006). Fifty-four consecutive patients were enrolled and assigned to the two study arm alternatingly. Twenty-seven patients received immunomodulation with IFN- α (4 doses 9 Mio IU. s.c. a week before operation, followed by a daily dose of 3 Mio. IU until a day before the operation), and 27 did not. The study groups did not differ with respect to age, sex, tumor stage and grade, histological type, operation time and technique. IFN- α related toxicity was WHO grade 0 (11%), 1 (59%), 2 (26%), and 3 (4%). During IFN- α administration leukocytes, CD19, HLA-DR and VEGF dropped significantly, while no difference was

observed in T-cell and NK-cell markers, and IL-10. All patients showed postoperatively elevated leukocyte counts. T-cell and activation markers decreased, but CD3, CD4 and CD28 alterations were significantly less accentuated in patients who had been treated with IFN- α . IFN- α did not improve tumor-specific survival in this study. Possibly, however, the time course of IFN- α action is slower than of IL-2, and beginning immunomodulation with IFN- α earlier before surgery would increase the effect. Clearly, more studies are needed here. A concise synopsis of perioperative immunomodulation in patients with IFN- α in patients has been compiled (Böhm et al. 2003). A Medline search yielded no further results (July 14, 2009).

OTHER AGENTS

Episodical reports are available on other substances used in perioperative immunomodulation in man, of which a selection is reviewed below. However, comparison is hampered because the reports are largely unsystematic and vary widely in terms of scientific approach, study design, and parameters assessed. Interferon- γ (100 μ g s.c. 7, 5, and 3 days before surgery) was given to 24 anergic, i.e., with a negative delayed-type hypersensitivity skin test, patients undergoing major surgery in a randomized study (Schinkel et al. 2001). This regimen caused mild toxicity and resulted in elevated prostaglandin E₂, tumor necrosis factor alpha, and IL-6 levels in supernatants of mitogen stimulated whole blood cultures. No clinical effect was observed in this study, in particular no reduction of infectious complications.

Twelve patients undergoing abdominal surgery were given ranitidin (50 mg every 6 h for 72 h after skin incision) (Nielsen et al. 1989). This resulted in a mitigated attenuation of delayed type cutaneous hypersensitivity and an increased stimulated NK cell activity and mitogen-stimulated lymphocyte proliferation in vitro. The results were reproduced with a similar regimen (100 mg ranitidine i.v. twice daily for 4 days after skin incision followed by 150 mg p.o. for a further 5 days). Here, infectious postoperative complications were less frequent in treated patients (Nielsen et al. 1994). Perioperative administration of cimetidin (400 mg preoperatively and 33 mg/h until the fifth postoperative day) in ten patients undergoing coronary bypass surgery resulted in an attenuated postoperative decrease of T suppressor (CD8+) cells and NK cell activity (Katoh et al. 1998).

Supplementation of parenteral nutrition with 0,18 g/kg/day glutamine until the sixth postoperative day in ten patients was accompanied by an increased T cellular DNA synthesis, but T cellular IL-2 production, and mononuclear IL-6 and TNF production remained unchanged (O'Riordain et al. 1994). Supplementation of glutamine 24 h before until 72 h after major abdominal surgery restored ex vivo lipopolysaccharide-stimulated TNF-alpha production earlier than in controls (Exner et al. 2003), from which the authors deduced a reduced perioperative immunosuppression in verum patients. Supplementation of polyunsaturated *n* - 3 fatty acids had a similar effect (Weiss et al. 2002) and reduced postoperative IL-6 serum levels but improved granulocyte/monocyte function (measured as monocytic HLA-DR expression) in 12 patients undergoing major abdominal surgery. Preoperative treatment with

thymostimulin of 18 patients with ENT tumors was associated with increased T cell infiltration of the connective tissue surrounding adjacent to the tumor and an improved cluster formation of dendritic cells with T cells (Kerrebijn et al. 1996). The authors concluded that pretreatment with thymostimulin improves cellular immunity. Perioperative application of mistletoe (Isorel) for two pre- and two postoperative weeks attenuated postoperative immunodysfunction in an uncontrolled study of 40 patients undergoing surgery for gastrointestinal tumors (Enesel et al. 2005).

Preoperative administration of the T cell activator lentinan (2 mg 7 days before coronary bypass surgery) in ten patients resulted in a quicker postoperative return to baseline of the percentage of T helper (CD4+) cells and prevented a decrease of NK cell activity (Hamano et al. 1999). The anti-inflammatory cytokine IL-1 receptor antagonist, leukocytes and soluble TNF receptors (p55 und p75) were elevated postoperatively in 19 patients undergoing open resection of an oesophageal carcinoma who had received granulocyte colony stimulating factor (G-CSF) 2 days before until 7 days after the operation (Hubel et al. 2000). No secondary wound healing was observed in treated patients, compared with 30% in an untreated historical control group. All these episodic reports are not systematic, and no guidelines of perioperative immunomodulation can be deduced from them. They do, however indicate that perioperative immunomodulation may improve outcome both in terms of wound healing and – to date less clearly demonstrated – in terms of survival, which is the crucial parameter in oncology.

CONCLUSIONS AND FUTURE DIRECTIONS

Complex immunodysfunction occurs in patients early after open renal surgery (Böhm et al. 2001). Some of these alterations are associated with the surgical trauma itself and related procedures. However, T cellular dysfunction and early postoperative elevation of cytokines IL-6 and IL-10 which have immunosuppressive properties are more pronounced in renal cell carcinoma patients. Pretreatment with IL-2 appears to be a feasible, yet investigational approach to complement surgical therapy and modulate some alterations of the immune system that occur peri-operatively in patients undergoing tumor nephrectomy. It modulates, in particular, T cellular immunity and attenuates the early postoperative increase of tumor-suppressive cytokines IL-6 and IL-10. Pretreatment should be commenced about a week before the operation in order to allow the immune system enough time to respond to the challenge and toxicity to subside. Functional data are needed to corroborate the phenotypic data presented here, before a definite recommendation can be given for these patients. In particular, the concept of seeding and circulation of tumor cells during an operation with subsequent formation of micro-metastases, however compelling, is speculative (Klatte et al. 2006) and needs corroboration with clinical survival data. Nonetheless, perioperative immunomodulation with IL-2 appears to prolong tumor-specific survival. Optimal IL-2 dose, duration of administration and possible beneficial co-stimulatory agents remain to be determined. Individualized neoadjuvant regimens (Bex et al. 2006) take advantage of a similar approach to counteract perioperative immunodysfunction in renal cancer patients.

Perioperative immunomodulation may be a promising approach to close the therapeutic gap between neoadjuvant and adjuvant immunotherapy. Although preoperative administration of IFN- α modulates several perioperative immune parameters, no study to date has shown an improvement of survival in man.

The combination of cytokines and angiogenesis inhibitors holds promise for the future. Recently, the FDA approved the tyrosine kinase inhibitors sunitinib and sorafenib, which both inhibit angiogenesis through blockade of both VEGF receptor and PDGF receptor (Klatte et al. 2007b). IFN- α lowers VEGF levels and toxicity of IFN- α in the perioperative setting is low. This implies that the combination of IFN- α with tyrosine kinase inhibitors may have an additive effect. This concept is supported by a recent report, which showed that combination sorafenib and IFN- α produces a response rate of 19%, which is greater than expected with either sorafenib or IFN- α alone (Ryan et al., 2006). Future studies are underway to clarify the question of patient survival with these combined regimens, which may also be applicable in a perioperative setting. IL-2, which appears to be the more potent cytokine in terms of response and survival, might also be combined with tyrosine kinase inhibitors and/or mTOR inhibitors. This possible combination could be of particular value due to the unfavorable toxicity of IL-2. In summary, perioperative immunomodulation might benefit patients with renal cell carcinoma, because sophisticated surgery, including nephron-sparing and laparoscopic or robotic techniques, alone or in combination with radio- or chemotherapy cannot cure metastasized disease.

This promise retains its appeal even in times when the advent of targeted therapies, tyrosine kinase inhibitors, mTOR-inhibitors, and others has upheaved many long-standing paradigms of the therapy of renal cancer.

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12

Metastatic Renal Cell Carcinoma: Use of Bcl-2 and Fas to Predict Responses to Immunotherapy

Yoshihiko Tomita, Ryo Maruyama, Toshiyuki Itoi, and Vladimir Bilim

INTRODUCTION

Because conventional chemotherapy, radiotherapy, and hormonal therapy have no benefit in terms of prolonging survival in patients with metastatic renal cell cancer (mRCC), almost all mRCC patients eventually succumb to disease. Intriguingly metastatic lesions have been known to disappear after the resectioning of the primary RCC although such cases are very rare (Kavoussi et al. 1986). In addition, immunotherapy, such as the administration of interferon-alpha (IFN-alpha) or interleukin-2 (IL-2), has shown promise, although the efficacy varies among cases and the total response rate reaches only approximately 20% (Krown 1987; Fyfe et al. 1995). Immunotherapy should be performed only in selected responders, because the treatment is sometimes accompanied by severe adverse effects especially with IL-2 (Fyfe et al. 1995). The demography favoring a response to immunotherapy includes a good performance status, prior nephrectomy, metastasis predominantly to the lung (Atkins et al. 1993; Fyfe et al. 1995; Figlin et al. 1997; Rosenberg et al. 1998), and only one

site of metastatic disease (Negrier et al. 1998), with a clear cell histological variant (Wu et al. 1998). To date, however, no reliable molecular markers have been identified that can predict susceptibility to immunotherapy.

The present study was performed to elucidate the potential role of the apoptosis-related molecules Bcl-2 and Fas as predictors of the susceptibility of metastatic RCC to immunotherapy. An immunohistochemical examination of tumor tissue from 40 patients with metastatic RCC undergoing postoperative immunotherapy after radical nephrectomy was performed. Patients with progressive disease after immunotherapy had a decreased rate of survival ($p = 0.006$). Progressive disease correlated with a higher proliferation index (PI) in the primary tumor. All primary tumors in cases of complete response or partial response were negative for Bcl-2, while 40.6% of no change + progressive disease patients, were positive for Bcl-2 ($p = 0.0373$). Patients in whom the primary tumors were both Bcl-2 and Fas-negative showed significantly better responses to immunotherapy than the remaining group ($p = 0.0022$). The Bcl-2 and Fas status of

the primary lesion may become useful for the selection of patients with metastatic RCC for immunotherapy.

APOPTOTIC MACHINERY AND TUMOR CELLS

Tumor cells usually evade apoptosis when responding to any anticancer treatment except surgical removal. However, the apoptotic machinery has extreme redundancy among tissues. For instance, the main apoptotic pathway for epithelial cells is different to that for immune cells (Roset et al. 2007). Accordingly, tumor cells derived from any tissue are likely to have the apoptotic pathway of their normal counterparts. Moreover, several genetic or epigenetic alterations in apoptosis-related molecules enable tumor cells to evade apoptotic stimuli (Reed 1999). Therefore, it is of importance to investigate the apoptotic machinery in each malignant tumor, including RCC.

Two major triggers of apoptosis are death receptors and mitochondria. Representative of death receptor is Fas/Apo-1/CD95 activated through polymerization by its Fas ligand and composed of a death inducing signaling complex (DISC) with Fas-Associated protein with Death Domain (FADD) and caspase-8 initiating signal transduction for apoptosis. Mitochondria contain lethal substances in the inter-membrane space, one of which, cytochrome-c, an important component of the electron transport system, is released into the cytosol when the cell is exposed to 'apoptotic stimuli', including ionizing radiation and anticancer drugs. Cytochrome-c composes the 'apoptosome' with Apaf-1 and caspase-9

and also initiates apoptosis. Both molecular complexes activate the initial caspase leading to the processing of the proforms of cell death executor caspases: caspase 3 and 7. The active form of caspase 3 and/or 7 cleaves several substrates leading to cell death with the apoptotic phenotype (Kischkel et al. 1995; Herr and Debatin 2001).

These cascades are modified in several ways. Bcl-2, initially found on chromosome 17p having a translocation of the immunoglobulin gene in B cell lymphoma turned out to be highly homologous with ced-9, coding for an anti-apoptotic molecule in *Caenorhabditis elegans*. Subsequent studies identified members of the Bcl-2 family as either 'pro-apoptotic' or 'anti-apoptotic' (Reed 1998). It is conceivable that the upregulation of 'anti-apoptotic' members renders tumor cells difficult to kill. Indeed, B cell type chronic lymphocytic leukemia cells readily survive without higher proliferative activity and accumulate in blood (Tsujimoto and Shimizu 2000).

FAS-DRIVEN APOPTOSIS AND BCL-2 IN RENAL CELL CANCER CELLS

Metastatic RCC resistant to immunotherapy may have a disadvantage when conducting apoptotic stimuli. Some *in vitro* studies have shown that Fas (CD95/Apo1) promotes and Bcl-2 prevents apoptosis in renal carcinoma cells. Previously, we demonstrated the sensitization of RCC cells to Fas-mediated apoptosis by IFN-gamma *in vitro* (Tomita et al. 2003). This effect was attributed to the regulation of downstream caspases. Cytokines,

including interferons, upregulate Fas expression in RCC cells (Nonomura et al. 1996), and also increase the susceptibility to Fas-mediated apoptosis. In RCC cell lines, a reduction in Bcl-2 has been shown to be associated with increased sensitivity to anti-Fas (Hara et al. 2001). We found that Fas stimulation significantly induced apoptosis in cells with low Bcl-2 levels compared with high Bcl-2 expressers (Tomita et al. 1996). Furthermore, it was shown that the down-regulation of Bcl-2 expression sensitizes interferon-resistant renal cancer cells to Fas stimulation (Kelly et al. 2004). Based on these studies, Bcl-2 may be associated with the resistance of RCC to immunotherapy *in vivo*.

BCL-2 OR FAS AND PROGNOSIS OF RENAL CELL CANCER PATIENTS

Since 1987, we have accumulated specimens from about 500 RCC patients and followed their clinical course. We investigated several apoptotic factors, together with their signaling mechanism and reported the prognostic value of serum soluble Fas in RCC patients (Kimura et al. 1999). Furthermore, frequent expression of Bcl-2 and an absence of p53 gene alterations were found in these RCC specimens (Tomita et al. 1996). These findings indicate that in RCC, Bcl-2 may evade apoptotic stimuli, and RCC patients with high levels of Bcl-2 would have a worse prognosis.

Tumor burden is determined by the balance of cell proliferation (input) and apoptosis (output). It can be assumed that tumors with much higher proliferation

rates result in progression even though they have greater susceptibility to apoptotic stimuli. Therefore, the simultaneous evaluation of proliferating ability, as well as apoptosis, is mandatory. We investigated the expression of Bcl-2 protein, the proliferation index (PI), the apoptotic index (AI), caspase-3, and p53 in 101 RCC specimens. Immunohistochemical methods were applied to Bcl-2, caspase-3, and p53 protein expression. To investigate the proliferative activity and apoptosis of tumor cells, PI and AI were calculated based on Ki-67 and terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labelling (TUNEL)-positive cells, respectively. Bcl-2 expression was detected in 72 out of 101 (71.3%) specimens. Bcl-2 positivity was inversely correlated with PI ($P < 0.0001$) and AI ($P = 0.0074$). Furthermore, Bcl-2 positivity was significantly correlated with better survival ($P = 0.0014$), and was associated with lower stage ($P = 0.0301$) and grade ($P = 0.0020$). Thus, in RCC, frequent Bcl-2 expression was correlated with a favorable character without a higher PI and AI (Figure 12.1a).

This result is contradictory to our hypothesis, indicating that frequent Bcl-2 expression might be a predictor of a better prognosis in RCC patients (Itoi et al. 2004). However, an important finding was that the degree of Bcl-2 expression was inversely correlated with a high PI, indicating that RCC expressing little Bcl-2 is likely to have high proliferative activity. In other words, for RCC cells to survive and progress in the host, RCC should have one of each demographic, ‘calmly progress but die-hard’ or ‘easy to die, but more rapidly proliferates enabling to avoid tumor shrinkage.’

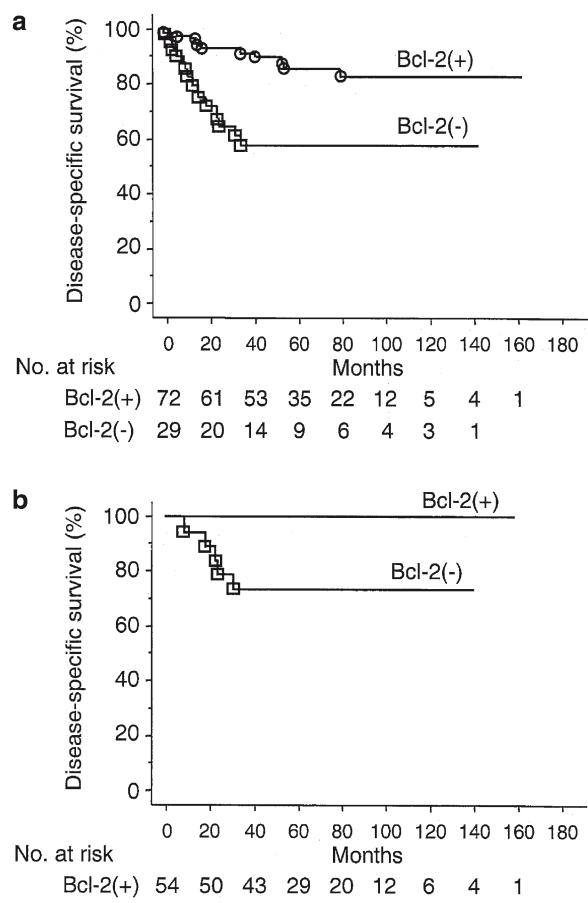


FIGURE 12.1. (a) Disease-specific survival in all cases according to Bcl-2 expression. (b) Disease-specific survival of curatively operated cases according to Bcl-2 expression

**ABSENCE OF BCL-2 AND
FAS/CD95/APO-1 PREDICTS
THE RESPONSE TO
IMMUNOTHERAPY IN
METASTATIC RENAL CELL
CARCINOMA**

The role of Bcl-2 appeared to be equivocal, because the previously mentioned study was performed in a mixed population of RCC patients, i.e., with or without metastatic disease. Indeed, there were no deaths in a patient group with high level of

Bcl-2 in the primary tumor but who were successfully operated on (Figure 12.1b). However, in patients with metastasis at nephrectomy, deaths were occurred even among high Bcl-2 expressers.

More recently, molecular targeted drugs were launched, but clinically available treatments include cytokine therapy using IFN-alpha or IL-2 is still available, and in this series, patients also underwent cytokine therapy. Thus, our interest focused on the detailed clinical course, including responses to immunotherapy in addition to the expression of apoptosis-associated

molecules Bcl-2 and Fas, Ki-67 indicating cell proliferation activity, and the TUNEL apoptotic index in metastatic RCC, which is not a rare clinical problem despite recent advances in diagnostic modalities and techniques detecting in early stage.

Clinical Course of the Patients

Forty patients with metastasis were participants in this study. Various types of therapies were employed, and they contained cytokines IFN-alpha and/or IL-2 as a key drug, and the use of anticancer drugs (such as 5FU or tegafur-uracil) was minimal and at the lowest dose for the sake of modulating tumor cells more susceptible to immunotherapy. Among 40 patients, a clinical response was observed in eight (20.0%), three of which showed a complete response and five a partial response. The metastasis sites in responders were as follows: the lungs in four, and one each in the following sites: brain and lung (which was removed surgically before the start of immunotherapy, and the patient subsequently achieved complete response),

bone and lung (as above), liver only, and lung and mediastinal lymph nodes. Patients with complete responses and/or partial responses survived from 2 months to 49 months. The presence of metastases only in the lung vs. other locations correlated with a longer survival ($p = 0.0333$). These results were not surprising and comparable with previous reports regarding immunotherapy.

The responders (complete response + partial response) had better prognosis than non-responders (no change + progressive disease) though the difference was not statistically significant ($p = 0.0657$) (Figure 12.2).

Expression of Bcl-2

Bcl-2 was expressed in 13 of 40 (32.5%) primary specimens and 2 of 12 (16.7%) metastatic lesions. Bcl-2 expression was not related to T stage or tumor grade. Bcl-2 staining was positive in 13 of 32 (40.6%) primary tumors from patients with no response (no change + progressive disease) to immunotherapy and in

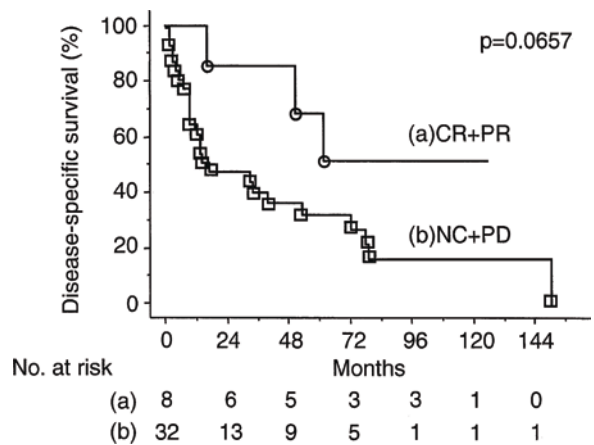


FIGURE 12.2. Comparison of disease-specific survival between complete response + partial response vs. no change + progressive disease in 40 mRCC patients subjected to cytokine-based therapy

zero (0%) of eight responders (complete response + partial response) ($p = 0.0373$) (Table 12.1). In addition, four of the five responders examined were negative for Bcl-2 at metastatic sites. In this series of patients, there was no correlation between Bcl-2 expression and disease-specific survival. In Bcl-2-negative cases, responders to immunotherapy showed a better prognosis than non-responders ($p = 0.0575$). Nevertheless, there was no difference in disease-specific survival between responders and non-responders.

Expression of Fas

Fas was detectable on the cell membrane and within the cytoplasm of cancer cells. It was expressed in 15 of 40 (37.5%) primary specimens and three of 12 (25%) metastatic lesions. Fas expression was not related to T stage or tumor grade. Although there was no correlation between responders to immunotherapy (complete response + partial response) and Fas expression, patients with progressive disease after immunotherapy expressed Fas more frequently in primary tumors than other patients (complete response + partial response + no change) ($p = 0.0484$). Among the eight responders, only one primary tumor expressed Fas (Table 12.1), and all five metastatic tumors available

from responders were negative for Fas, including a metastatic tumor from a patient positive for Fas in the primary RCC. The disease-specific survival of patients characterized according to Fas staining was not different; responders in the Fas(−) group tended to have a longer survival, although it was not statistically significant. The expression of Fas was not correlated with Bcl-2; however, Fas expression was significantly higher in the selected group of Bcl-2(−) nonresponders (91.7%) than responders (8.3%) ($p = 0.0433$). Both the Bcl-2 and Fas negative status correlated with the response (complete response + partial response) to immunotherapy ($p = 0.0022$) (Table 12.1).

DETECTION OF CELL
PROLIFERATION AND
APOPTOSIS

Ki-67 was expressed in the nuclei of cancer cells. The proliferation index ranged from 0.66% to 20.52% (mean 5.28%). It was significantly higher in G3 than G1–2 stages ($p = 0.0287$) and Bcl-2-negative than -positive cases ($p = 0.0390$). Although there was no correlation between proliferation index and response to immunotherapy (complete response + partial response), a higher proliferation index correlated with decreased survival ($p = 0.0189$) and patients with progressive disease presented with a significantly higher PI in the primary tumor ($p = 0.0087$). The apoptotic index ranged from 0% to 2.74% (mean 0.56%). No clinicopathological parameter correlated with the index. We found that patients with metastatic RCC whose primary tumors were both Bcl-2 and Fas-negative demonstrated a better response

TABLE 12.1. Correlation of Bcl-2 and Fas Expression with Response to Immunotherapy.

	Response (%)*		
	CR or PR	NC or PD	<i>p</i> Value
Bcl-2(−) and Fas(−) (n=15)	7 (46.7)	8 (53.3)	0.0022
Bcl-2(+) and/or Fas(+) (n=25)	1 (4.0)	24 (96.0)	

*CR, complete response; PR, partial response; NC, no change; PD, progressive disease.

to immunotherapy in the clinical setting (Maruyama et al. 2006).

CONCLUSION

These series of studies concerning RCC and apoptotic molecules indicate the presence of subgroups of RCCs. As mentioned above, one might ‘calmly progress but die-hard’ and the other ‘easy to die, but more rapidly proliferates’, and as a small subgroup, ‘easy to die but modestly proliferates’ might exist. The first subgroup may have a longer survival just because they do not proliferate, but elude attack by immune cells, and so the patients eventually succumb to disease. The second category may temporarily show a response to systemic treatment including immunotherapy, but overcome this afterwards. The presence of this second subgroup explains why a temporary remission of metastatic disease does not guarantee improved survival in such patients. A third subgroup, possibly up to 5% of metastatic RCC have an unique character susceptible to immune cells but not strong enough proliferative activity to spread and progress in the host, so that patients in this category have a practical and durable response to cytokine therapy (Table 12.2). The prognosis of RCC patients varies, and this hypothesis is not able to explain the response to treatments and clinical course in all

cases. However, especially when we consider cytokine therapies and benefits to metastatic RCC patients, examination of apoptosis-related molecules such as Bcl-2 and Fas may provide a clue for treatment strategy.

Newly developed, so-called molecular targeting drugs, such as sunitinib, sorafenib, bevacizumab, everolimus and temsirolimus have achieved drastic responses and/or prolongation of survival (Escudier et al. 2007; Hudes et al. 2007; Motzer et al. 2007). However, it seems that a complete response, free of cancer, is rarely achieved by these new drugs, whereas IL-2-based therapy renders complete response, though it is experienced in very few patients. That is the reason why IL-2 therapy has not been forsaken in a recent treatment algorism. Furthermore, more recent results revealed that IFN-alpha with a new drug, bevacizumab, represented comparable or even better progression-free survival (Szczylik et al. 2007). These findings indicate that cytokines are not ‘fossils’ yet. However, cytokine therapy should only be performed in those likely to have benefit.

The aim of this series of studies on RCC is on this point. The data shown in the chapter is based on detailed clinical observations and laboratory findings and may be useful for constituting treatment strategies in each case, though confirmation of these findings by others is warranted.

TABLE 12.2. Putative Patient Category Based on Response to Immunotherapy and Proliferation Activity.

Patient category	Proliferation activity <i>Ki-67 positive</i>	Anti-apoptotic property <i>Bcl-2 positive Fas positive</i>	Response to immunotherapy	Prognosis (% of patients)
1	-	+++	-	Better (comprise 80-85%)
2	+++	-	+	Poor (10-15%)
3	+	-	+++	Best (<5%)

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13

Wilms Tumor: Prognosis Using Microvessel Density

Yasemin Ozluk

INTRODUCTION

Wilms tumor (WT), the most common malignant neoplasm of the kidney in infants and children, is a triphasic tumor that mimics various stages of nephrogenesis, composed of epithelial, blastemal and stromal cells. More than 90% of the cases occur in children less than 6-years of age, mostly between 2 and 5 years. WT has overall survival rates exceeding 90% by the improvement of surgical and anesthetic procedures, and the combination of chemotherapy and radiotherapy (D'Angio et al. 1989; Argani and Beckwith 2004).

PROGNOSTIC FACTORS IN WILMS TUMOR

The most important conventional adverse prognostic features are advanced stage, the presence of anaplasia and age of <2 years at the time of diagnosis (Beckwith 1994).

Stage The NWTS staging system for WT is recommended (Beckwith 1998;

Qualman et al. 2003). The staging parameters are as follows:

Stage I

Tumor limited to kidney and completely resected

Renal capsule intact

Tumor not ruptured or biopsied prior to removal

No residual tumor apparent beyond margins of resection

Renal vein contains no tumor (intrarenal vessel involvement may be present)

No lymph node involvement or distant metastases

Stage II

Tumor extends beyond kidney but completely resected

Regional extension of tumor (vascular invasion outside the renal parenchyma or within the renal sinus, and/or capsular penetration with negative excision margin)

Operative tumor spill confined to flank (no peritoneal contamination)

Tumor biopsy (except fine-needle aspiration) prior to surgery

Stage III

Nonhematogenous metastases confined to the abdomen (e.g., tumor in regional lymph nodes), including tumor implants on or penetrating the peritoneum

Gross or microscopic tumor remains post-operatively (tumor at margins at resection)

Tumor spill before or during surgery not confined to flank

Piecemeal excision of the tumor (removal in > 1 piece)

Stage IV

Hematogenous metastases or lymph node metastases outside the abdominopelvic region (beyond renal drainage system, e.g., lung, liver)

Stage V

Bilateral renal involvement at diagnosis (Each side should also be staged separately, according to above criteria)

Anaplasia Tumors displaying anaplasia criteria, which are hyperchromasia, atypical mitoses (multipolar or otherwise recognisably polyploid) and nuclear enlargement with the major dimension at least three times those of adjacent cells, are regarded as unfavorable histology, whereas those that do not fulfill these criteria are defined as favorable histology. Anaplasia is believed to be correlated with responsiveness to therapy rather than with aggressiveness. In this point of view, it is clear that anaplasia confined to kidney does not effect the prognosis, since those tumors can be entirely excised. For this reason, anaplasia was divided into two categories of focal and diffuse. Diffuse anaplasia, which is an adverse prognostic feature, is defined as anaplasia in tumor in

any extrarenal site (vessels in renal sinus, extracapsular sites, lymph node or distant metastases), anaplasia in a random biopsy, or anaplasia unequivocally expressed in one region of the tumor but with extreme nuclear unrest (nuclear pleomorphism or atypia approaching the criteria of anaplasia) elsewhere in the lesion. Those tumors displaying features of anaplasia other than the ones mentioned above are defined as focal anaplasia (Qualman et al. 2003; Argani and Beckwith 2004).

Improvement in new protocols of oncologic treatment lessens the importance of the widely known prognostic factors. Therefore, new prognostic factors have been investigated for various types of cancers in the literature in recent years. Angiogenesis, which is defined as new blood vessel formation from pre-existing vasculature, is an important factor in the growth of solid tumors (Folkman 1971, 1986; Sköldenberg et al. 2001). During the period of tumor cell proliferation, when the cells fall apart from the vasculature, the tumor cannot grow since it has its own new vasculature (Folkman 1971).

ANGIOGENESIS

Quantification Methods

With the invention of antibodies against vascular endothelium, the assessment of tumor vascularity by detecting microvessel density (MVD) depends on two previously introduced quantification methods: counting the vessels in hotspots and concluding an average count on light microscope (Weidner et al. 1991) or obtaining the highest count by using Chalkley grid which is a circle with 25 randomly placed dots (Chalkley 1943).

The principle of the method introduced by Weidner et al. (1991) was based on identifying the most densely vascularized areas (hot spots) under light microscope, and counting the maximal MVD. The average of counts for four or five hot spot areas is recorded for each tumor. In the second method, Chalkley method, with the use of a Chalkley eyepiece graticule, an average of three Chalkley counts is used to represent the tumor vascularity. This method seems to be more objective in identifying microvessels. Chalkley count is related to area and number of vessels (Offersen et al. 2003).

Counting of stained structures per field of view within hot spots, MVD, is the most practically and widespread used method (Weidner et al., 1991, 1992, 1993). The minimum criteria of a countable microvessel is defined as any brown-stained endothelial cell, individually or in cluster, that can be easily separated from adjacent microvessels (Weidner et al. 1991; Hansen et al. 2004; Mineo et al. 2004). Although quantification of MVD by counting blood vessels in hot spots is a widely used method in several studies, it has some limitations such as the subjectivity in the distinction of individual vessels and low reproducibility (Vermeulen et al. 1996; Fox et al. 2000). Computerized analyses have been developed in microvessel assessments to eliminate the subjectivity (Lehr et al. 1997). In computerized analysis, first the area to be measured is selected, and then the program calculates MVD. Chantrain et al. (2003) introduced a software program that was used in quantification of MVD by CD31-immunostaining. They showed that “whole section scanning” method was more reproducible than the quantification on vascular hot spots or on randomly chosen fields.

To my knowledge, there are not many studies comparing the two methods of quantification of MVD in the literature (Fox et al. 1995; Hansen et al. 2004; Offersen et al. 2003). Offersen et al. (2003) compared two methods on 977 various carcinomas. They detected a moderate correlation between the methods. Furthermore, the prognostic impact of the methods was detected to be different according to the type of carcinoma. A comparison of those two methods was also done by Hansen et al. (2004) on 330 invasive breast carcinomas and the prognostic value of MVD was tested. Chalkley count was shown to be more reproducible in their study. The difference of those two methods is that Chalkley count is a measurement of the relative area of vessel profiles. Since Chalkley count is expected to be related to prognosis, microvessels are needed to reach a certain size of metastases (Hansen et al. 2004). On the other hand, Weidner (1995) reported that accurately following the published protocols and properly selecting hot spots by an experienced pathologist increased the reproducibility of conventional method. Since studies on angiogenesis in WT in the literature are in limited number, I have not found a study using Chalkley count or comparing two methods in WT.

Different endothelial cell markers, such as anti-factor VIII-related antigen/von Willebrand's factor, anti-CD34, -CD31 and -CD105, have been used in the evaluation of microvessel density in various human cancers (Sköldenberg et al. 2001; Salvesen et al. 2003; Mineo et al. 2004; Vieira et al. 2004). In a meta-analysis on breast cancer, it was shown that a possibility of staining failure occurs with anti-CD31, whereas anti-CD34 is expressed

more strongly (Uzzan et al. 2004). In another comparative study on cervical cancer, anti-CD34 was detected to be highly sensitive when compared with anti-CD31, although anti-CD34 also marked other tissue compartments (Vieira et al. 2004). Sköldenberg et al. (2001) recommended anti-CD31 for its higher specificity for capillary endothelium than anti-CD34 in a comparative study using different endothelial cell markers in WT. On the other hand, in my experience the use CD31 or CD34 did not matter in a study on 33 WT cases (Figure 13.1) (Ozlu et al. 2006).

Hlatky et al. (2002) discussed the clinical application of MVD in a review article with an emphasis on antiangiogenic therapy. They highlighted that, although MVD is a useful prognostic marker, it cannot be used as an indicator for therapeutic efficacy or as a guide for the stratification of patients. MVD does not reflect the angiogenic

activity or angiogenic dependence of a tumor. In summary, they stated that MVD reflects the metabolic burden of a tumor. Therefore, it was proposed that tumors having more metabolic burden also have high vascular density. In the same article, it was also stated that MVD cannot be used as a predictive factor for antiangiogenic treatment and also can not visualise the efficacy of antiangiogenic agents. Conclusively, they mention that although MVD has prognostic importance, MVD is not individually sufficient to reveal the functional status of tumor vasculature.

Angiogenesis and Wilms Tumor

WT, the most common malignant neoplasm of the kidney in children, is also studied for angiogenic mechanisms and antiangiogenic therapies. Using antiangiogenic therapy as an alternative therapy or a synergistic therapy in medical oncology has gained importance in recent years.

A physician needs a tool to screen the patient population with WT to identify the group of patients who unexpectedly have adverse prognosis and needs aggressive and/or assistant therapies. There are not many studies on angiogenesis in WT in the literature, and the available studies are mostly experimental (Kayton et al. 1999; Rowe et al. 2000; Huang et al. 2001; Kim et al. 2001; Soffer et al. 2001; Frischer et al. 2004)

Rowe et al. (1999) demonstrated a pathological neovascular network related to VEGF in a murine model of WT. Tumor angiography revealed irregular vascular network and no recognizable vascular pattern when compared with the renal parenchyma adjacent to the tumor. The same investigators presented a significant association between VEGF and tumor growth and

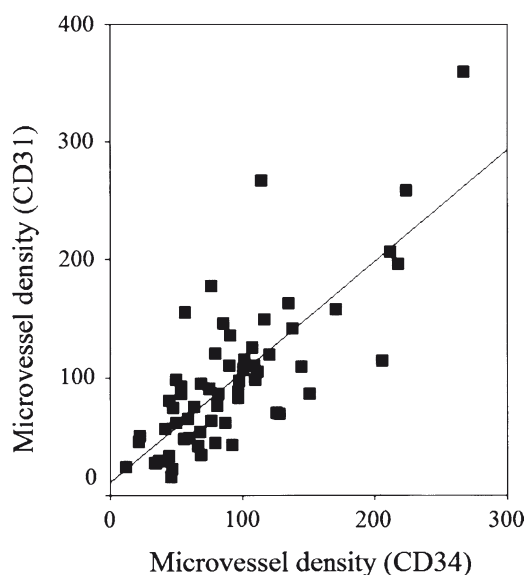


FIGURE 13.1. Microvessel density by anti-CD34 plotted against anti-CD31 in a scatterplot with a regression line illustrating the correlation ($p < 0.001$) (Ozlu et al. 2006)

metastasis in another paper (Kayton et al. 1999). In this study, they used a polyclonal antihuman VEGF antibody by immunohistochemistry to localise the expression of VEGF around the tumor and also used ELISA to identify the VEGF levels. They suggested that VEGF is secreted by tumor cells and subsequently binds to heparan sulfate residues in extracellular matrix where it may activate host endothelium. Furthermore, they demonstrated an increasing frequency of VEGF as the tumor grew. The same investigators showed that anti-VEGF antibodies can significantly reduce the tumor weight (Rowe et al. 2000). They speculated that this reduction occurs by targeting both the induction of neovasculature and tumor cell invasion/intravasation. Although angiogenesis and angiogenetic mechanisms seem to be similar in various tumors, one must keep in mind that response to antiangiogenic therapy may not be much the same. Kim et al. (2001) showed distinct patterns of response in experimental neuroblastoma and WT. Their results demonstrated that antiangiogenic therapies may require modification for different tumors to improve the success of treatment. Specific antiangiogenic therapy was tested by Huang et al. (2001) with RT-PCR. They used anti-VEGF165 aptamer and achieved an effective suppression in primary tumor growth without any adverse effect. They also demonstrated different vascular network and loss of blood vessels in treated tumors when compared with the control group. Their study supported the proposal of modified antiangiogenic therapy by Kim et al. (2001).

Another modification of antiangiogenic therapy is the combination of an antiangiogenic agent (anti-VEGF) with a conven-

tional chemotherapeutic agent. In Wilms tumor, combination therapy has been found to be more effective in a murine model of Wilms tumor in suppressing tumor growth and metastases (Soffer et al. 2001). An alternative target for therapy was shown to be HIF1 α /VEGF cascade (Karth et al. 2000). HIF1 α is a hypoxia activated transcriptional factor, and the product of one of its target genes is VEGF. The coexpression of HIF1 α and VEGF was demonstrated in this study. The authors speculated that HIF1 α and VEGF both play role in tumor angiogenesis in WT. Most studies on angiogenesis in WT are based on VEGF expression and anti-VEGF agents. MVD evaluation is rarely studied. The most comprehensive study on angiogenesis in WT was presented by Sköldenberg et al. (2001). The results of their interpretation of endothelial cell markers were mentioned above. They found an association between high MVD and poor outcome, and their results did not alter the decision of whether to use hot spot counting or stereological estimates. The evaluation of angiogenic growth factors, which are bFGF, TGF- α , TGF- β 1-3, TNF- α and VEGF, revealed a relation between the absence of TGF- α in the epithelial component and poor prognosis. TGF- α , besides being an angiogenic growth factor, is also known as a marker for normal tubular epithelium in human kidney (Derynck 1992). The association between the loss of TGF- α and prognosis may lead to a conclusion that TGF- α expression is lost during tumorigenesis in WT. In addition to all those conclusions, the elevated serum levels and expression of VEGF in the tumor tissue in this study calls anti-VEGF therapy to mind as an alternative therapy after the conventional method failed.

In a recent study using MVD on WT, like that by Sködenberg et al. (2001), MVD was shown to be a predictive value of disease outcome in WT (Abramson et al. 2003). They also suggested that MVD can be used for identifying the patient population with FH who unexpectedly have progressive disease in the follow-up. With the introduction of various quantification methods in the literature discussed previously, MVD has been recommended as a tool for risk assignment, because MVD needs only immunohistochemistry, pathological assessment and lesser time.

Ghanem et al. (2003) studied MVD and also VEGF and its receptor (FLT-1) on 62 preoperatively treated WTs. They showed that high MVD correlated positively with the degree of VEGF expression and poor prognosis significantly. MVD had prognostic value for tumor progression but not for tumor related death. They demonstrated VEGF as the most important angiogenic agent in WT, like similar studies in the literature (Kayton et al. 1999; Rowe et al. 1999).

We studied 33 WT cases for MVD, both by anti-CD31 (Figure 13.2) and -CD34

(Figure 13.3), and a significant relationship was demonstrated between MVD and poor prognosis in FH group (Figure 13.4) (Ozluk et al. 2006). In contrast to the data in the literature, there was no correlation between VEGF expression and angiogenesis. Our results support the theory that angiogenesis is not regulated by only one molecule but by a balance between angiogenic and angiostatic factors. Furthermore, we speculated that different angiogenic factors may be responsible for angiogenesis in different tumors. Sködenberg et al. (2001) detected immunopositivity with at least one angiogenic growth factor in each tumor. This finding is also identical to ours.

In conclusion, studies on angiogenesis and antiangiogenic therapies have become a subject of interest in recent years. They have been studied in various adult human cancers, however there are not many studies on pediatric tumors. The main object in these studies is not only to predict the prognosis but also to guide the treatment of the disease. WT, being the second most common neoplasm of childhood, needs further studies on angiogenesis in large number of cases.

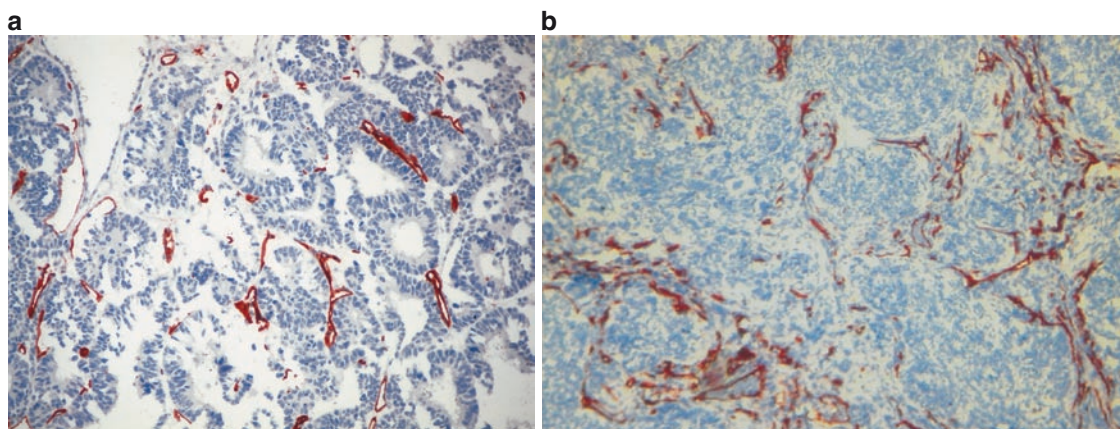


FIGURE 13.2. (a) The staining pattern of anti-CD31 displaying low vascularity (anti-CD31, $\times 200$). (b) A highly vascular Wilms tumor demonstrated by anti-CD31 (anti-CD31, $\times 125$) (Ozluk et al. 2006)

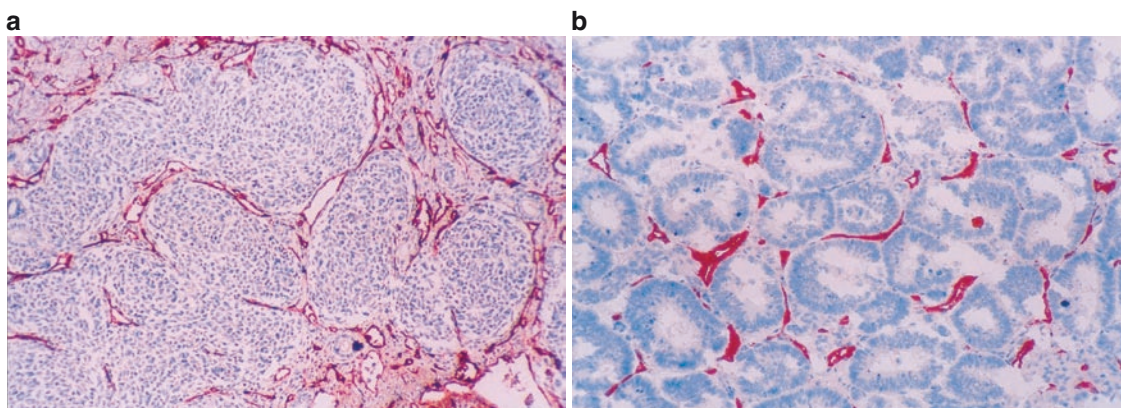


FIGURE 13.3. (a) Blastemal predominant Wilms tumor with a highly vascular stroma (microvessel density (MVD) was detected to be 218 in the average of five hot-spots per field of view at $\times 200$ magnification (0.09 mm^2)) (anti-CD34, $\times 310$) (Ozluk et al. 2006). (b) Epithelial predominant Wilms tumor displaying low vascular stroma (microvessel density (MVD) was detected to be 50 in the average of five hot-spots per field of view at $\times 200$ magnification (0.09 mm^2)) (anti-CD34, $\times 125$) (Ozluk et al. 2006)

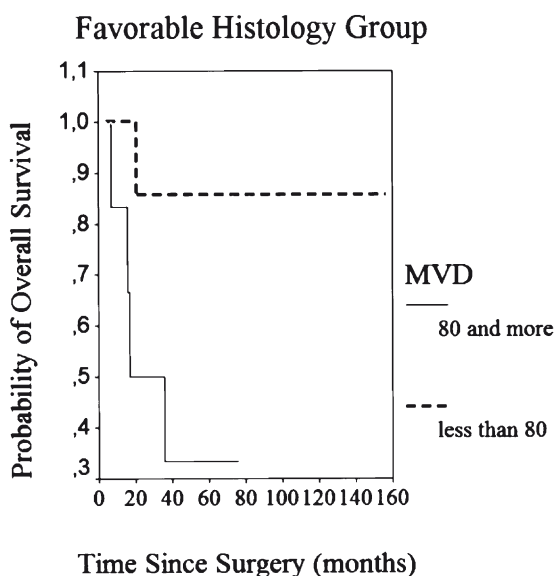


FIGURE 13.4. The difference between the overall survival rates of patients in the favourable histology group with high and low vascular tumours. Log-rank test showed a statistical difference ($p < 0.05$) (Ozluk et al. 2006)

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Part III

Urogenitary Tract Cancer

A. Adrenal

14

Adenomatoid Tumor of the Adrenal Gland: Differential Diagnosis Using Immunohistochemistry

Fanny Burel-Vandenbos, Nathalie Cardot-Leccia, Juliette Haudebourg, Damien Ambrosetti, and Jean-Francois Michiels

INTRODUCTION

Adenomatoid tumors (AT) are benign mesothelial tumors that usually affect both male and female genital tracts. They characteristically arise in the epididymis, testicular tunica, spermatic cord, fallopian tube, and in the uterus. Extragenital AT are uncommon. They have been reported in the omentum, skin, mesentery, pancreas, mediastinum, pleura, and heart. AT of the adrenal gland are very uncommon. Only 27 cases of AT of the adrenal gland have been reported (Evans et al. 1988; Travis et al. 1990; Raaf et al. 1996; Angeles-Angeles et al. 1997; Rodrigo Gasque et al. 1999; Glatz and Wegmann 2000; Chung-Park et al. 2003; Isotalo et al. 2003; Kim and Ro 2003; Schadde et al. 2003; Denicol et al. 2004; Burel-Vandenbos et al. 2005; Fan et al. 2005; Garg et al. 2005; Hamamatsu et al. 2005; Koren and Cunderlik 2005; Varkarakis et al., 2005; Furedi et al. 2007). In extragenital locations, such as the adrenal gland, AT may pose a diagnostic challenge, with a wide range of differential diagnoses, especially malignant

tumors. The challenge is due to the lack of radiological and clinical characteristics and to the histological heterogeneity of AT. In this chapter, we emphasize that immunohistochemistry plays an important role in reaching the diagnosis. The proper identification of this benign tumor in the adrenal gland and the knowledge of its differential diagnosis deserve attention to avoid invasive treatment.

GENERAL FEATURES

The age of patients with adrenal AT ranges from 24 to 65 years with marked male predominance. Involvement of the left adrenal gland seems more common. There are no specific clinical features. The tumor is usually found incidentally. Likewise, there are no specific radiographic features of AT in the adrenal gland (Rodrigo Gasque et al. 1999). Thus, it can be preoperatively confused with more common tumors, such as adenoma, pheochromocytoma or metastatic adenocarcinoma.

HISTOLOGY AND DIFFERENTIAL DIAGNOSIS

Histologically and immunohistochemically, adrenal AT are identical to those from the genital tract. Numerous immunohistochemical and ultrastructural studies support their mesothelial origin. Histologically, AT are heterogeneous (Figure 14.1). These tumors have a variable pattern ranging from anastomosing tubules or gland-like spaces lined by cuboidal and flattened cells to solid nests and strands of plump cells with abundant eosinophilic cytoplasm. The presence of dilated tubules lined by flattened cells may initially suggest an endothelial appearance. Usually one pattern predominates, so AT can present as a predominantly adenoid, angiomatoid,

solid or cystic tumor. More rarely, adrenal AT may be papillary (Glatz and Wegmann 2000). Vacuolated cytoplasm is a prominent feature of the cells, which gives them a signet-ring appearance. Nuclear pleomorphism, tumor necrosis, and mitotic activity are lacking. Mucosecretion is absent. The stroma is usually fibrous and sometimes hyalinized. It may contain lymphocyte aggregates and dystrophic calcification. AT are often ill-defined, with an infiltrative growth pattern and may spread to extra-adrenal adipose tissue.

The plethora of histologic patterns of AT complicate accurate diagnosis. The diagnostic challenge is illustrated by Isotalo et al. (2003), who reported five cases of adrenal AT. Among the five cases of AT, none was recognized on preliminary

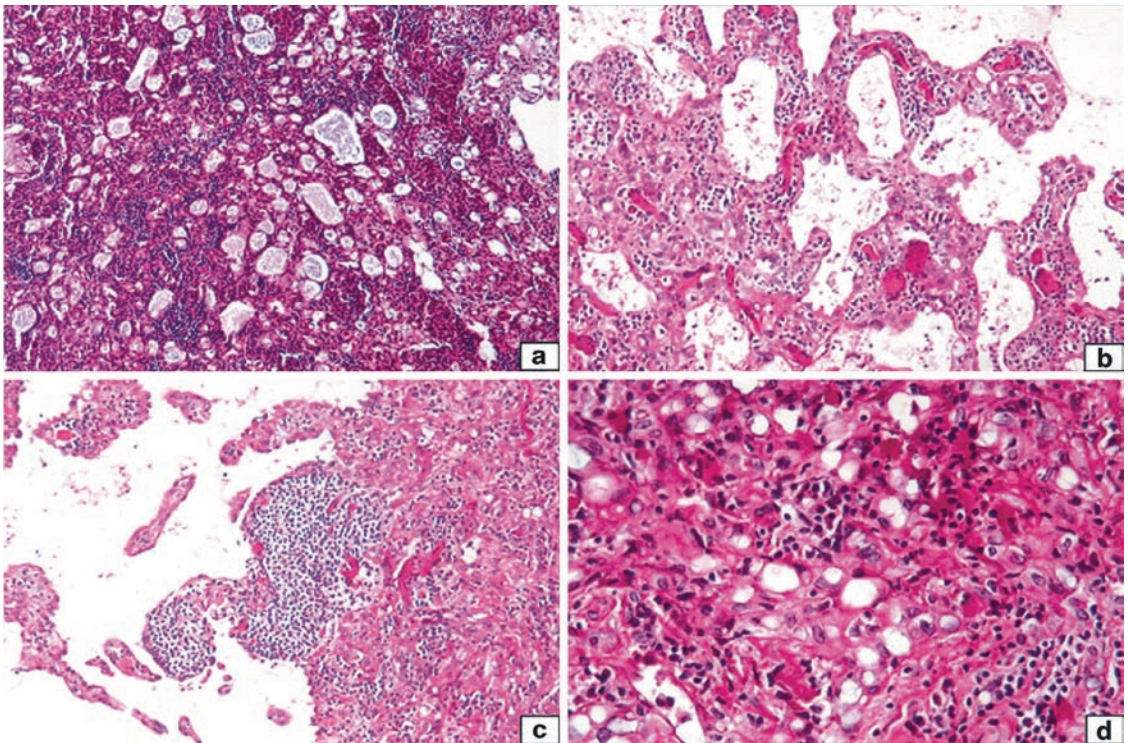


FIGURE 14.1. (a) Tubular pattern (HES $\times$ 100), (b) angiomatoid pattern (HES $\times$ 200), (c) cystic pattern with lymphoid nodule mimicking a lymphangioma (HES $\times$ 200), and (d) Signet-ring cells (HES $\times$ 400)

histological examination. They were interpreted as metastatic adenocarcinoma, adrenal cortical tumor, and lymphangioma, which represent the main differential diagnoses. Indeed, as AT can be predominantly angiomatoid or cystic and composed of endotheliiform cells, they may be misdiagnosed as hemangioma or lymphangioma. The vacuoles and gland-like differentiation may also simulate metastatic adenocarcinoma. The ill-defined limitation of AT increases the confusion with adenocarcinoma. However, the lack of atypical nuclei and mitotic activity should suggest a benign diagnosis.

IMMUNOPHENOTYPE

Immunohistochemistry is crucial for the diagnosis of adrenal AT. These are characterized by consistent expression of pan-cytokeratin, vimentin, and mesothelial markers. The antigens classically expressed in mesothelial neoplasms (Calretinin, HBME1, thrombomodulin, CA 125, cytokeratin 5/6, WT1) (King et al. 2006) are also detected in adrenal AT (Figure 14.2). In reported adrenal

AT cases, calretinin was always found to be strongly expressed, whereas CK5/6 expression might be only weak and focal. The patterns of immunostaining have been described in AT of the genital tract, but have not been described in reported cases of adrenal AT. In para-testicular AT, expression of the mesothelial markers CA 125, HBME1, and thrombomodulin was seen on the luminal surface of the cell membrane of tumor acini, and within tumor cell cytoplasm in solid AT (Delahunt et al. 2000).

Among epithelial markers, pan-cytokeratins (AE1/AE3, CAM 5.2, and KL1) are consistently expressed, whereas positivity for other keratins, especially cytokeratin 7, has been variably found in AT. For Isotalo et al. (2003) who reported the largest series of adrenal AT (5 cases), strong expression of cytokeratin 7 was consistently observed. Positive immunostaining for epithelial membrane antigen (EMA) is variably found. When detected, EMA expression was found in both cytoplasm and cell membrane.

If AT exhibits gland-like differentiation and signet-ring cells, the distinction with adenocarcinoma may be difficult, especially because both tumors express epithelial markers. Lack of a variety of carcinoma-related antigens (Ber-EP4, CD15, CEA, MOC-31, B72.3) has been found to be of assistance in the diagnosis of mesothelial tumors by excluding epithelial tumors of similar morphology (King et al. 2006). Mesothelial markers are very useful for accurate diagnosis. Among them, calretinin and CK5/6 are the most useful to distinguish AT from adenocarcinoma, because of their high specificity and sensitivity for mesothelial neoplasm (King et al. 2006). Furthermore, adrenal AT exhibits low MIB-1 proliferative activity, ranging

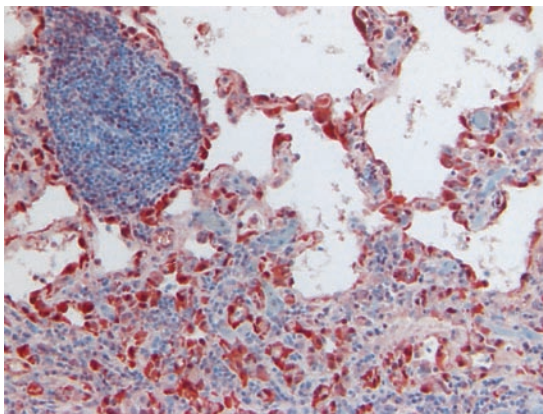


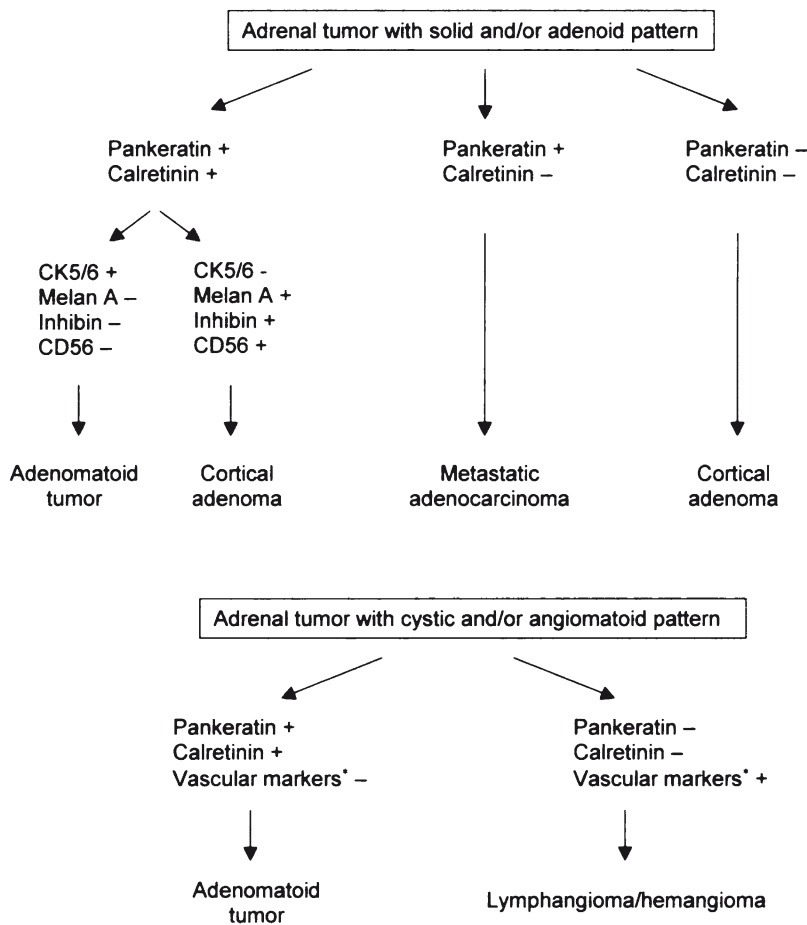
FIGURE 14.2. Calretinin immunostaining (×200)

from 0.2% to 2.7% (Isotalo et al. 2003), which constitutes an additional argument against adenocarcinoma. AT are negative for vascular markers, such as CD31 and Factor VIII, and for CD34, allowing to differentiate AT from lymphangioma or hemangioma. As calretinin has been found to be expressed in adrenal cortical tumors (Lugli et al. 2003; Zhang et al. 2003), other markers are required to differentiate between adrenal AT and adrenal cortical tumors. Adrenal cortical tumors are usually positive for melan-A, inhibin (Zhang et al. 2003) and CD56, whereas these markers are not or rarely detected

in mesothelial tumors. Conversely, CK5/6 expression usually found in AT is uncommon in adrenal cortical tumors (Chu and Weiss 2002).

The main immunohistochemical differences between AT and its differential diagnosis are summarized in the Figure 14.3.

In conclusion, AT should be included in the differential diagnosis of solid and/or cystic lesion of the adrenal gland. In the adrenal gland, AT may pose a diagnostic challenge with a wide range of differential diagnoses, especially malignant tumors, but diagnosis of AT can be easily established using immunohistochemistry.



* Vascular markers : CD31, CD34, factor VIII

FIGURE 14.3. Differential diagnosis of adenomatoid tumor using immunohistochemistry

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15

Testicular Cancer: Post-Chemotherapy Retroperitoneal Lymph Node Dissection

Philippe E. Spiess, MD, MS, FRCS(C), Nizar M. Tannir, MD, FACP,
and Louis L. Pisters, MD

INTRODUCTION

As reported by Jemal et al. (2006), an estimated 8,000 new cases of testicular cancer were diagnosed in 2005, with a reported 400 deaths due to this disease. Testicular cancer is the most common malignancy in men between 20 and 40 years of age. Major improvements in the prognosis of testicular cancer have occurred during the past 20 years, with overall disease-specific survival rates exceeding 90%, which is due largely to the sensitivity of these tumors to platinum-based chemotherapy. Patients with metastatic testicular cancer are frequently treated using a multimodal treatment approach consisting of systemic chemotherapy followed by post-chemotherapy retroperitoneal lymph node dissection (PC-RPLND). In this chapter, we will discuss the indications, technical details, treatment-related outcomes, and post-operative surveillance strategies following such dissection.

INDICATIONS FOR PC-RPLND

Before embarking on a detailed description of the clinical indications for PC-RPLND, several key concepts regarding testicular

cancer are reviewed. Testicular cancer can be divided into two major histologic subtypes: seminomatous (30–60% of cases) and non-seminomatous (40–70% of cases) germ cell tumors. The non-seminomatous germ cell tumors (NSGCT) can be further subdivided into mixed, embryonal, yolk sac, choriocarcinoma, and teratoma (mature and immature) histologies. The serum tumor markers α -fetoprotein (AFP), β -human chorionic gonadotropin (HCG), and lactate dehydrogenase (LDH) are used in the diagnosis, treatment, and surveillance of testicular cancer. The 1997 American Joint Committee on Cancer Staging System is the most frequently used method of staging testicular tumors. In this staging system, clinical stage I tumors are tumors that are confined to the testis, stage II tumors are confined to the retroperitoneal lymph nodes (stage IIA to retroperitoneal lymph nodes < 2 cm in diameter, stage IIB tumors to retroperitoneal lymph nodes between 2 and 5 cm in diameter, and stage IIC to retroperitoneal lymph nodes > 5 cm in diameter), and stage III tumors have metastasized to sites other than retroperitoneal lymph nodes.

Metastatic testicular cancer is usually treated using a multimodal approach consisting

of systemic chemotherapy (typically a platinum-based regimen) followed by surgical consolidation consisting of PC-RPLND and potentially, resection of disease at other sites. The most common indication for PC-RPLND is metastatic NSGCT for which normalization of serum tumor markers is demonstrated following systemic chemotherapy, and for which there is evidence of residual disease on radiographic imaging. Patients in this category who receive first-line chemotherapy (consisting frequently of either 3 cycles of bleomycin, etoposide, and cisplatin or 4 cycles of etoposide and cisplatin) and undergo PC-RPLND in this context have a 40% chance of fibrosis, 40% chance of teratoma, and 20% chance of viable germ cell tumor in the resected surgical specimen (Einhorn, 1981). As such, 60% of patients will derive a benefit from surgery as a consequence of the resection of viable cancer or teratoma. The risk of viable cancer in the surgical specimen increases to 50% in patients undergoing PC-RPLND following salvage chemotherapy (Donohue et al. 1998).

A second indication for PC-RPLND is the growth of a metastatic germ cell tumor during systemic chemotherapy despite normalization of serum tumor markers. This condition is termed growing teratoma syndrome (GTS) (Logothetis et al. 1982). In a recent surgical series by Spiess et al. (2007b), the excellent surgical outcomes achievable with PC-RPLND as a treatment for GTS were confirmed. These outcomes point to the importance of following serum tumor marker and radiographic responses of metastatic germ cell tumors to systemic chemotherapy. Because GTS is chemo-refractory, it should be managed by PC-RPLND within several weeks of suspecting this diagnosis as delay could result in a more difficult surgery with increased morbidity.

The third indication for PC-RPLND is for metastatic NSGCT treated with maximal doses of systemic chemotherapy in terms of toxicity profile. In this case, despite persistently elevated serum tumor markers, patients undergo a “desperate PC-RPLND” in an attempt to surgically resect all sites of disease. Although this approach is historically associated with a poor prognosis, Murphy et al. (1993) reported a surgical series of 48 patients undergoing “desperate RPLND” in which 79% of patients exhibited no evidence of disease at last follow-up.

In addition to these primary indications for PC-RPLND, there are 2 controversial indications that warrant mention. The first is for metastatic NSGCT in which complete radiographic regression of the metastasis and normalization of serum tumor markers occur. As reported by Beck and Foster (2006), the risk of relapse in this subset of patients is ~5% without PC-RPLND. In some instances, it is at a sufficiently low rate of recurrence amounting to negate PC-RPLND and observe patients, whereas other authors have been proponents of PC-RPLND in this context. Currently, there is no clear answer to whether PC-RPLND is the preferred treatment in these patients, but clearly both treatment options should be offered to patients explaining the merits and drawbacks to observation versus PC-RPLND.

Another controversial indication for PC-RPLND is in patients with clinical stage IIC or III seminomatous germ cell tumors with a residual mass > 3 cm in diameter following systemic chemotherapy. Some groups have advocated PC-RPLND in this context provided the mass is well delineated; however, others feel that these masses can be treated with external-beam radiotherapy or by observation alone. In addition,

PET scanning may be used to help clarify the likelihood that residual seminomatous masses > 3 cm in diameter harbor residual viable cancer, whereby PC-RPLND or external-beam radiotherapy may be offered only to patients with positive PET imaging.

PREOPERATIVE CONSIDERATIONS

Many preoperative considerations must be evaluated prior to performing a PC-RPLND for testis cancer. First, the patient must have had a complete metastatic evaluation recently (within the previous 3–4 weeks) including chest and abdominal/pelvic imaging and serum tumor markers, verifying that no sites of systemic metastasis have been missed, and also that complete normalization of serum tumor markers has occurred. It is quite possible that serum tumor markers remain elevated following chemotherapy in the absence of viable tumor (e.g., hepatotoxicity, hypogonadism, illicit drug use); however, one must always assume that persistent tumor marker elevation following chemotherapy signifies the presence of viable cancer elements until proven otherwise. Preoperatively, patients undergoing a PC-RPLND should have routine blood serology testing including a complete blood count, coagulation profile, renal function studies, liver function studies, and availability of an appropriate amount of blood for transfusion, if needed. The surgeon performing the PC-RPLND should review the previous surgical report of the orchiectomy to make sure this procedure was done using an inguinal approach and that there was no scrotal violation. Similarly, one must ensure that the spermatic cord was resected up to internal inguinal ring and must find out the

type of suture material (typically silk or PDS) used to ligate the cord as this suture will need to be identified and resected with the remaining spermatic vessels at the time of the PC-RPLND.

It is important that the surgeon review the preoperative imaging studies of the chest, abdomen, and pelvis as it helps plan the surgical approach and anticipated difficulty. On the preoperative imaging of the abdomen and pelvis, the surgeon must specifically identify the metastatic sites in terms of their size, location, and adjacent organs/structures. The review of the preoperative imaging may help determine whether adjacent organs, such as kidney, spleen, and segment of bowel may need to be resected at the time of PC-RPLND. The vascular anatomy must also be looked at in great detail, including the location of the metastatic mass(es) with regards to the inferior vena cava (IVC), aorta, superior mesenteric artery (SMA), and renal arteries and veins. Magnetic resonance imaging and angiography can help characterize the vascular anatomy prior to PC-RPLND particularly in terms of the renal and lumbar vessels (Corral et al. 2000). If the masses appear to encase vascular structures, the surgeon should also have the patient assessed by a vascular surgeon preoperatively and have this surgeon available at the time of surgery in case vascular surgical repair or grafting is needed. In patients with extensive retroperitoneal masses to be resected, the surgeon should consider the placement of preoperative ureteric stents to facilitate intraoperative localization of both ureters, thereby minimizing the risk of injury to these structures.

Patients with clinical stage III disease can be considered for simultaneous surgery at several sites within one setting, provided it is deemed by the surgeon that this does

not place the patient at risk of prolonged anesthesia and/or risk of bleeding. When multi-organ sites of resection are considered, it may be necessary for several surgeons to work together in a coordinated manner. When contemplating performing multi-organ resection, an important consideration is patient's preoperative comorbidities and anesthesia risk. The surgical team and anesthesiologist must keep in mind the type of chemotherapeutic regimen and number of cycles received preoperatively. Patients receiving bleomycin preoperatively are at risk of pulmonary fibrosis such that fluid management and ventilatory parameters must be carefully monitored. Preoperative pulmonary function studies should be performed in patients having received bleomycin preoperatively. In addition, cisplatin can cause nephro- and neurotoxicity, which needs to be considered preoperatively. The number of chemotherapy cycles received preoperatively can result in the surgical dissection, being more difficult due to scarring in the normal anatomical planes as well as decrease tissue vascularity and wound healing. Careful fascial closure using either PDS or prolene should be contemplated in an attempt to minimize the risk of evisceration and incisional hernias.

Prior to surgery (and ideally prior to initiation of systemic chemotherapy), patients undergoing PC-RPLND should be offered sperm banking if conception is a consideration, because injury to sympathetic nerves responsible for sperm emission could place patients at increased risk for infertility, particularly patients undergoing a full bilateral template dissection which is discussed in the next section.

Prior to surgery, the surgeon must have a detailed discussion with the patient regarding the risks and expectations of the surgery.

The surgeon must delineate the expected operative and postoperative course. Similarly, the potential complications of the surgery must be clearly explained including informing patients of the 1% risk of perioperative mortality, which was recently reported by Spiess et al. (2006a). Furthermore, all patient's questions and concerns should be addressed by the surgeon prior to PC-RPLND. The day prior to surgery, the patient should receive a full mechanical bowel prep using either magnesium citrate or Go-Lytely®, subsequent to fasting the night prior to surgery. If the patient takes anticoagulative agents, these should be discontinued at least 1 week prior to surgery. Failure to adhere to the pre-operative considerations cited here could result in poor cancer-related outcomes and increased morbidity.

TECHNICAL CONSIDERATIONS

PC-RPLND is a technically challenging operation that requires a detailed understanding and knowledge of retroperitoneal anatomy. One of the major morbidities associated with PC-RPLND is failure of antegrade ejaculation, which requires the coordination of 3 essential steps: 1) bladder neck closure, 2) semen emission, and 3) ejaculation. The sympathetic nervous system plays a key role in coordinating these events, with damage to sympathetic nerves or plexus resulting in ejaculatory disorders. Two technical modifications have been done in an attempt to minimize the risk of this complication: the use of a nervesparing procedures or a modified template dissection. Both of these modifications can be difficult to perform, particularly in the context of postchemotherapy surgery; furthermore, we feel strongly that at no

time should either of these approaches be performed when there is a risk of leaving residual cancer unresected. If both a nerve-sparing and a modified template dissection are feasible options, it is believed that the nerve-sparing procedure, when properly performed, may have a slightly better chance of preserving ejaculatory function.

When performing a PC-RPLND, two surgical approaches have traditionally been taken: a thoracoabdominal or a transabdominal approach. The thoracoabdominal approach provides excellent exposure of the upper abdomen particularly if there is suprahilar disease or if a simultaneous thoracic resection is considered in the same setting; however, we feel that this approach is quite morbid in terms of post-operative lung complications (i.e., atelectasis, pain, pneumonia). As such, we have adopted a transabdominal approach for all patients undergoing PC-RPLND. A midline incision is made from the xiphoid process to the pubis symphysis with the incision extended through the skin, subcutaneous tissue, fascia, lineal alba, and peritoneum. The falciform ligament is then ligated or resected in order to avoid hepatic tears and optimize hepatic cranial traction. A Bookwalter or Thompson retractor provides excellent retraction for this type of operation.

The small bowel is then retracted cranially after incising the posterior peritoneum on the right side lateral to the line of Toldt extending inferiorly below and around the ileocecal valve and then along the root of the small bowel mesentery on the left side medial to the inferior mesenteric vein (IMV). Division of the IMV assists the necessary exposure and reduces the risk of tearing the vein off of the spleno-portal confluence in the presence of very large retroperitoneal masses. The small bowel

is then reflected superiorly by placing it in a bowel bag with its color and peristalsis carefully inspected throughout the operation. The posterior surface of the pancreas and duodenum are then reflected superiorly, making sure to control lymphatics using hemoclips throughout the dissection in order to minimize the risk of postoperative chylous ascites. We then proceed in dissecting the anatomical structures constituting the boundaries of dissection.

As shown in Figure 15.1A, the boundaries of dissection for a full bilateral template dissection, which we perform in most patients at the time of PC-RPLND, includes the renal artery and vein superiorly, the right and left ureters (lateral boundaries), and the point where the ureter crosses the common ilial artery (the inferior boundary). Furthermore, the spermatic cord ipsilateral to the side of the orchiectomy is resected with the PC-RPLND specimen. All key vascular structures, including the aorta, IVC, renal artery and vein, gonadal vein, lumbar veins, SMA, inferior mesenteric artery (IMA), and ilial vessels, must be identified during the dissection. Similarly, an important surgical principle to remember is that proximal and distal control of key vascular structures, such as the aorta and IVC, help control excessive hemorrhage resulting from inadvertent vascular injuries.

As previously mentioned, it is important to obtain a preoperative consultation with a vascular surgeon who will be available at the time of surgery particularly when one expects the mass may be densely adherent to key vascular structures or if a vascular resection with grafting may be necessitated. The placement of pre-operative ureteric stents may also facilitate the intraoperative identification of the ureters, minimizing inadvertent risk of injury to these structures.

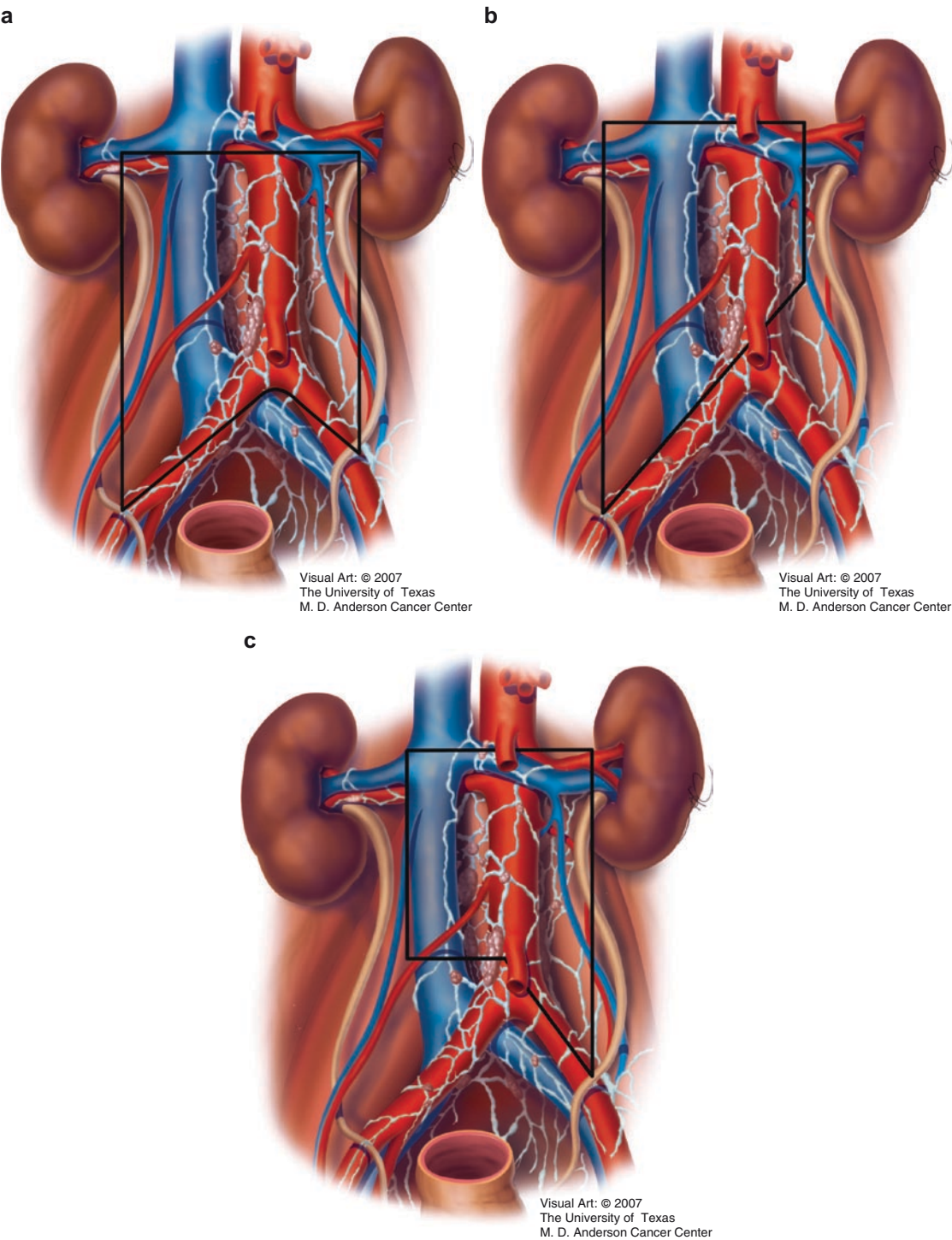


FIGURE 15.1. Schematic diagram illustrating the boundaries of a full bilateral (A), right modified template (B), and left modified template PC-RPLND (C)

When performing a full bilateral template PC-RPLND, several lymph node packets are resected as part of the surgical specimen including paracaval, precaval, interaorto-caval, and para-aortic lymph nodes with the “split and roll” technique allowing complete resection of all lymphatic tissue anterior, lateral, and inferior to the aorta and IVC. Using the “split and roll” technique, the vessel is retracted laterally while the lymphatic tissue is swept of the vessel and the lumbar vessels are doubly ligated (proximally and distally).

For patients with right-sided testis cancer, a right modified template PC-RPLND can be considered provided there is no evidence of disease either preoperatively or at the time of surgery outside of the surgical template. As shown in Figure 15.1B, the boundaries of dissection of a right modified template RPLND include the renal artery and vein superiorly, the right and left ureters (lateral boundaries), and the inferior boundary consists of a “dog leg” extending from the level of the IMA on the left side extending to where the right ureter crosses the common iliac artery on the right side. Using this modified template, the hypogastric plexus, which serves an important role in ejaculatory function, is preserved.

For patients with left-sided testis cancer, a left modified template dissection can be considered provided there is no evidence of disease outside of the surgical template either pre- or intraoperatively. As shown in Figure 15.1C, the boundaries of dissections of a left modified template include the renal artery and vein superiorly, the left ureter as the left lateral border, and the right lateral wall of the IVC as the right lateral border, and the inferior boundary consisting of a “dog leg” extending from the level of the

IMA on the right side and extending to the point where the left ureter crosses the common iliac artery on the left side.

Clearly, the modified template and nerve sparing approaches to PC-RPLND help minimize the risk of postoperative ejaculatory disorders; however, surgical oncologists must never compromise the adequacy of surgical resection. Thus, in patients with high-volume, bulky retroperitoneal disease, a full bilateral template dissection should be considered as the standard approach.

In recent years, increasing interest has been given to consideration of laparoscopic RPLND in patients with testis cancer, predominantly in the context of primary RPLND. Some centers have performed laparoscopic RPLND in the post-chemotherapy setting; however, in most cases, this approach has been offered only to patients with low-volume disease (clinical stages IIA and IIB), and the outcomes are difficult to compare to open PC-RPLND surgical series because most patients managed laparoscopically received adjuvant chemotherapy. As such, laparoscopic PC-RPLND should be considered experimental until future studies can validate that its oncological outcomes are comparable to open PC-RPLND surgical series.

TREATMENT-RELATED OUTCOMES

PC-RPLND is an essential diagnostic and therapeutic intervention in patients with metastatic NSGCT. Technical refinements and a better understanding of retroperitoneal anatomy and physiology have resulted in a significant improvement in the treatment-related outcomes, particularly reduced morbidity. As reported by Stephenson and

Sheinfeld (2004), the prognosis of patients undergoing PC-RPLND is most strongly correlated with pathologic findings at the time of surgery, with patients having fibrosis, teratoma, or both, with a 10–18% risk of relapse, and patients with viable germ cell tumor elements in the PC-RPLND having up to a 70% risk of relapse despite the majority of these patients receiving 2 cycles of adjuvant chemotherapy.

In the study by Spiess et al. (2006a), the treatment-related outcomes of 236 patients undergoing PC-RPLND in the context of metastatic NSGCT were reviewed. In this study, a multivariate analysis of potential predictors of DSS identified the presence of systemic symptoms at presentation, an elevated pre-RPLND serum AFP and HCG, post-operative complications, and recurrence predicting poorer DSS. Predictors of poorer recurrence-free survival (RFS) included advanced clinical stage (IIC-III) and the presence of viable tumor in the RPLND specimen. A pre-RPLND serum AFP > 9 ng/ml and HCG > 4.1 mIU/ml was found to predict a worst DSS.

Another study by Spiess et al. (2006b) evaluated the outcomes in patients harboring viable germ cell tumor elements at the time of PC-RPLND and attempted to determine whether the presence of viable tumor in the surgical specimen could be predicted. The incidence of viable tumor at the time of PC-RPLND in the good, intermediate, and poor-International Germ Cell Consensus Classification (IGCCC) risk categories were similar (17.8%, 15.6%, and 15.3%, respectively); however, the IGCCC disease-specific survival risk categories predicted and recurrence-free survival. On multivariate analysis, an elevated serum AFP level prior to PC-RPLND and the size of the retroperitoneal mass on

pathology review were predictive of viable tumor in the surgical specimen. Despite the use of these clinical parameters in predicting the presence of viable tumor at the time of PC-RPLND, these predictors are not sufficiently accurate to avoid performing surgery in any subset of patients. Therefore, PC-RPLND remains an essential component of the management of testis cancer patients with residual masses following systemic chemotherapy.

In a study by Fizazi et al. (2001), the treatment-related outcomes of 238 patients with viable residual disease following first-line chemotherapy were assessed, with an overall 5-year progression-free survival (PFS) rate of 64%. On multivariate analysis, predictors of PFS included incomplete surgical resection, viable malignant cell > 10%, and poor or intermediate IGCCC risk categories. Patients who did not have any of these adverse features had a 5-year PFS of 90% versus those with 2 or more adverse features had a 5-year PFS rate of 41%.

In a recent study by Spiess et al. (2007c), the treatment-related outcomes of patients with no viable tumor in the PC-RPLND specimen were reviewed. Of the 195 patients having fibrosis and/or teratoma at the time of PC-RPLND, 35 (18%) developed subsequent recurrences and 18 (9%) died of disease at a median follow-up of 45 months. On multivariate analysis, the only predictors of RFS in these patients was advanced clinical stage (stage IIC-III), and predictors of DSS included an elevated serum HCG prior to PC-RPLND, pathologic diameter of the retroperitoneal mass, and post-operative recurrence. An HCG > 1.2 mIU/ml prior to PC-RPLND trended toward statistical significance and a diameter of the retroperitoneal mass > 2.5 cm was a statistically significant,

predictor of poorer DSS. Therefore, patients with no viable germ cell tumor elements in the PC-RPLND remain at risk of disease recurrence and need to be routinely followed in the postoperative period, as shown in Table 15.1, summarizing our recently proposed surveillance strategy (Spiess et al. 2007a).

A subset of patients may continue to have elevated serum tumor markers despite receiving the maximal amount of preoperative chemotherapy, and in this context, patients may be offered “desperate PC-RPLND” in an attempt to remove all sites of visible disease. In a study by Beck et al. (2005), 114 patients underwent “desperate PC-RPLND” for metastatic NSGCT in the presence of persistently elevated preoperative serum tumor markers. Viable germ cell tumor

elements were present in > 50% of the patients and at a median follow-up duration of 6 years, 54% of patients remain alive. On multivariate analysis, several predictors of poorer DSS were identified, including the rate of change of HCG pre-op, an elevated AFP pre-op, a prior attempt at PC-RPLND, and the presence of viable germ cell tumor at the time of surgery. Clearly, patients undergoing “desperate PC-RPLND” constitute a high-risk population in terms of disease progression and surgical morbidity. Nevertheless, “desperate PC-RPLND” may offer a chance for long-term survival and cure in patients exhibiting chemo-refractory behavior; however, this operation should be performed at tertiary care referral centers with extensive experience in the surgical management of testis cancer.

TABLE 15.1. Recommended surveillance protocol in testicular cancer patients following PC-RPLND.

	Months Follow-up												
	3	6	9	12	15	18	21	24	27	30	33	36	Yearly x 5
Clinical stage IIA													
– History				x				x				x	
– Physical exam*				x				x				x	
– Serum tumor markers^				x				x				x	
– Chest X-ray				x				x				x	
– Abdo/Pelvic CT				x				x				x	
Clinical stage IIB													
– History		x		x		x		x		x		x	x
– Physical exam*		x		x		x		x		x		x	x
– Serum tumor markers^		x		x		x		x		x		x	x
– Chest X-ray		x		x		x		x		x		x	x
– Abdo/Pelvic CT		x		x		x		x		x		x	x
Clinical stage IIC													
– History		x		x		x		x		x		x	x
– Physical exam*		x		x		x		x		x		x	x
– Serum tumor markers^		x		x		x		x		x		x	x
– Chest X-ray		x		x		x		x		x		x	x
– Abdo/Pelvic CT		x		x		x		x		x		x	x
Clinical stage III													
– History	x	x	x	x	x	x	x	x	x	x	x	x	x
– Physical exam*	x	x	x	x	x	x	x	x	x	x	x	x	x
– Serum tumor markers^	x	x	x	x	x	x	x	x	x	x	x	x	x
– Chest X-ray	x	x	x	x	x	x	x	x	x	x	x	x	x
– Abdo/Pelvic CT		x		x		x		x		x		x	x

* Physical exam includes a head and neck examination and brief neurologic evaluation. ^ Serum tumor markers include AFP, HCG, and LDH; CT, computed tomography. PC-RPLND, post-chemotherapy retroperitoneal lymph node dissection.

POTENTIAL COMPLICATIONS

Despite important technical modifications (e.g., nerve sparing, template dissection, early vascular control) in an attempt to minimize patient morbidity without affecting cancer-related outcomes, PC-RPLND constitutes a major surgical procedure with significant risks. In a study by Baniel and Sella (1999), an overall complication rate of 20–35% was reported, with a peri-operative mortality rate of 0.8–1%. Pulmonary insufficiency secondary to bleomycin-induced interstitial fibrosis was the most important cause of significant side-effects and operative mortality. Chylous ascites resulting from surgical trauma to the lymphatic system (thoracic duct, cisterna chyli, or one of its major tributaries) in combination with increased chyle production and obstruction to the lymphatic drainage from the abdomen was reported in this series and in another series by Baniel and Sella (1995) to occur in 2–3% of patients following PC-RPLND. In the series by Baniel et al. (1999), resection of the IVC was found to be significantly associated with the development of post-operative chylous ascites.

In the series by Evans et al. (2006), the incidence of postoperative chylous ascites was reported to be 7%, with increasing amounts of preoperative chemotherapy and intraoperative blood loss predictive on multivariate analysis of developing this postoperative complication. Furthermore, in our surgical series, conservative management of chylous ascites using total parenteral nutrition, medium chain triglycerides, paracentesis, or a combination of these resolved 77% of cases of chylous ascites, with the remaining patients requiring a peritoneovenous shunt, which was associated with an eighty percent revision rate. Based on the

experience gained in the management of chylous ascites, we proposed a treatment algorithm for the management of chylous ascites as shown in Figure 15.2. In our study (Spiess et al. 2006a), which retrospectively reviewed our PC-RPLND experience in the management of metastatic NSGCT, we reported our series of 236 patients undergoing PC-RPLND with 28% of patients developing postoperative complications. The most common postoperative complications were chylous ascites ($n = 21$), ileus ($n = 15$), atelectasis ($n = 13$), and sepsis ($n = 6$). There were 3 perioperative deaths (1.3%), with 2 resulting from postoperative sepsis and 1 from pulmonary embolism. However, the majority of patients had an uneventful postoperative course with a median duration of hospitalization of 9 days (range 3–86 days). Despite patients having uneventful intraoperative courses in most large surgical series, it nevertheless remains that approximately one-quarter of patients will develop a postoperative complication, and there is a reported 1–3% risk of perioperative mortality, which must be discussed with the patient when obtaining consent for surgery. Early recognition and treatment of most perioperative complications will potentially limit their long-term consequence on patient outcome and quality of life.

POSTOPERATIVE FOLLOW-UP

Following PC-RPLND, patients remain at risk of disease recurrence, with the chest (49%), abdomen (22%), and supraclavicular lymph nodes (13%) being the most frequent sites of disease recurrence (Spiess et al. 2007a). In this recent retrospective review of our patterns of recurrence at M. D. Anderson Cancer Center following

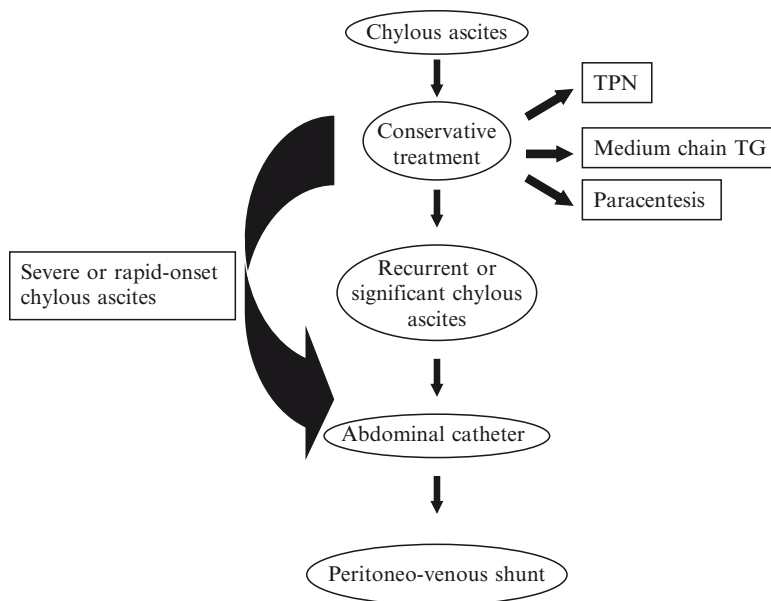


Figure reproduced from Evans JG, et al. J Urol 146:1463, 2006 (with written permission from Journal of Urology).

FIGURE 15.2. Proposed treatment algorithm for the management of chylous ascites

PC-RPLND, we noted that the risk of disease recurrence was strongly associated with the patient's clinical stage. On the basis of the patterns of recurrence following PC-RPLND, we have proposed stage-specific guidelines for the follow-up of patients with testicular cancer who undergo PC-RPLND in the context of normalized preoperative serum tumor markers as shown in Table 15.1. Although our proposed surveillance strategy is based entirely on our patterns of recurrence during the past 25 years during which systemic chemotherapeutic regimens have changed, we feel that this surveillance strategy, which uses a combination of medical history, physical examination, serum tumor markers, and radiographic imaging (chest X-ray, abdominal/pelvic CT imaging) provides a framework that can be used by clinicians to help identify recurrences at their likely sites and timing of occurrence,

nevertheless, our results require validation at other centers.

When performed by experienced surgeons, the risk of in-field recurrence remains acceptably low (1%) as we reported in this series. Clearly, patients are at risk of disease recurrence following PC-RPLND, and this must clearly be emphasized to patients following surgery. Failure to recognize postoperative recurrence may result in their delayed recognition, and thus potentially missing the window of opportunity when patients might be cured.

CONCLUSIONS

Patients with metastatic testicular cancer have a high probability of cure with multimodality treatment consisting of systemic chemotherapy and PC-RPLND. Careful attention to patient and disease-related

parameters may optimize the oncologic outcomes while minimizing the morbidity of surgery. PC-RPLND should be performed by a skilled surgeon with extensive experience in retroperitoneal surgery, and the involvement of vascular surgeons should be preemptively considered in patients with masses suspected to involve important vascular structures. Preoperative imaging should be carefully reviewed by the surgeon, because this provides key information in terms of the anticipated difficulty of the operation. Important predictors of treatment-related outcomes following PC-RPLND include elevated serum AFP and HCG prior to surgery, the presence of viable tumor in the surgical specimen, advanced clinical stage, and development of post-operative complications.

Despite attempts in predicting the presence of viable cancer elements in a post-chemotherapy retroperitoneal mass, no clinical parameters alone or in combination are sufficiently accurate to predict the pathology of these masses such that PC-RPLND could be withheld in a subset of patients. We, therefore, strongly encourage all urologists and oncologists to advocate PC-RPLND in all patients with residual retroperitoneal masses following systemic chemotherapy. We also believe that surgeons should follow-up patients with serum tumor markers and radiographic imaging at specific time points following PC-RPLND, which may vary depending on the clinical stage of their disease. Despite the favorable treatment-related outcomes of most patients with testicular cancer, there remains a subset of patients for whom aggressive tumor biology places them at risk of recurrence and disease-specific mortality. We hope future basic science and clinical studies will help better define ways of optimizing the outcomes in this young patient population.

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Survivors of Germ-Cell Testicular Cancer: Increased Risk of Second Primary Tumors

Lorenzo Richiardi and Milena M. Maule

INTRODUCTION

Germ-cell testicular cancer is a highly curable disease, with a 5-year survival >90%. For instance, among patients diagnosed in the beginning of the 2000s, mean survival was 97% in Europe and 95% in the United States (Verdecchia et al. 2007). It improved dramatically when cisplatin-based chemotherapy regimens were introduced at the end of the 1970s (Einhorn and Donohue 1977). As a consequence of the improvements in treatment, a secular rise in incidence of testicular cancer, which constantly doubled every 20 years, is nowadays associated in most developed countries with the lowest mortality rate in the last 50 years (Bray et al. 2006) (Figure 16.1). Therefore, the prevalence of survivors from testicular cancer has been increasing with time with a parallel increasing concern on the long-term health consequences of the disease and the treatments. These include mainly infertility, long-term cardiovascular effects, and increased incidence of second primary cancers.

In this chapter we will review evidence on the risk of second malignancies among testicular cancer survivors. Moreover, we will provide a brief review of methodological issues in the study of second cancers,

which will facilitate understanding and interpretation of the available evidence.

METHODS TO INVESTIGATE SECOND PRIMARY CANCERS

Cohort Studies

The most frequent approach used to investigate the epidemiology of second primary cancers is the cohort study. In a cohort study, a population of patients with a given first primary cancer is identified through specific inclusion criteria, such as registration in a population-based cancer registry or minimum survival time of a given length. The patients are then followed over time to estimate the risk of developing a second primary cancer and to assess if it is higher than what would be expected, according to the background rates experienced by the general population.

1. Standardized incidence ratio

Standardized incidence ratios (SIRs) are a common relative risk measure used to compare the incidence of second primary cancers in the cohort of cancer survivors (e.g., secondary thyroid cancers) with the incidence of primary cancers (e.g., first primary thyroid cancers) in the general

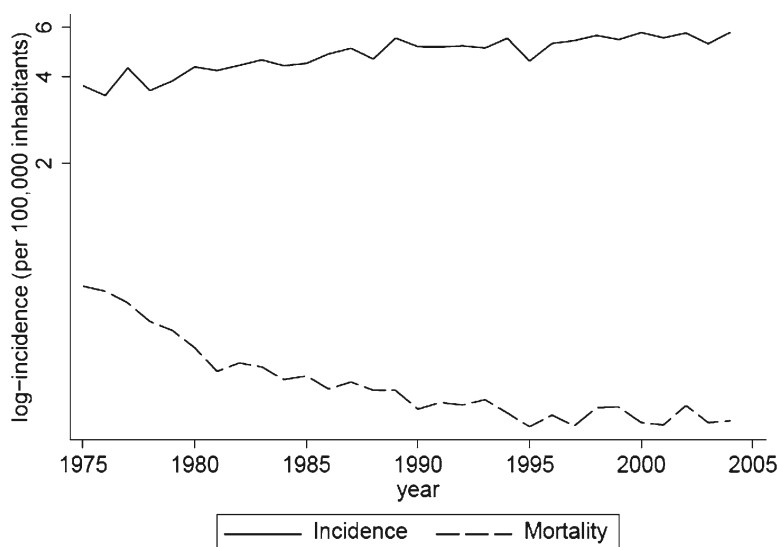


FIGURE 16.1. Diverging time trends in testicular cancer incidence and mortality. Surveillance Epidemiology and End Results, USA, 1975–2004 (SEER Program 2007)

population. SIRs are calculated as the ratio of the observed to the expected number of second primary cancers. When the source of the study population is a population-based cancer registry, observed and expected numbers are derived from the same source. Expected number of second primary cancers is obtained by applying the age-, sex-, and calendar year-specific incidence rates of first primary cancers to the cohort of survivors (Breslow and Day 1987). SIRs may vary with sex, length of follow-up, age at first cancer diagnosis, calendar period of first cancer registration, and other factors. They are interpreted as relative risks and are useful to identify groups at increased or decreased risk and to test etiological hypotheses.

2. Absolute excess risk

The absolute excess risk (AER) is the additional risk of developing a second primary cancer that having suffered from a first primary cancer adds on top of the background

risk. AER is calculated as the difference between the number of observed second primary cancers and the expected number of cases, divided by the total number of person-years at risk. The expected number of cases is calculated as for the SIRs. AER provides a useful measure of the cancer burden in a population due to second primary cancers. The information conveyed by an absolute risk measure such as AER is complementary to that provided by relative risk measures such as SIRs. For instance, a large SIR for a second primary cancer that is rare in the general population denotes increased risk in the survivors cohort but translates into a small number of excess cases in the population (Travis 2006).

3. Multiple regression analysis

Multiple regression analysis can be used to investigate the effects of clinical and demographic factors on the risk of second primary cancers. The standard approach is to perform within-cohort comparisons

using the Cox regression model. This implies the choice of a time scale that typically is time-since-study-entry. However, it has been pointed out that this approach does not take into account the natural age-associated increase of cancer risk, leading to biased or even reversed analytical conclusions. Yasui et al. (2003) suggest to use age rather than time-since-study-entry as the time scale for the Cox model. Another proposed approach is to model modifications of SIRs with the desired covariates using Poisson regression models (Yasui et al. 2003). This approach naturally accounts for the increase of cancer risk with age by using external age-specific reference rates.

4. Cumulative incidence

The cumulative incidence is another useful absolute measure of risk used to denote the probability of experiencing a second primary cancer by a specified time since first cancer diagnosis. In order to estimate the incidence of second cancers, patients are followed from study entry until the outcome of interest, and all patients that do not develop the event of interest are treated as censored. Among these are both patients that do not develop the second cancer and those that die from other causes. However, the latter type of censoring is informative because it implies that an intervening event occurred preventing the development of the outcome of interest (Gooley et al. 1999). These events are known as competing risk events and must be taken into account when computing the cumulative incidence. This can be estimated nonparametrically in a two-step process: both the Kaplan–Meier estimate of the overall survival from any event (second primary cancer or death) and the

conditional probability of second primary cancer are computed. The cumulative incidence of second primary cancers is then computed by summing the products of the overall survival and the hazard rate of second primary cancer over all time intervals (Satagopan et al. 2004). Failing to account for competing risks (which may include events other than death) generally leads to an overestimation of the cumulative incidence: the higher the frequency of the competing risk events, the larger the overestimation (Kim 2007).

Cumulative incidence of second primary cancers in a survivors cohort is most useful if compared to the expected cumulative incidence, that is the cumulative incidence of primary cancers in the general population. However, computing the expected cumulative incidence taking into account competing risks may be difficult because the exact time of occurrence of the competing risk event may not be available for the reference population (for example, cancer registries do not record time of death of the general population). Exceptionally, when the effect of competing risks in the general population may be assumed to be negligible, the expected cumulative incidence may be calculated using the life-table method (Woodward 1999) without considering competing risks. This method was applied to compare the observed and expected cumulative incidence in a young cohort of childhood cancer survivors assuming that death rate in the reference population was very small (Maule et al. 2007).

Nested Case-Control Studies

When the main interest of the study is to analyse the effect of treatment on the risk of second primary cancers, a useful study design is the case-control study nested in a

cohort of cancer survivors. The reason for choosing this design is that detailed information on the type, dose, and modalities of treatment may not be readily available for the whole cohort. The idea is then to retrieve this information for second primary cancer cases in the cohort and for a randomly selected sample of controls, usually stratified and matched to cases by selected characteristics (e.g., age, sex, first cancer type, period of first cancer diagnosis). Treatments are then compared between cases and controls. It has been pointed out that this study design has some weaknesses, such as the choice of the reference category (it is usually difficult to identify a group of unexposed patients) and overmatching (which occurs when matching is performed on a non-confounder that is associated with the exposure but not with the event – such as cancer stage) (Travis 2006).

Methodological Limitations

1. Classification of second primary cancers

A crucial point in the study of multiple cancers is the appropriateness of the definition of second primary cancer. The fundamental assumption is that individual malignancies are biologically independent (Curtis and Ries 2006). Most cancer registries have a set of rules for defining when a tumor is an independent second primary but there is no consensus over which rules are the most appropriate. The International Association of Cancer Registries and the International Agency for Research on Cancer (IARC) have proposed a common set of rules (Muir and Percy 1991) for a recent series of international multicenter studies of second malignant neoplasms including data from 13 population-based

cancer registries (Scelo et al. 2006). These rules aim at (1) distinguishing between true second primary cancers and extensions, recurrences and metastases of the first primary cancer, and (2) providing a correct classification of multiple primary cancers originating at the same site or in paired or contiguous organs.

2. Sample size and multiple comparisons

Sample size and power may pose serious limitations to the study of second primary cancers. If the study aims at analyzing different types of first and second cancers, and stratification by factors such as age, sex, period of diagnosis, length of follow-up, a large sample of initial primary cancers followed-up for many years is needed to ensure sufficient numerosity in each stratum.

The analysis of second cancer risk by type and various relevant covariates results in a large number of multiple comparisons for each original cancer site. As the number of tests increases, it becomes increasingly likely to find statistically significant outcomes due to random variability, even in the absence of real effects. Rather than using methods of adjustment, it has been suggested that the identification of real findings must rely on the biological plausibility of the association and consistency with previous studies (Curtis and Ries 2006).

3. Biases

Some biases affecting the study of second primary cancers have been reported (Curtis and Ries 2006). Cancer patients usually undergo closer medical surveillance than the general population. This may lead to the early detection of indolent second primary cancers which would have not been discovered in less scrutinized persons, and

hence in overreporting of second primary cancers among survivors. On the other hand, also underreporting of second primary cancers may occur among survivors with short life expectancy. Second cancer risk for specific types of cancer may result artefactually reduced if surgery for the first cancer removed one or more organs which would have been susceptible to develop a second primary cancer. A possible source of underascertainment of second primary cancers may occur if the geographical area covered by the cancer registry used to enrol cases is characterized by large out-migrations. The underestimation would be particularly relevant for long duration of the follow-up.

SECOND PRIMARY CANCERS AMONG SURVIVORS OF TESTICULAR CANCER

Results on second primary cancers should be interpreted in the context of the standard therapies for the first cancer and its genetic and environmental risk factors, as both long-term effects of the treatment and shared risk factors can explain the increased risks. Germ-cell testicular cancers are classified in two histological groups, seminomas and nonseminomas. This histological classification has a recognized prognostic relevance, as nonseminomas are more aggressive tumors (Schmoll et al. 2004). The standard treatment procedure for seminomas is surgery, possibly followed by radiotherapy, whereas patients with nonseminomas are treated with chemotherapy after orchiectomy. In addition, nonseminomas have a higher probability of relapse, which, in turn, may require salvage chemotherapy.

Chemotherapy regimens are platinum-based, and often include etoposide which has been introduced at the end of the 1980s (Williams et al. 1987). Radiotherapy is usually delivered in the infradiaphragmatic fields, either para-aortic alone or in association with iliac fields, whereas mediastinal radiotherapy was frequently used in the past. Organs exposed to the highest radiation doses are the stomach, the small intestine, the rectum, the liver, the pancreas, and the bladder (Travis et al. 2005). If supradiaphragmatic radiotherapy is used, the oesophagus, the lung, and the thyroid are also highly exposed.

Little is known regarding testicular cancer etiology, cryptorchidism, familial history of testicular cancer, ethnicity, and infertility being the only established risk factors (Richiardi et al. 2007b). It has been suggested that testicular cancer originates in the fetal life, although to date no single prenatal or perinatal exposure has been established as causal. Similarly, despite the observation of strong familial risks, linkage and association studies have failed to identify markers for the genetic susceptibility. A number of studies found higher incidence of testicular cancer among tall men and men who had an early puberty (Richiardi et al. 2007b). The risk of testicular cancer is increased among individuals affected by some genetic disorders, including Klinefelter's, Turner and Down syndromes and recessive X-linked ichthyosis (Lutke Holzik et al. 2003).

All Testicular Cancers

Several studies on survivors of testicular cancer have been conducted based on specific cancer registries or clinical series including at most a few hundred second

tumors (Moller et al. 1993; Wanderas et al. 1997; van den Belt-Dusebout et al. 2007). Some collaborative studies pooled data from different cancer registries to increase the precision of the estimates and study rarer second cancers. In 1987, data have been published on 18,000 cases from several populations followed up until the beginning of the 1980s (Kaldor et al. 1987); in 1997, Travis et al. (1997) reported the results on 29,000 cases from North America and Northern Europe followed-up until the beginning of the 1990s; two independent and partially overlapping studies followed up 40,000 (Travis et al. 2005) and 29,000 (Richiardi et al. 2007a) survivors of testicular cancer until the end of the 1990s.

Results of the most recent collaborative studies, which are summarized in Table 16.1, show a good consistency. There is an overall 40–60% increased risk of second malignancies compared with the general population. Specifically, survivors of testicular cancer had an increased risk of cancers of the gastrointestinal tract (including those of the oesophagus, stomach, small intestine, and colorectum), gallbladder and pancreatic cancer, malignant melanoma, cancers of the urinary tract (bladder and kidney cancer), lung cancer, soft tissue sarcoma, thyroidal cancer, non-Hodgkin's lymphoma and myeloid leukemia. In both studies, cancers of the bladder and stomach were those contributing most to the absolute excess number of cases.

Seminomas and Nonseminomas

Because seminomas and nonseminomas have different treatments, estimate of the risk of second malignancies by histology

is of great importance. This kind of analysis was possible only in the largest studies which had enough power for subgroup analyses. In these studies, for all malignancies and for most specific cancer sites, survivors of seminoma and nonseminoma had similar increased risks (Travis et al. 1997, 2005; Richiardi et al. 2007a). However, striking differences have been found for myeloid leukemia. In the collaborative study of Richiardi et al. (2007a), seminomas had a SIR of 2.4 (95% CI: 1.4–3.8), whereas nonseminomas had a SIR of 6.8 (95% CI: 4.1–10.5). These differences were larger for cases diagnosed in 1980 or later, among whom having had a seminoma was associated with a SIR of 3.6 (95% CI: 1.7–6.9), whereas a nonseminoma was associated with a SIR of 12.2 (95% CI: 6.8–20.1). A higher excess risk among nonseminoma (SIR: 5.4) than seminoma (SIR: 2.3) survivors was also found for acute nonlymphocytic leukemia in the collaborative study of Travis et al. (1997). These results support that the excess risk of myeloid leukemia can be explained by the effect of chemotherapy, as further discussed in the next section.

Chemotherapy and Radiotherapy

In the recent collaborative study by Travis et al. (2005), information on the initial treatment was available for ~10,000 cancer patients surviving at least 10 years, among whom the relative risks of solid tumors were 2.0 (95% CI: 1.9–2.2) for radiotherapy alone, 1.8 (95% CI: 1.3–2.5) for chemotherapy alone and 2.9 (95% CI: 1.9–4.2) for radiotherapy and chemotherapy combined. Smaller studies based

TABLE 16.1. Standardized incidence ratios (SIRs) and corresponding confidence intervals (CIs) of second primary tumors after testicular cancer in the two largest collaborative studies based on Cancer registry data.

Tumor site	Travis et al. (2005) ^a	Richiardi et al. (2007a) ^b
	Number of testicular cancer cases: 40,576 Number of second malignancies: 2,285 SIR (95% CI)	Number of testicular cancer cases: 29,511 Number of second malignancies: 1,811 SIR (95% CI)
All tumours	–	1.65 (1.57–1.73)
All solid tumours	1.41 (1.35–1.47)	–
All buccal	1.13 (0.89–1.41)	1.19 (0.89–1.56)
Oesophagus	1.44 (1.02–1.98)	1.79 (1.20–2.57)
Stomach	2.16 (1.84–2.53)	2.37 (1.97–2.82)
Small intestine	2.60 (1.56–4.06)	2.19 (0.94–4.31)
Colon	1.36 (1.18–1.57)	1.51 (1.25–1.82)
Rectum	1.46 (1.23–1.73)	1.37 (1.09–1.71)
Liver	1.08 (0.69–1.63)	1.23 (0.67–2.07)
Gallbladder, bile ducts	1.58 (0.90–2.56)	2.01 (1.10–3.37)
Pancreas	2.30 (1.90–2.76)	2.56 (2.03–3.19)
Larynx	1.13 (0.78–1.57)	1.16 (0.73–1.76)
Lung	1.19 (1.07–1.32)	1.33 (1.17–1.52)
Pleura	2.80 (1.57–4.62)	–
Bone	1.66 (0.66–3.42)	1.92 (0.62–4.48)
Soft tissue sarcoma	2.65 (1.83–3.73)	2.63 (1.58–4.11)
Melanoma of skin	1.48 (1.23–1.77)	1.62 (1.29–2.01)
Other neoplasm of skin	–	2.26 (1.97–2.57)
Breast	1.21 (0.24–3.53)	2.81 (0.91–6.55)
Prostate	1.05 (0.95–1.17)	1.07 (0.91–1.24)
Bladder	1.93 (1.70–2.18)	2.12 (1.80–2.47)
Kidney	1.42 (1.16–1.72)	2.05 (1.62–2.56)
Eye	0.91 (0.29–2.11)	1.34 (0.44–3.13)
Brain, nervous system	1.14 (0.88–1.45)	1.16 (0.78–1.66)
Thyroid gland	2.17 (1.46–3.10)	2.86 (1.69–4.51)
	Travis et al. (1997) ^c	Richiardi et al. (2007a) ^b
	Number of testicular cancer cases: 28,843 Number of second malignancies: 1,406 SIR (95% CI)	Number of testicular cancer cases: 29,511 Number of second malignancies: 1,811 SIR (95% CI)
Hodgkin's lymphoma	1.26 (0.67–2.15)	1.13 (0.56–2.02)
Non-Hodgkin's lymphoma	1.88 (1.46–2.39)	1.65 (1.27–2.10)
Multiple myeloma	0.81 (0.39–1.50)	1.22 (0.73–1.93)
Lymphoid leukemia	1.12 (0.64–1.83) ^d	0.99(0.53–1.69)
Myeloid leukemia	1.95 (1.36–2.70) ^d	3.62 (2.56–4.97)

Note: The study of Travis et al. (2005) is an update of the study of Travis et al. (1997). Both studies of Travis et al. (2005) and Richiardi et al. (2007a) include Cancer Registries from the Nordic countries and therefore have a 65% overlap. Travis et al. (2005) study included non germ-cell cancers, and analysed second solid malignancies only.

^aRegistries (period): United States, SEER program (1973–1999), Denmark (1943–1998), Sweden (1958–2001), Canada, Ontario (1964–2000), Norway (1953–1999), Finland (1953–2001).

^bRegistries (period): Australia, New South Wales (1972–1997), Canada, British Columbia (1970–1998), Canada, Manitoba (1970–1998), Canada, Saskatchewan (1967–1998), Denmark (1943–1997), Finland (1953–1998), Iceland (1955–2000), Norway (1953–1999), Singapore (1968–1992), Slovenia (1961–1998), Spain, Zaragoza (1978–1998), Sweden (1961–1998), United Kingdom, Scotland (1975–1996).

^cRegistries (period): United States, SEER (1973–1993), Denmark (1943–1991), Sweden (1958–1992), Canada, Ontario (1964–1992), The Netherlands (1971–1993), New Jersey (1979–1991), Finland (1953–1993), Connecticut (1935–1972).

^dSIRs and 95% CIs calculated by us on the basis of data reported in the original article.

on series of testicular cancer patients have less statistical power, but more detailed information on treatment. In a recently published cohort of ~2,700 5-year Dutch testicular cancer survivors, patients treated only with orchiectomy had a SIR of 0.7 (95% CI: 0.4–1.3) compared with the general population. Compared with surgery alone, patients had a relative risk of 2.6 (95% CI: 1.7–4.0) for treatment with infra-diaphragmatic radiotherapy, of 3.6 (95% CI: 2.1–6.0) for infradiaphragmatic and mediastinal radiotherapy, and of 2.1 (95% CI: 1.4–3.1) for chemotherapy alone (van den Belt-Dusebout et al. 2007). In a series of patients treated in Oslo between 1953 and 1990, the SIRs of second malignancies were 1.3 (95% CI: 0.4–3.4) for surgery alone, 1.6 (95% CI: 1.3–1.9) for radiotherapy alone, 1.3 (95% CI: 0.4–3.4) for chemotherapy alone, and 3.5 (95% CI: 2.0–5.9) for radiotherapy and chemotherapy combined.

Since the beginning of the 1990s, several studies have documented chemotherapy related myeloid leukemia after treatment for testicular cancer (Pedersen-Bjergaard et al. 1991; Kollmannsberger et al. 1999). In the collaborative study by Travis et al. (1997), nonseminoma survivors of 5 to 9 years after diagnosis of testicular cancer had a SIR of 14.3 if initially treated with chemotherapy, and of 6.7 if initially treated with radiotherapy alone (data on salvage chemotherapy were not recorded). A subsequent case-control study was nested within this cohort, including 36 cases of second leukemia (Travis et al. 2000). Compared with patients who received only orchiectomy, radiotherapy alone was associated with a threefold increased risk, whereas chemotherapy, either alone or in combination with radiotherapy, was associated with a fivefold increased risk. In

the same study, among leukemia patients ($n = 7$) and controls ($n = 17$) previously treated with platinum-based chemotherapy without chlorambucil, cases received a higher cumulative dose of cisplatin and for a longer duration. Other studies suggested that intensity and frequency of chemotherapy may be associated with the magnitude of the risk of second leukemia (Kollmannsberger et al. 1999). Some studies suggested that the increased risk of leukemia among testicular cancer patients treated with chemotherapy is due to etoposide (Pedersen-Bjergaard et al. 1991; Bokemeyer et al. 1995). Consistently with this hypothesis, most of the studies from the 1980s, which included cases treated mainly without etoposide, did not find an excess risk of leukemia (Wanderas et al. 1997). However, etoposide is usually given in combination with cisplatin and no study had enough power to solve this collinearity problem adequately and estimate separate risks for etoposide and cisplatin. A review of the International Agency for Research on Cancer concluded that there is sufficient evidence in humans for carcinogenicity of etoposide given in combination with cisplatin and bleomycin (group 1) (IARC 2000).

Long-term effects of radiotherapy can explain the excess risks of tumors of the gastrointestinal tract, pancreatic cancer, tumors of the urinary tract, and perhaps sarcomas found among testicular cancer survivors. These organs are in the infra-diaphragmatic field which receives the highest radiation doses during therapy for testicular cancer. In addition, a carcinogenic susceptibility to radiations of most of these organs has been previously documented among atomic bomb survivors (Preston et al. 2003) and patients treated for ankylosing spondylitis (Weiss et al. 1994).

In the collaborative study by Travis et al. (2005), the risk among 10-year testicular cancer survivors treated with radiotherapy alone was 2.7 (95% CI: 2.4–3.0) for cancer sites included in the infradiaphragmatic field, and 1.6 (95% CI: 1.4–1.8) for the other sites. Mediastinal irradiation, used especially in the past to treat advanced testicular cancer, is consistent with the observations of increased risks of lung, oesophageal, and thyroid cancers. In the nested case-control study of 36 second leukemias described above, testicular cancer survivors treated without chemotherapy had an excess risk of leukemia, which increased significantly with the total radiation dose to active bone marrow. This supports that ionising radiations also have a role in the development of second leukemias among testicular cancer patients.

In conclusion, in the last 15 years, a great effort has been devoted to the study of the risk of second malignancies to provide valid evidence and solve inherent methodological difficulties. Overwhelming evidence indicates that testicular cancer survivors have a 40–60% increased risk of second malignancies. The risk is increased in several sites, most of which are consistent with long-term effects of radiotherapy and/or chemotherapy. Because testicular cancer is a highly curable disease that affects young men with a very long life expectancy, the issue of an increased risk of second tumors will have to be carefully taken into account when evaluating advantages and disadvantages of alternative treatment approaches.

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Part IV

Urinary Bladder Cancer

Diagnosis

Urothelial Bladder Cancer: Screening with Urine-Based Tumor Markers

Robert S. Svatek and Yair Lotan

RATIONALE

Urothelial bladder cancer is the fourth most prevalent cancer in males and the ninth most prevalent cancer in females (NCI Cancer Screen 2007). An estimated 67,160 new cases of bladder cancer and 13,750 deaths from bladder cancer are expected in 2007 in the United States (Jemal et al. 2007). In addition, bladder cancer is considered the most costly cancer from diagnosis to death (Botteman et al. 2003). In the United States, bladder cancer is predominately transitional cell carcinoma subtype and it affects a defined population with established risk factors: age, tobacco, and chemical exposures.

The natural history of bladder cancer provides a distinct opportunity for benefits to be gained from early detection with screening. Low-grade, noninvasive (Ta), lesions exhibit high recurrence rates but progression to invasive disease is uncommon (Malkowicz 2002). As a result prognosis for these patients is excellent with long-term survival greater than 95% (Malkowicz 2002). Invasion of the lamina propria (T1) portends a worse prognosis than Ta disease defined by an increased

rate of recurrence (80%) and progression (40–50%) (Malkowicz 2002). However, bladder preservation is possible in up to 55–75% of patients with T1 high-grade lesions (Foresman and Messing 1997). Moreover, with aggressive surveillance, prognosis of patients with high grade T1 disease is significantly better compared to patients presenting with muscle-invasive disease (Foresman and Messing 1997). Unfortunately, with current detection methods, muscle invasion is present in 15–25% of patients diagnosed with bladder cancer (Messing 2002). In addition, many patients will have occult metastasis at presentation. Because of the large discrepancy in morbidity/mortality which is clearly dependent on stage, detection of bladder cancer at an earlier stage, prior to muscle invasion or metastasis, could render a significant improvement in patient morbidity and disease-specific survival.

A statement from the National Cancer Institute lists three requirements that must be met in order to prove that a screening program is efficacious: (1) the disease can be detected earlier than if the cancer were detected by development of symptoms, (2) treatment initiated early can render an

improved outcome, and (3) when evaluated in a prospective manner, screening results in a decrease in cause-specific mortality (NCI Cancer Screen 2007). In this chapter, we will examine previous efforts at bladder cancer screening; discuss available markers and methodology associated with marker selection; and evaluate the potential cost-effectiveness of bladder cancer screening.

PREVIOUS SCREENING PROGRAMS

To date, a limited number of attempts have been made to prospectively evaluate bladder cancer screening. However, bladder cancer has known risk factors that improve identification of populations at higher risk. It is estimated that exposure of aryl amines from tobacco causes 50% of incident bladder cancer, and approximately 20–25% is related to occupational exposure to chemicals such as including beta-naphthylamine (BNA), benzidine, and benzapyrene (Hemstreet et al. 2001). In people with occupational exposures (such as aniline dye workers, petroleum workers, rubber workers, leather finishers, hairdressers, and paint sprayers), the incidence of bladder cancer is up to 50 times higher than an unexposed population. As a result, the National Institute for Occupational Safety and Health has recommended bladder cancer screening and notification programs for such persons (Hemstreet et al. 2001). Several studies have been conducted in populations at high-risk from occupational exposures. In addition, 3 studies have examined the role of dipstick hematuria testing of asymptomatic individuals with one including a significant proportion of older smokers.

SCREENING IN PEOPLE WITH OCCUPATIONAL EXPOSURE

In 1986, the Drake Health Registry Study initiated bladder cancer screening using urinalysis, cytology, and quantitative fluorescence image analysis in 366 persons at high risk because of occupational exposure to beta-naphthylamine at the Drake Chemical Company, Lock Haven, PA (Marsh and Cassidy 2003). Of the 40 persons who underwent cystoscopy for positive test(s), one was diagnosed with carcinoma in situ and 2 with transitional cell carcinoma. In addition, other bladder abnormalities such as dysplasia were detected in a large proportion of a relatively young cohort which may indicate changes prior to the development of bladder cancer (Marsh and Cassidy 2003). Others (Bi et al. 1992; Hemstreet et al. 2001) have examined a high-risk Chinese cohort with occupational exposure to benzidine from three different Chinese cities. A study by Hemstreet et al. (2001) evaluated 1,788 workers exposed to benzidine and 373 nonexposed workers over a 6 year period. They assayed urothelial cells from voided urine samples for DNA ploidy, the bladder tumor-associated antigen p300, and a cytoskeletal protein (G-actin). Bladder cancer was diagnosed in 28 exposed workers and 2 nonexposed workers. For risk assessment, DNA 5CER had 87.5% sensitivity and 86.5% specificity and p300 had 50.0% sensitivity and 97.9% specificity. The risk of developing bladder cancer was 19.6 (95% CI = 8.0 to 47.9) times higher in workers positive for either the DNA 5CER or p300 biomarkers than in workers negative for both biomarkers and 81.4 (95% CI = 33.3 to 199.3) times higher in workers positive for both biomarkers. G-actin was a poor marker of individual risk.

HEMATURIA SCREENING

Screening for bladder cancer has been conducted using repeated chemical reagent strip for hemoglobin (Britton et al. 1992; Messing et al. 2006). Messing et al. (2006) tested 1,575 men ages 50 years and older who were solicited from well patient rosters in clinics in and around Madison, Wisconsin, in 1987 and from 1998 to 1992. Of these patients, 16% were current and 44% were former smokers. Participants who had positive test results underwent standard urologic evaluation. They compared their patient cohort with patients in the Wisconsin Tumor Registry in 1988 (n = 509 men). Two hundred and fifty-eight screening participants (16.4%) were evaluated for hematuria, and 21 participants (8.1%) were diagnosed with bladder cancer. Proportions of low-grade (Grade 1 and 2) superficial (Stage Ta and T1) versus high-grade (Grade 3) superficial or invasive (Stage $\geq$ T2) cancers in screened men (52.4% vs. 47.7%) and in men from the tumor registry (60.3% vs. 39.7%) were similar ($P = .50$). The proportion of high-grade superficial or invasive bladder cancers that were invasive were lower in screened men (10%) than in unscreened men (60%; $P = .002$). At 14 years of follow-up, no men with screen-detected bladder cancer had died of bladder cancer, whereas 20.4% of unscreened patients had died of bladder cancer ($P = .02$) (Messing et al. 2006).

The second notable study evaluating bladder cancer screening using hematuria testing was carried out in the UK by Britton et al. (1992). In their study, 2,356 men over the age of 60 were screened with repeated hematuria dipstick analysis. A total of 474 (20%) of men had evidence

for hematuria and 319 of these men underwent further testing for urothelial cancer. A total of 17 men were diagnosed with bladder cancer and the positive predictive value (PPV) (see Table 17.1) of hematuria testing was 5.3% (17 out of 319). After 3 years of follow-up (Whelan et al. 1993), only 1 (11%) of the 9 patients with T1 disease progressed to muscle-invasive disease, which is less than the 40–50% progression rate observed in non-screened populations (Malkowicz 2002).

A more recent study investigated the relevance of dipstick hematuria testing for bladder tumour screening in a random selection of men, age 60–70 years (Hedelin et al. 2006). In that study, screening was performed in 1,096 men who agreed to participate after invitation by mail. In concordance with the aforementioned studies, the PPV

TABLE 17.1. Definitions and formulas of statistical terms associated with 2 × 2 contingency tables

Term	Definition	Formula
Sensitivity	Proportion of diseased subjects who test positive with the screening test	$TP/TP + FN$
Specificity	Proportion of non-diseased subjects who test negative with the screening test	$TN/TN + FP$
Positive predictive value	Proportion of subjects with a positive screening test who are correctly diagnosed	$TP/TP + FP$
Negative predictive value	Proportion of subjects with a negative screening test who are correctly diagnosed	$TN/TN + FN$
False positive rate	Proportion of non-diseased subjects who incorrectly test positive with the screening test	1-specificity
False negative rate	Proportion of diseased subjects who incorrectly test negative with the screening test	1-sensitivity

TP – true positive; TN – true negative; FP – false positive; FN – false negative.

of hematuria dipstick testing for detecting bladder cancer was relatively low at 1.6–3.6%, corresponding to a RBC concentration cut-off of 10–25 RBCs/ μ l, respectively.

Home urine dipstick to assess for hematuria is convenient, inexpensive, and noninvasive. This classic study by Messing et al. (2006) demonstrated that bladder cancer screening can detect tumors prior to the progression to muscle-invasion thereby reducing cancer-specific mortality. Although that study lacked a prospective control arm, the data are convincing and support the rationale for bladder cancer screening. Unfortunately the low positive predictive value of hematuria testing limits the applicability for widespread bladder cancer screening. Indeed, among the studies evaluating hematuria dipstick for screening (Britton et al. 1992; Hedelin et al. 2006; Messing et al. 2006), only 3.6–8.3% of men who tested positive were actually found to have bladder cancer. Therefore, many men underwent presumptive unnecessary workups and may have incurred unnecessary anxiety and cost.

URINE-BASED TUMOR MARKERS

Screening for bladder cancer aims to detect disease early in an asymptomatic population. This is distinguished from bladder cancer surveillance which is used to detect tumors in patients with a history of cancer. Although cystoscopy and cytology are the standard of care for cancer detection, their use as screening agents is limited due to the invasiveness of cystoscopy and the low sensitivity of cytology. Recently the exploration of various molecular pathways implicated in cancer development has led to the identification of markers of disease

presence or progression. Some of these markers are actively utilized in clinical practice (Lewandowski 2003). These markers are designed to detect various changes thought to be associated with the development of bladder cancer, such as tumor protein expression or chromosomal abnormalities. Of the various tests, Hemastix (hematuria detection; Bayer Corp., Elkhart, IN), ImmunoCyt/uCyt (DiagnoCure Inc., Quebec, Canada), bladder tumor antigen (BTA stat Test; Polymedco Inc., Redmond, WA); nuclear matrix protein (NMP)–22 BladderChek (Matritech, Newton, MA), and urinary bladder cancer (UBC)–Rapid (IDL Biotech, Borl  bger, Sweden) are point-of-care tests that can be performed and provide results quickly in the clinician's office (Lokeshwar et al. 2005). Other tests, such as BTA-TRAK (Polymedco), NMP-22 (original test; Matritech), UroVysion (Vysis), hyaluronic acid–hyaluronidase (HA-HAase), BLCA-4, microsatellite DNA alterations Quanticyt (Gentian Scientific Software, Niawier, the Netherlands), nuclear karyometry, telomeric repeat amplification protocol assay (TRAP), human telomerase reverse transcriptase (hTERT), reverse transcriptase-polymerase chain reaction (RT-PCR), uCyt (Diagnocure, Quebec City, Quebec, Canada), and the DD23 marker are enzyme-linked immunosorbent assays (ELISA), RT-PCRs, or microscopic image analyses, and these must be sent to a central laboratory for reading (Lokeshwar et al. 2005).

Compared to conventional cytology, some of these markers have demonstrated superior sensitivity for low grade tumors and equivalent sensitivity for high grade tumors and carcinoma in situ (Lotan and Roehrborn 2003). Similar to dipstick hematuria testing, urine-based tumor markers are non-invasive, requiring a simple urine

collection for analysis. In addition, some of the markers can be tested with point-of-care assays providing quick results in the clinician's office. If found to have improved performance compared to hematuria testing, a urine-based marker could be a prime candidate for prospective evaluation of screening efficacy.

METHODOLOGICAL ASPECTS OF MARKER EVALUATION

The usefulness of a bladder cancer marker is determined by its performance characteristics. Avoidance of false positive tests results is critical for bladder cancer screening because false positive tests yield unnecessary costs, potential complications associated with confirmatory testing (i.e., cystoscopy and upper tract evaluation), and undue patient anxiety. False negative tests, on the other hand, potentially render false reassurances which can result in adverse outcomes should the patient delay seeking evaluation. Marker accuracy is often characterized by sensitivity, specificity, and positive predictive value (Table 17.1).

These performance characteristics are not completely determined by the intrinsic properties of the marker; rather they are also dependent on the cohort undergoing marker testing. For this reason, sensitivity, specificity, positive predictive value, and negative predictive value for any given marker can change depending on the population being evaluated. For example, the positive and negative predictive values of a marker change as the prevalence of the disease in a given population changes. In a high risk population any given marker should demonstrate an improvement in its positive predictive value of a marker

compared to a population with a lower prevalence of disease. Indeed, Grossman et al. (2005) reported an improvement in PPV from 20% to 37% in detecting bladder cancer using the NMP22 BladderChek Test among patients referred to urologists for evaluation due to hematuria or symptoms of bladder cancer. As pointed out by Nielsen et al. (2006), the characteristics of the control arm in a study population is a key factor in determining the performance features of biological markers for bladder cancer detection. For example, exclusion of certain patients from the study, such as healthier patients, may generate a control arm from the study population that does not reflect the real life scenario where many confounders are present. As a result, the prevalence of the disease may be increased and the presumed marker accuracy is artificially inflated (Nielsen et al. 2006). For this reason, in order to obtain an accurate gauge of marker accuracy, it is important that the "spectrum of control conditions (or subjects) reflects the conditions in the general population" (Nielsen et al. 2006).

Initially most cancer markers are analyzed on a continuous scale with overlapping values for patients with and without cancer. To be clinically useful, a cut-point needs to be established which dichotomizes the result into a positive or negative test. A useful method for evaluating the performance characteristics of a marker is the receiver operating characteristic (ROC) curve. This is a graphical display of the false positive rate (1-specificity) on the horizontal axis relative to the true positive rate (sensitivity) on the vertical axis (Figure. 17.1). Continuous marker levels are examined in a patient population with and without cancer and then the sensitivity and specificity for

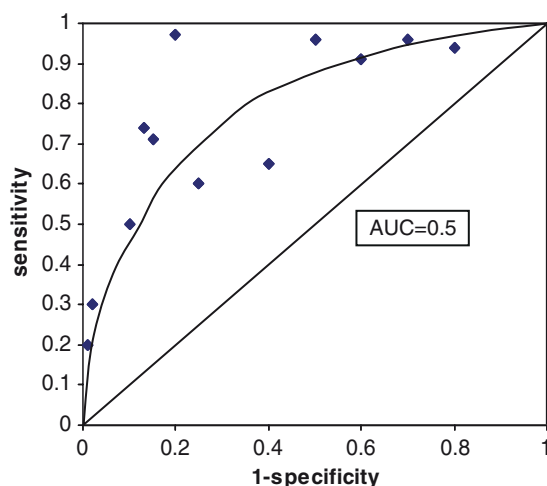


FIGURE 17.1. Hypothetical receiver operating characteristic curve for a marker

various selected marker levels are calculated, and plotted. Subsequently, a line can be drawn to connect these plotted points. The area under the curve (AUC) generated from drawing this line is equivalent to the utility of the marker. The marker's performance improves as the line moves higher and to the left. For example, a perfect marker with 100% sensitivity and 100% specificity would have an $AUC = 1$. On the other hand, an $AUC = 0.5$ indicates that the marker has a 50–50 chance of correctly detecting the presence of cancer.

Various methods to designate the “optimal” cut-point have been proposed (Zweig and Campbell 1993; Nielsen et al. 2006), but the clinical application of the marker greatly influences the appropriate cut-point. For example, the appropriate marker cut-point for detecting cancer in an asymptomatic population might be different than the marker cut-point for detecting the same cancer in a surveillance scenario. In a screened population, the false positive rate should be very low to avoid unnecessary diagnostic work-ups, especially if these

work-ups are expensive or invasive, as in bladder cancer (Nielsen et al. 2006). In a surveillance protocol, however, the consequences of failing to detect cancer outweigh the consequences of false-positive tests in patients with a known history of bladder cancer. As a result, specificity becomes more important for markers used to screen an asymptomatic population whereas sensitivity is more important for cancer detection during surveillance (Nielsen et al. 2006). In addition, when screening for a disease with a low prevalence, the false positive rate has a greater impact on the predictive value of the test than in a disease with a higher prevalence (Baker 2003). For example, screening a population for bladder cancer with a prevalence of 0.6%, a marker false positive rate of 1% and sensitivity of 75% would yield a positive predictive value of 43%. If the false positive rate were slightly higher at 5%, the positive predictive value would drop to 7% (Baker 2003).

Development of ROC curves and subsequent marker cut-points are subject to bias from unrealistic control populations (see above) and overfitting (Baker 2003). Overfit bias occurs because so many different marker cut-points can be generated for the available, sometimes limited data. By chance alone, one of these cut-points is bound to provide excellent accuracy. In order to avoid overfitting bias, internal validation is often performed. Internal validation can be performed by randomly splitting the data into a training set and a validation set. A few of the marker cut-points with the best performance obtained from the training set can then be selected to be tested in the validation set. In addition, further confirmation of appropriate marker cut-point can be evaluated in a prospective analysis from a separate population (Baker 2003).

SPECIFIC URINE-BASED TUMOR MARKERS

There are currently multiple urine-based tumor markers in various stages of development (Lokeshwar et al. 2005). There are four commercially available markers at this time including BTA stat[®] test (Polymedco Inc., Redmond, WA), NMP22[®] BladderChek[®] Test (MatriTech Inc, Newton, Mass), UroVysion (Vysis Downer's Grove, IL) and ImmunoCyt/uCyt[™] (DiagnoCure Inc., Quebec, Canada). Only the NMP22[®] BladderChek[®] test is FDA-approved for detection of bladder cancer in high risk patients, but the Urovysion test is approved for detecting bladder cancer in patients with hematuria. The other markers are currently approved for surveillance purposes.

BLADDER TUMOR ASSOCIATED ANTIGEN TEST

The bladder tumor associated antigen (BTA) test measures a basement membrane protein that is released into the urine as result of proteolysis by the tumor as it invades the bladder wall. BTA stat[®] test and BTA TRAK[®] assay, detect the complement factor H-related protein and have improved sensitivity over the original BTA test (Chao et al. 2001). BTA stat[®] is a point-of-care test while the BTA TRAK[®] assay requires a formal assay which is adaptable to many automated systems and can be performed in 2.5 h. It has been found to have significant association with the presence of bladder cancer in surveillance studies and has been tested in at least one study among a cohort without a history of bladder cancer (Nasuti et al. 1999). In that analysis, 100 patients without a history of bladder cancer but who

had signs and symptoms of dysuria, incontinence or hematuria were tested. Of the 19 cases which tested positive for BTA stat, 3 (15.8%) were found to have bladder cancer. Although it has an improved sensitivity over voided cytology, BTA has a high false positive rate especially in the presence of other urinary pathology such as infections, stones, or BPH. It is unknown how BTA would perform in a completely asymptomatic population.

NUCLEAR MATRIX PROTEIN-22

Bladderchek[®] is a nuclear matrix protein (NMP-22) marker that utilizes monoclonal antibodies to detect levels of mitotic apparatus proteins that are elevated in cancer cells. It is a point-of-care test which provides results within 30 min. Researchers have investigated the role of NMP-22 in the detection of bladder cancer among patients without a history of bladder cancer. A multi-institutional study by Grossman et al. (2005) utilized NMP-22 in a large cohort of patients at elevated risk for bladder cancer due to factors such as age, history of smoking, or hematuria. They found cancer in 6% of their cohort, and NMP-22 rendered a sensitivity and specificity of 55.7% and 85.7%, respectively. In their study, the proportion of patients with a positive test who were correctly diagnosed with bladder cancer (positive predictive value) was 19.7%. This is comparable to a 25–33% PPV afforded by TRUS biopsy for a PSA >4 ng/mL (Murphy et al. 2004). However, Grossman et al. (2005) reported an improvement in the positive-predictive value of the NMP-22 test from 19.7% to 37% when targeting only patients at highest risk for having bladder cancer

(men older than 60 years of age with a history of smoking). While these results are promising, most patients in this study had hematuria, and an evaluation in an asymptomatic population is necessary to validate the utility of such a test in screening asymptomatic patients.

UROVYSION

The accumulation of genetic alterations in a stepwise process is an accepted model of carcinogenesis. It follows that genetic alterations may help predict the future development of cancer. Homozygous loss of chromosome band 9p21, the location of tumor suppressor gene p16, and chromosomal instability are known early genetic events in the development of urothelial-cell bladder carcinoma (Sokolova et al. 2000; Kruger et al. 2003). Fluorescence in situ hybridization (FISH) assay (UroVysion®) is designed to detect aneuploidy of chromosomes 3, 7, 17, and loss of the 9p21 locus (Sokolova et al. 2000; Kruger et al. 2003). Several studies (Sokolova et al. 2000; Placer et al. 2002; Junker et al. 2006) have demonstrated FISH to be useful in the detection of recurrent tumors among patients with a history of bladder cancer. A large review found FISH to have a median sensitivity of 79% (Range 70–86%) and a median specificity of 70% (Range 66–93%) in predicting recurrent bladder tumors (van Rhijn et al. 2005). FISH has not been studied as a primary screening agent in a cohort of asymptomatic individuals. However, a large, multi-institutional study was performed to evaluate the performance of FISH in a symptomatic (hematuria) population without a history of bladder (Sarosdy et al. 2006).

In that series, as expected, FISH outperformed cytology as a detection tool (sensitivity of 69% compared to 38% for cytology), but the more impressive finding was the high positive predictive value (PPV = 65%) of FISH for detecting cancer in the high-risk sub-group (those with greater than 40 pack-year smoking history). It is uncertain if this performance would hold true in a completely asymptomatic population, but a high PPV would be ideal in a screened population and those results urge further testing of FISH in an asymptomatic population. Unfortunately, at a current cost of greater than \$300 per test, FISH is unlikely to be cost-effective in the setting of screening.

IMMUNOCYT/UCYT

ImmunoCyt/uCyt™ (DiagnoCure Inc., Quebec, Canada) uses fluorescent-labeled antibodies to 3 markers (LDQ10, M344, and 19A211) that are commonly found on malignant exfoliated urothelial cancer cells. The largest series evaluating ImmunoCyt/uCyt™ included 942 patients with a history of transitional cell carcinoma of the bladder. The study found ImmunoCyt/uCyt™ to have an increased sensitivity for detecting low grade cancer (sensitivity of 8.3% for cytology alone compared to 79.3% for combined ImmunoCyt/uCyt™ and cytology) and high grade cancer (sensitivity of 75.3% for cytology alone compared to 98.9% for combined ImmunoCyt/uCyt™ and cytology). A recent review provides a range of sensitivity 38.5–92.1% and specificity of 62–84.2%, emphasizing the influence that the population under study has on the performance characteristics of the marker. To date,

ImmunoCyt/uCytTM has not been tested in an asymptomatic population, but it is approved by the FDA for monitoring bladder cancer recurrence in conjunction with cystoscopy and cytology.

COST-EFFECTIVENESS

Before widespread implementation of an efficacious screening program can be implemented, policy makers must consider the value of screening for bladder cancer compared with other health-related and non-health-related issues. The methodology for doing this is with cost-effectiveness analysis. From a theoretical standpoint, screening for bladder cancer would seem to be a sound policy. Detection of cancer at an earlier stage would aim to decrease mortality from cancer-related death and/or to avoid treatment-related morbidity. However, acceptance of wide-spread screening strategies requires careful consideration of the competing risks, benefits, and costs associated with such policies. Due to the fact that there are no prospective studies to evaluate the cost-effectiveness of screening for bladder cancer, decision-analytic modeling must be utilized to estimate the cost-benefit of screening. This is an accepted means for cost-effectiveness analysis since randomized trials of screening versus not screening do not exist for any other cancer (Krahn et al. 1999; Shen and Parmigiani 2005).

Several studies were undertaken to evaluate the cost-effectiveness of utilizing NMP-22 as a screening agent (Lotan et al. 2006; Svatek et al. 2006). Initially, a study was performed to determine the cost of cancer detection (Svatek et al. 2006). A decision tree analysis was constructed

to evaluate the total cost of screening a low- and high-risk population for bladder cancer using NMP-22. It was noted that the cost-per-cancer detected would be in the \$2,000–\$5,000 range if used in a high-risk population but would be greater than \$100,000 if used in the overall population (Svatek et al. 2006). The cost per cancer-detected in the high risk population was similar or superior to screening for prostate, breast and colon cancer. Subsequently, a Markov model was created to estimate cumulative cancer-related costs and efficacy of screening (vs. no screening) of a high-risk population for bladder cancer using a urine-based tumor marker over a 5 year period (Lotan et al. 2006). This analysis found that in a population with greater than a 1.6% cancer incidence, screening with Bladderchek would result in both improved overall survival and a cost-savings. This is a unique finding because most evaluations of screening policies for other cancers such as prostate, breast, colon, and cervical are greater than \$50,000 per life year saved. The model can be applied to other markers utilizing their sensitivity and specificity and costs.

There are several reasons bladder cancer screening was found to be a superior strategy in terms of both survival and cost. Most importantly, there is a survival benefit to earlier cancer detection from muscle-invasive disease to non muscle-invasive disease even if only a small proportion of persons develop cancer. Cost advantage is also generated because of the significant cost associated with treatment of muscle-invasive disease and metastatic bladder cancer. At only \$24, Bladderchek is relatively inexpensive compared to screening with colonoscopy or mammography. In addition, even those persons with false

positive testing only have the additional cost of cystoscopy, cytology and physician visit (\$402).

BIASES AND PITFALLS
IN BLADDER CANCER
SCREENING

Several implications from a screening program should be considered against the potential benefits of screening for bladder cancer. As pointed out previously, false positive tests lead to unnecessary invasive diagnostic procedures. A test with a poor specificity is not cost-effective unless the population has a higher incidence of cancer because at a low incidence more cost is accrued for unnecessary testing (Figure. 17.2). For bladder cancer this includes cystoscopy and cytology. For some people, office-based cystoscopy is poorly tolerated and they may require global or regional anesthesia for evaluation. Furthermore, a small number of

patients may require transurethral bladder biopsies for suspicious but indeterminate lesions (Svatek et al. 2005).

False-negative screening test results can cause a delay in diagnosis and effective treatment because patients are falsely reassured from the screening test but subsequently develop clinically significant cancer (NCI Cancer Screen 2007). Conversely, some bladder cancer screening tests may detect malignancy prior to the development of a tumor large enough to be seen on cystoscopy, which are so called “anticipatory positive” results (Panani et al. 2006). These uncertainties may render unnecessary anxiety and aggressive clinical surveillance.

Interpretation of survival data from studies evaluating the efficacy of bladder cancer screening markers must consider lead time and length time bias. Lead time bias favors the screened population because screening in this cohort detects the disease before it would normally be diagnosed, thereby artificially increasing the survival time. Length time bias reflects the tendency for screening tests to detect the slower growing, less aggressive tumors which renders an artificial improved survival in the screened cohort. These biases are likely to be less significant in bladder cancer than slow growing tumors such as prostate cancer. Messing et al. (2006), using hemoglobin dipsticks, found a greater proportion of high grade noninvasive cancers because of the tendency of tumor markers including hemoglobin dipsticks to have a higher sensitivity for high grade disease. This would argue against a length time bias in bladder cancer screening. The issue of lead time bias is yet to be determined but the long term study by Messing et al. (2006) suggests that there is a significant difference in survival with no screened patient dying

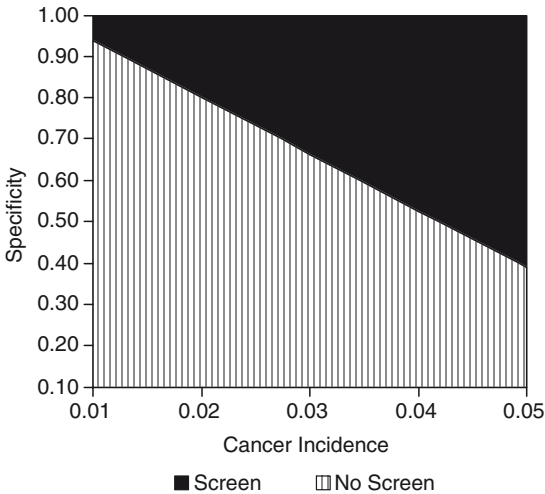


FIGURE 17.2. Two-way sensitivity analysis. The impact of varying the annual incidence of cancer and the marker specificity on cost-effectiveness of different detection strategies

vs. 20% of the control group. This would not be explained by lead time bias alone.

Another issue that may have critical implications in proving efficacy for bladder cancer screening is the pathway of bladder cancer development. Most epithelial tumors are thought to progress along a single pathway from benign to malignant disease which is characterized by alterations of critical genes which regulate cell growth and survival. Urothelial carcinoma, on the other hand, is considered to develop along two separate pathways. Low-grade papillary disease, which may include urothelial papilloma, papillary urothelial neoplasm of low malignant potential, and non-invasive low-grade papillary urothelial carcinoma is often multifocal and recurrent but has limited invasive capacity. As a result, patients with low grade disease, who represent 70–80% of patients with bladder cancer, have excellent survival outcome and are unlikely to benefit from bladder cancer screening. Invasive bladder cancer accounts for only 20% of all urothelial carcinoma and most patients with invasive disease have no previous history of low-grade non-invasive tumors. Unfortunately up to 50% of patients have lymph node metastasis at the time of diagnosis. The benefit of bladder cancer screening depends on the ability to identify high grade disease at a pre-invasive pathologic state. The study by Messing et al. (2006) suggests that this is feasible and further studies are needed to support this data.

FUTURE CONSIDERATIONS

The promising results from two large screening programs for hematuria have ignited interest in the field of bladder

cancer, and further steps are underway to evaluate the role of urine-based bladder tumor markers for bladder cancer screening. Currently a screening study is ongoing at UT Southwestern which uses Bladderchek® to screen asymptomatic patients at high risk (age older than 50 and heavy tobacco usage) for bladder cancer. Patients with a positive screening test undergo formal testing with cystoscopy and cytology. Bladderchek® is also being utilized by Dr. Stoller and colleagues as an annual screening agent for the employees of the San Francisco Fire Department given their higher risk from occupational exposures. Furthermore, there is a screening study to be carried out by the MD Anderson SPORE in which subjects will use multiple Hemastix tests. Everyone with a positive Hemastix test will undergo cystoscopy and 3 marker tests (NMP22® BladderChek® Test, UroVysion FISH and Immunocyt (Diagnocure Inc., Quebec, Canada)).

CONCLUSIONS

In conclusion, bladder cancer is a disease with a relatively high prevalence and unique dichotomous behaviour that lends itself toward potential benefits to be gained from early detection. Urine-based testing with blood or tumor markers is exciting given the potential for rapid, cheap, and non-invasive testing. Development of urine-based tumors requires careful attention to proper cut-points selection and proper evaluation in trials with appropriately controlled cohorts. Efficacy for bladder cancer screening requires proof of improved survival for the screened cohort and the potential benefits of screening need to be weighed against the potential

pitfalls. These include false positive tests leading to unnecessary expensive, invasive diagnostic procedures and undue patient anxiety, unclear incidence of cancer in an asymptomatic population, and uncertain cost-effectiveness. Further studies to evaluate the role of bladder tumor markers in high risk populations are on-going. In addition, more studies will be necessary to assess the cost-effectiveness of this approach prior to widespread utilization of screening for bladder cancer.

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Detection of OCT-4 in Bladder Cancer: Role of Cancer Stem Cell

Seyed Javad Mowla, Seyed Mehdi Jafarnejad, and Yaser Atlasi

INTRODUCTION

There exist several lines of evidence in support of the idea that malignant tumors contain a subpopulation of cells with biological properties similar to normal adult stem cells (Al-Hajj and Clarke 2004; Chang 2006; Kamstrup et al. 2007). The cancer stem cell (CSC) hypothesis is based on the observation that tumors arise from cells exhibiting the abilities to self-renew and to give rise to partially differentiated tissue cells, resembling an abnormal tissue development (Reya and Clevers 2005; Reya et al. 2001). The cancer stem cells can be generated whenever the mechanisms regulating the self-renewal process in normal stem or early progenitor cells goes awry; thus the changes could end up in illegitimate proliferation or impaired differentiation of the altered cells (Bjerkvig et al. 2005; Clarke and Fuller 2006; Wicha et al. 2006).

The hypothesis that cancers arise from the transformation of tissue-specific adult stem cells is based on several rationales. Firstly, stem cells are long-lived entities; thus, subjected to the accumulation of multiple genetic alterations (i.e., muta-

tions) that pave the way for the transformation process. Secondly, cancer stem cells share several important properties with their normal counterparts (Lou and Dean 2007; Prindull 2005; Wicha et al. 2006; Zhang and Rosen 2006). These characteristics include: (1) the sustained telomerase activity, (2) virtually unlimited self-renewal capacity, (3) the ability to partially give rise to multiple differentiated progenies, (4) increased membrane transporter activity through ATP-binding cassette transporters, and (5) the intrinsic ability to migrate and reside (metastasize) in other tissues or organs.

During the past few years, there have been considerable advances in deducing the biology of normal stem cells, along with the recent application of these concepts to experimentally define stem cell-like cancer cells (Mayani 2003; Pardal et al. 2003). These progresses have resulted in identification of CSCs in several human malignancies including leukemia (Daley 2004; Somervaille and Cleary 2006), breast cancer (Dontu et al. 2003), prostate cancer (Collins and Maitland 2006), and brain tumors (Fomchenko and Holland 2005; Hemmati et al. 2003). In addition,

development of cell sorting techniques in conjunction with new animal models, which enabled researchers to measure the self-renewal and tumorigenic abilities of various subpopulations of tumor cells, has simplified further analysis of CSCs (Hadnagy et al. 2006).

The CSC hypothesis raises several important implications. Firstly, if there were a population of CSCs, non-CSC tumor cells would probably not be able to initiate tumors, regardless of their differentiation status or their proliferative capacity (Al-Hajj et al. 2004; Schulenburg et al. 2006). The aforementioned idea would have crucial consequences in recruitment of experimental strategies to define CSCs. Secondly, the CSC hypothesis implies that any anti-cancer therapy must meet the criterion of complete eradication of the CSC population in order to eliminate the chances of tumor recurrence (Jones et al. 2004; Lou and Dean 2007; Sperr et al. 2004).

One of the major characteristics of stem cells is their self-renewal capacity, which is tightly regulated by several transcription factors. The OCT-4 transcription factor, a member of the POU family that is also known as Oct-3 and Oct3/4, has a unique and important role in sustaining the self-renewal in totipotent embryonic stem cells (ESCs) (Rosner et al. 1990). The expression of OCT-4 is spatially and temporally restricted to early embryonic stages, including: the inner cell mass, primitive ectoderm, primordial germ cells, as well as in ESCs, embryonic germ, and embryonic carcinoma cells (Nichols et al. 1998; Niwa et al. 2000; Takeda et al. 1992). Despite the traditional belief in restricted expression of OCT-4 in embryonic tissues, recent reports demonstrated that the expression of OCT-4 takes place in several adult tissues

(Tai et al. 2005), most likely in tissue resident adult stem cells.

Recently, it has been suggested that OCT-4 acts as a multi-functional factor in cancer and stem cell biology. Based on the reports that OCT-4 increases the malignant potential of ES cells in a dose-dependent manner, a possible oncogenic role has also been attributed to OCT-4 (Gidekel et al. 2003). Moreover, ectopic expression of OCT-4 in epithelial tissues could lead to a dysplastic induction through interfering with differentiation of epithelial stem/progenitor cells. The effect seems very similar to the primary role of OCT-4 in embryonic cells (Hochedlinger et al. 2005), providing a further evidence for oncogenic function of OCT-4.

Although the expression of OCT-4 in germ cell tumors has been extensively studied (Looijenga et al. 2003), little is known about the expression of this important factor in different types of somatic cancers. In this chapter, we are presenting some of the data obtained in our laboratory (Atlasi et al. 2007; Atlasi et al. 2008), aiming to explore the potential expression of OCT-4 mRNA and protein in bladder cancer. In addition, considering the crucial inhibitory role of OCT-4 in normal stem cells' differentiation, we also have been investigating whether OCT-4 expression might have any correlation with different grades of tumors, which negatively correlates with the differentiation status of cancer cells.

MATERIALS

1. Tissue biopsies: Fresh tissue biopsies were obtained from patients whom had been referred to Labbafi-nejad medical center in Tehran-Iran. The tissues were immediately snap-frozen

- in liquid nitrogen and stored for later analysis.
2. 0.01 M Phosphate buffered saline (PBS): 8.7 g NaCl, 0.272 g potassium dihydrogen phosphate, and 1.136 disodium hydrogen phosphate. Bring to 1 L by adding ddH₂O.
 3. RNX plusTM RNA extraction solution (Cinagene, Iran).
 4. DEPC (diethylpyrocarbonate) treated water: add 100 μ L of DEPC into 100 mL of ddH₂O, incubate overnight and autoclave to degrade the residual DEPC.
 5. RNase free plasticware: incubate all plasticware including tubes, tips, etc., in 1 μ L/mL DEPC water overnight. Place the plasticware in a clean container and remove the residual DEPC by autoclave.
 6. Chloroform.
 7. Isopropanol.
 8. Tris-borate EDTA (TBE) buffer (5x): prepare a stock containing 54 g Tris-base, 27.5 g boric acid, 20 mL of 0.5 M EDTA solution (pH 8.0) and 800 mL ddH₂O.
 9. EDTA (0.5 M): add 186.1 g Na₂-EDTA.2H₂O to 800 mL ddH₂O and adjust pH to 8 by using NaOH and bring to 1 L by adding ddH₂O.
 10. Agarose gel, 1%: dissolve 0.5 mg of agarose in 50 mL of boiling TBE buffer (1x), cool and cast the gel.
 11. 6X loading dye: 50% of glycerol, 1mM EDTA, 6% bromophenol blue and 6% xylene cyanol.
 12. DNA size marker.
 13. RNase-free DNase (Fermentase, Lithuania).
 14. RNase inhibitor (Fermentase, Lithuania).
 15. Oligo-dT18 primer (MWG, Germany).
 16. RevertAidTM MMuLV Reverse Transcriptase (Fermentase, Lithuania).
 17. 5x Reverse transcriptase buffer.
 18. PCR primers: forward and reverse PCR primers were designed using human OCT-4 and beta-2 microglobulin (β 2m) sequences (GenBank accession numbers: NM_002701 and NM_004048, respectively) by means of Genrunner software (version 3.02; Hastings Software Inc.) as follows: hOCT-4F: 5'- GAGAATTTGTTTCCTGCAGTGC- 3' hOCT-4R: 5'- GTTCCCAATTCCTTCCTTAGTG- 3' β 2m-F: 5'- CTACTCTCTCTTTCTGGCCTG- 3' β 2m-R: 5'- GACAAGTCTGAATGCTCCAC -3'
- All sequences were checked by BLAST analysis to avoid any nonspecific binding of the primers.
19. Taq polymerase (Cinnagen, Iran).
 20. 10x Taq polymerase buffer.
 21. 1.5 mM MgCl₂.
 22. 20 mM dNTPs mix containing all 4 deoxiribonucleotides three phosphates in equal concentration.
 23. Ethidium bromide solution: 50 mg Ethidium bromide powder in 100 mL TBE buffer.
 24. Modified RIPA buffer: 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM PMSF (Phenylmethylsulfonyl fluoride), 1 mM EDTA, 1% Triton x-100, 1% sodium deoxycholate, 0.1% SDS.
 25. Bradford solution (5x): 25 mg Coomassie brilliant blue, 12.5 mL ethanol, 25 mL metaphosphoric acid and bring to 50 mL by ddH₂O. Store at 4°C.
 26. Bovine Serum Albumin (BSA) serial dilution: make a serial dilution of 0.1 mg/mL to 1.7 mg/mL (with 0.2 intervals) of BSA in PBS. Store at -20°C.
 27. Stacking gel buffer: dissolve 6.1 g Tris Base and 0.4 g SDS in 50 mL ddH₂O. Adjust pH to 8.8 by 2 M HCl and bring to 100 mL with ddH₂O.

28. Resolving gel buffer: dissolve 18.2 g Tris base and 0.4 g SDS in 50 mL ddH₂O. Adjust pH to 8.8 by 2 M HCl and bring to 100 mL with ddH₂O.
29. Polyacrylamide stock solution (30.8%): dissolve 30 g polyacrylamide powder and 0.8 g bis-acrylamide powder in 100 mL ddH₂O. Filter with watman paper and store at 4°C in a dark container.
30. TEMED 10%: add 0.1 mL TEMED to 0.9 mL ddH₂O and mix. Prepare the solution immediately before use.
31. Ammonium persulfate (APS) 10%: dissolve 1 g of ammonium persulfate in 10 mL of ddH₂O. Prepare the solution immediately before use.
32. Polyacrylamide stacking gel (4%): 0.625 mL stacking gel buffer, 0.450 acrylamide stock solution, 1.45 mL ddH₂O, 0.025 mL APS and 0.008 mL TEMED.
33. Polyacrylamide resolving gel (12.5%): 1.5 mL resolving gel buffer, 2.45 mL polyacrylamide stock solution, 2.05 mL ddH₂O, 0.025 mL APS and 0.015 mL TEMED.
34. Sample buffer (5x): 10 mL stacking gel buffer, 5 mL glycerol, 1 g SDS, 0.5 mL bromophenol blue solution (1% in ethanol) and 1 mL 2-mercaptoethanol. Bring to 20 mL by ddH₂O.
35. Electrophoresis buffer: dissolve 3 g Tris base, 14.4 g glycine and 1 g SDS in 1 L ddH₂O.
36. Prestained protein size marker (Fermentas).
37. Hyband-PPVDF membrane (Amersham Biosciences Europe GmbH, Germany). Place the membrane in methanol immediately prior to use for 10 s and wash with ddH₂O.
38. Transfer buffer: 4.5 g tris base, 21.6 g Glycine and 225 mL methanol. Bring to 1.5 L by ddH₂O.
39. One percent PBS-Tween 20 (PBS-T) washing buffer: add 1 ml of Tween 20 detergent in 99 mL of PBS buffer and dissolve by vigorous pipetting.
40. ECL Advance Blocking solution (Amersham Biosciences Europe GmbH, Germany).
41. Anti-OCT-4 antibodies (SC-5279 and SC-8629; Santa Cruz Biotechnology Inc, CA)
42. Anti-beta actin antibody (Prosci Inc, CA).
43. HRP-conjugated anti-mouse IgG (Sigma, Germany).
44. HRP-conjugated anti-goat IgG (Abcam, UK).
45. HRP-conjugated anti-rabbit IgG (Dako-Cytomation, Denmark).
46. ECL Advance Western Blotting detection kit (Amersham Biosciences Europe GmbH, Germany).
47. Stripping solution: add 0.7814 g of 2-mercaptoethanol and 2 g of SDS in 90 mL of 62.5 mM Tris-HCl (pH = 6.7) and bring to 100 mL.
48. Formalin-fixed paraffin-embedded tissue sections.
49. Poly-L-lysine coated slides: incubate slides in poly-L-lysine solution at 37°C for 1 h, then remove the excess fluid and store at 4°C.
50. Xylene.
51. Four percent formaldehyde fixative: add 1 g formaldehyde powder into 20 mL PBS and warm to 60°C. Stir the solution and make it clear by gradual addition of 10 N NaOH. Filter the solution, adjust the pH to 7.4 and bring to 25 mL with PBS.
52. PBS-Triton x100 wash buffer: add 200 µL Triton x100 to 100 mL of PBS and pipette vigorously.
53. Ethanol: Prepare a serial dilution of ethanol with concentrations of 70%, 80%, 90% and 100%.

54. Citrate buffer (10 mM, PH 6.0): add 1.92 g $\text{H}_2\text{CO}_3 \cdot \text{H}_2\text{O}$ into 1 L ddH₂O, adjust the pH to 6 by 1 N NaOH and add 0.5 mL Tween 20. Mix gently and store at room temperature.
55. Three percent H_2O_2 : dilute desired amount of 30% stock H_2O_2 in ddH₂O immediately prior to use.
56. Normal goat serum.
57. Santa Cruz anti-OCT-4 polyclonal antibody (SC-8629; 1:50 dilution; Santa Cruz Biotechnology Inc, CA).
58. Goat ABC staining system kit (Santa Cruz Biotechnology Inc, CA).
59. Diaminobenzidin (DAB) 0.05%: Dissolve 50 mg of DAB in 100 mL ddH₂O. Add 1 μL H_2O_2 30% per mL of DAB solution immediately before use.
60. Entalin mounting solution.

METHODS

Here, we report a step-by-step protocol for detection of OCT-4 expression at mRNA and protein level by means of RT-PCR and Western blotting/immunohistochemistry techniques, respectively.

Human Clinical Samples

Fresh tissue biopsies were obtained from patients whom had been referred to Labbafi-nejad medical center. The tissues were immediately snap-frozen in liquid nitrogen and categorized in three groups: 32 tumor samples prepared by transurethral resection from 32 patients with transitional cell carcinoma of the bladder (group A), 13 non-tumor tissues, which were taken from the margin of tumors (cystoscopically normal appearing, group B) and 9 bladder samples from

patients with no symptoms and signs of bladder cancer, receiving surgical treatments for benign prostatic hyperplasia (group C).

Histopathological parameters were evaluated according to WHO criteria for grade and TNM system for stage classification. The experimental design was approved by the Ethics Committees of Tarbiat Modares University and Urology-Nephrology Research Center and the patients' written informed consent were collected prior to participation.

Total RNA Extraction

RNA extraction was done by means of RNX plusTM kit. The instruction includes multiple step procedure as follow:

1. Lyse the frozen sample in RNX solution in conjunction with homogenizing in a sterile RNase free 1.5 mL test tube and incubate at room temperature for 5 min.
2. Add 200 μL of chloroform to the test tube, mix gently and incubate on ice for 5 min.
3. Centrifuge the test tube in 12,000 g at 4°C for 15 min.
4. Transfer the upper phase into a new RNase free test tube.
5. Add equal amount of isopropanol, mix gently and incubate on ice for 20 min.
6. Centrifuge the test tube in 12,000 g at 4°C for 15 min.
7. Remove the supernatant and dissolve the pellet in 1 mL of 75% ethanol.
8. Centrifuge the tube in 7,500 g at 4°C for 8 min.
9. Discard the supernatant and dissolve the pellet in 50 μL DEPC-treated water.
10. Immediately use the RNA solution or store at -70°C for future use.

Analyzing the Quality of Extracted Total RNA

To determine the quality and integrity of the extracted RNA, it is recommended to use agarose gel electrophoresis. To this aim, resolve 5 μ L of the RNA sample in 1% agarose gel. Intact RNA samples show a typical banding pattern, which includes 3 bands of 28, 18 and 5s rRNAs.

Determining the Concentration of Extracted RNA

Optical spectrophotometry is used to determine the concentration of the extracted RNA. To this aim, first calibrate the spectrophotometer by pure DEPC-treated water at 260 nm wavelength, then make a dilution of 1:100 of RNA solution in DEPC-treated water then calculate the concentration of the stock solution by the formula:

$$C (\mu\text{g}/\mu\text{L}) = \varepsilon \times A_{260} \times d/1,000.$$

C = Concentration

A = Optical absorption at 260 nm wavelength

E = Molar extinction coefficient which is 40 for RNA samples

D = Dilution times

Semi-Quantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Reverse Transcription

To synthesize the complementary strand of mRNAs, poly-dTs oligomers are used as primer for extension by reverse transcriptase. Also, to avoid amplification of any probable DNA contaminate, treat the total RNA sample by RNase-free DNase as follow:

1. Add 1 μ g total RNA, 1 μ L DNase buffer, 1 μ L DNase enzyme, 0.25 μ L RNase

inhibitor and DEPC-treated water up to 10 μ L in a RNase free tube and mix.

2. Incubate at 37°C for 30 min.
3. Add 1 μ L EDTA.
4. Incubate at 65°C for 10 min to inactivate the DNase enzyme.
5. Add 1 μ L oligo dTs.
6. Incubate at 60°C for 5 min.
7. Add 4 μ L 5x buffer, 2 μ L dNTPs mix, 0.5 μ L RNase inhibitor and 0.5 μ L DEPC-treated water and mix.
8. Incubate at 37°C for 5 min.
9. Add 1 μ L RT enzyme and mix.
10. Incubate at 42°C for 60 min.
11. Incubate at 70°C for 10 min to inactivate the RT enzyme.
12. Store at -20°C.

PCR

Add the following materials in a sterile test tube:

Note: these quantities are for one PCR experiment of OCT-4 and β 2m genes on one template. For additional samples, multiply the quantities.

1. 4 μ L of cDNA
2. 2 U of Taq polymerase (Cinnagen, Iran)
3. 3 mM MgCl₂
4. 200 μ M dNTPs
5. ddH₂O up to 48 μ L

Mix gently, and then divide the mixture into equal amounts in two separate test tubes. Add 0.4 μ M of hOCT-4F and hOCT-4R into one test tube and 0.4 μ M of β 2m-F and β 2m-R to another one.

Set the following PCR programs for each tube:

OCT-4:

Initial denaturation (1 cycle): 94°C for 4 min.

Amplification (35 cycles): 94°C for 30 s, 62°C for 40 s, 72°C for 45 s.

Final extension (1 cycle): 72°C for 10 min.
β2m:

Initial denaturation (1 cycle): 94°C for 4 min.

Amplification (26 cycles): 94°C for 30 s, 57.5°C for 40 s, 72°C for 45 s.

Final extension (1 cycle): 72°C for 10 min.

These primer sets would amplify 470 bp and 191 bp fragments from OCT-4 and β2m cDNAs, respectively.

Agarose Gel Electrophoresis

1. Add 5 μL of OCT-4 and 5 μL of β2m PCR products into a tube along with 2 μL of 6X loading dye, mix and load in a 1% agarose gel.
2. Set the current until the leading dye pass 2/3 of the gel.
3. Stain the gel with etidium bromide solution for 15 min.
4. Wash by ddH₂O.
5. Visualize by UV documentation instrument.
6. Analyze the ratio of intensity of bands corresponding to OCT-4 per β2m by densitometer softwares.

WESTERN BLOTTING

Total Protein Extraction

1. Lyse the liquid nitrogen frozen tissue samples in modified RIPA buffer with simultaneous homogenization. Incubate on ice for 30 min with occasional shaking.
2. Centrifuge the lysate in 10,000 g at 4°C for 10 min.
3. Collect the supernatant and discard the pellet.
4. Store samples at -20°C.

Quantification of the Concentration of Extracted Protein

1. Add 195 μl of Bradford dye to 5 μl of protein sample in a 96-well plate. Also add 195 μl of Bradford dye to 5 μl of each BSA serial dilution in separate wells. Use one sample of pure Bradford dye as blank control.
2. Incubate for 5 min.
3. Read the absorption of each sample by ELISA reader on 595 nm wavelength.
4. Use the BSA serial dilution standard curve to determine the concentration of each protein sample.

SDS-PAGE

1. Assemble a mini-gel apparatus with an upper 4% stacking gel and a lower 12.5% resolving gel.
2. Load 25 μl of total protein with a 1:4 ratio of protein to sample buffer into each well by using Hamilton syringe.
3. Load 5 μl of prestained protein size marker in a separate well.
4. Set the current at 100 V until the leading dye passes the stacking gel.
5. Increase voltage to 120 V until the leading dye exits from the other side of resolving gel.
6. Disassemble the apparatus.
7. Remove the stacking gel and the lanes of the gel that have not been used.
8. Incubate in transfer buffer.

Transfer

1. Cut the PVDF membrane so that to completely cover the trimmed gel surface.
2. Permeabilize the membrane by incubating in methanol for 10 s.

3. Soak the membrane in ddH₂O for 5 min.
4. Move the membrane into ice-cooled transfer buffer and incubate for 10 min.
5. Soak 4 pieces of Watman paper and the pads in ice-cooled transfer buffer for 5 min.
6. Assemble the transfer sandwich on the bottom plate of the apparatus in the following order: pads – two pieces of Watman paper – trimmed SDS-PAGE gel – membrane – two pieces of Watman paper – pads. Remove the air bubbles by rolling a clean pipet over the sandwich.
7. Put the sandwich in transfer buffer filled transfer apparatus.
8. Set the current at 100 V for 2 h.
9. Disassemble the apparatus and check the membrane for proper transfer of pre-stained protein size marker.
10. Trim the edge of the membrane to avoid the high background edge effect.
11. Soak the membrane in PBS-T washing buffer.
- anti-mouse IgG) in a dilution of 1:50,000 for 1 h.
7. Wash the membrane with PBS-T for 3 × 10 min.
8. Place the membrane on a horizontal surface and add enough amount of Advance Western Blotting detection kit solution to cover the membrane and incubate for 5 min.
9. Remove the excess amount of solution and air dry the membrane.
10. Wrap the membrane in a saran wrap.
11. In dark room, expose the sensitive film to the membrane in a sealed cassette. Start with 30 s of exposure time and extend it in the subsequent rounds to 1 min in each round of exposure.
12. Develop the negatives.
13. Store the membrane in saran wrap in a cold and dry place.

Blotting

The following step must be done with continuous shaking of the membrane, preferably on a rolling stirrer.

1. Wash the membrane with PBS-T for 2 × 5 min.
2. Incubate the membrane in ECL Advance Blocking solution for 2 h.
3. Wash the membrane with PBS-T for 2 × 5 min.
4. Add the anti-OCT-4 in a dilution of 1:2,000 and incubate for 3 h.
5. Wash the membrane with PBS-T for 3 × 10 min.
6. Add the HRP-conjugated anti-goat IgG antibody (or HRP-conjugated
- Stripping and Reprobing the Membrane
- In order to ensure that equal amounts of samples are loaded in each well, it is desired to use the level of a housekeeping gene (beta actin in this case) as an internal control in each well. To this aim, it is recommended to first strip the anti OCT-4 stained membrane and reprobe it with the beta actin antibody.
1. Remove the saran wrap.
2. Wash the membrane with PBS-T for 3 × 10 min.
3. Incubate the membrane in excess amounts of stripping buffer in a 50°C bath for 30 min with occasional shaking.
4. Wash the membrane with PBS-T for 3 × 5 min.
5. Incubate the membrane in ECL Advance Blocking solution for 2 h.
6. Wash the membrane with PBS-T for 2 × 5 min.

7. Add the anti-beta actin antibody in a dilution of 1:2,000 and incubate for 3 h.
8. Wash the membrane with PBS-T for 3×10 min.
9. Add the HRP-conjugated anti-rabbit IgG antibody in a dilution of 1:50,000 for 1 h.
10. Wash the membrane with PBS-T for 3×10 min.
11. Place the membrane on a horizontal surface and add enough amount of Advance Western Blotting detection kit solution to cover the membrane and incubate for 5 min.
12. Remove the excess amount of solution and air dry the membrane.
13. Wrap the membrane in a saran wrap.
14. In dark room, expose the sensitive film to the membrane in a sealed cassette. Start with 30 s of exposure time and extend it in the subsequent rounds to 1 min in each round of exposure.
15. Develop the negatives.
9. Wash with PBS-Triton for 3×5 min.
10. Block nonspecific binding of the antibody by means of incubating the samples with normal goat serum for 1 h.
11. Incubate the samples with anti-OCT-4 polyclonal antibody in 1:50 dilution for 2 h at room temperature. Leave one slide without addition of anti-OCT-4 antibody as negative control.
12. Wash with PBS-Triton for 3×5 min.
13. Incubate with secondary anti-goat antibody at 4°C overnight.
14. Wash with PBS-Triton for 3×5 min.
15. Incubate with avidin-HRP at room temperature for 1 h.
16. Wash with PBS-Triton for 3×5 min.
17. Add the DAB solution, so as to cover the slides in a dark room for 25 min.
18. Wash with PBS-Triton for 3×5 min.
19. Incubate with hematoxylin for 10 s.
20. Wash with running tap water for 10 min.
21. Dehydrate in increasing concentrations (70%, 80%, 90% and 100%) of ethanol, 5 min each.
22. Incubate in xylen for 5 min.
23. Mount with entalin.
24. Analyze the slides under light microscope.

Immunohistochemistry

1. Prepare 5 μ m sections from formalin-fixed paraffin embedded (FFPE) tissues.
2. Place the sections on poly-L-lysine coated slides and air dry.
3. Remove paraffin by dipping the slides in xylene for 2×10 min.
4. Rehydrate in descending concentrations (100%, 90%, 80% and 70%) of ethanol for 5 min each.
5. Wash with PBS-Triton for 5 min.
6. Boil the slides in citrate buffer for 15 min (antigen retrieval).
7. Wash with PBS-Triton for 2×5 min.
8. Suppress the endogenous peroxidases activity by incubating the slides with 3% H_2O_2 for 20 min.

Statistical Analyses

Replicate all experiments two or three times and analyze the results by performing Analysis of Variance (ANOVA) test to determine the difference of OCT-4 expression among different biopsy groups. Additionally, we used Pearson's correlation coefficient so as to examine the correlation of OCT-4 expression and tumor/non-tumor state of the samples (SPSS software for windows, version 11, Chicago).

RESULTS

Expression of OCT-4 in Tumor and Non-Tumor Tissues of Human Bladder

We designed specific PCR primers to amplify a segment of OCT-4 gene, which is shared by both spliced variants of the gene (GenBank accession numbers: NM_002701 and NM_203289). As expected a 470 bp DNA fragment of OCT-4 was amplified in the PCR reaction. We detected the expression of OCT-4 in the great majority (96%) of the examined tumor samples of bladder taken from the patients diagnosed with transitional cell carcinoma (Group A, 31/32). Nevertheless, the expression of OCT-4 was also detected in 23% of nontumor tissues, which were taken from the margin of tumors (group B, 3/13) as well as in 33% of samples from patients with no symptoms and signs of bladder cancer. The latter group was receiving surgical treatments for benign prostatic hyperplasia (group C, 3/9).

Because we used a semi-quantitative RT-PCR approach, a densitometric evaluation and comparison of relative expression of OCT-4/ β 2m among different tissue samples was feasible. The intensity of OCT-4 expression was significantly higher in neoplastic tissues (group A) compared to the nonneoplastic (Group B and C) samples ($p < 0.001$). There was also a strong correlation of 0.6 between the expression of OCT-4 and the tumor vs., nontumor state of the samples ($p < 0.001$) whereas none of the investigated clinico-pathological variables (tumor grade, stage and size) showed a statistically significant correlation with the expression levels of OCT-4.

Figure 18.1a shows the results of RT-PCR of the expression of OCT-4 and β 2m (as an

internal control) in six representative primary bladder carcinoma samples (T) and their nontumor marginal tissues (M) from the same patients. It should be noted that the expression of OCT-4 is significantly higher than the corresponding nontumor marginal tissues from the same patients. Figure 18.1b shows the relative (β 2m-normalized) intensity of OCT-4 expression in tumor and nontumor bladder tissues. Relative band intensities for OCT-4 for each sample were quantitated by densitometry, normalized to β 2m expression, and the mean of expression in the different groups is shown as histograms. Statistical analysis revealed that the expression of OCT-4 is significantly higher in tumor samples compared with nontumor ones ($p < 0.001$).

The pattern of OCT-4 expression was also examined at protein level in the bladder samples. As a positive control, we used the embryonic carcinoma cell line NTERA2 (NT2), which has previously been shown to express the OCT-4 protein. As shown in Figure 18.2e, we detected an expected 45 kDa band in NT2 cell line, using a polyclonal anti-OCT-4 antibody. We also detected a single strong band in tumor and a single weaker band in nontumor bladder tissues; however, the size of the latter bands was noticeably higher (~52 kDa in size) than the expected size. To ensure that the observed ~52 kDa band is not a false signal detected by the antibody due to nonspecific binding, the same experiment was repeated with a monoclonal anti-OCT-4 antibody. The experiment produced a similar 45 kDa band in NT2 cell line and a single ~52 kDa band in bladder tissues (Figure 18.2f). Additionally, further analysis revealed a weaker band in NT2 cells. This band was similar in size to the band detected in bladder tissues.

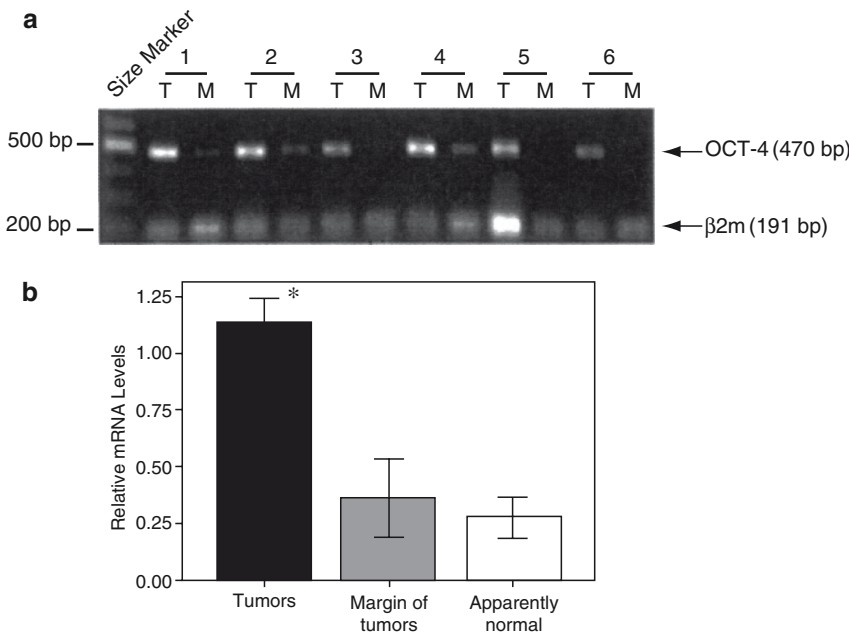


FIGURE 18.1. Detection of OCT-4 mRNA in bladder tumors. (a) RT-PCR analysis of the expression of OCT-4 and β2m (as an internal control) in bladder tissues of six representative primary bladder carcinoma samples (T) and their nontumor marginal tissues (M) from the same patients. Note that the expression of OCT-4 is significantly higher than the corresponding nontumor tissues from the same patients. (b) Relative (β2m-normalized) intensity of OCT-4 expression in tumor and non-tumor bladder tissues. Relative band intensities for OCT-4 for each sample were quantitated by densitometry, normalized to β2m expression, and the mean of expression in the different groups is shown as histograms. Statistical analysis revealed that the expression of OCT-4 is significantly higher in tumor samples compared with non-tumor ones ($p < 0.001$)

Tissue Distribution and Intracellular Localization of OCT-4 Protein in Bladder Tumors

Using the polyclonal anti-OCT-4 antibody, we examined the tissue distribution and subcellular localization of OCT-4 protein in bladder tissues. As a positive control, Formalin-Fixed Paraffin-Embedded (FFPE) sections of seminoma and embryonic carcinoma of testis, which are known to have a nuclear localization for OCT-4, were used. As shown in Figure 18.2c, we detected the same (nucleus restricted) subcellular localization for OCT-4 in positive control cells. Further, we examined the expression and subcellular localization

of OCT-4 protein in FFPE sections of bladder carcinoma. OCT-4 was primarily localized in the nuclei of tumor cells, with no immunoreactivity in normal cells adjacent to the tumors (Figure 18.2a). Strikingly, the intensity of immunoreactivity was variable among positive cells, suggesting heterogeneity among the cells within a single tumoral tissue in term of OCT-4 expression. A cytoplasmic distribution of OCT-4 was also detected in certain samples (Figure 18.2b). Nevertheless, no immunoreactivity was observed in negative immunohistochemistry controls, which were incubated in the absence of primary antibody (Figure 18.2d).

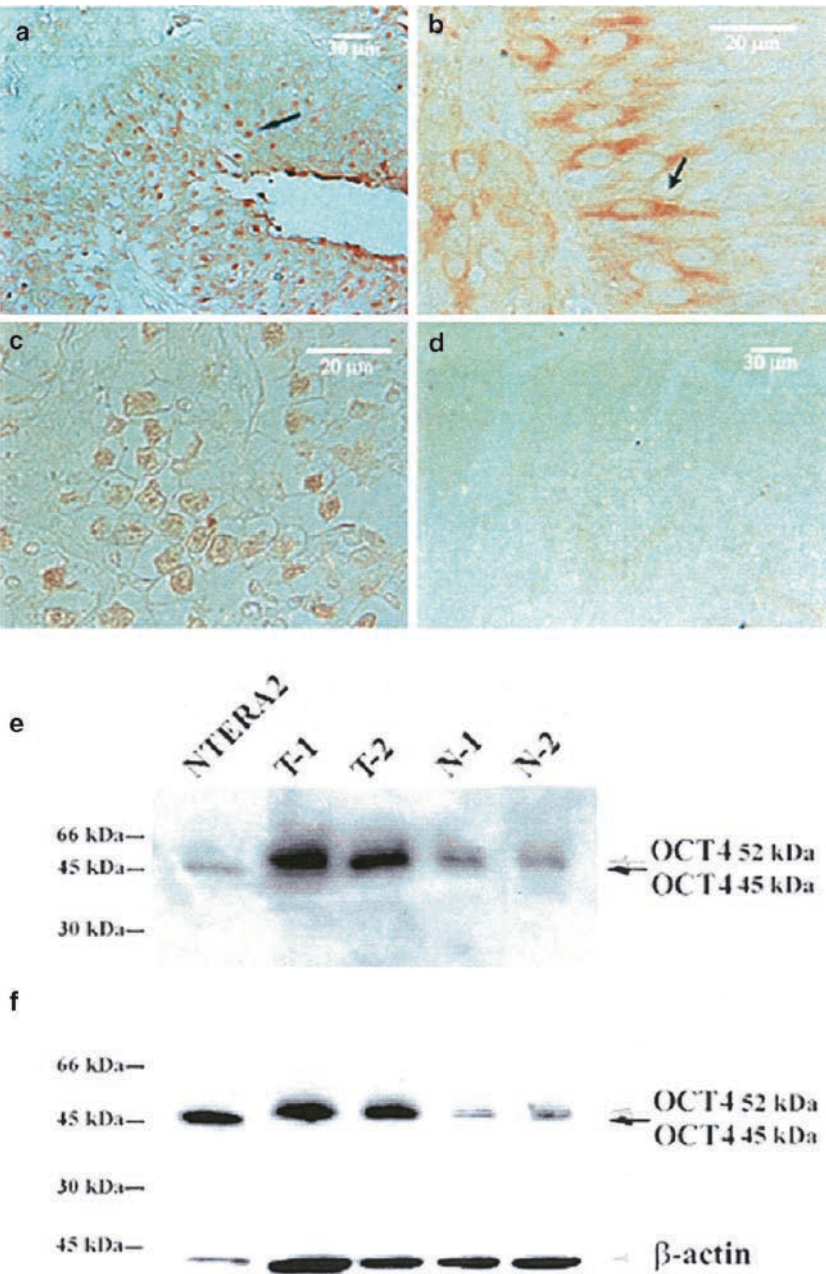


FIGURE 18.2. Detection of OCT-4 protein in bladder tumors. (a–d) Representative IHC data of OCT-4 protein expression in FFPE sections of tumor tissues. (a,b) Bladder sections were deparaffinized and subsequently incubated with OCT-4 antibody and avidin-HRP, before being visualized with DAB. (a) OCT-4 expression was primarily localized in the nuclei (in *brown color*; *arrow*) of the cells, or in some samples localized in the cytoplasm of the cells located adjacent to the basal lamina of urothelium (b, *arrow*). (c) Detection of OCT-4 in the nuclei of a human seminoma specimen (used as a positive control). (d) The negative control, sections from the same specimen in (a) and (b) was identically processed, except for the omission of the first (OCT-4) antibody. (e and f) Western blot analysis of OCT-4 protein expression in bladder tissues. Total proteins were isolated from

DISCUSSION

Tumor recurrence and multifocality are two common features of bladder tumors. Currently, several lines of evidence support a clonal nature for multifocal and recurrent urothelial carcinomas, suggesting that these tumors are derived from a primary transformed progenitor cell (Denzinger et al. 2006; Junker et al. 2005; Sidransky et al. 1992). Based on the newly proposed CSC hypothesis, this primary mutant cells are possibly transformed stem/early progenitor cells, with a dysregulated self-renewal capacity generating more CSCs similar to the mother cells as well as phenotypically diverse partially differentiated cancer cells with less stem cell-like properties; hence, leading to the reduction of tumorigenesis potential. The new concept could change our understanding of tumor initiation and progression. It may also modify our diagnostic and therapeutic approaches by allowing better identification of CSCs and development of target driven cancer therapy.

Given the recent proposed role for CSCs in tumorigenesis and the notion that these cells are generated through uncontrolled self-renewal of normal stem/progenitor cells, it is very important to examine the expression and involvement of genes which regulate the unique properties of stem cells (i.e., self-renewal and developmental potential) in carcinogenesis processes. To achieve such goals, we examined

the expression of a well-known upstream self-renewal regulatory factor, OCT-4, in human bladder cancer and further determined the correlation between the expression of this gene with the tumor state of the samples.

Prior to our study, the expression of OCT-4 had been reported in germ cell tumors, a small number of human kidney and lung cancer samples (Looijenga et al. 2003), human breast cancer and osteosarcoma biopsies (Ezeh et al. 2005; Gibbs et al. 2005; Jin et al. 1999) and in some human cancer cell lines (Tai et al. 2005; Wang et al. 2003). However, the data presented here are the most comprehensive study of the expression of OCT-4 in tumor vs. nontumor tissues of a somatic cancer.

Based on our observations, OCT-4 is proportionally expressed in bladder tumors comparing to its significantly lower expression in a subset of nontumor tissues. This result might be interpreted as the expansion of bladder cells that intrinsically express OCT-4 or through acquisition of self-renewal capacity by other cancer cells that may lead to an increase in OCT-4 expression.

The sensitivity and specificity of OCT-4 expression as a molecular marker in detection of bladder tumors were determined as 96% and 66%, respectively. We found no significant correlation between the expression level of OCT-4 and the grade/stage of the tumor samples. However, further quantitative approaches might be required

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FIGURE 18.2. (continued) NT2 cell line (used as a positive control) and 2 representative tumor (T1 and T2) and 2 representative non-tumor bladder biopsies (N1 and N2). 25 mg of total protein from each sample was assessed for OCT-4 expression using an anti-OCT-4 polyclonal antibody (sc-8629; a) or an anti-OCT-4 monoclonal antibody (sc-5279; b). Both experiments confirmed the expression of a 52 kDa form of OCT-4 protein in bladder tissues and a smaller, 45 kDa, form of the protein in NT2 cell line. The expression of β -actin was used as a loading control

in this regard in order to elucidate the significance of OCT-4 expression in this type of cancer.

Work on archival collection of FFPE samples of bladder tumors is in progress in our laboratory to assess the correlation between OCT-4 expression and prognostic parameters such as tumor progression, tumor recurrence, and cancer survival rate.

Interestingly, in addition to the tumor samples, we also observed a low expression of OCT-4 in some nontumor bladder tissues obtained from individuals with no symptoms and signs of bladder cancer (group C). Furthermore, we observed no significant difference in OCT-4 expression between group B and group C samples. Recently, Tai et al. (2005) have shown that OCT-4 is expressed in several human adult stem cells (e.g., breast, pancreas and liver stem cells). Also, Matthai et al. (2006) have recently reported the expression of OCT-4 in normal human endometrium. The observed expression of OCT-4 in normal bladder tissues might reflect the presence of rare normal bladder cells with stem cell-like properties in these samples which express OCT-4. For that reason, we expect that evaluating the expression of OCT-4 in exfoliated cells of urine samples would potentially decrease the presence of normal bladder stem cells in the sample and increase the specificity of OCT-4 as a molecular marker for bladder cancer.

Both antibodies used in our study, detected a slightly higher molecular weight of OCT-4 protein in bladder tumors compared to the one in NT2 cells. We suggest that differential post-translational modifications of OCT-4 in these two systems might be responsible for the observation. However, this post-translational modification and its potential correlation with

bladder carcinogenesis need to be further investigated.

According to our IHC results, OCT-4 positive cells were not distributed equally in different tumors, ranging from several scattered cells to aggregated clusters. Similar observation has been reported previously by Gibbs et al. (2005), who observed a variable OCT-4 expression in different bone tumors ranging from 1% to 25% of the cells. The reported variation was even higher for Nanog (another key regulator of stem cell self-renewal) positive cells, which comprised 1% to 50% of the cells in different samples. In contrast to nuclear staining, the cytoplasmic distribution of OCT-4 was mainly restricted to the cells located adjacent to the basal lamina. Using immunohistochemical analysis on tissue microarrays, Looijenga et al. (2003) had previously reported no expression of OCT-4 in a panel of somatic tissues such as bladder tumors. The latter finding is in contrast to our finding and the reason for this inconsistency might be due to the heterogenous nature of tumors, and the fact that tissue sampling for microarrays might not be a good representative of the whole tumor. Recently, using immunohistochemical analysis of the whole tissue sections, several groups have reported the expression of OCT-4 protein in different somatic tissues (Gibbs et al. 2005; Matthai et al. 2006).

In conclusion, our data are the first report on the expression of the embryonic stem cell marker, OCT-4, in bladder cancer and would add more weight to the findings that candidate OCT-4 as a multifunctional factor involving in major biological processes such as embryonic development, control of differentiation, and stem cell based carcinogenesis. More specifically, OCT-4 can potentially be regarded as a new mole-

cular marker for bladder tumors, and its expression might indicate the existence of stem-like cancer cells in these tumors. This is also a further evidence to support the concept of stem cell origin of cancer. Moreover, the data might provide valuable information on the nature and behavior of bladder tumors, leading to a new strategy for targeting the CSCs and perhaps one step closer to cure cancer recurrence and metastasis. However, further studies are required to isolate and characterize the putative CSCs from bladder tumors in order to elucidate the role of OCT-4 in carcinogenesis of the tumor.

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Part V

Cervical Uterine Cancer

Diagnosis

Uterine Cervical Glandular Lesions: Differentiation Using Immunohistochemistry of Mucins

Allyson C. Baker, William E. Grizzle, and David Chhieng

INTRODUCTION

Mucins are the major glycoprotein components of mucus. These large, filamentous glycoproteins are present in the cytoplasm of the epithelium that produces them and the surrounding extracellular milieu. Mucins are produced by a variety of cells including the epithelia of the gastrointestinal, respiratory, and reproductive tracts. Mucins have many different functions including protecting the epithelium, providing a transport material for cells, aiding in epithelial renewal, differentiation, and integrity. They can also play a role in carcinogenesis.

Genes that code for the protein component of mucin are termed MUCs. Currently, 14 structurally different mucin glycoproteins have been assigned to the MUC gene family. The MUC genes are located on several different chromosomes. MUC 1 is on the long arm of chromosome 1 at locus 21 (1q21). The long arm of chromosome 3 contains MUC 4 at locus 29 (3q29). Both MUC 2 and MUC 5AC are located on the short arm of chromosome 11 at locus 15.5 (11p15.5) (Dekker et al. 2002). The expression of MUC genes is relatively tissue-specific. For example, MUC 1, a

membrane associated mucin gene, forms a major component of gastric mucin; it is also found in a variety of other gastrointestinal organs such as the pancreas and has also been found in colorectal neoplasias. MUC 2 codes for a secretory type of mucin found in the intestinal epithelia and trachea (Manne et al. 2000). MUC5AC is present in the tracheobronchial tree and is also in the gastric mucosa as a surface mucin. The human female reproductive tract contains many of these MUC genes. The mucin composition is known to vary slightly during the menstrual cycle (Gipson et al. 1997). However, with malignant transformation of the glandular epithelium, the mucin composition can vary greatly, including the production of mucins not normally present in the female reproductive tract.

MUC 4 and MUC 5 are the predominant endocervical mucins, which are secretory in nature (Gipson et al. 1997; Gipson et al. 1999; Wells and Brown 2002). The epithelium of the endocervix also expresses membrane associated mucins, MUC 1 and MUC 6. The squamous epithelium of the ectocervix and vagina show weak expression of MUC 1 in the cytoplasm of the basal cells and focal, strong MUC 4 staining

in the cytoplasm of the parabasal cells, primarily in the nonkeratinizing squamous epithelium, while the endometrial glandular epithelium expresses MUC 1 and low amounts of MUC 6 (Gipson et al. 1997).

The mucin genes can undergo aberrant expression when an epithelium undergoes malignant transformation. This can cause reduced production of an expected mucin and/or production of a structurally different and unexpected mucin within the transformed epithelium. Benign and malignant endocervical lesions can be difficult to differentiate at times due to the overlap in morphology among these lesions. Few studies have examined the expression of mucins in various benign and malignant glandular lesions involving the uterine cervix (Audie et al. 1995; Riethdorf et al. 2000). The objective of this study was to evaluate the expression of MUC 1, MUC 2, MUC 4, and MUC 5AC in various non-neoplastic and neoplastic glandular lesions involving the uterine cervix, and to determine whether a distinct phenotypic pattern exists to differentiate between benign, non-neoplastic, and dysplastic lesions and between endocervical and endometrial adenocarcinomas involving the endocervical canal.

MATERIALS

Solvents, Media, and Solutions

1. Xylene (histologic grade).
2. 70%, 95%, 100% ethanol.
3. Deionized water (dH_2O).
4. Tris buffer: 0.05 M Trizma base, 0.15 M NaCl, 0.01% Triton X-100 (4 drops/L), and dH_2O (pH 7.6). To make 4 L, add 24.23 g Trizma base, 35.06 g NaCl, 16 drops Triton X-100, and dH_2O (up to 4 L), then adjust the pH to 7.6. Record the date of preparation and store at room temperature. Use within 3 months.
5. Phosphate buffer saline (PBS): 137 mM NaCl, 2.7 mM KCl, 8.1 mM anhydrous Na_2HPO_4 , 1.5 mM anhydrous KH_2PO_4 , and dH_2O (pH 7.44). To make 1 L, add 8.00 g anhydrous NaCl, 0.2 g KCl, 1.15 g anhydrous Na_2HPO_4 , 0.2 g anhydrous KH_2PO_4 , and dH_2O (up to 1 L), then adjust the pH to 7.44. Record the date of preparation and store at 4°C . Use within 3 months.
6. PBE buffer: 1.0% bovine serum albumin (BSA), 1.0 mM ethylenediamine tetraacetic acid (EDTA), 1.5 mM NaN_3 , and PBS (pH 7.6). To make 100 mL of PBE, add 1.0 g BSA, 0.0202 g EDTA, 0.00975 g NaN_3 , and PBS (up to 100 mL), then adjust the pH to 7.6. Record the date of preparation and store at 4°C . Use within 3 months. For better results, sprinkle the BSA around the beaker, add the other reagents, and leave at room temperature for ~1 h. Add the stir bar after the BSA is completely dissolved. Mixing the reagents in this manner reduces the chance for the BSA to aggregate.
7. Three percent hydrogen peroxide (H_2O_2): Dilute commercial 30% H_2O_2 solution to 3% H_2O_2 in dH_2O (tenfold dilution). To make 100 mL 3% H_2O_2 , add 10 mL 30% H_2O_2 to 90 mL dH_2O . Record preparation date and store at 4°C . Limit the use of 3% H_2O_2 to 1 week after preparation.
8. Three percent goat serum: Add 600 μL goat serum (Sigma-Aldrich Co., St. Louis, MO) to 20 mL PBE and filter through 0.2 μm filter. Record the date of preparation and store at 4°C . Use within 1 month.

9. Antigen retrieval solution (0.01 M citrate buffer): 0.01 M citric acid monohydrate. To make 1 L of citrate buffer, add 2.1 g of citric acid monohydrate to dH₂O (up to 1 L), then adjust the pH to 6.0 with NaOH. Record the date of preparation and store at 4°C. Use within 3 weeks.
10. Mayer's hematoxylin (Sigma-Aldrich Co.) filtered using Whatman paper #1. Record date of preparation and store at room temperature. Use within 3 months.
11. Permount (Fisher Scientific, Hampton, NH) mounting medium. Store at room temperature.
12. MUC antibodies: mouse monoclonal anti-MUC 1 (Clone Ma695, Novocastra, New Castle upon Tyne, United Kingdom), mouse monoclonal anti-MUC2 (Clone Ccp58, Novocastra), mouse monoclonal anti-MUC 4 (Clone 1G8, Zymed, San Francisco, CA), and mouse monoclonal anti-MUC 5AC (Clone 45M1, Zymed). Antibodies are used at dilutions of 1:200 each. To make the dilutions, add 4 µL of anti-MUC to 800 µL of PBE. The dilutions should be prepared fresh for each use.
13. Link and Labeling Secondary Detection Reagents: A multi-species (mouse/rabbit) detection system obtained from Signet Laboratories.
14. Diaminobenzidine (DAB) tetrachloride substrate: Prepared according to the manufacturer's instructions using a liquid DAB concentrated substrate pack (Biogenex Co., San Ramon, CA). This solution should be prepared fresh for each use. Caution: DAB is a suspected carcinogen, so the use of gloves is recommended.

Other Materials and Equipment

15. Histoprep Marker (Fisher Scientific, Pittsburgh, PA).
16. Superfrost Plus slides (Fisher Scientific).
17. Glass staining dishes with slide insert and handle (Fisher Scientific).
18. PAP pen, hydrophobic slide marker (Biogenex).
19. Staining racks (Fisher Scientific) and corresponding plastic containers.
20. Glass coverslip (the size of the coverslip should be large enough to cover the entire tissue section on the slide).
21. Humidity chamber.
22. Oven heated to 58°C (57–60°C).
23. Plastic hemostats for handling slides out of xylene prior to attaching coverslips to slides.
24. Coplin jars, plastic and microwave safe.
25. Plastic, microwave safe container (at least 8 × 8 in.²).
26. Microwave oven.
27. Microtome.
28. Light microscope equipped with bright-field optics (40× and 100×).

METHODS

Formalin-fixed, paraffin-embedded tissues of 51 gynecologic cases were selected from the years 1996 to 2003 from the archives of the Department of Pathology at the University of Alabama at Birmingham. These cases included ten endocervical adenocarcinomas (three well differentiated, six moderately differentiated, and one poorly differentiated), four adenosquamous carcinomas of endocervical origin, eight endometrial adenocarcinomas of endometrioid type (five moderately differentiated

and two poorly differentiated) including one adeno-squamous carcinoma, eight endocervical adenocarcinomas in situ (AIS), two glandular dysplasias, six tubal metaplasias, ten microglandular hyperplasias, and three normal endocervical specimens. All AIS and endocervical adenocarcinomas were endocervical subtype and the endometrial adenocarcinomas were endometrioid subtype. The adenocarcinomas of endometrial origin involved the endocervix through contiguous spread in all cases. The hematoxylin-eosin sections were reviewed and representative tissue blocks containing the lesions were retrieved and diagnostically confirmed.

Sectioning of Tissues and Slide Preparation

1. Label Superfrost Plus slides with solvent resistant Histoprep marker.
2. Cut 5 μm thick tissue sections from paraffin blocks using a microtome.
3. Heat slides in a 57–60°C oven for 1 h to make the tissue more adherent to the slides and aid in the deparaffinization process.
4. Deparaffinize with three separate baths of xylene followed by rehydration with absolute (100%), 95% and 70% ethanol for 5 min each.

Antigen Retrieval

1. Heat a water bath (square microwave safe container almost filled with water) for 8 min in the microwave oven.
2. Place four Coplin jars, filled with citrate buffer (antigen retrieval solution), into the water bath. We recommend placing the jars near the four corners.
3. Heat the buffer to a boil in the microwave, ~6 min.

4. Add the slides to the Coplin jars and bring the citrate buffer back to a boil (~5 min).
5. Once boiling, pause the microwave, reset the cooking time to 5 min, and continue the retrieval process.
6. Once the 5 min are complete, add additional citrate buffer to the jars so that the jars are completely filled, continue the cooking time for an additional 5 min.
7. Pull out the water bath with the four Coplin jars and run cool water into the bath for 15 min, avoiding any splashes into the Coplin jars.
8. Pour out the citrate buffer from the Coplin jars and fill the jars with cold water. Repeat this rinse process two additional times.
9. Pour Tris buffer into each Coplin jar and allow to stand for 5 min. From this point on, the tissue must never dry out.

Delineating Tissue Sections

1. After draining excess buffer off the slide, delineate the tissue section by using a PAP pen (hydrophobic slide marker). This reduces the amount of reagents needed and reduces the risk of drying.
2. Immediately recover the tissue with Tris buffer taking care not to cover the PAP pen line. Place the slide on a slide rack.
3. Repeat this procedure for all slides.

Inactivation of Endogenous Peroxidase

1. Drain the slides of Tris buffer by moving the slide from a horizontal, resting position to a vertical position and tap the slide lightly on the rack.
2. Reposition the slide on the horizontal rack and cover the tissue section with 3% aqueous H_2O_2 for 5 min.

3. Remove the aqueous H_2O_2 by rinsing the slide well with Tris buffer for 1 min, repeat this procedure two times.

Blocking Non-specific Binding of Proteins

1. Drain slides by gently tapping off excess Tris buffer.
2. Place slides in humidified chambers.
3. Cover each tissue section with 3% goat serum and incubate at room temperature for 20 min. Do not rinse off the goat serum, proceed directly to the next step (primary antibody step).

Primary Antibody Step

1. Prepare the primary antibody (all four mouse monoclonal antibodies: anti-MUC 1 (Clone Ma695), anti-MUC 2 (Clone Ccp58), anti-MUC 4 (Clone 1G8), and anti-MUC 5AC (Clone 45M1) are prepared by the same method): mouse monoclonal antibody (MUC 1, MUC 2, MUC 4, or MUC 5AC) at a concentration of 1:200. This is performed by diluting 4 μ L of antibody by 800 μ L of PBE buffer. This concentration (1:200) is very sensitive, and higher concentrations result in non-specific staining. This solution should be made fresh.
2. Drain the goat serum from the slides by gently tapping off the excess liquid.
3. Place the slides in humidified chambers.
4. Cover each tissue section with the primary antibody (anti-MUC 1, anti-MUC 2, anti-MUC 4, anti-MUC 5AC), and incubate at room temperature for 1 h.

Note: Negative controls remain in 3% goat serum and are also incubated at room temperature for 1 h in the humidified chamber.

5. Remove the primary antibody by rinsing the slide well with Tris buffer for 1 min. Repeat this rinsing process two additional times.

Amplification of Primary Antibody

1. Drain the buffer from the slides by gently tapping off the excess liquid.
2. Place the slides on the slide rack.
3. Cover each tissue section (including the control sections) with the linking reagent (biotinylated goat anti-mouse/rabbit antibody (Link)) and incubate for 20 min at room temperature.
4. Rinse the slide well with Tris buffer for 1 min. Repeat this step two additional times.
5. Drain the Tris buffer from the slide.
6. Cover each tissue section with the labeling reagent (streptavidin peroxidase (Label)) and incubate for 20 min at room temperature.
7. Rinse the slide well with Tris buffer for 1 min. Repeat this step two additional times.

Develop Color with Peroxidase Substrate

1. Drain the slides of Tris buffer by gently tapping off the excess liquid.
2. Place slides on the slide rack.
3. Cover each section of tissue with liquid DAB and incubate for 7 min at room temperature.

Note: The liquid DAB undergoes oxidation and forms a stable brown end product at the site of the antigen-antibody complex.

4. Rinse the DAB off the slide with dH_2O .

Counterstaining

1. Immerse the slides in hematoxylin for 30 s.
2. Immerse the slides in running tap water for 4 min.

Note: Tap water usually has enough calcium carbonate in it to act as a bluing agent.

Mounting the Tissue Specimens

1. Dehydrate the tissue sections by immersing the slides in baths of 70% and then 95% ethanol for 3 min each, followed by 100% ethanol for 5 min.
2. Immerse slides in three baths of xylene for 5 min each.
3. Using a plastic hemostat, remove one slide at a time and drain excess xylene.

Note: Avoid drying the tissue specimen. It should still have a thin covering of xylene for the mounting media to flow evenly over the tissue section.

4. With a glass wand, place a drop of Permout on the edge of the slide. Allow the coverslip to slowly cover the tissue section, starting at the edge where the Permout has been placed.

Note: When the coverslip comes into contact with the Permout, slowly decrease the angle between the coverslip and the slide allowing the capillary action of the xylene and Permout to seal the coverslip to the slide, thus avoiding any unnecessary air bubbles.

The slides were evaluated using a light microscope equipped with brightfield optics. Each slide was examined independently by at least two investigators to minimize bias. The degree of expression of each marker was evaluated according to the distribution, intensity, and pattern of staining. The distribution of staining was based on the percentage of the lesion involved by staining ranging from 0% to 100%. The pattern of staining was characterized as luminal/apical, membranous, and/or cytoplasmic. If more

than one staining pattern was seen in the same lesion, all patterns identified were recorded. The intensity of staining ranged from 1+ (weak staining) to 3+ (intense staining). The presence of any staining was considered positive. Fisher exact test was used for statistical analyses. The level of significance was set at 0.05.

RESULTS

Sections of normal endocervical glandular epithelium, whether they were associated with other lesions, benign or malignant, stained strongly for MUC 1. The staining was in the cytoplasm and lumens of the glandular epithelium. MUC 2 staining was absent in all normal glandular epithelium. The normal glandular epithelium of the endocervix was strongly positive for MUC 4 with a predominately luminal pattern. MUC 5AC staining was present in the cytoplasm and lumens of normal endocervical epithelium.

The ectocervix was also examined for MUC staining. MUC 1 was absent in the squamous epithelium, including the basal layer, of the non-inflamed ectocervix. On the contrary, foci of squamous epithelium with inflammatory infiltrates showed diffuse and intense MUC 1 staining. MUC 4 showed staining of the parabasal layer of the squamous epithelium. MUC 2 and MUC 5AC were consistently negative in the ectocervix. In cases with associated squamous dysplasia (ranging from mild to severe), MUC 4 showed intense staining of the dysplastic epithelium as opposed to the parabasal staining of uninvolved epithelium. MUC 2 and MUC 4 also showed focal staining of immature squamous metaplasia, but not mature squamous metaplasia.

The benign lesions of the endocervix showed similar staining patterns to normal endocervical glandular epithelium. Microglandular hyperplasias showed positivity for MUC 1, MUC 4, and MUC 5AC, while MUC 2 was negative. The mucin profile of tubal metaplasias paralleled that of normal endocervical glandular epithelium. The only difference was a slightly stronger intensity in staining for the MUC 5AC antibody in the cases of tubal metaplasia. This increase in intensity was demonstrated in four of the six cases; however, all cases did stain positively for MUC 5AC.

When comparing benign cases to lesions with dysplastic glandular elements, we noticed that there was really no difference in the staining characteristics for MUC 1, MUC 4, and MUC 5AC between the two (benign vs. glandular dysplasia). We did note that the 2 cases of glandular dysplasia showed positivity for MUC 2. However, the staining was intense, yet focal.

When compared to benign cases, endocervical adenocarcinoma in situ (AIS) showed intense MUC 1 staining; however, it was focal as compared to the diffuse staining of the benign lesions. Only two of eight cases were

positive for MUC 2. MUC 4 and MUC 5AC were positive in all benign cases (Figure 19.1) and negative in the majority (5/8) of AIS cases. When MUC 5AC staining was present in cases of AIS, it was very focal (<10%).

In contrast to AIS of the endocervix, the majority of endocervical adenocarcinomas showed positive MUC 4 (six cases out of ten) and MUC 5AC (eight cases out of ten) staining. MUC 5AC showed intense and focal (20% to 30%) staining and was more often positive in the better differentiated adenocarcinomas. The poorly differentiated endocervical adenocarcinomas were negative for MUC 4 and MUC 5AC. MUC 2 positivity was also seen in four cases (4/10) of endocervical adenocarcinoma.

Endocervical adenosquamous carcinoma expressed MUC 5AC in all cases (4), but the staining was diminished not only in intensity, but also in distributional percentage when compared to benign endocervical glands. This finding looked as though it may have conveyed some meaning until we looked at the adenosquamous carcinoma of endometrial origin, involving the endocervix by contiguous spread. Adenosquamous carcinoma of endometrial origin had identical

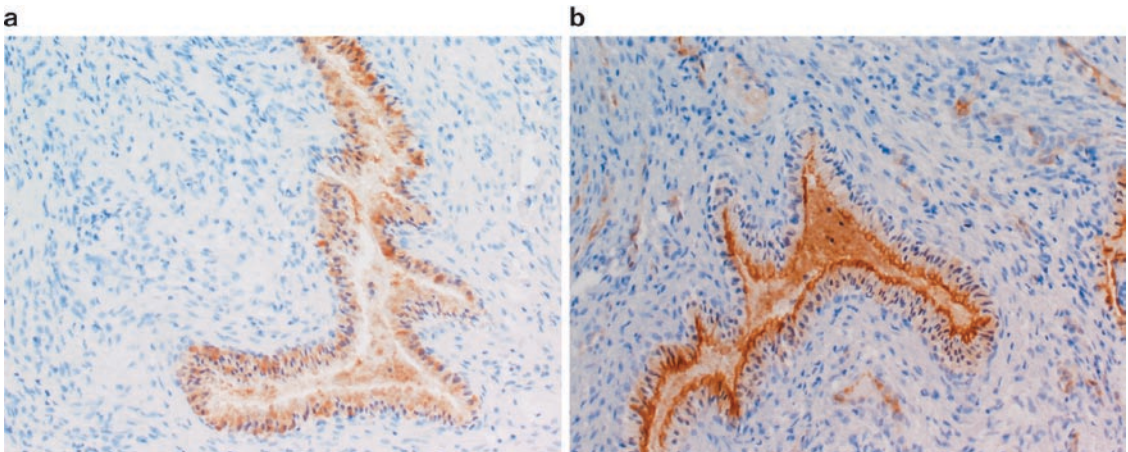


FIGURE 19.1. Demonstrates the staining pattern of MUC4 and MUC 5AC in benign endocervical glands. All slides are viewed at 200X. ((a) MUC 4, (b) MUC 5AC)

MUC 4 and MUC 5AC staining patterns. Two cases (2/4) of adenosquamous carcinoma of the cervix showed focal cytoplasmic MUC 2 positivity in the glandular component. The squamous component was negative for all MUC stains in the adenosquamous carcinomas regardless of origin.

Similar to benign and malignant endocervical lesions, adenocarcinomas originating in the endometrium and involving the endocervix by contiguous spread showed strong MUC 1 staining. Only a minority (2/9) of endometrial adenocarcinomas were positive for MUC 2. However, both of the positive cases were moderately differentiated and had focal papillary areas, and it was in the papillary areas that the MUC 2 stain was present. Focal MUC 4 positivity was present in only four out of nine cases of endometrial adenocarcinoma; the remaining five cases were negative. The positive cases consisted of the two moderately differentiated cases mentioned above that had MUC 2 positivity with focal papillary features, while the remaining two positive cases were poorly differentiated adenocarcinomas. The majority of endometrial adenocarcinomas (7/9) were negative for MUC 5AC. The two endometrial adenocarcinomas that were positive for MUC 5AC showed markedly diminished staining (<5%) when compared to normal endocervical glandular epithelium and even malignant tumors that originated in the endocervix.

The majority of endocervical adenocarcinomas exhibited intense staining with MUC 5AC. The better differentiated forms had more diffuse staining when compared to the moderate and poorly differentiated ones. MUC 5AC was positive in only two endometrial adenocarcinomas with very focal (<5% of tumor cells) staining. Endo-cervical ade-

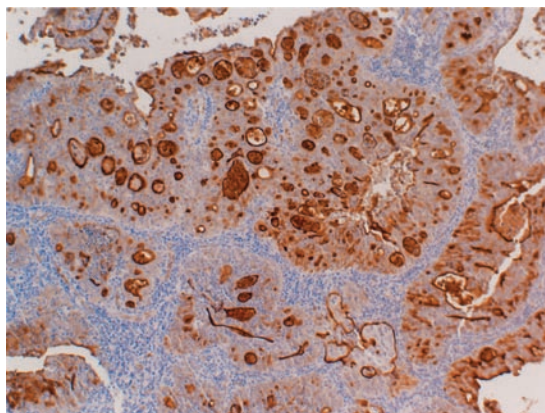


FIGURE 19.2. Demonstrates the staining of MUC5AC in adenocarcinoma of endocervical origin. The slide is viewed at 200 $\times$

nocarcinomas were more likely to express MUC 5AC when compared to endometrial adenocarcinomas (Figure 19.2). The difference in MUC 5AC staining between endocervical and endometrial adenocarcinomas was statistically significant ($p = 0.001$, Fisher exact test). There were no significant differences in the expression of MUC 1, MUC 2, and MUC 4 between endocervical and endometrial adenocarcinomas.

DISCUSSION

In this study, we have observed that all endocervical epithelium expressed MUC 1, MUC 4, and MUC 5AC. MUC 2 was not appreciated in any of the normal or benign lesions of the endocervix. Our findings with MUC 1, MUC 2, and MUC 5AC staining patterns agree with the previously published results (Audie et al. 1995; Gipson et al. 1997; Riethdorf et al. 2000; Zhao et al. 2003). Only a few studies have explored the expression of MUC 4 in normal endocervical epithelium. These studies reported the expression of MUC 4 ranging from 40% to 75% using

various techniques including polymerase chain reaction, in situ hybridization, and immunohistochemistry (Audie et al. 1995; Gipson et al. 1997, 1999).

Benign endocervical lesions, including tubal metaplasia and microglandular hyperplasia, showed similar mucin expression profiles when compared to normal endocervical glandular epithelium (Baker et al. 2006). Both lesions strongly expressed MUC 1, MUC 4, and MUC 5AC and showed no staining with MUC 2. Riethdorf et al. (2000) examined the staining pattern of MUC 2 and MUC 5AC among these lesions. They determined that MUC 2 was not expressed in either of these lesions, thus agreeing with our observations. However, they noticed the presence of MUC 5AC expression in all microglandular hyperplasia cases but not in any tubal metaplasia cases. From this discovery, these authors suggested that MUC 5AC may be helpful in differentiating tubal metaplasia from endocervical neoplasms, particularly, adenocarcinoma in situ which often expresses MUC 5AC and can be confused with tubal metaplasia. Unfortunately, our observations do not support this argument. In our study, all cell types of tubal metaplasia, which included ciliated cells, non-ciliated cells and intercalated cells stained strongly with MUC 5AC. Riethdorf et al. (2000) did note that sporadic expression of MUC 5AC was present in rare single cells of incomplete tubal metaplasia. The authors explained that these rare, MUC 5AC positive cells may have represented residual “normal endocervical” cells. We are not able to comment further on their finding as we did not have any cases of incomplete tubal metaplasia. All six cases in our study had no residual normal endocervical cells at the base of the glands. One possible explanation regarding the

discrepancy in the staining patterns of MUC 5AC between our study and others is that although the antibodies used in the Riethdorf et al. (2000) study and ours were of the same clones, they were from different suppliers. Therefore, the staining pattern with MUC 5AC in tubal metaplasias warrants further study to determine the expected staining pattern.

Another interesting finding was the expression of MUC 2 by immature squamous metaplasia (Riethdorf et al. 2000; Baker et al. 2006). MUC 1 reportedly should show weak expression in the basal layer of the squamous epithelium of the ectocervix, which we did not identify in any of our cases that contained squamous epithelium. However, we observed the presence of intense MUC 1 staining in squamous epithelium of the ectocervix involved by cervicitis. It was intriguing to see strong expression of MUC 1 in inflamed squamous epithelium but not in non-inflamed squamous epithelium. Our study also showed the presence of strong MUC 4 staining in dysplastic (mild to severe) squamous epithelium at the transformation zone.

Our series contained two cases of endocervical glandular dysplasia. Both cases expressed MUC 1 and MUC 4 diffusely and MUC 2 and MUC 5AC focally. Riethdorf et al. (2000) reported no staining with MUC 2 in three cases of glandular dysplasia and positive staining with MUC 5AC in two out of three cases. Because of the limited number of cases in this category, it is difficult to generalize the results, and further investigations are warranted. However, our observations suggest the progression from benign to dysplastic lesions may be associated with increased MUC 2 expression and reduced MUC 5AC expression.

The possibility of MUC 2 gene expression preceding neoplastic transformation is further implied by studying endocervical AIS (Baker et al. 2006). Our results as well as others (Riethdorf et al. 2000) showed that between 25% and 46% of endocervical AIS expressed MUC 2. Although others reported 100% of the AIS, irrespective of the histologic subtypes, expressed MUC 5AC, we only observed MUC 5AC expression in half of the cases. In the AIS cases which were positive for MUC 5AC, the expression was diminished when compared to the normal endocervical epithelium (Baker et al. 2006). We also noticed a majority of AIS expressed MUC1 and <40% of AIS expressed MUC 4. The differences in the MUC 4 and MUC 5AC expression among benign lesions (tubal metaplasia and microglandular hyperplasia) and AIS were statistically significant. The morphologic differentiation of AIS from non-neoplastic lesions, particularly tubal metaplasia, can be difficult at times. Both AIS and tubal metaplasia can show nuclear stratification, variable degree of nuclear atypia and the presence of mitotic figures (Oliva et al. 1995).

Identification of ciliated cells and intercalated cells would favor a diagnosis of tubal metaplasia, but can be difficult to recognize because of poor preservation or obscuring inflammation (Kruman et al. 1992). Therefore, the relative lack of MUC 4 and MUC 5AC staining in AIS might be useful in differentiating AIS from normal and benign endocervical lesions which were found to frequently express MUC 4 and MUC 5AC.

Riethdorf et al. (2000) observed *de novo* MUC 2 expression in neoplastic endocervical glands and postulated that MUC 2 expression may accompany neoplastic

transformation of endocervical glands. Our findings support this theory. In our case, only two cases of glandular dysplasia expressed MUC 2, and there were no significant differences in the proportion of benign endocervical epithelium, AIS, and endocervical adenocarcinoma that expressed MUC 2. Lau et al. (2004) in their study of differential MUC expressions in carcinomas of various sites showed that endometrial and endocervical adenocarcinomas, nine and ten cases, respectively, were negative for MUC 2. These discrepant findings suggest the need for a larger study to verify which postulation is correct.

Endometrial adenocarcinoma may involve the endocervix via direct extension and thus be confused with an adenocarcinoma of endocervical origin (Kruman et al. 1992). The presence of mucinous differentiation favors endocervical origin but may also occur in endometrial adenocarcinoma. Therefore, the differentiation between endocervical adenocarcinoma and endometrial adenocarcinoma involving the endocervical canal can be a diagnostic challenge, particularly when dealing with small biopsies. From a surgical point of view, the origin of the tumor would be important to know in deciding whether parametrial soft tissue margins should be examined. We studied ten cases of endometrial adenocarcinoma (including one case of adenosquamous carcinoma) which involved the endocervical canal by direct extension to determine if there was any difference in the phenotypic expression of mucins between endocervical and endometrial adenocarcinomas. Our findings with MUC 1, MUC 2, and MUC 5AC were similar to that reported in the literature (Riethdorf et al. 2000; Zhao et al. 2003; Baker et al. 2006). Lau et al. (2004) noted the expression of MUC 5AC in the

majority (70%) of endocervical adenocarcinomas studied, as opposed to only occasional expression by adenocarcinomas of the endometrium (22%). In our series, all endometrial adenocarcinomas expressed MUC 1, about half expressed MUC 4, and only a small proportion (less than one-third) expressed MUC 2 and MUC 5AC. The majority of the infiltrating endocervical adenocarcinomas, including adenosquamous carcinoma, expressed MUC 1, MUC 4, and MUC 5AC whereas only two out of nine (22%) cases of infiltrating endometrial adenocarcinomas expressed MUC 5AC. Well differentiated endocervical adenocarcinomas showed stronger and more diffuse expression of MUC 5AC when compared to the more poorly differentiated forms. The importance of MUC 5AC negativity in endometrial adenocarcinomas is further implied when one realizes that our two cases of endometrial adenocarcinoma that were positive for MUC 5AC were very focally positive (<5% of the malignant cells). The differences in staining of MUC 5AC between endometrial adenocarcinoma and endocervical adenocarcinoma were statistically significant (Baker et al. 2006). This finding suggested that if an adenocarcinoma involving the endocervical canal demonstrated positive MUC 5AC staining, an endocervical origin would be favored over an endometrial origin.

Gipson et al. (1997) have studied the expression of mucins in normal endometrium. In our study, normal endometrial epithelium expressed MUC 1 but not MUC 2, MUC 4, and MUC 5AC. As mentioned previously, MUC 4 expression was noted in about half of the endometrial adenocarcinomas we studied, suggesting the possibility of up-regulation of MUC 4 during the neoplastic transformation of endometrial epithelium.

All of our endocervical adenocarcinomas were of the usual/endocervical type. We did not include any other histologic subtypes such as intestinal or endometrioid variants. Other studies have reported that adenocarcinomas of the mucinous/intestinal subtype express predominantly MUC 2 and to a lesser extent MUC 1 and MUC 5AC (Riethdorf et al. 2000; Zhao et al. 2003). Other histologic subtypes such as the endometrioid variant demonstrate phenotypic patterns of mucin expression much closer to that of usual/endocervical variant (Riethdorf et al. 2000; Zhao et al. 2003).

In conclusion, both benign and malignant endocervical lesions as well as endometrial adenocarcinomas expressed MUC 1. Only neoplastic endocervical lesions expressed MUC 2. Immunohistochemical reactivity with MUC 4 and MUC 5AC may be helpful in differentiating benign endocervical epithelium and non-neoplastic lesions from AIS. Finally, the phenotypic expression of MUC 5AC may be useful in differentiating endometrial adenocarcinomas from those of endocervical origin. This discrimination may be particularly helpful in small biopsies, such as endocervical curettage. Similar experimental designs are warranted to further confirm this phenotypic expression of MUC 5AC in differentiating adenocarcinomas of endocervical versus endometrial origin.

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Uterine Cervical Carcinoma: Preoperative Magnetic Resonance Imaging Staging

Hak Jong Lee and Seung Hyup Kim

INTRODUCTION

Even though the frequency is different from country to country, uterine cervical carcinoma is one of the common malignancies affecting female genital tract and leading cause of death of women. The increased knowledge in surgical techniques, radiation treatment, and recent advances in chemotherapy requires a more clear staging of the disease. Because it is easy to find abnormal squamous cells by using a Papanicolaou smear, the prevalence of invasive squamous cell carcinoma has decreased. Cervical cancer is an important disease from clinical, surgical, radiologic, and sociologic perspectives and can serve as a model of how the combined use of screening, staging, surgery, and therapy can control and possibly even eradicate this cancer. Despite the controversies regarding the choice of therapy, there is uniform agreement on the need for information regarding the size and the stage of the disease at presentation. The value of magnetic resonance (MR) imaging is the precision with which it can stage cervical cancer, and it is better than computed tomography (CT) or ultrasound, and even better than clinical staging. In this

chapter, we consider MR imaging of uterine cervical cancer, focusing on the role of MR in the preoperative staging of cervical cancer.

NORMAL ANATOMY OF UTERINE CERVIX

The cervix is a cylindrically shaped structure 2 to 4 cm long extending from the uterine body to the vagina. The cervical canal is lined with a single layer of columnar epithelium resting on a basement membrane. There are numerous glands penetrating the epithelium, which create the thick secretion of the cervical canal. If the ducts of these glands become obstructed, retention cysts are formed, which are seen commonly on MR imaging and called nabothian cysts (Disaia and Creasman 1993). The external surface of the vaginal portion of the cervix (portio vaginalis) is covered with stratified squamous epithelium similar to that which lines the vagina (Mezrich 1994). With age, squamous epithelium grows back to cover the columnar cells of the endocervical gland. This transitional area is called the squamocolumnar junction (SCJ).

It is at the squamocolumnar junction that 90% of squamous cell cancers of the cervix develop. In younger women, the SCJ is located outside the cervical canal, and the tumor tends to grow outward, and shows exophytic growth pattern. In contrast, in elderly patients the SCJ is located within the cervical canal. In these patients, cervical cancer tends to grow inward along the cervical canal, showing endophytic growth pattern (Okamoto et al. 2003).

On T1-weighted image, uterine body, and cervix show homogeneous low signal intensity. On T2-weighted image, the uterus and cervix have three layers of different signal intensities. Endometrium and mucosal layer of cervix show very high signal intensity, whereas inner layers of the uterine myometrium and cervical stroma show very low signal intensity. Outer myometrium and outer stroma of the cervix show intermediate signal intensity (Cho 2002). The preponderance of collagenous tissue is important physiologically, allowing the cervix to dilate remarkably during delivery, and is important for radiologists because it causes the characteristic hypointense appearance of normal cervical stroma seen on MR images. This hypointense appearance serves as a high-contrast background to the relatively hyperintense appearance of cervical cancer.

The vagina has two layers, high signal intensity of mucosal layer and intermediate signal intensity of muscle layer. The parametrium is loose connective tissue mixed with abundant vessels surrounding the uterine cervix and vaginal fornix. The signal intensity of parametrial tissue surrounding the uterus is variable, reflecting the variable makeup of the tissues, which include fat, vascular structures, and ligaments (Mezrich 1994). On T2-weighted

image, the parametrium manifests high signal intensity of fatty tissue and vessels with slow flow. The cardinal and sacrouterine ligaments show hypointense on T1-weighted images and hyperintense on T2-weighted images. It is speculated that this appearance is related to the prominent vascularity and rich fibrous connective tissues within ligaments, with the connective tissue and blood flow accounting for the low signal on T1-weighted images and the long T2 of blood and rephasing of the blood flow causing the high signal on T2-weighted images (Mezrich 1994).

GENERAL CONSIDERATION OF UTERINE CERVICAL CANCER

Squamous cell carcinoma is the most common histologic type, followed by adenocarcinoma. Other rare histologic types of the malignant uterine cervical tumors include small cell carcinoma, adenosquamous cell carcinoma, and lymphoma. Gross appearances of uterine cervical carcinomas are variable and may be fungating, ulcerative, or infiltrative (Nicolet et al. 2000). The usual patterns of tumor spread in uterine cervical carcinomas include direct extension to adjacent structures such as uterine corpus, vagina, or parametrium and lymphatic spread to regional lymph nodes (Cho 2002). The prognosis of the cervical carcinoma depends on tumor size and spread of tumor at the time of diagnosis. Exact pretreatment tumor staging is very important to decide treatment and to predict the prognosis, and MR is a very important tool in the evaluation of pretreatment tumor staging.

The prognosis of cervical cancer is determined primarily not only by stage but

also by nodal status, tumor volume, depth of invasion, and invasion of capillary-like space in parametrium (Choi et al. 2004). One of the most important parameters is the presence or absence of parametrial involvement. Cancer without parametrial involvement is a primary candidate for surgery, whereas the treatment in more advanced cases mainly consists of radiation therapy. Although there are numerous prognostic factors that may affect treatment planning, clinical International Federation of Obstetrics and Gynecology (FIGO) staging for tumor extension is usually the main determinant in guiding therapy choices. Nevertheless, clinical FIGO staging also has limitations. When correlated with surgical staging, clinical staging for cervical cancer has an error rate of 26–66% (Sheu et al. 2001).

In cervical cancer, tumor size has been regarded as an important prognostic factor correlating with the frequency of lymph node metastasis (Kovalic et al. 1991). MR is considered to be a more reliable modality in measuring tumor size (Hricak et al. 1988). In 85.3% of the tumor >1 cm, the discrepancy between the tumor size determined by MR imaging and the measured surgical specimen was <5 mm (Sheu et al. 2001). One of the most important factors in treatment planning is parametrial status. On MR imaging, the preservation of a low-signal intact rim of cervical stroma on T2 weighted images between the hyperintense tumor and the paracervical tissue is a reliable sign of intact parametrium (Kim et al. 1990). In applying these criteria, the accuracy in the assessment of parametrial invasion by MR imaging was reported as high as 95% (Sheu et al. 2001). Although it is not included in the clinical FIGO staging system, the presence of lymph node

metastasis has been shown to be an important prognostic factor for cervical cancer (Hricak et al. 1993).

MAGNETIC RESONANCE IMAGING TECHNIQUE FOR UTERINE CERVICAL CANCER

Magnetic Resonance images of the uterine cervix are acquired usually in axial and sagittal projections. Sagittal images are useful for evaluating the relationship between tumor and surrounding uterine structure or vagina. Usually, axial images have an important role in evaluating parametrium. Although coronal images are not necessary for evaluation of the uterine cervical carcinoma, in cases of retroverted uterus it may be helpful for evaluating parametrial tumor extension. For high-resolution MR imaging of the uterus, pelvic phased-array coil with small field of view is needed. However, for evaluating lymph node extension, body coil with large field of view image is better. In selected cases, endorectal or endovaginal coils can be used for imaging the uterine cervix or rectum.

Fast spin echo (FSE; or turbo spin echo, TSE) techniques, which considerably reduce scan time, have replaced T2-weighted conventional spin echo (CSE) imaging in the pelvis. Recently, breath-hold fast recovery FSE (FRFSE) has also been used in the pelvis and may soon replace nonbreath-hold conventional FSE. The Fast spin echo T2-weighted imaging is the most important for diagnosing uterine cervical carcinoma. It can accurately display cervical stroma, the tumor, and surrounding structures. The FSE or TSE sequence acquires up to 128 echo trains by applying multiple 180°

pulses in rapid succession after each 90° pulse. A different phase-encoding gradient is used for each echo. By acquiring multiple images within one echo time (TE), the FSE reduces the imaging time by a factor equal to echo train length. With the short imaging time of FSE, motion artifacts can be reduced thus improving image quality (Kim 2002).

In defining anatomic and pathologic details, the FSE is known to be superior to the CSE in the overall image quality. Compared with CSE technique, FSE images can show higher fat signals and lower signal intensity of the solid tissues such as uterus and ovarian stroma. The structures with fluid, such as ovarian cysts and follicles are more prominent and sharper on FSE. More recently, the breath hold FRFSE technique has been reported to show a comparable accuracy in the detection of pelvic diseases compared with that of nonbreath-hold FSE technique (Masui et al. 2001).

The field of view can be reduced and the matrix number can be increased up to 512 × 256 or higher in the presence of pelvic multicoils. However, the high-intensity fat in near fields beneath the anterior and posterior coils of pelvic multicoils often causes respiratory phase ghosting artifacts, thus degrading the image quality. Thus, the pelvic multicoils are not recommended in obese patients, and in those cases the body coil can be used instead.

Although there are several studies reporting advantages of contrast enhancement with the use of dynamic MR imaging, its role is still under discussion because of the small number of series undertaken and lack of routine use in the diagnostic workup (Seki et al. 1997). Contrast-enhanced MR imaging has limited value for evaluation of

the uterine cervical carcinoma. But, it may be helpful in the evaluation of advanced diseases with suspected bladder and rectal invasions. However, with dynamic imaging, in which images are acquired every 30 s beginning immediately after a bolus injection of 0.1 mmol gadolinium-diethylenetriaminepenta-acetic acid (Gd-DTPA) and continuing for a total of 4 min, the tumor-cervix contrast in the early dynamic phase was higher and the ability to evaluate parametrial invasion was better than with conventional T2-weighted images. There is a marked difference in the rate of contrast enhancement between tumor and cervix but little or no difference in the steady state or equilibrium degree of enhancement between tumor and stroma (Yamashita et al. 1992).

Magnetic Resonance Findings of Uterine Cervical Cancer

Due to fine contrast resolution, MR imaging can provide highly accurate information regarding the exact extent of tumors. Cervical cancers appear as hyperintense masses on T2-weighted images regardless of histopathologic type. The usefulness of dynamic contrast enhanced studies in diagnosing parametrial invasion and predicting radiosensitivity has been reported (Hawighorst et al. 1997), although sagittal T1-weighted and T2-weighted images and oblique axial T2-weighted images obtained perpendicular to the uterine axis are sufficient for staging in most cases (Shiraiwa et al. 1999).

The normal cervical stroma is hypointense on all spin echo pulse sequences, and is more prominent on T2-weighted images. Like many other tumors having a long T1 and T2, cervical cancer shows relatively hyperintense on proton density

and T2-weighted images. The marked signal intensity difference of normal and abnormal uterine cervix on MR images makes the conspicuity of invasive cervical cancer. Three of the most important prognostic factors for patients with invasive cervical cancer are the size or invasion depth of the tumor, the presence of parametrial extension, and the presence and extent of lymphadenopathy (Mezrich 1994).

Magnetic Resonance Staging of Uterine Cervical Cancer

The FIGO staging system is based on clinical evaluation consisting of bimanual pelvic and rectal examination, chest radiography, excretory urography, cystoscopy, proctoscopy, and cervical biopsy. The greatest difficulties in clinical evaluation are in the assessment of invasion of the parametrium and the pelvic side walls, in the estimation of tumor size, and in the evaluation of lymph node metastasis (Özsarlak et al. 2003).

In general, the staging of cervical carcinoma with MR imaging is also based on the classification system of FIGO. Although, the imaging studies of the cervix including CT and MR imaging are not recommended for clinical staging by FIGO, MR imaging has been described as the most accurate and useful imaging modality in determining the stage of uterine cervical carcinoma. Even if CT is not as accurate as MR imaging in local staging of the cervical carcinoma, the former is a reliable modality for determining regional lymph node assessment, demonstrating advanced disease, monitoring distant metastasis, and planning radiation therapy. Magnetic resonance is very helpful to evaluate the parametrial involvement because normal cervical stroma appears as

a ring of distinct hypointensity on axial T2-weighted images. The presence of a low signal intensity stripe of peripheral cervical stroma surrounding a cervical tumor on T2-weighted MR images is a highly reliable indicator that parametrial invasion is not present (Kim et al. 1993). However, the limitations such as false-negative and false-positive findings are also recognized. Tumor foci are occasionally demonstrated only microscopically, which is beyond the resolution of current MR imaging (false-negative findings), and linear stranding around the cervical mass is suggestive of parametrial invasion but may be a result of peritumoral inflammatory tissue (false-positive findings) (Nicolet et al. 2000).

(1) Stage 0. This stage is detected by vaginal (Papanicolaou) smear and clinical inspections. The MR imaging has no real role to play at this stage, but it could be used to confirm the lack of significant stromal invasion, and such imaging of the cervix at this stage is almost always normal (Mezrich 1994).

(2) Stage I. Carcinoma in this stage is confined to the cervix. This stage is subdivided into stages IA and IB depending on the depth of invasion. Stage IA is defined as a microinvasive tumor, and usually cannot be demonstrated at MR imaging. Cancer at the stage IB includes tumor with invasion depth > 5 mm that is still contained within the cervix. Accurate measurement of this stage can have a profound and direct effect on the choice of method for patient therapy. The combination of the normal low signal intensity cervical stroma and high signal intensity of cervical carcinoma on T2-weighted images makes MR imaging an almost ideal modality in determining whether or not a tumor is confined to the cervix. Numerous studies have shown

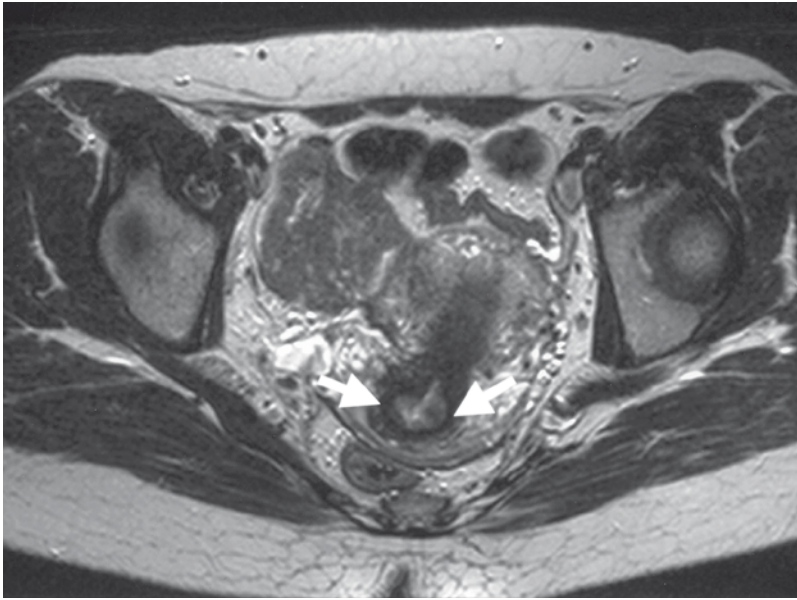


FIGURE 20.1. Stage IA cervical carcinoma in a 58-year-old woman. T2-weighted axial MR image shows high signal intensity lesion with the cervical canal (*arrows*). The normal low signal intensity cervical stroma is noted. This patient underwent radical hysterectomy and the lesion was confirmed as stage IA cervical cancer

the presence of a dark stripe around the tumor means it is unlikely that tumor has extended into surrounding parametrium, with reported sensitivities for determining confinement to the cervix ranging from 93% to 100% (Mezrich 1994). However, the lack of visualization of the hypointense stromal ring surrounding the tumor is not by itself evidence that the tumor has extended beyond the cervix (Figure 20.1).

(3) Stage II. Tumors in stage II extend beyond the cervix but not to the pelvic wall. The tumor may involve the upper two thirds of the vagina. Depending on whether or not the parametrial tissues are involved, stage II is divided into IIA or IIB. Stage IIA is defined as a tumor that invades the upper two-thirds of the vagina without involvement of the parametrial tissue. Magnetic resonance images reveal segmental disruption of the hypointense vaginal wall on T2-weighted images. When

the tumor invades beyond the uterus with parametrial invasion but does not invade the pelvic wall, it is defined as stage IIB. At MR imaging of IIB patients, triangular protrusion of the tumor through the disrupted hypointense ring of cervical stroma is seen (Togashi et al. 1998) (Figure 20.2).

Because the preferred treatment option is different in stage IIA and IIB, it is very important to decide whether or not there is parametrial involvement. Even though the presence of hyperintense signal within the parametrium is recognized, this observation is not enough evidence to conclude that parametrial invasion is present. The reasons for such false-positive findings include surrounding tissue edema, prominent peritumoral or normal parametrial vasculature, inflammation of the intracervical tumor, and large, exophytic appearance of tumors distending the vaginal fornix.

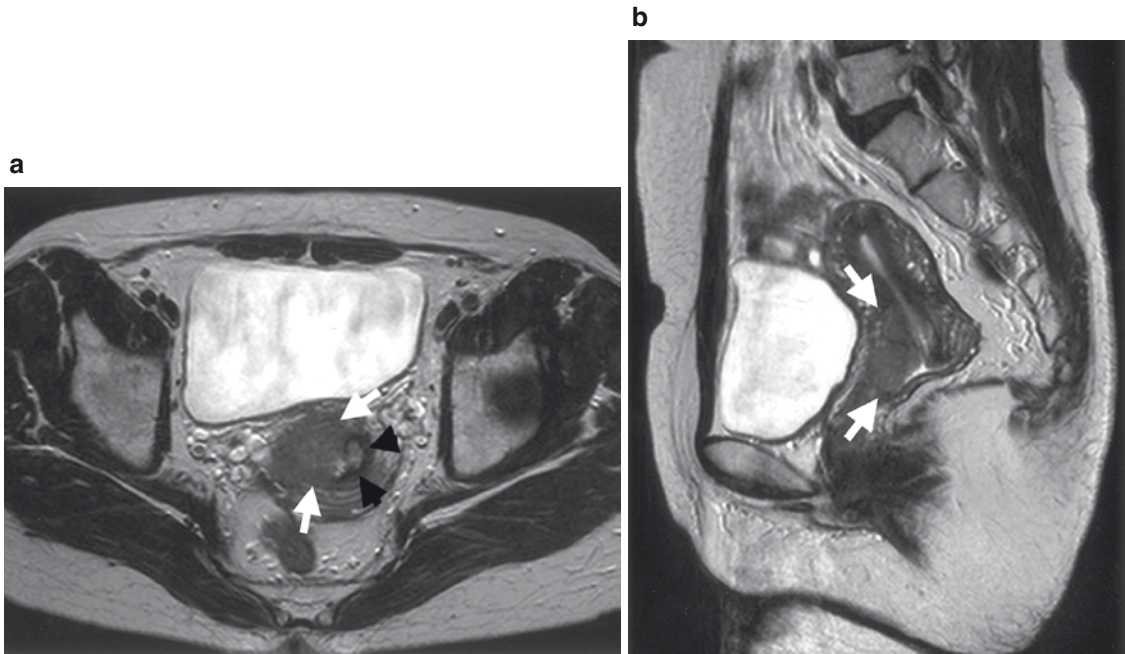


FIGURE 20.2. Stage IIB cervical carcinoma in a 43-year-old woman. (a) T2-weighted axial MR image shows high intensity mass in the uterine cervix (*arrows*). Note that low intensity cervical stroma is preserved in the left side (*black short arrows*) but is destroyed in full thickness in the right side. (b) T2-weighted sagittal MR image reveals high signal intensity mass in the uterine cervix and upper vagina (*arrows*)

Magnetic resonance detection of vaginal extension is highly effective, with accuracies ranging from 83% to 93%. Vaginal extension is recognized on sagittal and axial T2-weighted images, with the tumor seen as a high intensity mass replacing or invading the normal low intensity vaginal wall (Kim et al. 1993).

(4) Stage III. Tumors in stage III involve the lower-third of the vagina or extend to the pelvic sidewall. In stage IIIA vaginal involvement reaches the lower-third of the vaginal canal without extending to the pelvic wall. Occasionally, the anterior vaginal wall is partly disrupted and the tumor infiltrates the bladder wall but not the vesical mucosa. Stage IIIB tumors extend to the pelvic wall or cause hydronephrosis. At MR imaging, the tumor obliterates the entire cardinal ligament and extends to the pelvic mus-

cles including levator ani, piriformis, and obturator internus. Sometimes inflammatory changes or edema of the musculature of the pelvic sidewall can lead to false-positive findings. Hydronephrosis caused by tumor invasion of the ureter is also classified as stage IIIB. Magnetic resonance urography can clearly demonstrate hydronephrosis caused by tumor extension.

(5) Stage IV. Tumors in this stage extend outside the reproductive tract and are subdivided into those that involve the mucosa of the bladder or rectum (stage IVA) and those with distant metastases or extension out of the true pelvis (stage IVB). If the tumor invades the vesical or rectal mucosa, it is classified as stage IVA. In analyzing MR imaging, the diagnosis of stage IVA cervical carcinoma should be made when the loss of normal low

signal intensity of the bladder or rectal wall contiguous to the cervical carcinoma on T2-weighted images is seen. When a cervical cancer invades only the serosa or muscular layer of the bladder without reaching the mucosal surface, endoscopy cannot detect the invasion.

When an extravesical tumor or inflammation extends to the bladder, cystoscopy usually reveals vesical lesions that are slightly raised and shaggy, somewhat irregular, and often surrounded by bulbous edema or telangiectasia and hemorrhage, which are termed herald lesions. The MR imaging findings of irregularity, nodularity, and high signal intensity in the anterior aspect of the posterior wall are highly suspicious of invasion of the bladder wall, analogous to the herald lesion seen with cystoscopy. However, these findings may also be seen with edema caused by nonneoplastic processes (Kim and Han 1996). When any distant metastases have occurred, the stage is defined as IVB, which means distant metastasis or disease outside the true pelvis.

PELVIC COMPUTED TOMOGRAPHY VERSUS MAGNETIC RESONANCE

CT scanning has been reported to be of value in staging of cervical carcinoma and assessment of nodal status. Although in some cases large tumors often show a rim of high attenuation or appear as a low-attenuation mass, most of the small tumors are isodense with normal cervical tissue, and the cervical enlargement may be the only CT finding of cervical cancer (Özsarlak et al. 2003). The advantage of CT is its wide availability and its

ability to show anatomy with high detail. The disadvantage of CT for the evaluation of cervical cancer is that the tumor is recognized primarily only as a change in morphology and not as a distinct entity. Thus, for example, in the case of stage I tumor confined to the cervix, MR imaging shows the tumor as a high signal lesion surrounded by low signal cervical stroma, whereas CT may show a slightly enlarged cervix, or more likely a morphologically normal cervix.

In the staging of cervix cancer, MRI is more accurate than CT, but both are superior to clinical findings in evaluating the regional extension and preoperative staging of cervix cancer (Özsarlak et al. 2003). The advantages of MR imaging, in which both signal and morphology help define the presence of tumor, have been demonstrated in several studies (Cobby et al. 1990). One study of 99 patients found MR imaging superior to CT in tumor detection (75% versus 51%), in accuracy of parametrial evaluation (87% versus 80%), in overall staging (77% versus 69%), and even in pelvic lymph node evaluation (Kim et al. 1993).

Boss et al. (2000) reviewed 12 studies describing staging accuracy with MR imaging in cervical carcinoma. Among 502 patients with histological diagnosis, the mean percentage of overall staging accuracy was 79% (range 47–90%). At CT examination correlated with histological diagnosis, the staging accuracy was 62% (range 32–80%).

Although a number of CT criteria for parametrial invasion, including irregular cervical margin, thick parametrial strands, and eccentric parametrial soft tissue mass have been described, the only reliable finding is the obliteration of the periureteral fat plane. However, the preservation of

a hypointense fibrous stromal ring at T2-weighted MR imaging has been reported to have a high negative predictive value for parametrial invasion (Sheu et al. 2001; Nicolet et al. 2000).

Complete disruption of the ring with nodular or irregular tumor signal intensity extending into the parametrium is a reliable sign of invasion. It has been reported that vaginal involvement can be easily accessed by clinical examination. On T2-weighted imaging, vaginal involvement is seen as replacement of normal low-intensity vaginal wall by high-intensity tumor mass.

Evaluation of Pelvic Lymph Nodes

Although regional lymph node involvement is not included in tumor staging, evaluation of the regional lymph node is important because prognosis of the cervical carcinoma is closely related to lymph node status. Evaluation of the lymph node is based on the size and location of the nodes. The modalities for detection of metastatic pelvic lymph nodes are CT, MRI, lymphoscintigraphy, and lymphangiography with or without percutaneous needle biopsy (Kim et al. 1994). One of the limitations of CT or MR imaging in the evaluation of pelvic nodes is that it is impossible to differentiate metastatic nodes from nonmetastatic hyperplastic nodes of similar size.

Surgical lymph node assessment is the gold standard for the diagnosis of lymph node metastases. However, surgical lymphadenectomy is specialized and increases the time and cost of the procedure, with an increased risk of immediate and delayed complications to the patient. Therefore, a noninvasive technique that accurately identifies lymph node metastases would be beneficial (Schlaerth et al. 2002).

According to several studies, lymph nodes with a short-axis diameter <1 cm were considered for diagnosis of lymph node metastasis. The accuracy of MRI in detecting lymph node metastasis varies from 75% to 100% with a mean accuracy of 86%, and CT varies from 75% to 86% with a mean accuracy of 81% (Boss et al. 2000; Hawighorst et al. 1997; Nicolet et al. 2000). A lymph node-specific MR contrast agent has been developed, allowing the identification of malignant nodal infiltration independent of the lymph node size. This MR contrast agent is composed of an iron oxide core and a low molecular weight dextran coating. It is known as ultrasmall particles of iron oxide (USPIO). The particles being administered intravenously are taken up by macrophages in the reticuloendothelial system, especially within the lymph nodes. Uptake of USPIO results in marked loss of signal intensity of the lymph node on T2- and T2*-weighted sequences because of a susceptibility artifact caused by the iron (Rockall et al. 2005). So, normal lymph nodes reveal dark signal intensities, whereas abnormal lymph nodes, which are unable to take USPIO, showed high signals in T2- or T2*-weighted images.

Ultrasmall particles of iron oxide-MRI have several potentials for the improvement in the management of cervical cancer patients. First, the preoperative localization of malignant nodes may allow planning of the surgical lymphadenectomy by directing the surgeon to a node suggestive of metastasis at an unusual site. This may reduce both surgical time and morbidity without a reduction in diagnostic yield. Second, when USPIO-MRI is negative, surgical lymph node sampling could be avoided altogether, with a high degree of confidence. This could be of particular value

in patients with high surgical risks, such as obesity or diabetes. Third, USPIO-MRI could help in defining the radiotherapy fields by mapping the extent of lymph nodes in both the pelvis and retroperitoneal regions accurately (Rockall et al. 2005). Thus, MR contrast agents using USPIO may improve the preoperative evaluation of lymph node metastases compared with standard MRI. This information may provide the clinician with important information in planning the optimal surgical or radiotherapy treatment.

In conclusion, we have discussed the present and potential accuracy of MR imaging for assessing the size and extent of tumor compared to clinical or CT staging. Unlike CT, MRI can accurately measure the size of the tumor, determine whether or not it is confined within the cervix, and determine extension to the vagina, parametrium, or myometrium. With the rapid improvements in MR techniques, the diagnostic accuracy of MR will be improved. In addition, MRI using new contrast agents such as nanoparticles, which are administered intravenously and taken up by macrophages, may improve the preoperative evaluation of lymph node metastasis, and help clinicians in planning the optimal surgical and radiotherapy treatment.

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Treatment

21

Cancer Imaging and Intracavitary Brachytherapy for Cervical Cancer

Shingo Kato and Tatsuya Ohno

INTRODUCTION

Carcinoma of the uterine cervix is the second most common malignant tumor among women worldwide, with an estimated 493,000 new cases and 274,000 deaths in the year 2002. It is more common in developing countries, where 83% of cases occur, with high rates in sub-Saharan Africa, Melanesia, Latin America, the Caribbean, South Central Asia, and South East Asia (Parkin et al. 2005).

Radiation therapy plays a major role in the treatment of cervical cancer. The combination of external beam radiation therapy and intracavitary brachytherapy (ICBT) is the standard treatment for this disease. The principal advantage of ICBT is its excellent dose distribution. When radioactive sources are appropriately placed, they can deliver a very high dose directly to the cervical tumor and a lower dose to the surrounding normal structures, resulting in high local tumor control with minimum normal tissue damage.

Brachytherapy is classified by its dose rate into low-, medium-, and high-dose rate treatment according to the International Commission for Radiation Units and

Measurements (ICRU) Report No. 38: (1) low-dose rate (LDR): 0.4 to 2.0 Gy/h, (2) medium-dose rate (MDR): 2 to 12 Gy/h, (3) high-dose rate (HDR): more than 12 Gy/h. In LDR-ICBT for cervical cancer, 10–20 h are needed for one treatment. In contrast, the treatment time of HDR-ICBT is approximately 10–20 min. Cervical cancer has traditionally been treated with LDR-ICBT using ^{226}Ra or ^{137}Cs source. However, this modality has some disadvantages due to the longer treatment time, such as the need for hospitalization, radiation exposure to medical personnel, physical and psychological stress to patients, and difficulty of maintaining good applicator geometry during treatment. HDR-ICBT was developed to overcome these disadvantages (Henschke et al. 1964; O'Connell et al. 1965).

The primary disadvantage of HDR brachytherapy is the potential late normal tissue toxicity. Treatment with large HDR fractions reduces the potential for recovery of sublethal normal tissue injury and may therefore narrow the therapeutic ratio between tumor control and late complications. To overcome these radiobiological disadvantages, various dosages and fractionation schedules of HDR-ICBT have

been examined. Consequently, several studies, including nonrandomized and randomized clinical trials, have demonstrated that HDR-ICBT is comparable with LDR-ICBT in terms of local control, survival, and morbidity (Teshima et al. 1993; Toita et al. 2003; Nakano et al. 2005). HDR-ICBT with ^{60}Co or ^{192}Ir source has been widely used in East Asia and Europe for over three decades, and recently it has been increasingly adopted in the United States (Nag et al. 2000).

The objectives of imaging in the treatment of cervical cancer are as follows: tumor detection, diagnosis, evaluation of tumor extension, staging, determination of the target volume of radiation therapy, assessment of the treatment, and follow-up. Recently, some imaging modalities have been used to assist in optimal dose delivery of radiation therapy (three-dimensional (3D) image-based 3D treatment planning of ICBT). This article describes the brachytherapy techniques and the imaging modalities used for cervical cancer brachytherapy.

INTRACAVITARY BRACHYTHERAPY FOR CERVICAL CANCER

Applicator Insertion

The most common type of applicator of ICBT for cervical cancer is the combination of an intrauterine tandem and vaginal colpostats (ovoids). The sources in the intrauterine tandem applicator deliver the dose to the cervix, endometrium, upper vagina, and medial parametrium. The lateral vaginal sources in the ovoid applicators increase the lateral spread of the dose in the parametrium. When the vaginal fornices

are asymmetric or absent, the combination of an intrauterine tandem and a ring applicator is useful, because it has a reproducible geometry and is easy to insert. If a tandem and ovoids or tandem and ring applicator cannot be inserted because of a narrow vagina, the absence of fornices, or vaginal extension of the disease, a tandem and a vaginal cylinder may be used. However, it should be realized that the use of a vaginal cylinder results in lower parametrial doses and higher bladder and rectal doses than the previous two methods.

A Foley catheter is placed into the bladder and a balloon is filled with 7 cc of radio-paque contrast material to identify the posterior bladder wall in the radiographs. Vaginal packing using radiopaque gauze is performed to displace the bladder and rectum away from the applicators and to identify the anterior rectal wall.

Optimal applicator placement and attention to detail are essential for maximizing local tumor control and minimizing normal tissue toxicity. Magnetic resonance imaging (MRI) is helpful for safe and precise applicator insertion. Ultrasound-guided applicator insertion is sometimes necessary particularly in patients with altered cervical anatomy, as routine applicator insertion may cause uterine perforation.

Dose Specification

For treatment planning of ICBT, x-ray-based two-dimensional (2D) treatment planning has traditionally been used. The International Commission on Radiation Units and Measurements (ICRU) Report 38 recommends several standardized dose specification points using two orthogonal x-rays. Point A is defined as 2 cm superior to the ovoid surface and 2 cm lateral from the axis of the intrauterine tandem.

The dose delivered to point A is used as the reference dose to the cervical tumor. The bladder reference point is defined as the posterior surface of a Foley balloon close to the bladder neck. The rectal reference point is defined as 0.5 cm behind the posterior vaginal wall on the anteroposterior line from the lower end of the intrauterine tandem sources (or from the middle of the ovoid sources). These reference point doses should be recorded and reported in each ICBT session.

MAGNETIC RESONANCE IMAGING FOR CERVICAL CANCER BRACHYTHERAPY

To provide optimum ICBT for cervical cancer, obtaining accurate information prior to treatment regarding tumor size, volume, shape, and the tumor extent to the parametrium, vagina, and endometrium is vital. It is also important to be aware of the uterine volume, the length of the uterine cavity, and the existence of hydrometrocolpos before ICBT. Physical pelvic exam is used to evaluate the tumor status and uterine volume. This exam is convenient and cost-effective, but the findings tend to be somewhat subjective. To obtain more accurate and detailed information, imaging modalities, such as ultrasound (US), computed tomography (CT), and magnetic resonance imaging (MRI), are considered to be beneficial.

Among these modalities, MRI has great advantages over US and CT on the basis of its superior soft-tissue contrast resolution. On T2-weighted images, the characteristic feature of cervical cancer is an intermediate signal intensity mass. Tumor signal intensity is usually higher than the

normal low signal intensity of cervical stroma, and the signal intensity of muscle tissue of the myometrium is lower than that of the cervical stroma. The paracervical soft tissues usually demonstrate higher signal intensity than cervical cancer. Thus, T2-weighted MR images can visualize the cervical tumor size and volume, distinguish the tumor from the normal cervix and uterus, and determine the parametrial and vaginal infiltration of disease (Figure 21.1a). On T1-weighted images, cervical tumors are usually isointense with the normal cervix and may not be visible, but after administration of intravenous contrast media, most tumors are clearly visualized as heterogeneously enhanced masses. Multiplanar scanning capabilities, including sagittal and coronal views, as well as axial views, are also useful in assessing tumor size, location, and spread to the surrounding normal tissues. Several reports have demonstrated that MRI findings correlate well with surgical findings in cervical cancer (Kim et al. 1990; Hawnaur et al. 1994; Hricak and Yu 1996). Because of the high accuracy for tumor staging, MRI is considered to be useful to determine the optimum treatment volume for external beam radiation therapy (Mayr et al. 1993). In ICBT, sagittal views of MRI are particularly helpful for the safe and precise insertion of the applicator, as they can accurately demonstrate the tumor volume and location as well as the anatomical relationship between the tumor and the uterine cavity.

Furthermore, MRI is useful for the evaluation of tumor response to radiation therapy (Figure 21.1b) and for the prediction of treatment outcome. Kodaira et al. (2002) reported that the pretreatment tumor size measured by MRI and lymph node swelling diagnosed by MRI were significant

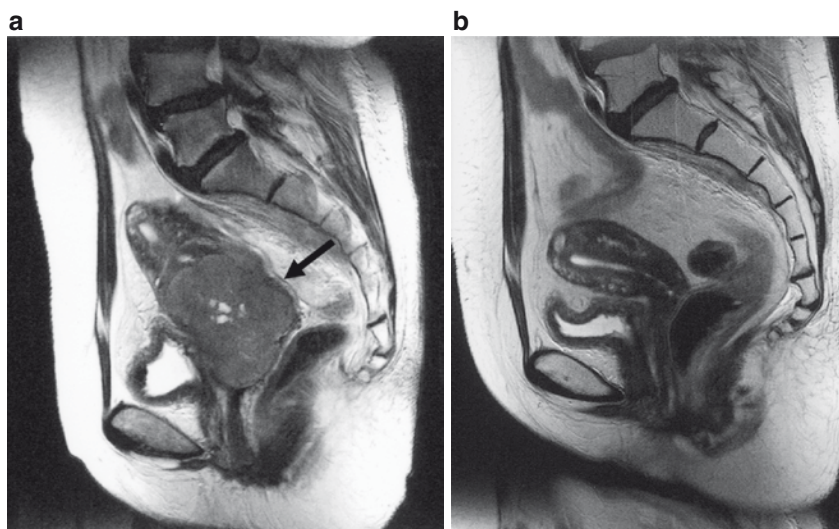


FIGURE 21.1. MRI findings before and after radiation therapy. The patient had a tumor sized $6.0 \times 7.0 \times 7.0$ cm in the uterine cervix. The tumor extended to the bilateral pelvic wall, and was classified as stage IIIB disease according to the International Federation of Gynecology and Obstetrics (FIGO) staging system. Sagittal T2-weighted image (TR/TE/FA = 5000/120/90°) clearly demonstrated the cervical tumor (arrow). The anatomical relationship between the tumor and the uterine cavity was also demonstrated (a). After chemoradiotherapy, the tumor completely disappeared (b)

prognostic factors for local control and/or survival in stage II cervical cancer patients treated with radiation therapy. Mayr et al. (2002) reported that the tumor volume regression rate during radiation therapy represented the best outcome prediction for local control and survival. The effect of ICBT is strongly influenced by the cervical tumor size. Therefore, measurement of the pretreatment and mid-radiotherapy (just before ICBT) tumor size is considered important.

IMAGE-BASED BRACHYTHERAPY

As mentioned above, the current clinical practice for cervical cancer brachytherapy is to prescribe the dose to point A. However, point A is an empiric point and does not reflect the actual tumor volume.

Furthermore, the ICRU bladder and rectal points may not represent the points delivered to the maximum doses to the bladder and rectum. Therefore, the conventional x-ray-based 2D treatment planning of ICBT may result in either under-coverage of the tumor extent or unnecessary dosage to the surrounding normal tissues.

Recently, CT and MRI have increasingly been used for treatment planning of ICBT for cervical cancer, as these imaging modalities provide more accurate information than orthogonal x-rays on the topographic relationship between the applicators and cervical tumor and organs at risk. Treatment planning using CT or MRI also allows assessment of 3D dose distributions and dose-volume evaluation for tumor and organs at risk (Figure 21.2).

Regarding 3D image-based treatment planning in cervical cancer brachytherapy, the Group Européen de Curiethérapie-European

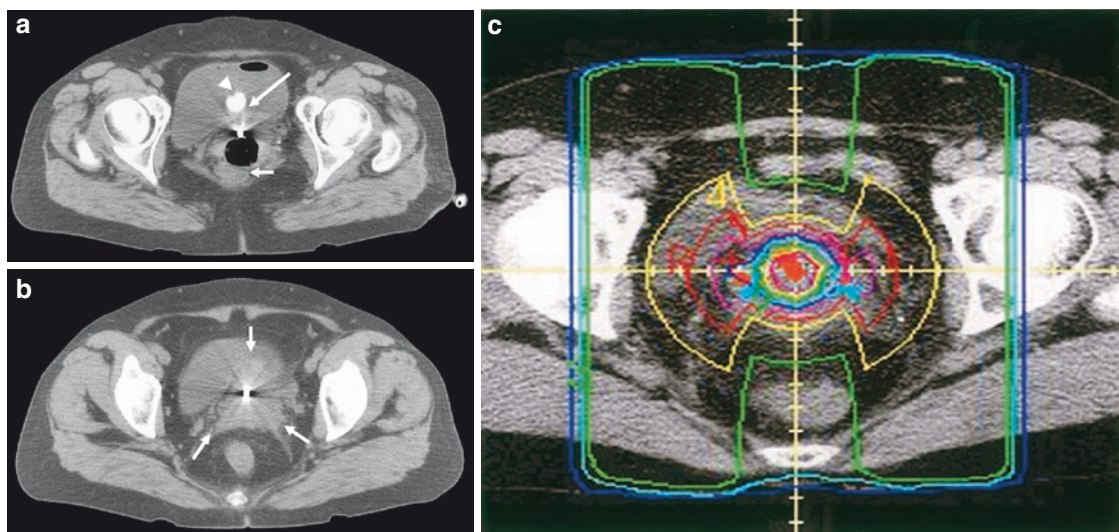


FIGURE 21.2. CT images taken at the time of brachytherapy. A Foley catheter is placed into the bladder and a balloon is filled with 7 cc of radiopaque contrast material to identify the posterior bladder wall (*arrowhead*). The images clearly demonstrate the anatomical relationships between the applicators and the rectum (*short arrow*) and bladder (*long arrow*) (**a**) and the tumor volume (*arrow*) (**b**), which enable calculation of the doses delivered to the rectum, bladder, and the tumor volume. Composite dose distribution of ICBT and EBRT projected onto a CT image (**c**)

Society for Therapeutic Radiology and Oncology (GEC-ESTRO) provided recommendations for the definition and delineation of target volumes and organs at risk (Haie-Meder et al. 2005). Recommendations were also given for recording and reporting the 3D dose-volume parameters for target volumes and organs at risk derived from dose-volume histograms (Pötter et al. 2006a). Based on the recommendations, MRI-based 3D treatment planning was proposed by the working group (Kirisits et al. 2005). Several authors have reported that MRI-based brachytherapy, according to the GEC-ESTRO recommendations, could improve target volume coverage and reduce doses to organs at risk (Pötter et al. 2006b).

3D image-based treatment planning of ICBT is considered to have the potential to improve the dose distributions in cervical cancer brachytherapy. Data collection on the relationship between 3D dose-volume

parameters for target volumes and organs at risk and clinical outcomes are needed to establish optimum ICBT for cervical cancer.

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Cervical Cancer: Methods for Assessing the Quality of Life

Elfriede Greimel

INTRODUCTION

The treatment of patients with cervical cancer has changed significantly in recent years. Pelvic surgery, radiotherapy, chemotherapy or combined regimens are successful treatment options in terms of survival (Photopulos 1990; Burke 1994; Landoni et al. 1997; Morris et al. 1999; Rose et al. 1999). For patients with disease of limited volume, radical abdominal hysterectomy is usually performed, which may cause quality of life (QoL) impairments due to physiological and psychological effects. Approximately, 25% of women with early stage cervical cancer reported vaginal changes that persisted 5 years after radical surgery (Bergmark et al. 1999). For locally advanced disease extensive radiotherapy including external pelvic irradiation and brachytherapy has been the standard treatment for several years (Keys and Gibbons 1996). More recently concurrent chemoradiotherapy has become the treatment of choice for locally advanced cervical cancer with promising results in terms of survival (Green et al. 2001). Neoadjuvant chemotherapy followed by radical surgery has been suggested as an alternative to irradiation (Buda et al. 2005). Intensive multimodal therapies are potentially cura-

tive treatments, but may produce adverse effects surfacing months or years later. Concomitant chemoradiation may double acute and late toxicity and cause severe or potentially life-threatening complications (Kirwan et al. 2003). Vaginal stenosis, atrophy, or loss of elasticity of vaginal tissues after radiotherapy are well-documented. Many women fear that intercourse may be painful or will cause damage. Sexual dysfunctions experienced by women after cervical cancer treatment have been reported (Jensen et al. 2003; Frumovitz et al. 2005; Vistad et al. 2006).

CONCEPT OF QUALITY OF LIFE

The QoL concept aims to incorporate the World Health Organization (WHO) definition of health including physical, emotional, and social well-being. The WHOQOL Group has defined QoL as “an individual’s perception of their position in life in the context of culture and value systems in which they live in relation to their goals, expectations, standards, and concerns” (The WHOQOL Group 1993). In clinical medicine QoL “represents the functional effect of an illness

and its consequent therapy upon a patient, as perceived by the patient” (Schipper et al. 1996). There is a general consensus that QoL is a multidimensional construct including physical functioning (e.g., the ability to perform activities of daily living); psychological functioning (e.g., emotional distress, mood changes); social functioning (social and recreation activities, family interactions), and disease- and treatment-related symptoms (Lepke and Hunt 1997). The term QoL refers to the subjective health status and includes all aspects of patients’ well-being. Based on this theoretical framework, researchers have generated a growing body of work with the development of reliable and valid instruments. However, there is no uniformly accepted gold standard for QoL measurement. Because the number of QoL instruments is expanding, it is difficult for researchers to select the most appropriate measures and to interpret the results.

SELECTING APPROPRIATE QUALITY OF LIFE MEASUREMENTS

Quality of life instruments can be used to study populations in randomized clinical trials, to improve patient–clinician interactions in routine practice, and to support policy making and economic evaluation of health care (Velikova et al. 1999). In selecting an instrument scientists should consider the different types of instruments that are available and how they meet the requirements of the proposed application. One key issue that frequently raised concerns is the appropriateness of the measures used. Quality of life instruments need to be examined in terms of their psychometric proper-

ties, in order to make useful interpretations of the results. There is an international consensus that QoL questionnaires should be (1) specific to the target population, (2) designed primarily for self-completion, (3) multidimensional in structure, (4) composed of multi-item scales, and (5) relatively brief (completion time 10–15 min). It is recommended to use an instrument with documented psychometric properties and one that is known to apply to the specific research question. Higginsons and Carr (2001) suggested two steps in choosing a QoL measure and introducing it for clinical practice.

First Step: Questions to Be Asked When Selecting a Quality of Life Instrument

- Are the QoL domains covered relevant?
- In what population and setting was it developed and validated, and are these similar to the research setting in which it is planned to be used?
- Is the measure valid, reliable, responsive, and appropriate?
- What were the assumptions of the assessors when determining validity?
- Are there floor and ceiling effects – that is, does the measure fail to identify deterioration in patients who already have a poor QoL (floor effect) or improvement in patients who already have a good QoL (ceiling effect)?
- Will it measure differences between patients or over time, and to what extent?
- Who completes the measure: patients, a relative, or a professional?
- How long does the measure take to complete?
- Do staff and patient find it easy to use?
- Who will need to be trained and informed about the QoL measure?

Second Step: Introducing a Quality of Life Instrument in Clinical Practice

- Review who is using which measures internally and externally.
- Choose a measure.
- Decide whether other outcomes also need to be monitored.
- Involve staff and patients.
- Adapt the measure for local use and requirements.
- Identify a leader of the project.
- Assign responsibilities.
- Agree on a time table.
- Test when and where the measure will be completed.
- Plan and begin training in both the use of the measure and associated clinical skills.
- Agree on start date and review period.
- Begin using the measure.
- Review its use in the first week and month and then at regular intervals.
- Review individual patients' results and group results to improve care.
- Modify measure as patients and staff feel appropriate.

PSYCHOMETRIC PROPERTIES OF A QUALITY OF LIFE INSTRUMENT

An understanding of the psychometric properties of QoL instruments is needed to select the best measurement for any given application. The most important psychometric criteria used to evaluate QoL instruments are reliability, validity and responsiveness to change (Hays and Revicki 2005).

Reliability

Reliability is the extent to which measurements remain consistent each time they

are administered to the same subject under identical conditions. It is a very important criterion of patient reported measures in clinical trials, because it is essential to establish that any changes observed in a study are due to the intervention and not to problems in the assessment tool. Quality of life instruments that yield consistent measurement allow scientists to draw conclusions or make claims regarding the generalization of their research. A range of analyses can be conducted to establish reliability estimations. The internal consistency of multi-item scales can be assessed by Cronbach's alpha coefficient. A magnitude of 0.70 is usually regarded as sufficient. An alpha coefficient below 0.50 indicates that the item is not derived from the same conceptual domain. Another aspect of reliability is the reproducibility or stability of a measure. This evaluates whether an instrument yields the same results on a repeated application under similar conditions and is assessed by test-retest using correlation coefficients. Reliability assessments often help researchers interpret data and predict the value of scores and the limits of the relationship among variables.

Validity

Validity of a QoL instrument refers to the degree to which a questionnaire accurately reflects the specific concept that the researcher is attempting to measure. Test validation typically includes construct validity, content validity, and criterion related validity. *Construct validity* refers to the postulated underlying construct of an instrument and seeks agreement between the theoretical concept and the QoL measurement. Construct validity can be distinguished between convergent validity and

discriminate validity. Convergent validity refers to the extent to which different ways of measuring the same trait inter-correlate with one another. Discriminate validity is the lack of a relationship among measures which theoretically should not be related. Construct validity involves the empirical and theoretical support for the interpretation of the construct. *Content validity* is based on the extent to which a measurement reflects the specific intended domain of content. In assessing content validity, a clear idea of what aspects of a concept are to be measured is essential. Content validity is the degree to which the content of a questionnaire matches a content domain associated with the construct. For cross-cultural studies, content validity forces the researchers to define the very domains they are attempting to study. Content validity evaluations include systematic comparison of a measure with existing standards, well-accepted theoretical definitions, or opinions of experts and patients. *Criterion related validity* is used to demonstrate the accuracy of a measure or procedure by comparing it with another measure which has been demonstrated to be valid. Criterion validity is the correlation of a scale with some other measure, ideally a 'gold standard'. It involves the correlation between the test and a criterion variable taken as representative of the construct.

Responsiveness to Change

Responsiveness implies that a QoL instrument is able to detect clinically relevant changes over time. This is important for clinical trials that aim to compare treatment outcomes in terms of the therapeutic effect. The simplest method is to calculate change scores for an instrument over time in a trial or longitudinal study and to examine the

correlations of such change scores with changes in other variables. Known-group comparisons can be used to evaluate the extent to which the instrument is able to discriminate between subgroups of patients differing in clinical status. Examples of clinical parameters to form subgroups include disease stage, performance status, or treatment modalities. Changes in QoL can be compared to change in clinical status, interventions of known or expected efficacy, or direct reports of change by patient or health care providers. Another common form of standardized expression of responsiveness is the effect size. The basic approach is that QoL scales are administered, for example, before and after a treatment. The size of change is calculated as the difference between mean scores at assessments, divided by the standard deviation of the baseline score.

TYPES OF QUALITY OF LIFE MEASUREMENTS

There are different types of QoL instruments which differ in content and their intended purpose or application. These include *generic measures*, *disease or condition-specific measures*, and *cancer site specific measures*.

1. *Generic measures* are designed to assess very broad aspects of subjective health. These instruments are potentially suitable for a wide range of patients with chronic disease as well as for the general population. Such instruments allow for comparisons of results across studies of different patient populations. The disadvantages of broad applicable measures are that they are less responsive to clinically important changes in health

status. Two examples of widely used generic instruments are the Medical Outcome Study (Short Form) MOS SF-36 (Ware and Sherbourne 1990) and the WHOQOL Assessment Instrument (The WHOQOL Group 1995). The SF-36 Health Survey comprises 36 items that assess health across eight dimensions: physical functioning, social functioning, role limitations due to physical problems, role limitations due to emotional problems, mental health, energy/vitality, pain, and general health perception. There is also a single item regarding perceptions of health changes during the past 12 months. Item scores for each of the eight dimensions are summed.

The WHOQOL is an international cross-culturally comparable QoL assessment instrument that was developed simultaneously in a wide range of languages and cultures. The 100 items are categorized in the following domains: physical well-being, psychological well-being, level of independence, social relationships, environment, and spirituality. Responses to questions are scored on a five-point Likert-type scale. A short version including 26 items also exists (WHOQOL-BRÉF), which measure physical health, psychological health, social relationships, and environment. The WHOQOL-BRÉF may be more convenient for use in large research studies.

2. *Disease specific measures* are designed for use in specific disease populations. Multiple instruments are available for common chronic diseases such as cancer. One of the most widely used instruments is the European Organisation for Research and Treatment of Cancer Quality of Life Questionnaire (EORTC QLQ-C30). This core questionnaire was

designed to be applicable to a broad range of cancer patients and is a psychometrically robust instrument. The questionnaire consists of 30-items comprised of five functional scales (physical, role, emotional, social, and cognitive) three symptom scales (fatigue, pain, nausea, and vomiting), and six single items assessing additional symptoms commonly reported by cancer patients. It also includes two questions on patients overall QoL and overall health condition, providing a global QoL score. All scales and single items have satisfactory levels of reliability and validity and are highly consistent across different cultural groups (Aaronson et al. 1993).

Another example of a disease specific instrument is the Functional Assessment of Cancer Therapy (FACT) measurement system (Cella and Bonomi 1996). The general version of the instrument (FACT-G) can be used with patients of any tumor type. It consists of 27 Likert-type items that make up five general subscales (physical well-being, social/family well-being, relationship with doctor, emotional well-being, and functional well-being). Disease specific instruments are usually responsive to clinically important changes due to treatment. A disadvantage of such specific instruments is that comparisons across different diseases and treatments are not possible.

3. *Cancer site-specific instruments* are designed to measure specific aspects of QoL. These instruments provide an assessment of particular QoL issues that are affected by a certain type of cancer or a certain therapy. The information gained is often more detailed than that provided by generic instruments that cover broader aspects of health. Cancer

site-specific modules are supplements to the generic questionnaires. Examples for site-specific scales for patients with cervical cancer are the FACT-Cx including 15 items and the EORTC QLQ CX-24 module. Such modules address additional relevant areas of QoL that are not sufficiently covered by a broader instrument. Both the EORTC QLQ-C30 with its modules and the FACT with its specific subscales for a certain cancer diagnosis, are examples that combine a more general core questionnaire with cancer-site specific scales. This has become the standard approach to QoL measurement in clinical trials.

DEVELOPMENT AND CROSS-CULTURAL VALIDATION OF QUALITY OF LIFE INSTRUMENTS

Many QoL instruments have been developed in the last decade, mainly in English. For non-English speaking populations careful translation and cross-cultural validation are required. The International Quality of Life Assessment (IQOLA) project has developed a standard method for cross-cultural application of QoL instruments (Bullinger et al. 1998; Blazeby et al., 2001). This includes the following three stages: (1) translation and cross-cultural evaluation to ensure the conceptual equivalence of questions across countries; (2) psychometric testing of the scoring algorithms to test the assumptions underlying items and the construction of multi-item scales; (3) reproduction of interpretations and examination of the validity and comparability of the scales across countries.

EORTC MODULAR APPROACH TO QUALITY OF LIFE ASSESSMENT

The modular approach to QoL assessment adopted by the EORTC QoL Group is the development of tumor site specific modules to be administered in addition to the core questionnaire. A module is defined as a set of items assessing QoL issues not sufficiently covered by the core questionnaire. These include (1) disease symptoms related to tumor site; (2) side effects and other issues related to treatment; or (3) additional QoL dimensions that are relevant across diagnoses and treatment modalities. The following section describes the methodology for developing disease-specific module according to the standardized guidelines of the EORTC Quality of Life Group (Blazeby et al. 2001). The module development process consists of four phases: (1) generation of relevant QoL issues; (2) operationalisation of the QoL issues into items; (3) pretesting the module; and (4) testing the psychometric properties (Table 22.1).

TABLE 22.1. The four phase module development process.

Phase I: Generation of quality of life issues
Literature search
Review of QoL instruments related to cervical cancer
Interviews with health care professionals
Interviews with patients
Selection of relevant QoL issues
Phase II: Operationalisation
Construction of items for the provisional module
Items worded to be compatible with the QLQ-C30 format
Translation process using forward-backward procedure
Phase III: Pre-testing
Testing the translations in a pilot sample
Cognitive debriefing (patients complete the module and undergo debriefing questions related to the items)
Data analysis according to preset criteria
Phase IV: Testing the psychometric properties
Evaluation of reliability, validity and cross-cultural acceptability

DEVELOPMENT OF THE CERVICAL CANCER MODULE (EORTC QLQ-CX24)

Phase I: Generation of QoL Issues

In phase I relevant QoL issues were identified using three sources: literature, patients, and health care professionals. Literature searches were conducted using electronic databases. Existing QoL instruments were also reviewed. A list of 74 QoL issues related to cervical cancer patients was derived. Issues were excluded if they were too general or if they were covered by the EORTC QLQ-C30. Each issue was rated for relevance and priority by patients and health care providers. In addition, they were asked if there were issues missing from the list that patients and health professionals considered relevant, or if issues were included which they considered irrelevant for the target group. Sixty eight patients diagnosed with various stages of cervical cancer (26 stage I, 22 stage II, 11 stage III, 4 stage IV) and 132 health care professionals (51 gynecologists, 29 medical and radiation oncologists, 31 nurses, 7 psychologists, 7 social workers, 7 others) rated each issue on a four-point Likert scale (1 = lowest relevance, 4 = highest relevance). Issues with high relevance ratings (mean score ≥ 2) and high priority ratings for inclusion in the module (ratings of at least 30%) were considered for inclusion in the module. The list of issues was also presented to the EORTC Gynecologic Cancer Trial Group. Members provided feedback on the appropriateness of content and breadth of coverage to achieve content validity. On the basis of their responses, the list of issues was adapted. The health care professional ratings were largely in agreement with the patient ratings.

Phase II: Construction of Items and Translation

The module is comprised of issues pertaining to symptoms of cervical cancer, treatment related issues and any additional dimensions of QoL that are relevant for cervical cancer patients. The module included gastrointestinal symptoms, genito-urinary symptoms, vaginal symptoms, body image, issues related to sexual functioning, and menopausal symptoms. For the identified issues, questions were generated and a provisional module established. The module was developed in English and then translated following a forward-backward procedure into 13 languages. Particular care was given to the translations in order to achieve wording which would be widely understood, socially acceptable, and equivalent across countries and cultures.

Phase III: Pretesting

In phase III, the primary focus was to identify and solve potential problems in the translations, and to determine the need for additional items or the elimination of items. The applicability, relevance, and comprehensiveness of the module items were tested in 12 languages. Patients completed the questionnaire in the presence of an interviewer. They were then asked to comment on the appropriateness of the instructions and the wording of the questions. Items found to be problematic were rephrased or removed if participants considered them ambiguous or difficult to understand. Some linguistic modifications were made, and a provisional version of the cervical cancer module was established.

Phase IV: Testing the Psychometric Properties

In phase IV, the psychometric properties of the provisional version were tested on a cross cultural sample including patients with cervical cancer from 12 countries. One hundred and ninety patients with histological confirmed cervical cancer FIGO stages I–IV completed the EORTC QLQ-C30, the QLQ-CX24 module, and debriefing questions. The purpose was to assess the scale structure, internal consistency, and validity. The hypothesized scale structure including five multi-item scales (gastro-intestinal symptoms, genito-urological symptoms, vaginal symptoms, body image, sexual functioning) was assessed using multi-trait scaling analyses. Internal consistency of the multi-item scales was calculated using Cronbach’s alpha coefficient. This indicates the degree to which items are related within a subscale. A value of .70 or above was taken as adequate internal consistency. The initial exploratory scale analysis did not confirm the hypothesized five multi-item scales structure. For the symptom scales the internal consistency was poor (below .70). Therefore, all symptom items

were combined in one multi-item scale. The scale structure was re-analyzed confirming a three multi-item scale structure: *Symptom Experience* (items 31–37, 39, 41–43), *Body Image* (items 45–47), and *Sexual/Vaginal Functioning* (items 50–53) and five single item scales: *Lymphoedema* (item 38), *Peripheral Neuropathy* (item 40), *Menopausal Symptoms* (item 44), *Sexual Worry* (item 48), *Sexual Activity* (item 49), *Sexual Enjoyment* (item 54). Items in the Symptom Experience scale can be used in two ways: as a subscale representing cumulative treatment effects or as single items assessing individual symptoms. The three EORTC QLQ CX-24 multi-item scales have good internal consistencies with Cronbach’s alpha coefficients above .70 (Table 22.2).

Validity was assessed by comparing different clinical groups. Known-group comparisons were used to explore whether the QLQ-CX24 was able to discriminate between subgroups of patients differing in terms of their clinical status. The Karnofsky Performance Status scores correlated significantly with the *Symptom Experience* scale ($r = -.20, p = .010$) and

TABLE 22.2. Scale structure and reliability of the EORTC QLQ-CX24.

Scales	Number of items	M	SD	Cronbach’s alpha coefficient
Multi-item scales				
Symptom experience	11 items	14.06	13.05	.72
Body image	3 items	27.61	29.49	.86
Sexual/vaginal Functioning	4 items	25.98	27.98	.87
Single item scales				
Lymph oedema	1 item	20.04	32.61	n.a.
Peripheral neuropathy	1 item	18.18	27.90	n.a.
Menopausal symptoms	1 item	26.15	31.07	n.a.
Sexual worry	1 item	25.21	29.90	n.a.
Sexual activity	1 item	30.50	37.26	n.a.
Sexual enjoyment	1 item	59.49	32.32	n.a.

the single item scales *Lymphoedema* ($r = -.16$, $p = .047$) and *Sexual Worry* ($r = .16$, $p = .044$). The QLQ-CX24 module discriminated well between patients with early cancer stage (FIGO I) versus patients with advanced stages of disease (FIGO II–IV). On the *Symptom Experience* scale, patients with FIGO I had significantly less symptoms compared to patients with FIGO stages II–IV ($p = .029$). Similarly, on the *Body Image* scale, patients with FIGO I stage had significantly lower impairments than patients with FIGO stages II–IV ($p = .030$). Concerning treatment status, statistically significant differences between patients on-treatment compared to patients off-treatment were found on the *Symptom Experience* scale and the *Sexual/Vaginal Functioning* scale. Women under active treatment reported significantly more symptoms compared to patients who were off-treatment ($p = .036$). On the *Sexual/Vaginal Functioning* scale, patients under active treatment had significantly lower scores compared to patients after completion of treatment ($p < .001$).

The EORTC-CX24 has been developed according to the formal guidelines of the EORTC Quality of Life Group. The final module contains 24 items including three multi-item scales and six single item scales (Appendix 1). It is intended to be used in conjunction with the EORTC QLQ-C30. All major dimensions of QoL as well as specific symptoms related to cervical cancer are addressed. The psychometric analyses confirmed a three subscale structure with high construct validity and reliability (Greimel et al. 2006). Based on psychometric grounds, the QLQ-CX24 module can be recommended for assessing the QoL of cervical cancer patients in clinical trials. The module can pro-

vide additional information on top of the EORTC core questionnaire and allows accurate assessment of the effectiveness of interventions from the patient's perspective. The module should be scored according to the EORTC conventions, i.e., the average of the items that contribute to each scale is taken as the raw score. The evidence to date shows that the core questionnaire and the 24 item cervical cancer module can be completed in less than 20 min; and are therefore highly practical and acceptable to patients. Translations are available in Croatian, Chinese (Mandarin), Danish, Dutch, English, German, Italian, Korean, Norwegian, Polish, Portuguese, Sinhala, Swedish, and Turkish. The EORTC QLQ-CX24 module is a copyrighted instrument.

Requests for permission to use the module and for scoring instructions should be addressed to the EORTC Quality of Life Unit, Avenue E. Mounierlaan 83/11, 1200 Brussels, Website: <http://www.eortc.be/home/qol>.

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Cervical Cancer: Positron Emission Tomography and Positron Emission Tomography/Computed Tomography

Lilie L. Lin and Perry W. Grigsby

INTRODUCTION

Worldwide, cervical cancer is the second most common cause of cancer-related deaths in women. Imaging has not traditionally played a large role in the diagnosis and staging of cervical cancer. Staging of cervical cancer, due to the high incidence in developing countries, is largely based on clinical examination per the Internationale de Gynecologie et d'Obstetrique (FIGO) staging system. Conventional imaging has limited ability to detect small foci of disease. Positron Emission Tomography (PET) capitalizes on the increased metabolism of malignant cells and is useful in distinguishing benign from malignant disease, staging, and monitoring of response to therapy. Though the use of PET has only recently extended to cervix cancer, recent publications demonstrated an important role for PET in patients with cervix cancer. This chapter discusses the current role of PET in the evaluation and management of patients with cervix cancer.

Background and Staging

Cervical cancer was expected to account for ~9,710 new cancer diagnoses, as well

as 3,700 deaths in 2006 within the United States (Jemal et al. 2006). Though the incidence of cervical cancer has been decreasing within the United States due to improved screening, it still remains a significant global health issue. Squamous cell carcinomas represent over 90% of cervical cancers and originate in the surface epithelium of the cervix; adenocarcinomas represent ~5% to 9% of cervical cancers and originate in the cervical glandular tissue. Adenosquamous carcinoma is relatively infrequent and represents ~2–5% of all cervical carcinomas. Rare cervical sarcomas and small-cell carcinomas account for the remainder.

Cervical cancer typically disseminates in a predictable fashion, with initial spread to local structures and regional lymphatics and later hematogenous spread to distant organs, such as bone and lung. The pattern of nodal metastasis is also predictable: tumor spreads from the primary lesion sequentially to pelvic lymph nodes, paraaortic lymph nodes, and supraclavicular lymph nodes. Cervical cancer is staged clinically based on the FIGO staging system. Involvement of pelvic or paraaortic lymph node does not alter the FIGO clinical stage

of disease, but signals a worse prognosis, and has important implications for therapy (Heller et al. 1990). Because of limitations of conventional radiologic techniques for evaluating lymph nodes, surgical assessment of paraaortic nodes is considered the “gold standard”. Moreover, because of the morbidity associated with surgical staging, this procedure is not widely used; thus, the search for an accurate noninvasive staging method is an ongoing process.

Positron emission tomography with the radiopharmaceutical agent ^{18}F -fluorode-

oxyglucose (FDG) is an imaging modality that appears to be well suited for imaging cervical carcinoma. Most primary tumors, except for very small lesions, are readily seen on PET images and exhibit intense FDG uptake (Figure 23.1). In our experience, primary squamous cell carcinomas and adenocarcinomas have similar FDG-avidity. In a review of 230 patients with cervical cancer (squamous cell carcinoma 200, adenocarcinoma 30), we found that the mean maximum standardized uptake value (SUV_{max}) of squamous cell carcinomas was

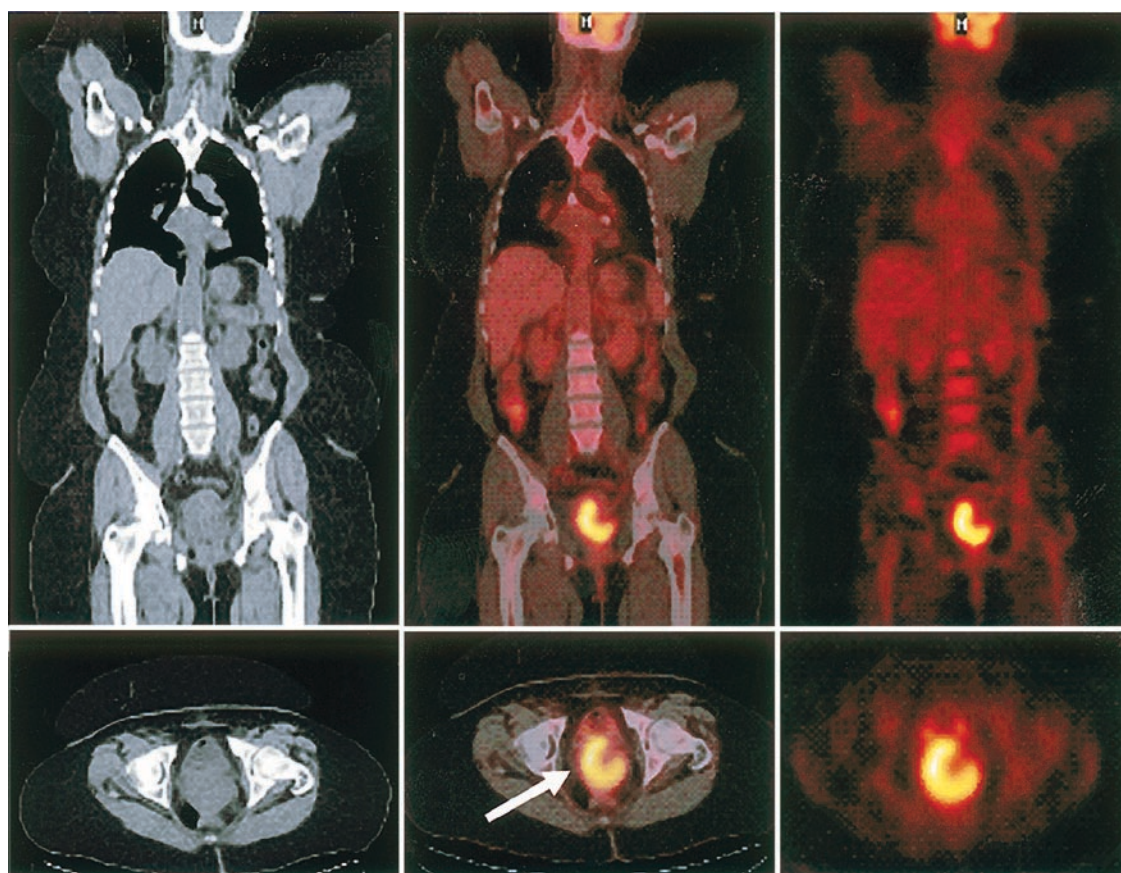


FIGURE 23.1. Advanced cervical cancer. Coronal (*top*) CT, PET/CT fusion, and PET images demonstrate intense FDG uptake within the patient's known primary cervical tumor, pelvic, para-aortic (*arrowhead*), and supraclavicular lymph nodes. Transaxial (*bottom*) CT, PET/CT fusion, and PET images demonstrate intense FDG uptake within subcentimeter supraclavicular lymph nodes (*arrow*). Advanced disease in such patients can be confirmed with fine needle aspiration biopsy of the supraclavicular lymph node

slightly higher than adenocarcinoma, but the difference was not significant (11.7 vs 9.6) (unpublished data). However, because of its relatively poor spatial resolution and inability to assess parametrial invasion or involvement of adjacent organs reliably, FDG-PET is of limited value for staging of the primary tumor.

A number of studies have shown that FDG-PET is superior to conventional imaging methods for detecting metastatic disease, particularly lymph node metastasis (Havrilesky et al. 2005). They reported a systematic review of the published literature up through 2003. They included only those studies involving 12 or more subjects who had PET performed with a dedicated scanner with specified resolution, and with clinical follow-up ≥ 6 months or histopathology as the reference standard. In patients with newly diagnosed cervical cancer, the pooled sensitivity of PET was 79% (95% CI 65–90%), and the pooled specificity was 99% (96–99%) for detection of pelvic lymph node metastasis (Havrilesky et al. 2005). Two studies were identified, and each compared PET to MRI and CT. Magnetic Resonance Imaging had a pooled sensitivity of 72% (53–87%) and pooled specificity of 96% (92–98%), whereas CT had a pooled sensitivity of 47% (21–73%) (there were insufficient data to calculate a pooled specificity). In four prospective studies in which histology after paraaortic lymphadenectomy was used as the reference standard, the pooled sensitivity of PET for the detection of paraaortic nodal metastasis was 84% (95% CI 68–94%) and the pooled specificity was 95% (89–98%) (Havrilesky et al. 2005). In three of these studies, the inclusion criteria for study entry included a negative CT or MRI of the abdomen. Thus, the accuracy of conventional imaging could

not be calculated. The fourth study did not require a negative abdominal imaging study prior to surgery. The sensitivity and specificity of MRI in the 12 patients who underwent aortic node sampling were 67% and 100%, respectively.

False-negative results for detection of nodal metastasis are chiefly related to the limited resolution of PET and, thus, its inability to detect microscopic disease and small macroscopic tumor deposits. Wright et al. (2005) evaluated the sensitivity of FDG-PET by comparison with surgical lymphadenectomy in patients with early-stage cervical cancer. The sensitivity and specificity of FDG-PET to detect pelvic lymph node metastases was 53% and 90%, respectively. They found that the mean size of tumor deposits was larger in PET-positive pelvic nodes (15.2 mm; range 2–35 mm) than in PET-negative nodes (7.3 mm; range 0.3–20 mm). In a prospective study to investigate the clinical benefits of FDG-PET imaging for lymph node staging of early stage cervical cancer prior to radical hysterectomy, 60 patients with MRI defined lymph node negative disease underwent preoperative FDG-PET (Chou et al. 2006). These authors found that with a sensitivity and specificity of 10% and 94%, respectively, dual phase FDG-PET had little additional value for early stage cervix cancer in patients that were preselected with MRI imaging.

The FDG-PET false negative lymph node metastases measured a median of 4.0×3.0 mm, while the single FDG-PET positive lymph node metastases measured 6×5 mm. False-positive results are most likely related to uptake of FDG in hyperplastic nodes or misinterpretation of physiologic activity in bowel or the urinary tract as nodal metastasis. The use

of PET/CT has been shown to improve the accuracy of staging in various cancers, because the combined functional and anatomical information allows for a significant improvement in lesion localization and differentiation of physiologic FDG uptake from pathologic FDG uptake (Antoch et al. 2004). This leads to a decrease in the frequencies of both false-positive and false-negative results.

A study by Grigsby et al. (1999) has shown that FDG-PET is superior to CT and lymphangiography in showing unsuspected sites of metastasis in pelvic lymph nodes, extrapelvic lymph nodes, and visceral organs in patients with newly diagnosed advanced cervical cancer. FDG-

PET showed abnormalities consistent with metastasis more often than did CT in pelvic lymph nodes (67% vs 20%) and in paraaortic lymph nodes (21% vs 7%). Positron Emission Tomography also showed disease in supraclavicular lymph nodes in 8% (Grigsby et al. 2001) (Figure 23.2).

The role of PET/CT in the staging of cervical cancer needs to be determined. The literature currently contains limited data on the use of PET/CT in cervical cancer; however, it is expected that PET/CT image fusion will allow for easier distinction of pathologic and physiologic tracer uptake and, thus, improve the accuracy of image interpretation (Subhas et al. 2005). In a study by Sironi et al. (2005),

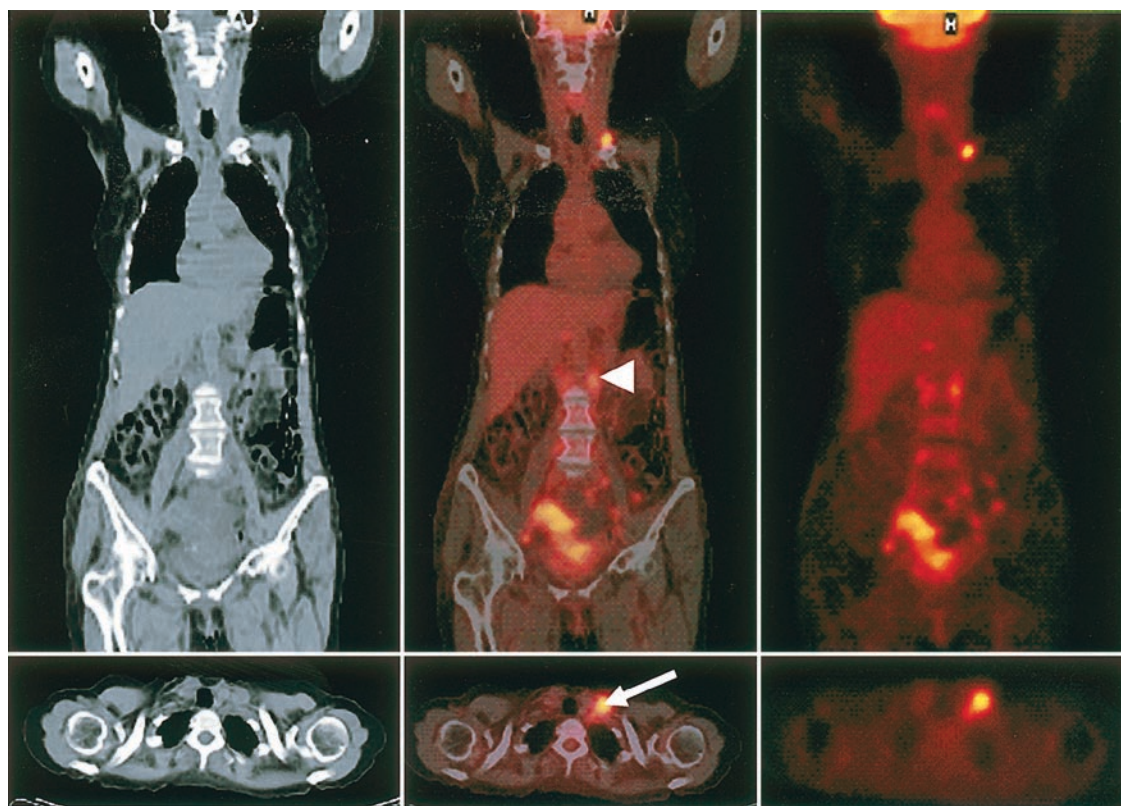


FIGURE 23.1. Early stage cervical cancer. Coronal (*top*) CT, PET/CT fusion, and PET images of a patient with newly diagnosed cervical carcinoma. Transaxial (*bottom*) CT, PET/CT fusion, and PET images demonstrate intense FDG within the cervix (*arrows*)

PET/CT imaging was compared to lymph node staging in patients with early stage cervical cancer. They found that the sensitivity, specificity, positive predictive and negative predictive value were 73%, 97%, 92%, and 89%, respectively. Based on the results in the literature to date, the United States Centers for Medicare and Medicaid Services in January 2005 approved coverage for use of FDG-PET in initial staging of patients with cervical cancer who have no evidence of extrapelvic metastatic disease on CT or MRI.

An evolving, competing imaging method for detection of metastatic disease in lymph nodes is MR lymphography with ultrasmall superparamagnetic iron oxide (USPIO) particles as the contrast agent. After intravenous injection, USPIO is taken up by macrophages in healthy lymph nodes and produces a loss of T1 signal, whereas the signal of metastatic tissue remains unchanged (Hamm 2002). Rockall et al. (2005) have shown that USPIO-MRI is superior to standard size criteria for detection of metastatic lymph nodes in patients with endometrial and cervical cancers. In 44 patients, 768 pelvic or paraaortic lymph nodes were sampled histologically, of which 335 were correlated on MRI; 17 malignant lymph nodes were found in 11 of the 44 patients. Magnetic Resonance Imaging using USPIO criteria had significantly higher sensitivity than the size criteria for detecting lymph node metastasis both on a node-by-node basis (93% vs 29%) and a patient-by-patient basis (100% vs 27%). However, no significant differences were noted in the specificity or positive- and negative-predictive values between the two methods. The false-negative rate was relatively high with both methods, resulting in low positive-predictive values

(61% vs 56%). The false-negative results were mainly due to failure in identifying involved lymph nodes in the parametrium and only occurred in patients with cervical cancer. Further studies in larger populations are needed to establish the use of this technique in clinical practice.

Directing Therapy

The use of FDG-PET in pretreatment clinical staging has had a significant impact on the therapeutic management of patients with cervix carcinoma. The standard treatment of advanced cervical carcinoma is radiotherapy with concurrent chemotherapy (Morris et al. 1999). Radiotherapy is directed at the pelvis to encompass primary disease as well as pelvic lymph nodes. The radiotherapy port is expanded to include the paraaortic lymph node region only in patients who have evidence of paraaortic nodal disease. Patients who have evidence of disease beyond the paraaortic lymph nodes at the time of initial diagnosis have little chance of a cure, and receive palliative therapy. Based on the findings by Grigsby et al. (2001), we now routinely administer curative paraaortic irradiation to patients with CT-negative, FDG-positive paraaortic nodal disease, whereas no irradiation to this region would have been administered to such patients in the past before the use of PET to assess for paraaortic disease. Fourteen patients in that analysis had paraaortic disease detected by FDG-PET that was not detected by CT. These patients had their radiation portals increased to include the paraaortic nodal region. Currently we are investigating the use of PET/CT-guided intensity-modulated radiotherapy (IMRT) to deliver higher doses to paraaortic nodes that have FDG-avid

disease by PET/CT (Esthappan et al. 2004). Fused PET/CT images can be used to differentiate tumor from adjacent normal structures more reliably and thus, allow for delivery of higher doses of radiation to the tumor while decreasing radiation dose to normal structures.

FDG-PET may also be useful in determining whether concurrent chemotherapy should be administered to patients with advanced-stage disease. A recent study from the Gynecologic Oncology Group (GOG 109) randomized patients with pathologically positive pelvic lymph nodes to either radiotherapy alone or to radiotherapy with concurrent and adjuvant cisplatin and 5-fluorouracil chemotherapy (Peters et al. 2000). The study demonstrated that there was both a superior disease-free and overall survival advantage when chemotherapy was added to radiation therapy. A subsequent report from this study demonstrated that there was no benefit to the use of concurrent chemotherapy in patients with only one positive lymph node (Monk et al. 2005). A similar finding was demonstrated by Grigsby et al. (2005). In this retrospective analysis of 65 patients, there was no apparent clinical benefit to the use of concurrent chemotherapy with primary irradiation in patients who had no evidence of lymph node metastasis by FDG-PET. Thus, FDG-PET may be useful to select a subgroup of patients with locally advanced cervical cancer without evidence of lymph node metastases who may not benefit from the administration of concurrent chemotherapy. Larger prospective studies need to be done to confirm these results.

Prognosis

Several prognostic factors have been identified for patients with carcinoma of the cervix.

These include patient age, tumor histology, tumor stage, tumor size, lymph node metastasis, and tumor hypoxia (Stehman et al. 1991; Hockel et al. 1993). In a study of 101 patients with newly diagnosed cervical cancer, Grigsby et al. (2001) demonstrated that the lymph node status determined by FDG-PET is predictive of progression-free and overall survival in patients with cervical cancer. The 2-year, disease-free survival was better predicted by PET evidence of lymph node involvement than by CT findings. Based on the imaging findings in the pelvic lymph nodes, the 2-year, disease-free survival was 84% for CT-/PET- patients, 64% for CT-/PET+ patients, and 48% for CT+/PET+ patients ($p = .05$). Based on the imaging findings in the paraaortic nodes, the 2-year, disease-free survival was 78% in CT-/PET- patients, 31% for CT-/PET+ patients, and 14% for CT+/PET+ patients ($p \leq .0001$). None of the patients with PET+ supraclavicular lymph nodes survived 2 years. The PET-determined status of the paraaortic nodes was the strongest predictor of survival in a multivariate logistic regression analysis. These results suggest an opportunity to salvage some patients with paraaortic nodal metastasis defined by PET, as described above. In an analysis of 256 patients in our registry, we also found that the extent of lymph node involvement is inversely correlated with survival (Grigsby 2005).

Miller and Grigsby (2002) evaluated the usefulness of tumor volume measurement with FDG-PET in 57 patients with cervical cancer. Tumor volume and lymph node status determined by PET and FIGO stage determined by clinical examination were predictive of progression-free survival; tumor volume and lymph node involvement

by PET predicted overall survival. The presence of lymph node involvement did not correlate with tumor volume. We have demonstrated that the results of posttreatment surveillance FDG-PET studies in patients with cervical cancer are strongly predictive of patient survival (Grigsby et al. 2004). We have also found that FDG-PET demonstrated metastatic involvement in the left supraclavicular lymph nodes in 8% of our patient population; this finding has a positive-predictive value of 100% and indicates a dismal prognosis, despite aggressive therapy (Tran et al. 2002). Similarly, we found that the cause-specific survival for patients with FIGO stage IIb carcinoma is highly dependent upon the extent of lymph node metastasis demonstrated by whole-body FDG-PET at initial presentation (Singh et al. 2003). The three-year estimates of cause-specific survival were 73% for those with no lymph node metastasis, 58% for those with only pelvic lymph node metastasis, 29% for those with pelvic and paraaortic lymph node metastasis, and 0% for those with pelvic, paraaortic, and supraclavicular lymph node metastasis ($p = 0.0005$). In a recent review of 96 patients with cervical cancer, we found that FDG uptake within the primary cervical tumor, as measured by the SUV_{max} , is predictive of disease-free survival in patients undergoing radiotherapy for cervical cancer (Xue et al. 2006). Thus, high FDG uptake may be useful in identifying patients who need more aggressive initial therapy.

We have also recently published a study examining the use of FDG-PET imaging during the course of chemoradiotherapy (Lin et al. 2006). In that analysis, 11 patients received between two and three FDG-PET imaging studies during the course of therapy. Three patients had complete

resolution of initial abnormal FDG-PET uptake prior to the completion of their chemoradiotherapy. All 11 patients remain free of disease, but future studies will need to be performed to determine whether subgroups of patients can be identified as either poor responders or early responders. Their subsequent treatment regimen could then be tailored accordingly.

Posttherapy Monitoring

Approximately 30% of cervical cancer patients will ultimately fail after definitive treatment (DiSaia and Creasman 2001). Clinical and radiological techniques have been used for early detection of recurrent disease with much success. FDG-PET has also been shown to have a role in the post-treatment monitoring of patients with cervical cancer. In a large retrospective study by Ryu et al. (2003), 249 women with previously treated cervical cancer without overt evidence of recurrence underwent FDG-PET as part of their routine follow-up. Eighty patients (32%) were found to have abnormal FDG uptake; 28 (11%) had clinically or histologically confirmed recurrent disease. The sensitivity and specificity of FDG-PET for detection of recurrent disease were 90% and 76%, respectively. The positive- and negative-predictive values were 35% and 98%, respectively. There was a high false-positive rate associated with FDG uptake in the pulmonary hila, lungs, and neck, as well as the inguinal and axillary regions. The majority of the recurrences were detected within 6–18 months after diagnosis. In another series by Unger et al. (2004), FDG-PET detected recurrences in 31% of asymptomatic patients and in 67% of symptomatic patients. In symptomatic patients, the sensitivity of FDG-PET was 100%, the specificity was 86%, and the positive- and

negative-predictive values were 93% and 100%, respectively. By comparison, in asymptomatic patients, the sensitivity of FDG-PET was 80%, the specificity was 100%, and the positive- and negative-predictive values were 100% and 89%, respectively. In a study by Grigsby et al. (2004), 152 patients previously treated with radiotherapy with or without concurrent chemotherapy who were free of FDG-avid sites on PET obtained an average of 3 months posttherapy, had 5-year cause-specific and overall survival of 80% and 92%, respectively. Persistent abnormal uptake in the cervix or lymph nodes was found in 20 patients, and their cause-specific survival was 32%. New areas of increased FDG uptake in previously unirradiated regions were found in 18 patients, none of whom were alive at 5 years. Posttreatment PET abnormalities were found to be the most significant predictor of death from cervical cancer in this study. Together, these results point to a significant impact of FDG-PET findings on treatment strategy after primary therapy.

Other groups have investigated the use of FDG-PET in combination with other biomarkers, such as squamous cell carcinoma (SCC) antigen. In a phase II study, 27 patients with previously treated cervical cancer underwent FDG-PET for unexplained elevation of serum SCC antigen levels found during follow-up evaluation (Chang et al. 2004). Positron Emission Tomography showed FDG-avid lesions in 17 of 18 patients with proven recurrent disease; 12 of these patients had distant recurrences only, two had local recurrences only, and three had both local and distant recurrences. As a result of PET imaging, only seven of the 18 patients with recurrent disease were treated with curative intent as compared to 16/30 patients treated with

curative intent in the historical control group. This imaging allowed better selection of patients for salvage therapy, which resulted in a trend toward increased overall survival of those treated compared with a historical control group. These promising results will need to be confirmed in further investigations to better define the role of FDG-PET in combination with molecular biomarkers. Additionally, Yen et al. (2004) in a prospective study examined the role of FDG-PET imaging in patients with biopsy confirmed relapse of disease or unexplained elevation of serum tumor marker levels with documented relapse after treatment. Fifty-five women were enrolled, 36 (66%) had treatment modifications as a result of the PET findings, and the remaining 19 were treated as originally planned. Of those patients whose plans were modified, 25% received salvage therapy with curative intent, but the modality or field of irradiation was changed, and 75% received only palliative treatment. Together, these results demonstrate the usefulness of FDG-PET for determining the intent and optimal scope of salvage therapy in patients with recurrent cervical cancer.

It is concluded that FDG-PET imaging plays large role in the initial staging of patients with cervical cancer. The imaging results have important implications for prognosis as well as therapeutic management. Further studies are ongoing to establish a role for FDG-PET imaging in post-treatment monitoring, restaging, and radiotherapy treatment planning.

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Endometrial Cancer: Indoleamine 2,3-Dioxygenase as a Prognostic Indicator

Kazuhiko Ino, Eiko Yamamoto, Kiyosumi Shibata, Hiroaki Kajiyama, Akihiro Nawa, and Fumitaka Kikkawa

INTRODUCTION

Endometrial cancer is the most common malignancy of the female genital tract. In three-fourths of cases of this disease, the tumor is clinically confined to the uterus at the time of diagnosis, and most patients with early-staged disease achieve a favorable clinical outcome with surgery alone (Morrow et al. 1991; Grigsby et al. 1992). However, a significant number of patients with early-staged disease develop localized recurrence or distant metastases, and the patients with recurrence show a poor outcome. Several clinicopathological parameters are currently used for the classification of risks for relapse, such as surgical stage, histological type, grade, depth of myometrial invasion, cervical stromal invasion, lymph node metastasis, lymphovascular space involvement, and peritoneal cytology. For patients belonging to high or intermediate risk groups defined by these parameters, either postoperative radiotherapy or chemotherapy has been used (Creutzberg et al. 2000; Keys et al. 2004). However, selecting the patients that will receive the adjuvant therapy and its effectiveness on survival remain controversial

(Cardenes and Randall 2003). Thus, the identification of new molecular markers more strictly related to the intrinsic biological behavior of endometrial cancer, and the individualization of adjuvant therapy based on more reliable prognostic indicators, may be helpful in further improving the survival of patients with this disease.

Indoleamine 2,3-dioxygenase (IDO) is a heme-containing intracellular enzyme that catalyzes the initial and rate-limiting step of metabolism of the essential amino acid tryptophan by cleavage of the pyrrole ring (Takikawa 2005). While only a small amount (less than 5%) of tryptophan from food is converted to serotonin and further converted into melatonin, most (more than 95%) dietary tryptophan is metabolized by IDO along the kynurenine pathway, leading finally to the biosynthesis of nicotinamide adenine dinucleotide (NAD) (Figure 24.1). This enzyme is expressed in a wide range of tissues such as the lung, intestine, brain, and placenta. A cDNA encoding human IDO has recently been cloned and its deduced primary structure was obtained by Tone et al. (1990). Human IDO cDNA encodes a protein of 403 amino acids with a molecular weight of ~45 kDa. The IDO

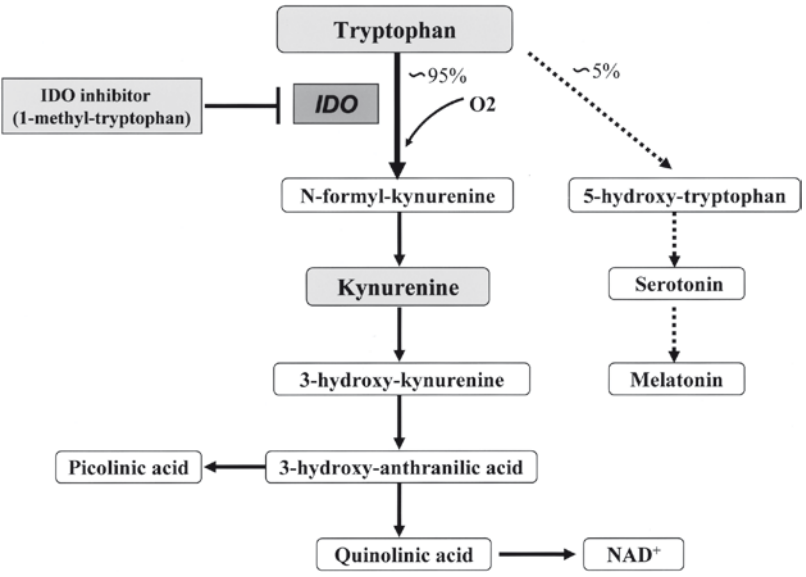


FIGURE 24.1. Catabolic pathway of tryptophan by IDO. IDO catalyzes the initial and rate-limiting step of tryptophan metabolism along the kynurenine pathway

protein is encoded by a tightly regulated gene that responds to inflammatory mediators such as interferon-gamma (IFN- γ), tumor necrosis factor-alpha (TNF- α), and lipopolysaccharide (LPS). Among them, IFN- γ is a major inducer of IDO expression (Takikawa et al. 1988).

Recently, evidence has been accumulating that indicates an immunosuppressive function of IDO. It was firstly reported by Munn et al. (1998) that IDO is expressed in placental trophoblasts and macrophages during pregnancy, and prevents rejection of the allogeneic fetus; thereby, suggesting involvement of IDO in fetal-maternal tolerance. This is based on the findings that a pharmacological inhibition of IDO activity by 1-methyl-tryptophan (1-MT) results in rejection of the fetus in pregnant mice. Subsequent studies by Mellor and Munn (1999) and Frumento et al. (2002) clarified the mechanism of IDO immunosuppression by locally depleting tryptophan and/or by producing toxic tryptophan catabolites

(e.g., kynurenine, Figure 24.1), which causes arrest of proliferation of alloreactive T-cells and natural killer (NK) cells that are very sensitive to tryptophan shortage. Secondly, it was reported by Mellor and Munn (2004) that IDO is expressed in antigen-presenting cells, especially certain subsets of dendritic cells, and regulates immune response and induces tolerance.

In malignancy, Uyttenhove et al. (2003) demonstrated for the first time that IDO was present in various human cancer tissues, and that it was expressed not only by immune cells but also by the tumor cells themselves. They clearly demonstrated using mouse models that IDO was involved in protecting tumors from attack and rejection by tumor-associated antigen-specific host cytotoxic T-cells. In contrast, it was reported by Munn et al. (2004) that IDO is expressed by CD19+ plasmacytoid DCs in tumor-draining lymph nodes in tumor-bearing mice, and these specific IDO-expressing DCs potently suppress host

antitumor T-cell responses and induce tolerance to tumor-derived antigens. More recently, Muller et al. (2005) have shown that IDO inhibitors (1-MT) potentiated the antitumor activity of chemotherapeutic agents in mice, suggesting the involvement of IDO in the refractoriness to chemotherapy. Based on the above findings from the mouse studies, considerable attention is now being paid to the role of IDO in the progression of human cancer and its therapeutic potential as a new prognostic marker or a molecular-target. However, there have been few studies on IDO expression in human cancers, and its prognostic or functional significance has not yet been well studied.

We have recently reported that IDO is expressed in human endometrial cancer tissues and this molecule is a reliable indicator for disease progression and poor prognosis of endometrial cancer patients (Ino et al. 2006). Here, we summarize the results of the immunohistochemical staining for IDO expression and its correlation with the patient survival in endometrial cancer. We also discuss the possible mechanism by which IDO contributes to the immune escape and cancer progression.

MATERIALS AND METHODS

Antibodies

Mouse monoclonal antibody against human IDO was prepared as previously described by Takikawa et al. (1988), and was kindly provided by Dr. Osamu Takikawa, National Center for Geriatrics and Gerontology, Japan. We previously confirmed the reactivity and specificity of this monoclonal antibody on Western blot analysis of tumor tissue samples obtained from endometrial cancer patients, where

IDO protein was detected as a 42 kDa single band (Ino et al. 2006).

Patients

Eighty patients with endometrial endometrioid adenocarcinoma who underwent surgical treatment at Nagoya University Hospital between 1992 and 2001 were included in this study. Initial diagnoses were made preoperatively by the pathological review of endometrial biopsy or curettage specimens. Surgical treatment consisted of total abdominal hysterectomy and bilateral salpingo-oophorectomy, followed by surgical staging, including peritoneal washing cytology and lymphadenectomy. Patients with the histological cell types other than endometrioid adenocarcinoma, such as papillary serous or clear cell, were not included in this study. The mean age of the patients was 57.2 years (range 31–86). All patients were staged according to the 1988 International Federation of Gynecology and Obstetrics (FIGO) criteria: 54 were stage I (7 were IA, 33 were IB, 14 were IC), 10 were stage II, 10 were stage III, and 6 were stage IV. Histological grade was assigned according to the criteria of the World Health Organization (WHO) classification: 40 were G1 (well-differentiated), 27 were G2 (moderately-differentiated), and 13 were G3 (poorly-differentiated). In this study, all patients with FIGO stage IC and more advanced-staged disease received postoperative adjuvant chemotherapy with six cycles of either cisplatin/doxorubicin/cyclophosphamide or cisplatin plus etoposide in 1992–1999, and carboplatin plus paclitaxel after 2000. Patients receiving postoperative radiation therapy or any preoperative treatment were excluded from this study because the number of these

patients was very small. Patients with recurrence were treated with the chemotherapy, local radiation therapy, or surgical tumor resection, if possible.

Immunohistochemical Staining

Informed consent was obtained from individual patients for the use of their tissue samples. Surgical specimens were fixed in 10% formalin and embedded in paraffin. Paraffin specimens were cut at a thickness of 4 μm . For heat-induced epitope retrieval, deparaffinized sections were soaked in Target Retrieval Solution consisting of 10 mM Tris and 1 mM EDTA (pH 9.0) (DAKO, Glostrup, Denmark), and treated at 95°C for 30 min in a microwave oven. Immunohistochemical staining was performed using the avidin–biotin immunoperoxidase technique. Endogenous peroxidase activity was blocked by incubation with 0.3% H_2O_2 in methanol for 15 min, and nonspecific immunoglobulin binding was blocked by incubation with 10% normal goat serum for 10 min. Sections were incubated at room temperature for 2 h with anti-IDO monoclonal antibody at 1:200 dilution. The sections were rinsed three times and incubated for 30 min with the biotinylated second antibody. After washing three times, the sections were incubated for 30 min with horseradish peroxidase-conjugated streptavidin, and finally treated with 3,3'-diaminobenzidine tetrahydrochloride in 0.01% H_2O_2 for 10 min. The slides were counterstained with Meyer's hematoxylin. As a negative control, the primary antibody was replaced with normal mouse IgG at an appropriate dilution. As a positive control, tissue sections of normal placenta were used as previously reported by Sedlmayr et al. (2002).

Evaluation of Indoleamine

2,3-Dioxygenase Expression

The IDO expression levels were classified semi-quantitatively based on the total scores of the percent positivity of stained tumor cells and the staining intensity. Namely, the percent positivity was scored as “0” if <5% (negative), “1” if 5–30% (sporadic), “2” if 30–70% (focal), and “3” if >70% (diffuse) of cells stained, while the staining intensity was scored relative to the known positive and negative controls as “0” if no staining, “1” if weakly stained, “2” if moderately stained (intermediate level between strong and weak), and “3” if strongly stained. The final IDO expression score was defined as follows; “IDO-” if the sum of the percent positivity score and the staining intensity score was 0–1, “IDO1+” if the sum was 2–3, “IDO2+” if the sum was 4–5, and “IDO3+” if the sum was 6. In this scoring system, IDO expression in the tumor stromal cells was not considered because the IDO immunostaining in nontumor cells was not remarkable or absent in all cases examined. In each case, at least three different areas were evaluated, and the mean of the results was considered the final expression score. The scoring procedure was carried out by two independent observers without any knowledge of the clinical data. The concordance rate was >95% between the observers. In the case of disagreement, the slides were reviewed by these two observers together with another, different observer, who were seated together at a multiheaded microscope, in order to resolve the difference of opinion.

Statistical Analysis

Fisher's exact test and Pearson chi-square test were used to analyze the correlation

of IDO expression with various clinicopathological parameters. Overall survival was calculated from the date of surgery to the date of death or date of last follow-up. Progression-free survival was calculated from the date of surgery to the date of progression/recurrence or date of last follow-up. Survival analyses were performed according to the Kaplan–Meier method. Comparison of the survival between groups was performed with the log-rank test. Cox proportional-hazard analysis was used for univariate and multivariate analysis to explore the impact of variables on survival. The SAS software (SAS Institute Inc., Cary, NC) was used for all statistical

analyses, and a p-value of <0.05 was considered significant.

RESULTS

Immunohistochemical Expression of IDO in Endometrial Cancer Tissues

We examined the IDO protein expression in endometrial cancer by immunohistochemical staining, using 80 surgical specimens. As shown in Figure 24.2, the immunoreactivity of IDO was detected at variable levels, and was localized in the cytoplasm of tumor cells. In contrast, the immunoreactivity of

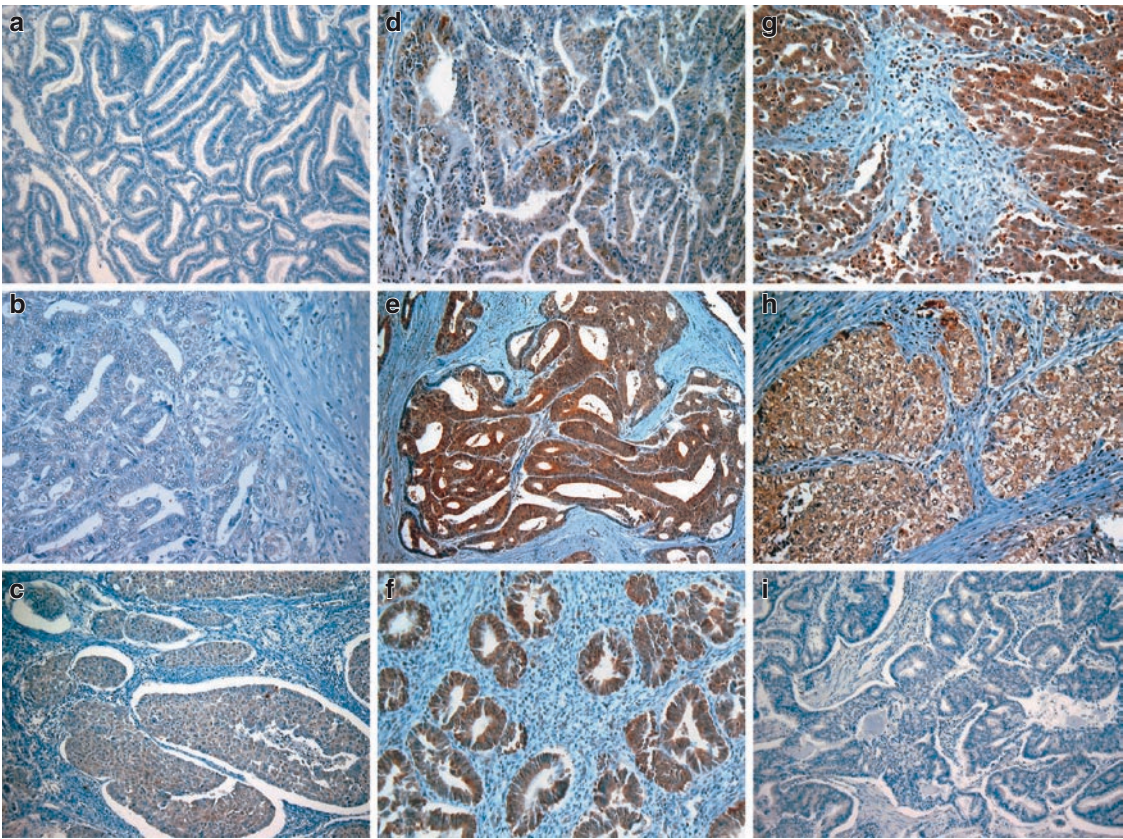


FIGURE 24.2. Representative immunohistochemical staining for IDO expression in endometrial cancer tissues. (a) IDO– (negative); (b) IDO1+ (sporadic/weak); (c) and (d) IDO2+ (focal/moderate); (e–h) IDO3+ (diffuse/strong); (i) negative control. Original magnification, $\times 100$

IDO was very faint or absent in the tumor stroma. Of the 80 specimens examined, the “high IDO expression” (IDO2+ or 3+) was found in 37 (46%) cases, of which 25 (31%) were IDO2+ and 12 (15%) were IDO3+. IDO– and IDO1+ tumors were found in 15 (19%) and 28 (35%) cases, respectively. Next, the correlation of the high IDO expression (IDO2+ and 3+) with currently-used clinicopathological parameters was analyzed in the 80 cases. The high IDO expression was positively correlated with the depth of myometrial invasion ($p = 0.001$), presence of lymph-vascular space involvement ($p = 0.001$) and lymph node metastasis ($p = 0.023$), but not with the histological grade ($p = 0.118$).

Association of IDO Expression with the Patient Survival

Follow-up data were available for all 80 patients. The median follow-up period was 71.6 months (range 5–148). During the follow-up period, disease progression/recurrence was observed in 14 cases (17.5%), in which 9 patients (11.3%) died. The median time to progression/recurrence and death were 10.5 and 17.8 months, respectively. To evaluate the impact of IDO expression on patient prognosis, overall survival and progression-free survival were analyzed using the Kaplan–Meier method. The 5-year overall survival rates of patients with IDO–/1+, IDO2+ and IDO3+ were 96.8%, 82.5% and 63.6%, respectively, while the 5-year progression-free survival rates for IDO–/1+, IDO2+ and IDO3+ were 97.7, 72.9 and 36.4%, respectively. Patients with high IDO expression (IDO2+ or 3+) had significantly impaired overall survival ($p = 0.002$) and progression-free survival ($p = 0.001$) as compared to patients with no or weak expression of IDO (IDO– or 1+). Next, we focused on

the patients with early-staged disease. In FIGO stage I/II patients ($n = 64$), the 5-year progression-free survival rates for IDO–/1+ and IDO2+/3+ were 100% and 75.0%, respectively, and there was a significant difference in the progression-free survival between the two groups ($p = 0.001$). Finally, we analyzed the correlation of IDO expression with the progression-free survival in the patients with FIGO stage Ic and greater ($n = 40$), because all these patients underwent postoperative adjuvant chemotherapy due to the risk of disease recurrence/progression. In these patients, the 5-year progression-free survival rates for IDO–/1+ and IDO2+/3+ were 92.9% and 58.8%, respectively, with a significant difference between the two groups ($p = 0.027$).

Multivariate Analysis of Prognostic Variables in Endometrial Cancer Patients

To compare the impact of IDO expression on survival with those of currently-used clinicopathological prognostic factors, Cox proportional-hazard analysis was performed. Among the six variables including IDO, age, FIGO stage, grade, myometrial invasion, and lymph-vascular space involvement, the FIGO stage was the only significant prognostic factor (hazard ratio = 5.59, $p = 0.021$) with respect to overall survival on multivariate analysis, although IDO expression, as well as stage, myometrial invasion and lymph-vascular space involvement, were significant prognostic factors on univariate analysis. In contrast, both IDO expression (hazard ratio = 12.04, $p = 0.020$) and FIGO stage (hazard ratio = 4.52, $p = 0.009$) were found to be independent prognostic factors with respect to progression-free survival on multivariate analysis.

DISCUSSION

Although many tumor-associated antigens have been identified in various tumor cells, the reason why tumor antigen-specific host T cells fail to control tumor progression remains obscure. Tumors are known to be able to successfully escape the host immune system by two possible mechanisms, proposed by Whiteside (2006): (1) a loss of surface antigens, rendering them invisible to immune cells, or (2) an attack on the immune cells, disabling their anti-tumor functions. Munn and Mellor (2007) suggested that one mechanism by which tumors create a state of tolerance may be tryptophan catabolism carried out by IDO. In the present study, we demonstrated the expression of IDO in endometrial cancer using 80 surgical specimens, and found that the IDO overexpression by tumor cells was positively correlated with disease progression and the impaired patient survival.

Our data showed that IDO was highly expressed in 46% cases, while IDO-negative tumors were found in only 19% cases. The localization of IDO was dominant in tumor cells, and its expression in the tumor stroma was not prominent or was absent. Consistently, Uyttenhove et al. (2003) reported that IDO was expressed in the tumor cell itself, in a variety of human tumor types. In contrast, several studies showed that IDO was also present in some macrophages (Friberg et al. 2002) and eosinophil granulocytes (Astigiano et al. 2005) in the tumor stroma, or in dendritic cells in tumor-draining lymph nodes (Munn et al. 2004). In gynecological cancers, two previous reports by Sedlmayr et al. (2003) and Uyttenhove et al. (2003) showed that IDO was expressed in tumor cells themselves,

although a very limited number of samples were analyzed in these studies. Recently, Okamoto et al. (2005) reported that IDO was expressed in 17 of 24 cases with advanced ovarian serous carcinoma. Schroecksnadel et al. (2005) also showed that the serum tryptophan concentration was lower in 20 patients with gynecological cancer. Taken together with our results, IDO is frequently expressed in gynecological cancers.

The present data clearly demonstrate that high IDO expression is positively correlated with the deep myometrial invasion, the presence of lymph-vascular space involvement, and lymph node metastasis. Furthermore, the patients with high IDO expression had an impaired clinical outcome by analyzing the rates of both overall survival and progression-free survival. Consistently, three recent reports showed the correlation between the high IDO expression and poor patient prognosis in ovarian cancer (Okamoto et al. 2005), lung cancer (Astigiano et al. 2005), and colorectal cancer (Brandacher et al. 2006), although they analyzed only the overall survival. More importantly, when focusing on the cases with early-staged disease (FIGO I/II), we showed that the patients with no or weak expression of IDO achieved 100% progression-free survival, whereas 5 of 24 patients with the high IDO expression developed recurrence (progression-free survival = 75%). These findings suggest that the patients with high IDO expression are very likely to recur and have poor prognosis, even in cases with early-staged disease at the time of surgery. Our multivariate analysis demonstrated that IDO expression is an independent prognostic factor for progression-free survival, suggesting that IDO may be a reliable prognostic parameter of endometrial cancer. Because >70% of endometrial cancer patients present

early-staged disease, and most of them are curable with surgery alone, it would be of substantial benefit to define the minority of patients who are likely to recur, and also to give aggressive adjuvant therapy to these patients alone. There have been several molecular markers identified showing the prognostic impact in endometrial cancer (Mell et al. 2004; Stefansson et al. 2004); however, their clinical application, instead of the currently-used prognostic factors, has not yet been realized. Our data suggests that IDO may become a useful indicator for the prognosis of endometrial cancer and may contribute to the individualization of the application of adjuvant therapy, not only in advanced staged, but also in early staged patients.

Finally, the mechanism by which IDO contributes to the tumor progression of endometrial cancer is discussed. Two

possible mechanisms for the immunosuppressive action of IDO in tumor-bearing hosts are proposed (Figure 24.3). Namely, IDO expressed by the tumor cells themselves can create a localized immunosuppressive status within the tumor microenvironment (“effector phase”) either by suppressing the proliferation and function of effector T-cells and NK cells due to tryptophan depletion, or by directly killing tumor-infiltrating T-cells and NK cells using toxic catabolites of tryptophan. Alternatively, host dendritic cells expressing IDO can pick up tumor-derived antigens and migrate into tumor-draining lymph nodes (“priming phase”), where these IDO-expressing dendritic cells cannot effectively prime naïve T-cells, resulting in T-cell deletion, failure of clonal expansion, or perhaps induction of regulatory T-cells. The correlation of the high IDO expression in tumor cells

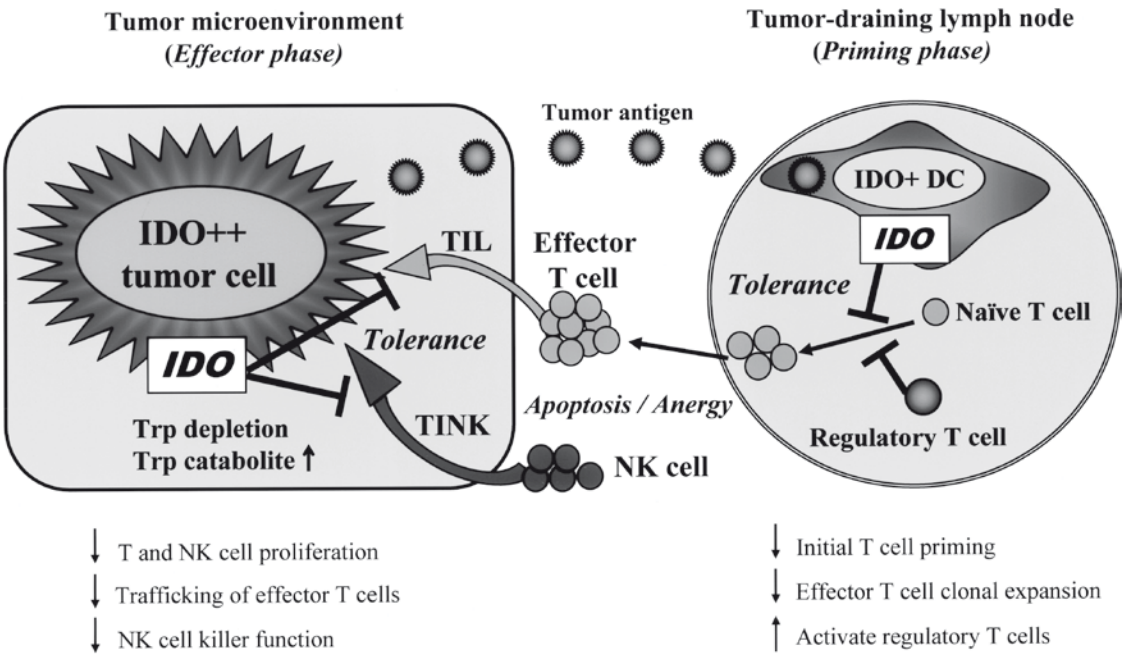


FIGURE 24.3 Schematic representation of the possible mechanism of immune tolerance induced by both IDO-expressing tumor cells within cancer tissues (“effector phase”) and IDO-expressing dendritic cells in tumor-draining lymph nodes (“priming phase”). DC, Dendritic cell; TIL, Tumor-infiltrating lymphocyte; TINK, Tumor-infiltrating natural killer cell; Trp, Tryptophan

with disease progression and the impaired patient survival shown by the present study may be attributable to the local tolerance by the tumor-mediated IDO activity within tumors. In fact, our preliminary studies showed that the high IDO expression is significantly correlated with the low number of tumor-infiltrating CD8+ T cells and CD57+ NK cells (unpublished data by Ino et al.). This may be supported by one recent report showing the association of IDO expression with a reduction of CD3+ lymphocytes in colorectal cancer (Brandacher et al. 2006).

Another possible mechanism by which IDO contributes to cancer progression was proposed by Muller et al. (2005), who demonstrated the involvement of IDO in a chemoresistance in cancer. Consistently, Okamoto et al. (2005) showed that IDO was overexpressed in paclitaxel-resistant ovarian cancer cell lines and tissues using a gene expression profiling study. Our results demonstrated that there was a marked difference in progression-free survival between the no or low IDO expression group and the high IDO expression group when focusing on the 40 patients with FIGO stage Ic and more who underwent postoperative chemotherapy. This might be due, at least in part, to the resistance of IDO-overexpressing endometrial cancer cells to chemotherapy. Further studies are needed to identify the functional roles of IDO in human cancer cells besides its enzymatic or immunosuppressive actions.

In conclusion, we demonstrate here that high IDO expression correlates with the disease progression and impaired clinical outcome in endometrial cancer patients. Furthermore, IDO is an independent prognostic factor for progression-free survival. These results indicate that IDO is a reliable and

promising prognostic indicator and may become a novel molecular target in the therapeutic strategy for endometrial cancer.

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Part VI

Skin Cancer

Melanoma

Neurofibromatosis Type 1-Associated Malignant Melanoma: Molecular Evidence of Inactivation of the *NF1* Gene

Albert Rübben

INTRODUCTION

Neurofibromatosis type 1 (NF1; MIM# 162200) is an autosomal dominant neurocutaneous disease that is aggravated by an enhanced incidence of malignant tumors deriving from the neural crest (Riccardi 1992). The birth incidence of NF1 is $\approx 1/3000$ to $1/3500$. Patients with NF1 display multiple café-au-lait macules (CALMs) at birth or during early childhood, and develop neurofibromas as adults. NF1-associated malignant tumors from neural crest origin comprise malignant schwannoma (neurofibrosarcoma), medulloblastoma, astrocytoma, and pheochromocytoma.

The disease is caused by germline mutations that inactivate one neurofibromin gene on the long arm of chromosome 17 (17q11.2). The protein neurofibromin encoded by the *NF1* gene is a RAS-specific GTPase-activating protein that functions as a negative regulator of the RAS pathway (Xu et al. 1990). It can be considered a tumor suppressor gene as inactivation of both copies of the *NF1* gene can be found

in NF1-associated malignant schwannoma and pheochromocytoma (Legius et al. 1993; Xu et al. 1992). Inactivation of both *NF1* alleles as well as loss of heterozygosity (LOH) of microsatellite DNA within the *NF1* gene could also be demonstrated in benign NF1-associated neurofibromas (Rutkowski et al. 2000; Sawada et al. 1996; Serra et al. 1997).

Melanocytes originate from the neural crest. Melanocyte abnormalities, as demonstrated by café-au-lait macules, are among the first signs of neurofibromatosis type 1. Surprisingly, melanoma incidence does not seem to be markedly elevated in NF1 (Guillot et al. 2004). Therefore, the role of *NF1* mutations in NF1-associated melanoma remains unclear.

Before discussing evidence that suggests a causative role of *NF1* mutations in NF1-associated melanoma, I would first like to focus on the definition of cancer genes and on different approaches for identification of cancer genes, as methodological considerations are important for the evaluation of the available data.

METHODOLOGY

Definition of Cancer Genes

Cancer genes are mutated genes that are causally implicated in oncogenesis (Futreal et al. 2004). In consequence, every search for cancer genes comprises the identification of mutated genes in human cancers and, in addition, the proof that the detected mutated genes are causally implicated in oncogenesis. As most malignant human tumors display genomic instability, a significant amount of the mutations within an individual malignant tumor will represent random mutations that do not play a role in the genesis or progression of the malignancy.

It can be expected that genes which are frequently mutated in a specific cancer entity do play a causal role in oncogenesis of this cancer but the reverse assumption might not be true in all cases. It must be considered that most cellular functions are not performed by a single molecule but by an array of different molecules which may constitute a complex functional unit or which may form sequential molecular pathways. In addition, cellular functions may not be sustained by a single molecular pathway but may be ensured by multiple redundant pathways. Respectively, mutations of different genes may have the same net effects on a cancer phenotype, and a low mutation frequency of a gene in a specific tumor entity does not per se rule out a causative role of this mutation in the carcinogenic process. With regards to the definition of cancer genes, it is, therefore, necessary to consider the individual malignant tumor. A more refined definition could then state that cancer genes are genes whose altered wild type expression, expression as a mutated molecule or fully

or partially reduced expression are requirements for the development and/or maintenance of an individual malignant tumor.

Cancer genes are further subdivided into oncogenes, tumor suppressor genes, gatekeeper genes, and caretaker genes (Kinzler and Vogelstein 1997; Vogelstein and Kinzler 2004). Oncogenes exhibit activating mutations during oncogenesis while tumor suppressor genes must be fully inactivated by a two hit process (Knudson 1971). Gatekeeper genes are genes that control cell proliferation and cell death and are often tissue specific. Caretaker genes ensure genomic stability. It has been pointed out by Pérez de Castro et al. (2007) that some caretaker genes do not fit into the definition of oncogenes and tumor suppressor genes as gene dosage effects may suffice for inducing genomic instability.

Identification of Genes Implicated in Oncogenesis

Starting from the aforementioned tentative definition of cancer genes, proof that a mutated gene is causally implicated in oncogenesis of an individual malignant tumor could best be achieved by deleting, silencing, activating, or reintroducing the putative gene or gene product in vivo and by then evaluating the clinical response. This approach might only function for mutated genes which actively maintain the malignant phenotype, such as oncogenes and gatekeeper genes, but not necessarily for genes whose inactivation has contributed to the development of the malignant phenotype, such as genes implicated in genomic stability. Still, when a therapeutic agent has been developed to target a cancer gene or the respective gene product, its clinical efficacy will supply ultimate proof of the role of the gene in

carcinogenesis. The clinical success of the tyrosinase inhibitor imatinib in the treatment of gastrointestinal stroma tumors and dermatofibrosarcoma protuberans may be regarded as an example of this ultimate proof (Rubin et al. 2002). A less stringent but more feasible approach to identify cancer genes consists in deleting, silencing, activating, or reintroducing putative cancer genes or their products in human cancer cell lines. Nevertheless, it has to be considered that cell lines may not match the *in vivo* cancer with regards to accumulated mutations and growth requirements.

Determination of mutation frequencies of putative cancer genes in a specific cancer entity represents a valuable approach to identify frequently mutated genes that are likely to play a causal role in oncogenesis of the analyzed tumor entity, but this approach may not be able to elucidate the role of rare mutations as explained before. Cancer incidence data of patients with germline mutations of putative cancer genes may also help to identify cancer genes of a specific cancer entity. Nevertheless, due to the redundancy of cellular pathways, the lack of statistical significance may not rule out that a cancer gene exerts a significant role in an individual human cancer of a given cancer entity. cDNA microarrays represent a modern technique to evaluate the impact of putative cancer genes for a specific cancer entity. The parallel expression analysis of thousands of genes in a sufficiently large number of cancer samples enables the identification of cancer-specific gene expression patterns. It can be expected that mutated genes specifically overexpressed in a cancer entity or within a subset of the cancer entity as identified by gene expression patterns, represent causally implicated oncogenes. Additional experiments might still be necessary

to elucidate the biological role of the identified genes. Moreover, the cDNA microarray analysis might not be able to confirm the causal role in oncogenesis of an inactivated tumor suppressor gene.

Complete inactivation of a putative tumor suppressor gene by deletion or silencing in a specific cancer entity or within an individual malignant tumor can be considered indicative of a causal role of the gene in oncogenesis as it represents the classical two-hit inactivation mechanism postulated by Knudson (1971). One of the oldest and most commonly used techniques to identify tumor suppressor genes is the search for loss of heterozygosity (LOH) within the genes or in the vicinity of putative cancer genes. Loss of heterozygosity is often demonstrated by analysis of heterozygous microsatellite DNA, which can easily be amplified by PCR and, therefore, allows the analysis of formalin-fixed archival tumor tissue. Nevertheless, detection of LOH alone is not sufficient for demonstration of a two-hit mechanism. In addition, inactivation of both copies of the tumor suppressor gene by homozygous loss or by an inactivating mutation of the remaining copy must be shown. In the case of malignant tumors arising in patients with germline mutations, it is sufficient to demonstrate loss of heterozygosity with deletion of the wild-type copy. Still, even complete loss of a tumor suppressor gene in an individual malignant tumor might not represent definite proof of a causal role of this mutation in oncogenesis as the mutation might still represent a byproduct of genomic instability that is not implicated in carcinogenesis. As a general rule, one may assume that inactivation of tumor suppressor function in an early primary tumor is a stronger indication of a causative role than mutation

detection in more advanced cancers or in their metastases.

A significant role of a mutated gene in carcinogenesis may also be deduced by its biological function. Obviously, genes implicated in growth, cell cycle, and apoptosis control, as well as genes ensuring the integrity of the human genome are likely to play a significant role in carcinogenesis. Still, due to the redundancy of cellular functions, it has to be demonstrated that a putative cancer gene plays a central role in the development and/or maintenance of a specific cancer entity or an individual malignant tumor. Indications for a causative role might be the activity of the gene in the normal tissue which gives rise to the malignant tumor, the activity of the gene during cell renewal of this tissue and the activity of the gene during embryogenesis or during fetal development of the tissue giving rise to the malignant tumor.

ROLE OF *NF1* GENE MUTATIONS IN NF1- ASSOCIATED MELANOMA

Melanoma Incidence in NF1

In Europe, melanoma incidence is $\approx 10/100,000/\text{year}$ (Garbe and Blum 2001), while the birth incidence of NF1 is $\approx 1/3000$ to $1/3500$. Less than 50 cases of NF1-associated cutaneous melanoma have been reported up to now, and the few published studies on the association between malignant melanoma and NF1 disclose variable data. The frequency of malignant melanoma in NF1 patients has been estimated between 0.1% and 5.4%, and 0.11% has been given as frequency of NF1 in melanoma patients (de Schepper et al. 2005; Guillot et al. 2004). Taken together,

epidemiological data do not demonstrate a grossly enhanced melanoma risk in NF1 patients, which argues against a causative role of *NF1* mutations in NF1-associated melanoma.

On the other hand, clinical peculiarities of patients with both diseases might hint towards a non-fortuitous association:

Guillot et al. (2004) reported that melanomas tend to develop at younger age in NF1 patients with a median age of 33 years compared to 46–53 years in larger melanoma series.

Nine of the 40 reported NF1-associated melanomas arose within a giant congenital melanocytic nevus (GCN) (Guillot et al. 2004; Doherty et al. 2006). As the birth incidence of GCN is estimated, between 1:20.000 and 1:500.000, melanomas on GCN seem overrepresented in NF1 patients.

Unusual cutaneous and extracutaneous melanomas, such as three cases of anal melanoma (Ishii et al. 2001) and 18 cases of uveal melanoma (Honavar et al. 2000) have also been described in association with NF1.

Biologic Role of Neurofibromin in Melanocytes

Patients with NF1 display multiple café-au-lait macules (CALMs) at birth but the number of these hyper pigmented macules does not seem to increase after the first decade. This indicates that haploinsufficiency of neurofibromin has a strong effect on melanocyte biology during embryogenesis or fetal development but does not seem to play an important role in melanocyte cell renewal later in life. The round shape of CALMs is compatible with a monoclonal melanocyte expansion within

the skin, and analysis of X-inactivation patterns suggests a monoclonal origin as demonstrated by Eisenbarth et al. (1997). On the other hand, melanocyte density seems only moderately and inconsistently elevated in NF1-associated CALMs (de Schepper et al. 2006a). Moreover, malignant melanomas in NF1 patients do not demonstrate a preferential association with CALMs (Guillot et al. 2004), which could be expected if melanocytes of CALMs would have undergone significantly more cell divisions than melanocytes of unaffected skin. In addition, cell culture studies performed by Eisenbarth et al. (1997) did not detect *NF1* allele loss in melanocytes of CALMs. Melanocytes and Schwann cells, thus, behave discordantly in NF1 patients as Schwann cells appear normal at birth but give rise to benign neurofibromas in adulthood through complete loss of neurofibromin function (Rutkowski et al. 2000), which later in life may progress to malignant schwannomas. Together, the molecular and clinical data suggest that neurofibromin exerts a different biologic function in melanocytes as compared to Schwann cells where it acts as a tumor suppressor and negatively regulates the RAS pathway (Xu et al. 1990). In concordance with this assumption, cell studies with melanocytes of NF1 patients did not detect enhanced RAS-GTP-levels but could demonstrate increased melanogenesis (Kaufmann et al. 1991). Boucneau et al. (2005) performed cDNA microarray analysis of cultured *NF1* heterozygous melanocytes from a NF1 patient and could demonstrate differential regulation of the melanocyte specific dopachrome tautomerase gene when compared to control melanocytes. Furthermore, de Schepper et al. (2006b) reported that neurofibromin

interacts with the amyloid precursor protein and colocalizes with melanosomes in normal melanocytes.

It may be assumed that formation of CALMs is caused primarily by the effect of neurofibromin haploinsufficiency on melanocyte differentiation and pigmentation and less by a RAS-mediated effect on cell proliferation. In mice, melanocytes precursors migrate during embryogenesis from the neural crest dorsolaterally and enter the skin where they proliferate clonally and finally differentiate into mature skin melanocytes (Yoshida et al. 1996; Wilkie et al. 2002). Modulation of pigmentation by neurofibromin haploinsufficiency during regular melanoblast expansion in the skin might be sufficient to form monoclonal and round shaped CALMs. In conclusion, the available data suggest that neurofibromin does not seem to strongly control proliferation of embryonic melanocyte precursors or mature skin melanocytes and might not act as a negative regulator of the RAS-pathway in melanocytes.

Mutations of the *NF1* Gene in NF1-Associated Malignant Melanoma

The *NF1* gene is rarely mutated in typical cutaneous malignant melanoma. Gómez et al. (1996) did not detect LOH at the *NF1* gene in 68 analyzed cutaneous melanomas and Gutzmer et al. (2000) found LOH only in one out of 20 malignant melanomas. On the other hand, four cases of melanoma cell lines with reduced neurofibromin levels and one melanoma cell line with complete loss of the *NF1* gene have been described (Johnson et al. 1993; Andersen et al. 1993). Johnson et al. (1993) could demonstrate that reduced neurofibromin levels did not affect RAS-regulation in the cell line, which further suggests that

neurofibromin does not act as a negative regulator of the RAS-pathway in melanocytes and in most melanomas. Interestingly, *NF1* was reintroduced in one of these cell lines (SK-MEL-28) and inhibited its growth, which must be regarded as strong indication for a tumor suppressor function of *NF1* in malignant melanoma that is independent of its GTPase activity (Johnson et al. 1994).

If one still assumes that neurofibromin exerts GTPase activity in some melanoma precursors, then the low *NF1* mutation frequency in typical cutaneous malignant melanoma might be explained by the high frequency of activating *BRAF* and *NRAS* mutations, which could be detected in more than 80% of all cutaneous melanomas (Davies et al. 2002). In the presence of activating *BRAF* and *NRAS* mutations, loss of NF1 GTPase activity might not be necessary for growth induction and might, therefore, not be positively selected during melanoma oncogenesis.

In contrast to the low mutation frequency of *NF1* in typical malignant melanoma, LOH at the *NF1* gene has been demonstrated in ten out of 15 analyzed cases of desmoplastic neurotropic melanoma (Gutzmer et al. 2000) which represents a rare cutaneous melanoma variant that displays neural features and markers (Winnepeninckx et al. 2003). It may be speculated that putative precursors of desmoplastic melanoma might display neurofibromin control of cell proliferation by GTPase activity due to a more neurogenic differentiation status. Uveal melanoma has also been analyzed for NF1 expression and it could be shown that 18 out of 38 uveal melanomas displayed downregulation of NF1 and that one uveal melanoma contained a deletion of the *NF1* locus (Foster et al. 2003).

Inactivation of the *NF1* Gene in

NF1-Associated Malignant Melanoma

Up to now, only two cases of NF1-associated malignant melanoma, one anal melanoma and one childhood cutaneous melanoma, have been analyzed for loss of the *NF1* gene, and both analyzed melanomas displayed LOH at the *NF1* gene. In the anal melanoma, LOH was detected at the intragenic microsatellite marker IVS38GT53.0 (Ishii et al. 2001). The patient had no relatives with NF1, which would have allowed linking the alleles of the IVS38GT53.0 microsatellite marker with the *NF1* germline mutation. Therefore, it was not possible to determine whether somatic allele loss resulted in complete *NF1* inactivation. The second case was an early stage cutaneous malignant melanoma on the leg of a 15-year-old boy (Rübben et al. 2006). LOH was detected at the intragenic microsatellite marker IVS27AC28 in microdissected formalin-fixed melanoma tissue. The germline *NF1* mutation c. 5546 G/A was identified in the patient and in the father of the patient, who also carried the disease. As microsatellite alleles could be determined in the patient, in the tumor tissue and in the patient's parents, it was possible to demonstrate that the somatic mutation in the melanoma targeted the wild-type maternal *NF1* gene. The inactivation pattern of the NF1 gene in the analyzed melanoma, thus, represented the classical two-hit inactivation of a tumor suppressor gene as postulated by Knudson (1971). It has been reported that LOH analysis of formalin-fixed tissue might lead to false positive LOH results due to the low quantity of amplifiable target DNA (Liu et al. 1999; Farrand et al. 2002). In order to avoid this bias, LOH analysis of the microdissected melanoma tissue was performed

in this study using digital PCR for quantification of microsatellite allele ratios, which allows correct statistical interpretation of the results (Vogelstein and Kinzler 1999; Zhou et al. 2001; Traverso et al. 2002; Pohl and Shih 2004).

CONCLUSION

The epidemiologic, biologic and molecular data procure conflicting evidences with regards to a causal role of *NF1* mutations in the oncogenesis of NF1-associated malignant melanoma. Melanoma incidence is not grossly elevated in NF1, and LOH at the *NF1* gene is a rare mutation in sporadic cutaneous malignant melanoma. Neurofibromin haploinsufficiency results in melanocyte abnormalities, which most probably develop during embryogenesis and fetal development and become clinically apparent as café-au-lait macules, but it seems that neurofibromin does not exert an anti-proliferative effect on melanocytes and melanocyte precursors. Furthermore, loss of GTP-ase mediated down regulation of the RAS-RAF-MEK-ERK-pathway could neither be demonstrated in melanocytes from NF1 patients nor in melanoma cell lines with downregulated neurofibromin levels. These findings argue against a role of *NF1* as a tumor suppressor in malignant melanoma.

On the other hand, NF1-associated melanomas seem to display unusual clinical features, such as younger patient age, atypical anal or uveal localization, and frequent association with giant congenital nevi. In addition, LOH has been frequently detected in desmoplastic melanoma that represents a rare variant displaying neural features, and reduction of neurofibromin expression could be demonstrated in uveal

melanomas. The strongest evidences of a causative role of *NF1* mutations in melanoma oncogenesis come from a study of melanoma cell lines (Johnson et al. 1994) and from a molecular analysis of an NF1-associated childhood melanoma. It could be demonstrated that reintroducing strong NF1 expression in a melanoma cell line expressing low neurofibromin levels resulted in growth inhibition, which indicates that neurofibromin deficiency contributed to the maintenance of the malignant phenotype. In the analyzed NF1-associated childhood melanoma, complete somatic deletion of the *NF1* gene could be detected, which strongly suggests that the *NF1* gene acted as a tumor suppressor in this melanoma (Rübben et al. 2006).

The conflicting results may be reconciled if one assumes that *NF1* inactivation represents an alternative pathway towards malignant melanoma only in a minority of all melanoma precursor lesions in the general population as well as in NF1 patients due to limitations imposed by melanocyte biology. Congenital nevi as well as precursors of desmoplastic, anal and uveal melanomas might be more susceptible for this oncogenic pathway.

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Malignant Melanoma: Localisation and Characterization Using Fluorodeoxyglucose-Positron Emission Tomography/Computed Tomography

Olivier Gheysens and Felix M. Mottaghy

INTRODUCTION AND CLINICAL BACKGROUND

Malignant melanoma (MM) is the most aggressive neoplasm among skin cancers and its incidence is increasing dramatically worldwide. The rise in incidence can be partially related to better screening programs and earlier detections of thin lesions. Although the disease accounts only for ~5% of total skin cancers, it is responsible for the majority of skin cancer deaths. Therefore, early detection and accurate staging of MM is crucial to optimize treatment planning and may potentially lead to longer survival. Several key prognostic factors have been identified along the history of MM such as lesion thickness, ulceration (both markers for vertical growth), Clark level, regional lymph node status, and age. The most important prognostic factors are the tumor thickness and the sentinel lymph node status (the first node draining the tumor), the latter especially in early-stage cancers. Regional lymph node invasion can lead to dissemination into any tissue or organ as seen with other cancers. There

are essentially three different pathways of dissemination: locally via satellite or in transit metastasis, involvement of regional lymph nodes and extension to visceral and nonvisceral organs. These patterns and the American Joint Committee on Cancer (AJCC) clinical stage determines the imaging work-up protocols for patients.

This chapter will focus on the role of nuclear medicine, namely positron emission tomography (PET) and integrated PET/CT (computed tomography), in the management of MM. Conventional single photon emission computed tomography (SPECT) or planar scintigraphy examinations will not be discussed. However, it is noteworthy to mention that bone scintigraphy for detecting osteoblastic metastases and sentinel lymph node (SLN) scintigraphy are routinely used in the work-up. The latter is based on the lymphatic drainage system of tumors, and the sentinel node is the first hypothetical node or group of lymph nodes reached by metastasing tumors cells. The SLN procedure has gained a high impact in early stage (AJCC stage I and II) melanoma.

POTENTIAL INDICATIONS
OF FLUORODEOXYGLUCOSE
POSITRON EMISSION
TOMOGRAPHY IMAGING
IN THE MANAGEMENT OF
MALIGNANT MELANOMA

Positron emission tomography and more recently integrated PET/CT are radionuclide-based imaging technologies that are routinely used for diagnosing, staging and evaluating treatment response of different cancers. PET is a technique that generates a three-dimensional image of functional processes in the body. In order to conduct a PET scan, a radioactive tracer or radioisotope needs to be systemically injected. The positron-emitting moiety of the tracer decays by emitting a positron from its nucleus and annihilates with an electron of a neighbouring atom; thereby, producing two 511 keV photons. The latter travels in exactly opposite directions and is detected by the PET system in a coincident fashion.

The radioisotopes used with PET are positron emitters and the most commonly used and available positron emitter is 18-F followed by 11-C, 13-N, 15-O and 124-I in

no particular order. The generation of these isotopes takes place in a cyclotron which accelerates charged particles for collision with a target to generate isotopes having a relatively short half-life (e.g., 18-F has a half-life of 110 min). Fluorodeoxyglucose (FDG) is the most widely used tracer in oncologic PET imaging and provides information on cell metabolism. FDG is a glucose analogue which can be transported by means of the glucose transporter and phosphorylated by the hexokinase enzyme to FDG-6-phosphate. The latter is no substrate for the enzymes involved in glycolysis and therefore gets trapped inside the cell (Figure 26.1). The rationale of using a radiolabeled deoxyglucose analogue for oncologic diseases is based on the increased glucose metabolism of cancer cells (upregulation of glucose transporters and/or hexokinase activity) compared to noncancer cells. Thus, the significant increased glucose metabolism of cancer cells allows to easily distinguish those from benign cells.

Indications for FDG-PET imaging in the management of MM mainly include detection of loco-regional and distant metastases and evaluation of treatment response.

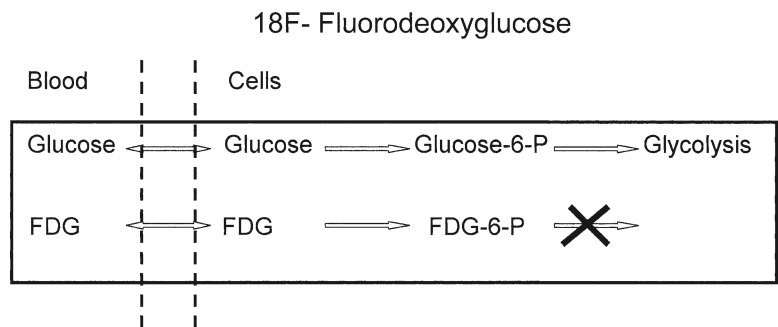


FIGURE 26.1. Schema of FDG uptake mechanism. Radiolabeled deoxyglucose is taken up by the cells and gets phosphorylated by the hexokinase enzyme just like glucose. However, the phosphorylated FDG-6P is no substrate for the glycolysis pathway and therefore gets trapped inside the cell in contrast to glucose-6P which gets further metabolized

Initial staging of MM is defined by the following factors: lesion thickness, the presence of lymph node and/or distant metastases. FDG-PET has limited or no value in the staging of the primary tumor which is based on the depth of invasion. Suspicious pigmented lesions are easily detected during a routine clinical examination, and diagnosis of MM is done by biopsy or surgical resection. On top of the clinical suspiciousness, the highest stage tumors (T4) having a depth invasion of >4 mm are beyond the spatial resolution of currently commercially available PET systems, and furthermore FDG-PET is usually performed after diagnosis has been confirmed.

DETECTION OF LOCOREGIONAL LYMPH NODE INVASION

As mentioned earlier, metastatic lymph node involvement is a very important prognostic factor. The imaging modality of choice for detecting lymph node metastases is highly dependent on the pretest likelihood which is defined by lesion thickness. Lesions thicker than 1 mm have a higher probability of malignant invasion. Sentinel lymph node (SLN) procedure has been a well validated and established technique to detect lymph node metastases (Morton et al. 1999; Topping et al. 2004), especially in early stage MM. However, this procedure does not provide any information on distant lesions. Whole-body FDG-PET may therefore be superior in both detecting locoregional and distant metastases.

One of the earliest and very important studies to evaluate the use of FDG-PET in detecting lymph node metastases was

performed by Crippa et al. (2000). This study reported a sensitivity and specificity of 95% and 84%, respectively. However, all the patients included in the study had known lymph node involvement based on clinical examination or other imaging modalities, and therefore, very high pretest likelihood. Subgroup analysis for node size showed a sensitivity of 100% for nodes bigger than 10 mm and only 23% for nodes smaller than 5 mm. These results point out the low sensitivity for smaller lesions on FDG-PET because of the intrinsic physical limitations of PET systems. The latter was also confirmed by Acland et al. (2001) who studied 50 consecutive patients with MM. The group reported a sensitivity of 0%, hereby providing evidence that PET alone is not sufficient to detect small or micrometastatic lesions and cannot replace the SLN procedure in early stage MM. However, the pre-test likelihood in this cohort was very low and might underestimate the accuracy and performance of PET.

Recent studies report a much lower sensitivity than initial studies and several hypotheses have been proposed. First, the big variation in results may be caused by the introduction of the SLN procedure in which the first hypothetical lymph node for metastasing tumor cells is screened for micrometastasis, and the diagnostic performance of FDG-PET to detect lymph node metastases is always compared to the gold standard which is histology. Another explanation might be the experience people have acquired over time to perform these examinations. The latter could be ruled out by two studies done by Wagner et al. (1997, 1999). Wagner et al. (1997) studied 11 patients with both clinical and no clinical evidence for lymph node

involvement and who underwent surgical lymphadenectomy. They reported a sensitivity and specificity of 100% for both.

The second study by Wagner et al. (1999) included 70 patients with stage AJCC I-III and showed a sensitivity of only 17% with PET compared to SLN biopsy. Because the same group and centre performed both studies, methodological differences or multi-centre variability could be ruled out. The largest study to date performed by Wagner et al. (2005) also reported a sensitivity of 21% for AJCC stage I-II. As shown in previous studies, PET was found to have a very low sensitivity in early stage MM or patients who had no evidence of locoregional lymph node involvement. The conclusion from these studies is that SLN procedure has a better sensitivity and remains the first choice among patients with stage I and II MM for locoregional lymph node staging. FDG-PET can be considered in cases with high pretest likelihood for lymph node metastases.

DETECTION OF DISTANT METASTASES

Malignant melanoma has a tendency to spread very unpredictably throughout the body and mean survival upon detection of distant metastases is approximately 6 months. It is, therefore, very important to have accurate imaging modalities to identify metastases as early as possible. Conventional imaging technologies such as ultrasound, computed tomography (CT) and magnetic resonance imaging (MRI) have limited sensitivity and specificity for single lesions. Physical examination and tumor markers (e.g., S100) provide useful information to guide further diagnostic evaluation.

FDG-PET is the most widely used and newest imaging modality to screen for distant metastases in patients with MM. PET has been proven to perform superior in detecting metastases compared to conventional imaging techniques (Figure 26.2). The sensitivity and specificity for detecting distant metastases with PET is ~90%. PET is very sensitive for detecting soft-tissue and lymph node invasion which could not be done with clinical examination and conventional methods. There are a lot of studies comparing the performance of CT and FDG-PET for diagnosing metastases. One of the first studies showing the added value of PET for distant metastases was reported by Gritters et al. (1993). They studied 12 patients with different stages of MM, and PET identified all the visceral and lymph node metastases. Several lesions were not detected on initial CT read-outs, but retrospective review showed several of the lesions seen on PET were clearly visible on follow-up CT scans. More important, PET imaging has been demonstrated to detect metastases up to 6 months earlier than CT which has implications for patient management.

PITFALLS AND ADDITIONAL VALUE OF INTEGRATED PET/CT IMAGING

Positron emission tomography outperforms CT especially for detecting abdominal and lymph node metastases. Most of the lesions missed by PET are either lesions <10 mm or lesions in the brain. The former is especially true for small pulmonary lesions, and the limited detection is due to the intrinsic physical limitations in terms of spatial resolution of commercial

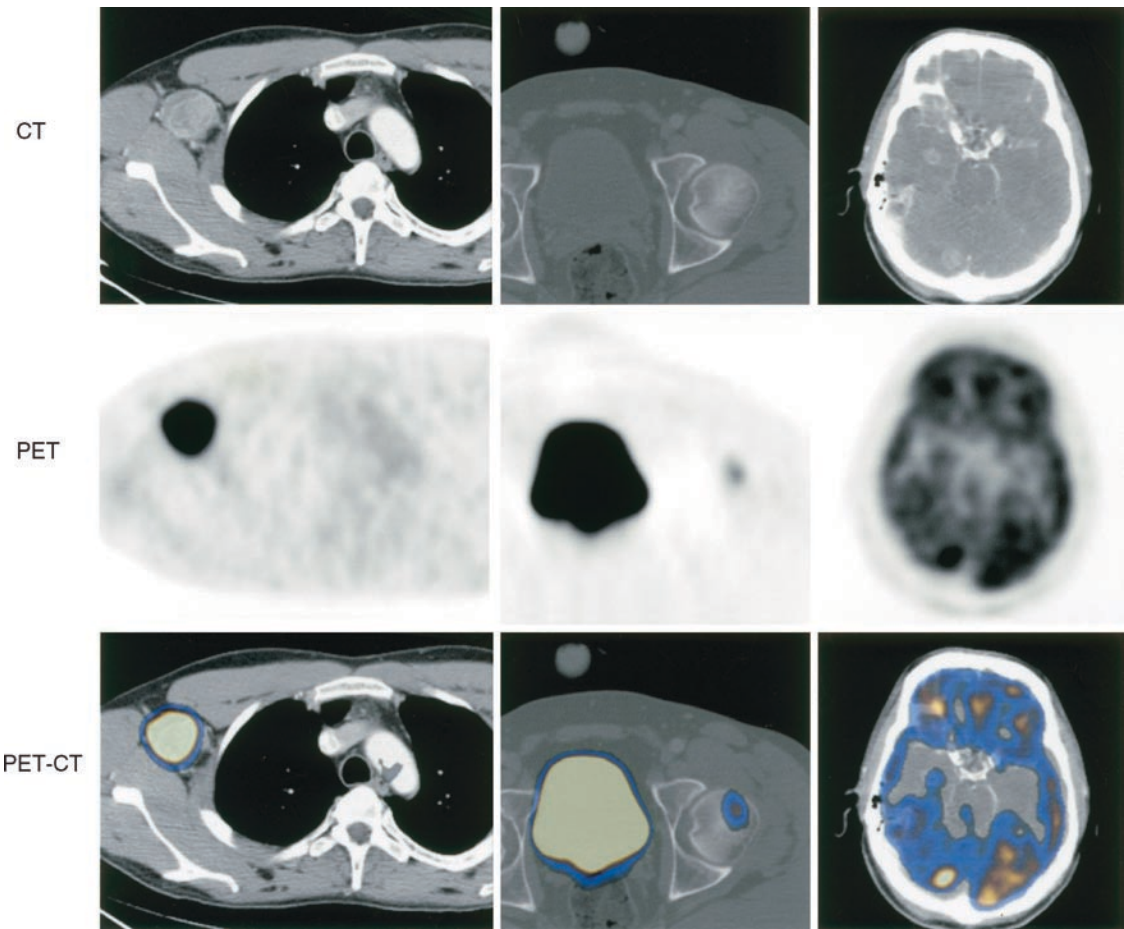


FIGURE 26.2. Staging of MM with FDG-PET. A 29-year-old male with right shoulder melanoma on biopsy and pathological lymph node in the right axilla (initial stage IIIB) was referred for a PET/CT scan. FDG-PET revealed a very intense FDG uptake in the right axillary lymph node and showed two additional areas of high FDG uptake in the left pelvic region and the brain. On the CT and fused PET/CT images the lesion in the pelvic region could be localized in the left femoral head whereas the brain uptake corresponded with a brain metastasis. Patient was classified as having AJCC stage IV disease based on the PET/CT findings

PET systems. CT is the diagnostic tool of choice for the detection of small pulmonary metastases. Due to the already physiologically high FDG uptake in the brain, small metastases are often missed (see also Figure 26.2, where some of the metastases can already be seen with PET, whereas other are not depicted). These lesions can be better visualized with CT due to the higher resolution and contrast enhancement,

but MRI is the diagnostic tool of choice for brain metastases. Thus, whole body FGD-PET should be seen as complementary to conventional imaging techniques in the management of patients with MM.

The recent development of hybrid PET/CT systems combining the functional information with anatomical details has dramatically altered the interpretation of PET. The additional anatomical information

increases the accuracy to distinguish abnormal uptake patterns versus normal physiologic variability. Therefore, both the false-positive and false-negative findings are dramatically reduced. The superiority of PET/CT compared to PET has already been proven for different cancers and is also valid for MM. A recent study by Reinhardt et al. (2006) reported the increased accuracy of PET/CT compared to PET or CT alone for detecting both non-visceral and visceral metastases. PET/CT was especially more accurate and specific for determining the N-stage than CT, but not more than PET alone. Restaging for metastatic disease was most accurately assessed with

combined PET/CT whereas CT scored much lower. The most important contribution of combined PET/CT compared to the different single modalities is the improved detection accuracy for visceral metastases especially in the lung. CT contributed significantly to detect pulmonary metastases and the specificity of CT even improved after fusion with PET data. PET/CT will be the modality of choice for melanoma patients with suspected recurrence and therapy follow-up (Figure 26.3).

Another study done by Mottaghy et al. (2007) compared the accuracy of combined PET/CT to PET alone or a coregistration of PET and CT data. The group reported that the

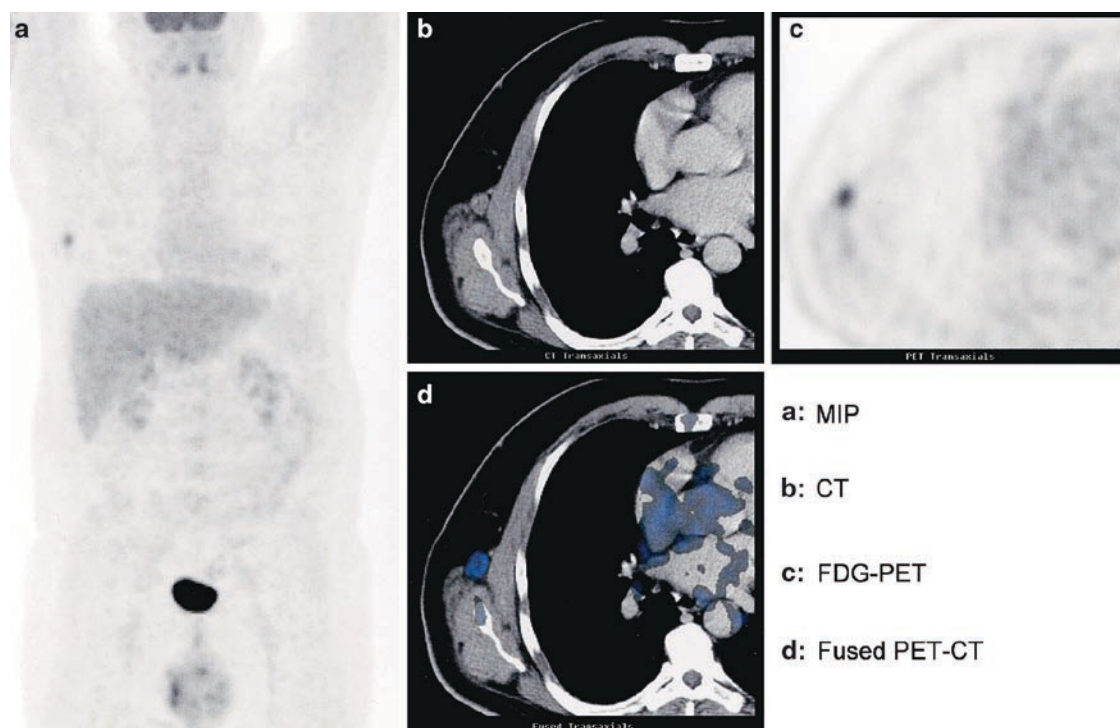


FIGURE 26.3. Restaging of MM with FDG-PET/CT. A 51-year-old male with a history of superficial spreading melanoma of the back (Breslow thickness 1,47mm; Clark level III, stage IIb) 11 years ago presented with an increased S100 tumor marker. Clinical exam showed no evidence for local recurrence or lymph node involvement. FDG-PET/CT revealed a focal FDG uptake in the right flank/low axillary region corresponding with a local lymph node enlargement on CT as shown on the fused PET/CT image. Fine needle aspiration cytology confirmed the presence of a metastatic lymph node and curative surgery was performed. A maximum intensity projection (MIP) image and transaxial PET, CT and fused PET/CT images are shown

number of lesions with an uncertain localization was significantly reduced with PET/CT or side by side PET and CT compared to PET alone. On top of the more accurate localization, they also reported an improved characterization of the lesions with PET/CT; however, the latter was not significantly increased compared to PET alone.

Several studies underline the significant added value of PET/CT compared to single modality imaging in terms of throughput, localization, sensitivity, and specificity. First, there is a reduction in acquisition time using CT for attenuation correction, thereby minimizing patient discomfort and reducing artifacts. Secondly, and the most obvious advantage of PET/CT compared to PET alone, is the accurate anatomical location of FDG-avid lesions with regard to neighbouring tissues. This holds mainly true for head and neck cancers in which there are few anatomical landmarks. Despite coregistration software with CT or MRI, patient repositioning and temporary anatomical changes might cause difficulties in interpretation which can easily be overcome by integrated PET/CT systems. Another potential advantage of combined anatomical and functional information might be a role in radiation therapy planning to precisely locate the area of interest. Thirdly, there is an increased specificity combining PET/CT datasets. The degree of radiotracer uptake in benign lesions is usually much lower compared to malignant lesions, although a gray zone exists. This is the case for structures or tissues possibly having an increased FDG uptake in normal conditions such as brown fat or intestines. The addition of CT data prevents a false-positive interpretation. The opposite could be true for lymph nodes detected on CT in which FDG information can distinguish

between reactively enlarged lymph nodes and metastatic lymph nodes. Last but not least, there is substantial evidence from the literature that PET/CT improves sensitivity in the detection of both primary and metastatic lesions. The latter has implications for staging and patient management as described below. With the combined PET/CT information, a lesion with very low FDG uptake with very suspicious characteristics is more likely to be correctly diagnosed than with PET alone. The limited spatial resolution of PET in combination with respiratory movements makes it very difficult to diagnose small lung nodules which are easily seen on CT. There are indeed cases when FDG uptake or CT findings alone are not sufficient to accurately characterize the lesion but the combination of both increases the diagnostic accuracy. Thus, there is increasing evidence that PET/CT adds complementary information in staging and treatment response assessment and, therefore, leads to changes in patient management earlier during follow-up.

ROLE OF FDG-PET IN MONITORING RESPONSE TO THERAPY

Whole body FDG-PET also plays a major role in the evaluation of treatment response especially in metastatic melanoma. FDG uptake relies on glucose utilization which is dependent on cell metabolism and proliferation. Chemotherapeutics, such as dacarbazine, nitrosoureas, vinca alkaloids or platinum compounds as well as isolated limb perfusion with melphalan, altering tumor metabolism should affect FDG uptake, and can therefore be monitored with PET. Several studies have assessed the

feasibility of noninvasive metabolic monitoring of chemotherapy using FDG. It was also reported that quantitative FDG-PET scans of different cancers showed a fast and significant decline in glucose metabolism after efficient treatment, even before a reduction in tumor size was detected on conventional imaging. These findings were absent in nonresponders and, therefore, FDG-PET has its value as an early metabolic marker for therapeutic efficacy. Major changes in tumor volume, evaluated by CT, tend to occur rather late after the start of therapy, which could impair therapeutic management in the case of nonresponders. In contrast, FDG-PET is a sensitive method to evaluate early response and is helpful for prognosis and follow-up. In the case of melanoma, FDG-PET might be very useful in patients with metastatic disease who are included in experimental protocols with new chemotherapeutics or immunotherapies. To date, no single treatment exists to prolong overall survival, and the search for new and alternative therapies is currently under active investigation. FDG-PET can help improve treatment regimens and dosage. FDG-PET is also valuable as a surveillance tool for patients with stage III-IV AJCC melanoma after treatment to detect recurrence. Schwimmer et al. (2000) showed that in these high-risk populations, FDG increased the detection of unexpected metastatic localizations thereby changing treatment regimens in one out of five patients.

ROLE OF FDG-PET IN PATIENT MANAGEMENT

The information obtained with PET can alter the treatment planning for MM in different ways. As mentioned above, PET imaging has been proven to increase the

detection of occult metastases leading to worse prognosis. Several studies that have used PET in the work-up of patients with melanoma have reported unexpected systemic invasion. A retrospective study by Stas et al. (2002) reported an additional value of PET on top of conventional imaging strategies by upstaging 10% of cases, downstaging in 24% and detection of more lesions within the same stage in 15%. Similar results were reported by Wong et al. (2002). Thus, FDG-PET has an important value to guide treatment planning and change therapies based on the up or downstaging.

The diagnostic performance of PET in the setting of melanoma will also have an impact on the general cost-effectiveness. There are currently no effective therapies for advanced melanoma disease and detection of systemic disease is most likely not going to change the outcome other than preventing more invasive treatments. Therefore, cost-effectiveness studies are needed to provide physicians with a useful algorithm in the management of MM.

ALTERNATIVE TRACERS FOR DIAGNOSING MM AND MONITORING THERAPY RESPONSE

Currently, fluorodeoxyglucose is the most commonly and widely used PET tracer used in clinical settings and is the modality of choice for studying oncologic diseases in terms of staging and treatment response assessment. Recent years have witnessed a huge revolution in the field of molecular imaging due to the identification of novel molecular targets. In order to assess these targets, novel probes have been developed and are under intense investigation and constantly optimized to obtain higher

precision and information density. Several of these novel probes such as fluorothymidine, fluorodopa and a radiolabeled RGD peptide, will be discussed.

Fluorodihydroxyphenylalanine (FDOPA) has been proven to be taken up by melanoma cells in small animal models and more recently in patients with MM. Tyrosine is an amino acid used in the synthesis of melanin and is transported and transformed in the cells to dihydroxyphenylalanine (DOPA). Because DOPA is a precursor of melanin, labelling DOPA with a positron emitter (18-F) provides a tool for specific tumor imaging. FDOPA mainly provides information on the transport of the tracer rather than metabolic information but is a marker for amino acid transport which is enhanced in malignant tissues. One of the first studies with fluorinated DOPA was performed by Dimitrakopoulou-Strauss et al. (2001) and included patients with pretreated metastatic melanomas. This group reported a sensitivity of 64% for FDOPA compared to 86% for FDG, whereas the combination of both tracers reached a sensitivity of 95%. Diagnostic problems with FDG-PET could occur in treated melanoma lesions because the uptake might be very low and similar to that of surrounding tissues. In those specific cases, an alternative tracer providing additional information to the glucose metabolism is needed and one of the markers for cell viability is the assessment of amino acid transport. Nevertheless, it still remains an open question whether FDOPA may be a useful first line tracer in the management of MM and further studies are needed to address this question.

Besides metabolism, cell proliferation is another important marker providing information on the growth kinetics of cells. There are several PET-based cell proliferation markers available such as 11C-fluoromethyluracil and fluorothymidine (FLT). FLT is an ana-

logue of thymidine that is phosphorylated by the mammalian thymidine kinase. Because the fluorine is placed in the 3'-position on the sugar, FLT is a terminator of the growing DNA chain. Only a small fraction of FLT is actually accumulated in DNA; most of it is retained intracellularly after phosphorylation (Shields et al. 1998). Thus, FLT is a marker for the evaluation of tumor growth, and can be used as a noninvasive tracer to evaluate the effect of an anti-proliferative drug. A study reported by Cobben et al. (2004) demonstrated the feasibility of using FLT for staging in AJCC stage III melanoma patients. The sensitivity of FLT was similar to FDG and has the same detection limit due to the limited spatial resolution. When comparing FLT to FDG for detecting visceral metastases, the detection of liver metastases might be impaired with FLT because of physiologic liver uptake. In contrast to FDG, brain metastases could be detected with FLT because a lack of physiologic brain uptake. The role of FLT for stage I and II melanoma patients remains to be determined.

Another example of developing a molecular probe against a new molecular target, the integrin $\alpha_v\beta_3$ expression, is a radiolabeled RGD peptide. The integrin $\alpha_v\beta_3$ is highly expressed in activated endothelial cells leading to angiogenesis, and is a key mediator in tumor growth and cell metastases. It has been shown in human melanoma that the level of integrin $\alpha_v\beta_3$ expression is a marker for the transition towards more invasive tumors. Thus, integrin $\alpha_v\beta_3$ is a very interesting molecular target for specific therapies and, therefore, assessing the expression level of integrin $\alpha_v\beta_3$ is of tremendous importance to select those patients who will benefit from these targeted therapies. Several radiolabeled $\alpha_v\beta_3$ antagonists have been synthesized and the first human studies were performed with

a fluorinated glycosylated cyclic pentapeptide galacto-RGD (arginine-glycine-aspartic acid). The first human study reported by Beer et al. (2006) showed a very good correlation between the radiotracer uptake and the immunohistological staining, demonstrating the feasibility to non-invasively assess the expression levels of integrin $\alpha_v\beta_3$. They also reported the highest uptake in lymph node metastases from MM, confirming the importance of the integrin $\alpha_v\beta_3$ expression for metastatic potential. Thus, RGD-PET might be a very promising tool to assess the metastatic potential of a given tumor, and can be used to determine the integrin $\alpha_v\beta_3$ expression level before starting a therapy targeted against $\alpha_v\beta_3$.

In conclusion, FDG-PET is the most widely used noninvasive imaging modality for detecting distant metastases and restaging in patients with MM. FDG-PET is more sensitive for diagnosing lymph node and visceral metastases compared to conventional

CT imaging except for detecting small pulmonary lesions. FDG-PET is of limited use in stage I and II melanoma compared to sentinel lymph node procedure that is more sensitive at detecting micrometastases. Patients with a stage III and IV melanoma definitely benefit from the higher sensitivity of PET compared to CT. Based on the literature, it would be recommended to implement FDG-PET for staging and restaging of AJCC stage III–IV melanoma patients and FDG-PET would be suggested for restaging stage I–II melanoma patients only if there is clinical suspicion. It is important to detect recurrence at a very early stage because surgery is the only curative approach and follow-up with FDG-PET might therefore prolong overall survival. A suggestion for the implementation of FDG-PET or FDG-PET/CT into the clinical work up of MM patients is shown in Figure 26.4.

Since the introduction of PET/CT systems, PET/CT has become the modality of choice

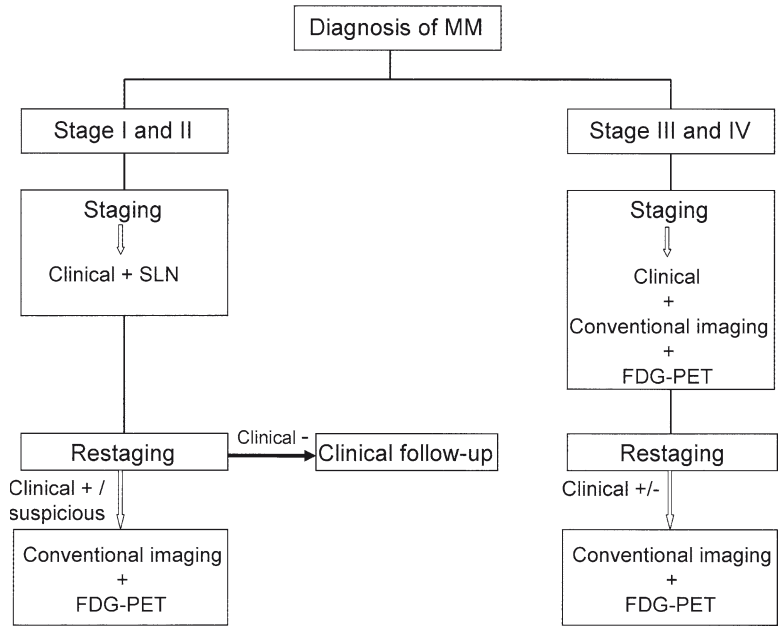


FIGURE 26.4. Flowchart to suggest implementation of FDG-PET in the work-up of patients with MM. Conventional imaging includes ultrasound, CT and MRI where applicable

offering additional and complementary information for staging malignancies. PET/CT has several advantages over PET alone in terms of throughput, increased accuracy, and specificity, all of which eventually have major implication towards patient management. However, while integrated PET/CT outperforms each single modality in staging and restaging, the additional value of PET/CT for treatment response assessment still needs to be determined. Further prospective clinical studies are needed to establish the role of PET/CT in the management of MM.

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Malignant Melanoma Versus Deep Penetrating Nevus: Diagnostic and Prognostic Immunohistochemistry of Dipeptidyl Peptidase IV (Methodology)

Alexander Roesch, Michael Landthaler, and Thomas Vogt

INTRODUCTION

Malignant melanoma is known to display a tremendous histologic diversity. Experience has shown that biomarkers determined in research laboratories can be adapted to everyday histopathology routine. However, the present immunohistochemical markers are not applicable for differentiation between malignant melanoma and melanocytic borderline lesions mimicking melanoma, such as dysplastic nevus, Spitz nevus or deep penetrating nevus (Roesch et al. 2006a). Thus, we intended to discriminate true malignant tumor progression from invasive, but non-metastasizing melanocytic growth applying our recently established algorithm for immunohistochemical marker evaluation (Roesch et al. 2005a, b, 2006b). This new approach could be helpful for further studies, because it allows straightforward and exact histomorphologic assessment of candidate markers.

THE DEEP PENETRATING NEVUS AS A MODEL OF PARADOXICAL MELANOCYTIC INVASION

For our immunohistochemical study on discrimination markers, we focused on a tumor entity that was first described by Seab et al. (1989) when they reported a series of benign invasive, but non-metastasizing pigmented melanocytic tumors, termed deep penetrating nevi (DPN). Two years later, Barnhill et al. (1991a, b) described a histomorphologic similar melanocytic lesion using the term plexiform spindle cell nevus. As independently reported by Seab et al. (1989) and Robson et al. (2003), typical DPN clinically appear as darkly pigmented papules or nodules with mild epidermal changes and are most frequently found in the face, on the upper trunk or proximal extremities of patients at the age of 10 to 30 years. Histopathologic growth patterns are often worrisome showing a

wedge-shaped invasive growth extending from the upper dermis into the subcutaneous fat tissue, not rarely following preformed structures, e.g., hair follicles or sweat glands. Cytological pleomorphism defined as a variation of cell size and shape together with a melanoma-like hyperchromasia was reported by Mehregan and Mehregan (1993) to occur also in the deeper, invasive portions of DPN. Further features that complicate the distinction of DPN from malignant melanoma are the presence of an inflammatory stroma reaction observed in 75% of DPN, lack of melanocytic maturation in the deep portion and even some possible degree of lesional asymmetry as previously described (Mehregan and Mehregan 1993; Robson et al. 2003; Ruiter et al. 2003). In some cases, only histological features together with clinical follow-up information can confirm that a lesion was truly benign. Estimates exist that, depending on the criteria used for classification, misdiagnoses as melanoma occur in 29% to 40% of the cases (Mehregan and Mehregan 1993; Robson et al. 2003; Seab et al. 1989). So, beyond the clinical demand for precise diagnosis, the DPN may also serve as a unique natural model for detection of new markers for discrimination between true metastatic progression and melanocytic invasion as an isolated phenomenon.

COMMON MELANOMA MARKERS FAIL TO SEPARATE BETWEEN MELANOCYTIC INVASION AND TRUE MELANOCYTIC MALIGNANCY

In previous studies, several immunohistochemical attempts with standard melanoma markers, such as S-100 or HMB-45, failed

to differentiate DPN from NMM (Seab et al. 1989; Skelton et al. 1991). Before we initiated our experiments, Mehregan et al. (1995) had already suggested PCNA (proliferating cell nuclear antigen) as a possible discrimination marker. PCNA represents an accessory protein of DNA δ -polymerase which is increased during the late G1 growth phase and peaks in the S phase of the cellular cycle. However, PCNA never entered routine diagnostics, perhaps due to a lack of studies with higher numbers of cases.

IMMUNOSTAINING OF DIPEPTIDYL PEPTIDASE IV DISCRIMINATES METASTATIC MALIGNANT MELANOMA FROM DEEP PENETRATING NEVUS – APPLICATION OF A NEW HISTOMORPHOLOGIC EXPRESSION ALGORITHM (METHODOLOGY)

In our study, we expanded on the search for new discriminating markers analyzing an empirical selection of common candidate markers either determining cell proliferation, such as MIB-1/Ki-67, retinoblastoma protein (pRb) and its inactivated form phospho-pRb Ser795, or melanocytic invasion, such as matrix metalloproteinases (MMPs) and integrin β 3. Since previous studies had shown that the dipeptidyl peptidase IV (DPPIV, CD26) is almost lost during melanoma progression affecting both melanoma proliferation and invasion (Houghton et al. 1988; Morrison et al. 1993; Wesley et al. 1999), this new marker was additionally chosen as a target for our immunohistochemical investigations.

For evaluation of immunolocalization and immunoreactivity, we applied an algorithm that we had successfully established for other molecular markers in former studies (Roesch et al. 2005a, b).

Tissue Sample Collection and Immunohistochemistry

Tumor material and clinical follow-up information of 14 deep penetrating nevi and a set of 12 matched nodular malignant melanomas was collected at our department. Conventional formalin-fixed, paraffin embedded tissue sections were deparaffinized and rehydrated according to standard protocols. After inhibition of endogenous peroxidase activity with hydrogen peroxide, three different protocols for antigen retrieval were performed: (1) For MIB-1/Ki-67, integrin $\beta 3$ -, phospho-pRb Ser795- and total pRb-staining, the sections were incubated at 100°C for 30 min with citrate buffer (pH 6.0). (2) For DPPIV-detection, EDTA buffer (pH 8.0) was used. (3) MMP-1-, MMP-2 and MT1-MMP-staining required pretreatment with 150 μ L pepsin for 25 min at 37°C. After washing with PBS, samples were blocked with SuperBlock™ (Zytomed, Berlin, Germany) and subsequently incubated with a 1:100 dilution of the primary antibody for 35 min at 37°C. For detection of total pRb and phosphorylated pRb, Rb (4H1) Monoclonal antibody and Phospho-Rb (Ser795) Monoclonal antibody, respectively, were used from Cell Signaling Technology/New England Biolabs GmbH, Frankfurt, Germany. Ki-67 was detected by the MIB-1 antibody, DakoCytomation, Glostrup, Denmark. Integrin $\beta 3$ -, MMP-1-, MMP-2- and MT1-MMP-detection was performed with anti-human-CD61 (CBL-479)-, anti-human-MMP-1 (MAB3307)-, anti-human-MMP-2 (MAB13405)- and anti-human-MT1-MMP (MAB3317)-antibodies

from Chemicon, Hampshire, UK. Anti-human-CD26 (DPPIV) D068-1 antibody from MBL, Woburn, USA was used for DPPIV-staining. For detection of all primary antibodies, the avidin-biotin complex method was performed using a biotinylated secondary antibody (ZytoChemPlus Anti-Broad Spectrum™) together with avidin-conjugated horseradish peroxidase (ZytoChemPlus HRP™, Zytomed, Berlin, Germany) and AEC Substrate Chromogen™ (DakoCytomation, Hamburg, Germany) according to the manufacturers' recommendations.

Immunohistochemical Evaluation

Immunostaining was semi-quantitatively assessed by two independent investigators in a blinded fashion to reduce bias. Inter- and intra-examiner reproducibility was 82% and 88%, respectively. Immunoreactivity was scored using uniform criteria to maintain the reproducibility of the method. The relative quantity of immunostaining was recorded considering the expression in tumor cells (nuclear, cytoplasmatic or membranous staining) and, in case of MMPs, also in the surrounding extracellular matrix.

Considering the markers with nuclear staining, like total pRb, phospho-pRb Ser795 and MIB-1/Ki-67, for each sample, three representative fields of vision were evaluated at 400x magnification in a tumor region with maximum and a region with minimum immunostaining as described previously (Roesch et al. 2005a, b). Afterwards, the number of positive stained nuclei was estimated as percentage of all nuclei (p) per field of vision. In addition, the staining intensities (i) of positive cells were scaled from grade 1, when the cells showed a slight granular staining pattern, to grade 4, when the cells had completely filled nuclei. Grades 2 and 3 were assigned to intermediate staining intensities.

Staining percentage and staining intensity were subsequently subsumed to a single expression score (ES) according to the formula

provided by Figure 27.1. To facilitate comparability between different samples, the expression information of the whole

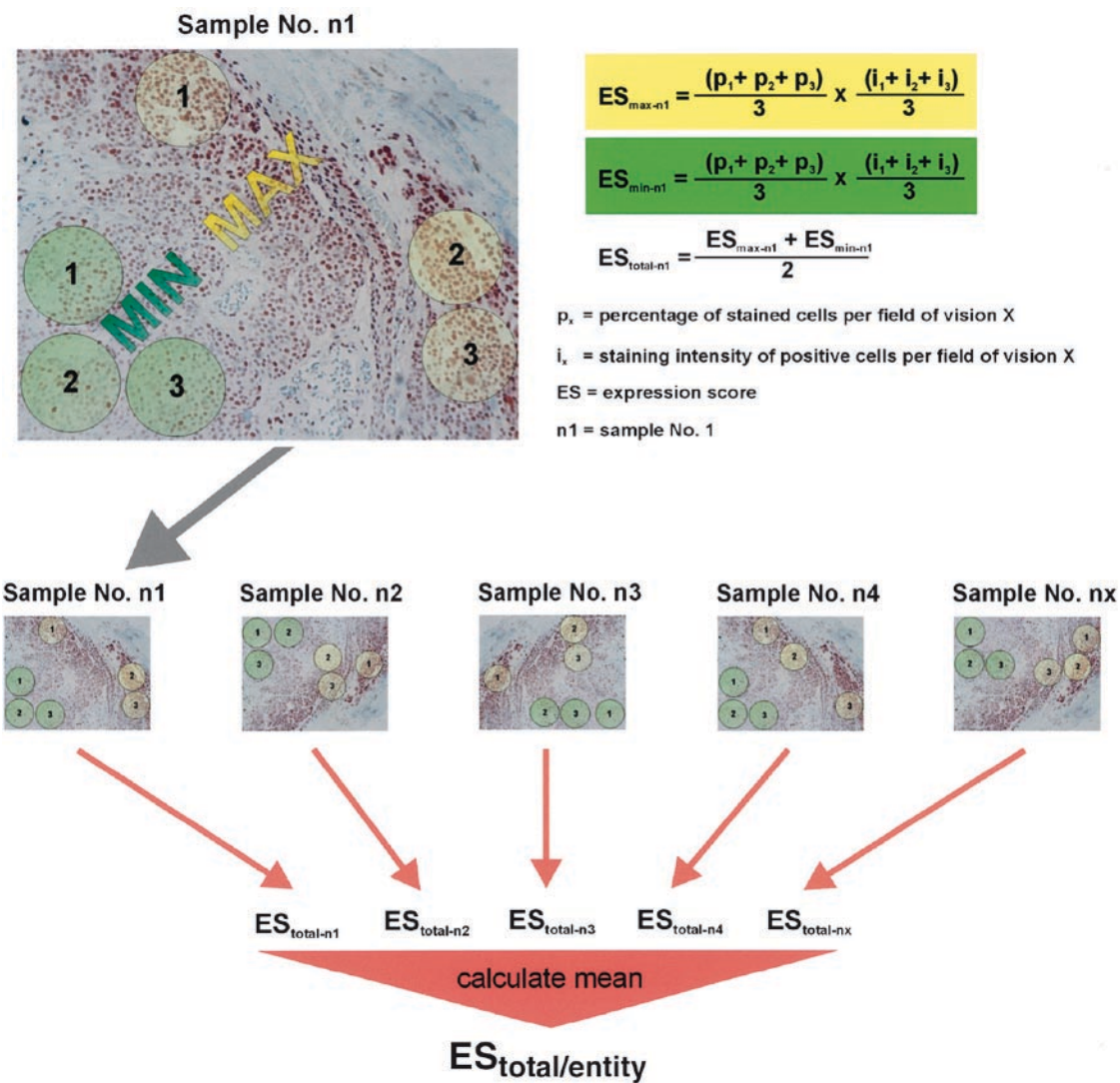


FIGURE 27.1. Methodology: Calculation of a histomorphologic expression score. For each sample, three representative fields of vision are evaluated at 400× magnification in a tumor region with maximum and a region with minimum immunostaining as described previously (Roesch et al. 2005a, b, 2006b). Afterwards, the number of positive stained nuclei is estimated as percentage of all nuclei (p) per field of vision. In addition, the staining intensity (i) of positive cells is scaled from grade 1, when the cells showed a slight granular staining pattern, to grade 4, when the cells had completely filled nuclei. Grades 2 and 3 were assigned to intermediate staining intensities. Staining intensity and staining percentage are subsequently subsumed to a single expression score (ES) according to the provided formula. To facilitate comparability between different samples, the expression information of the whole tumor (ES_{total}) is calculated as an average value from ES_{max} and ES_{min} . Based on the ES_{total} data of all samples (n1, n2, n3, ..., nx) of one tumor entity, entity-specific expression scores ($ES_{\text{total/entity}}$) are calculated as means

tumor (ES_{total}) was calculated as average value from ES_{max} and ES_{min} . Based on the ES_{total} data of all samples ($n_1, n_2, n_3, \dots, n_x$) of one tumor entity, entity-specific expression scores were calculated as means.

However, using this algorithm, neither immunolocalization nor immunoreactivity of MIB-1/Ki-67, pRb and phospho-pRb Ser795 revealed a consistent difference between DPN and the matched cases of NMM. Moreover, also the typical invasion-related markers matrix metalloproteinase-1, matrix metalloproteinase-2, membrane-type matrix metalloproteinase-1 and integrin $\beta 3$ showed no significant differences in expression. According to the highly invasive character of DPN, matrix metalloproteinase-1 and matrix metalloproteinase-2 immunostaining of some DPN even exceeded that of NMM, thereby, confirming their role in tumor infiltration, but, at the same time, questioning their importance for metastasizing. Of note, in the case of matrix metalloproteinases and integrin $\beta 3$, the staining intensity (i) was not taken into account for calculation of ES, because staining intensities remained consistent within all samples analyzed.

In contrast to the other markers, only DPPIV staining clearly discriminated the two entities (Figure 27.2). Since DPPIV exhibited a more complex staining pattern in all samples, the mean staining percentage (p) and intensity (i) were semi-quantitatively assessed considering whole tumor sections and not selected fields of vision. Total expression scores (ES_{total}) were calculated in accordance with the algorithm shown in Figure 27.1. The mean $ES_{total-DPPIV}$ for deep penetrating nevi (87) significantly exceeded the mean $ES_{total-DPPIV}$ of nodular malignant melanomas (nine) with $p < 0.001$. All deep penetrating nevi stained positive. 46% ($n = 5$)

of all deep penetrating nevi showed an $ES_{total-DPPIV} > 80$ and 36% ($n = 4$) revealed an ES_{total} between 80 and 40. 18% ($n = 2$) had an ES_{total} lower than 40. In contrast, the highest ES_{total} reached in a nodular malignant melanoma was 38. All other melanoma samples showed expression scores lower than ten. The intratumoral immunolocalization of DPPIV in both entities showed a diffuse expression pattern with focal accumulation close to cutaneous adnexes or at the tumor periphery.

DISCUSSION AND BIOLOGIC BACKGROUND

In our reported study, only DPPIV kept its promise to be a protein that possibly discriminates true melanocytic malignancy and deep penetrating nevi. The dipeptidyl peptidase IV (DPPIV, CD26) is a 110-kD, trans-membrane, cell surface peptidase expressed by normal melanocytes and common nevi, but primary and advanced malignant melanomas almost invariably lose their DPPIV expression (Houghton et al. 1988; Morrison et al. 1993; Wesley et al. 1999). It was reported by Pethiyagoda et al. (2000) that DPPIV has numerous functions including involvement in T-cell activation, cell adhesion, digestion of proline containing peptides in the kidney and intestines, HIV infection and apoptosis, and regulation of tumorigenicity in certain melanoma cells. Functionally, DPPIV re-expression leads to re-differentiation and an acquired dependence on exogenous growth factors, but it also favors loss of tumorigenicity and anchorage-independent growth as described by Wesley et al. (1999). Thus, high DPPIV expression was found by Pro and Dang (2004) to be

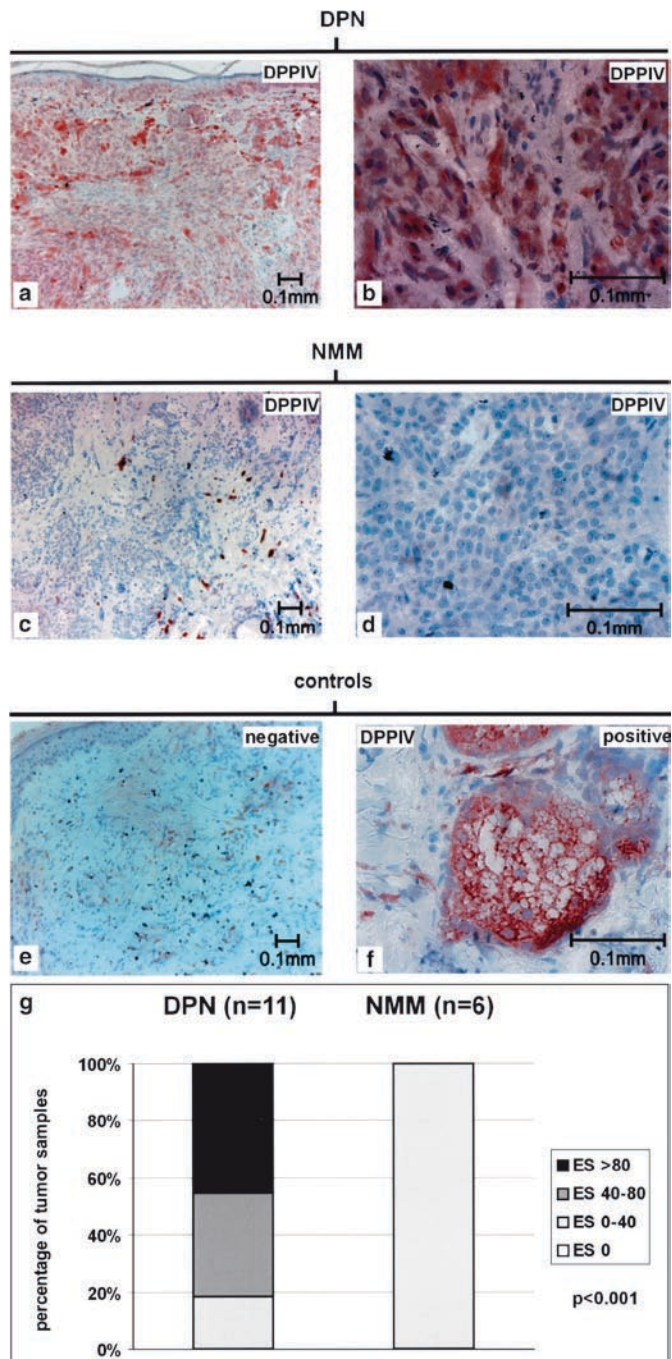


FIGURE 27.2. The dipeptidyl peptidase IV (DPPIV) discriminates between metastatic melanoma and benign deep penetrating nevus (modified from (Roesch et al. 2006b), with kind permission by the Nature Publishing Group). (a) DPPIV expression in a DPN with its typical diffuse staining pattern (100x). (b) 400x magnification. (c) Example of a NMM showing only single positive cells (100x), (d) 400x magnification. (e) Negative control (100x overview), (f) Positive control (sebaceous gland, 400x magnification). (g) Immunoreactivity was semi-quantitatively assessed regarding staining quantity and subcellular staining intensity in 11 DPN and 6 NMM. Staining quantity and intensity were summarized to a single expression score (ES) as recently published (Roesch et al. 2005a, b, 2006b). The ES values of each sample were grouped into one of four ES categories (ES 0, ES 0–40, ES 40–80 and ES > 80) and displayed as percentage of all samples of one entity. Differences were statistically significant with higher scores in DPNs (U-test $p < 0.001$)

correlated with less metastatic potential *in vivo*. The effect of DPPIV appears to be mediated through several mechanisms: (1) by up-regulation of other factors such as E-cadherin and tissue inhibitors of matrix metalloproteinases (Kajiyama et al. 2003), (2) by its ability to bind components of the extracellular matrix such as collagen or fibronectin (Abdel-Ghany et al. 1998; Pethiyagoda et al. 2000; Pro and Dang 2004) and, most interesting for melanoma biology, (3) by its inhibition of mitogen-activated protein kinase (MAPK)-extracellular signal-regulated kinase (ERK)1/2 activation (Wesley et al. 2005). Together with these previous findings, our current observations make DPPIV a prime candidate for future investigations in the context of melanoma progression.

For this study, we applied an evaluation algorithm that had been repeatedly approved to provide reliable and reproducible results acquiring both immunolocalization and immunoreactivity of marker signals (Roesch et al. 2005a, b). It is a particular advantage of this approach that two different sub-features are comprised for assessment of immunoreactivity, i.e., staining percentage and staining intensity. By this, staining signals can be accommodated at different levels, e.g., 10,000 cells that stained weakly positive, e.g., with a slight granular signal, are appraised differently from 10,000 cells with high expression signals, e.g., with completely stained nuclei. Consequently, this method is suited for many semi-quantitative applications, e.g., verification of differential gene transcription studies based on mRNA data or comparison of protein expression between different samples or tumor entities. To facilitate comparison of expression data, staining percentage and staining intensity data can be subsumed to a single expression

score, either for distinct tumor regions, such as maximum or minimum staining regions, or for whole tumor sections. This semi-quantitative method is highly informative but always flexible. Despite the dependence on subjective criteria, after a short phase of training, this method can reach an inter-observer reproducibility between 82% (Roesch et al. 2005a) and 92% (Roesch et al. 2006b). However, compared to more vague evaluation methods, such as simple staining classifications from 0 to +++, the presented algorithm is certainly more time-consuming.

Beyond the practical implications of this study, our observations add to the evidence that DPN could serve as a valuable, natural model of melanocytic progression and to dissect invasion mechanisms from metastatic potential. Against our expectations, semi-quantitative assessment of both immunolocalization and immunoreactivity of the proliferation markers MIB-1/Ki-67, pRb and phospho-pRbSer795 failed to show consistent differences between DPN and matched cases of NMM. Moreover, also the invasion-related markers MMP-1, MMP-2, MT-MMP-1 and integrin $\beta 3$ showed no significant differences in expression. This is surprising since, the induction of MMP-1 has been suggested to be a pivotal late event in the progression of advanced melanomas not compatible with any benign condition (Airola et al. 1999; Hofmann et al. 2000a; Yang et al. 2003) and even dysplastic nevi as well as early melanomas usually lack a significant MMP-1 and MMP-2 expression (Hofmann et al. 2000b; Vaisanen et al. 1996, 1998). Conclusively, the reported expression data confirm the importance of classic invasion markers for tumor infiltration, but, at the same time, question their influence for metastasizing.

We conclude that this new semi-quantitative algorithm for evaluation of immunohistochemical sections is highly informative and suited for research, but also for daily practice of histopathologists. Regarding the data acquired with this method, among the “major suspects” reflecting melanocytic tumor progress, DPPIV bears the highest potential to be further exploited as a marker in doubtful cases. Moreover, due to its emerging functional role in invasion/proliferation of various cancers, the molecular mechanisms downstream of DPPIV deserve to be studied in more detail.

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Nonmelanoma Skin Cancer: Use of EphA1 Receptor as a Prognostic Marker

Christian Hafner

THE EPH/EPHRIN FAMILY

The Eph receptors represent the largest family of receptor tyrosine kinases and interact with ephrin ligands. Since the identification of the first receptor of this family, EphA1, in an erythropoietin producing hepatocellular carcinoma cell line (Hirai et al. 1987), the evolving family of related receptors was termed Eph receptor tyrosine kinases. Based on the sequence homology, structure, and binding affinity, both Eph receptors and ephrin ligands are divided into the subclasses A and B (Eph Nomenclature Committee 1997). Presently, 14 Eph receptors and 8 ephrin ligands are known in humans (Figure 28.1). EphA10 has been recently described as a novel member of the family (Aasheim et al. 2005). Two receptors, EphA10 and EphB6, lack a tyrosine kinase activity (Gurniak and Berg 1996). EphA receptors promiscuously bind ephrin-A ligands, and EphB receptors likewise bind ephrin-B ligands. The binding affinity between the single members varies considerably. As an exception, EphA4 can also bind ephrin-B ligands, and ephrin-A5 can act with EphB2 at a high concentration level (Himanen et al. 2004). Ephrin-A ligands are tethered to the outer leaflet of the plasma membrane with a glycosyl-phosphatidyl-inositol anchor,

while ephrinB ligands share a trans-membrane domain and a short intracytoplasmatic tail. Because Eph receptors and ephrin ligands are both membrane-bound, a direct cell–cell contact is necessary for ligand binding and consecutive activation of intracellular signaling cascades. Structural data revealed that typically Eph-ephrin dimers form tetramers bridging the gap between neighbouring cells and promoting higher-order clustering of signaling centers at cell-cell interfaces (Himanen et al. 2001; Pasquale 2005). As a unique feature, bidirectional signaling is initialized in both the receptor and the ligand bearing cell upon receptor–ligand interaction (Bruckner and Klein 1998). This bidirectional Eph/ephrin signaling between cells is fundamentally involved in developmental processes which depend on organized patterning and movement of cells such as patterning of hind-brain rhombomeres, axonal guidance, and maintenance of cellular boundaries in the organogenesis of the central nervous system (Pasquale 1997) or during the remodeling of blood vessels (Cheng et al. 2002). Currently, the role of Eph-receptors and ephrins in adult human tissues beyond their well defined role in developmental processes is not well defined for most tissue types (Poliakov et al. 2004).

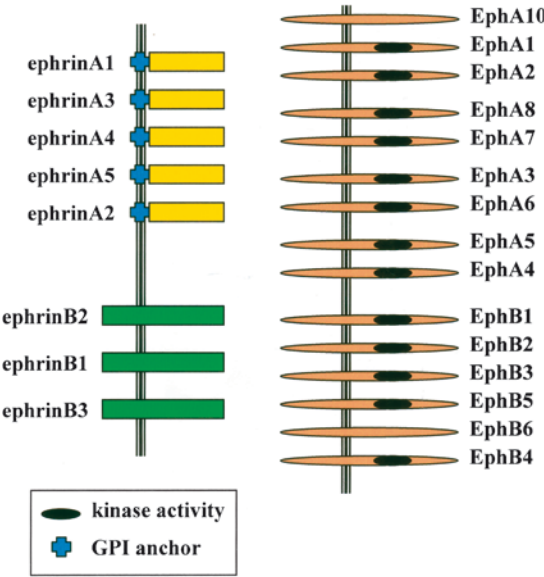


FIGURE 28.1. The family of Eph receptors and ephrin ligands (modified from Hafner et al. 2002)

EPH/EPHRIN EXPRESSION IN ADULT HUMAN TISSUES

The Eph/ephrin family is differentially expressed in various adult human cells (Hafner et al. 2004), suggesting an important role of this family in the homeostasis of adult tissues. Experimental evidence for this hypothesis has been gained in the intestinal epithelium (Batlle et al. 2002; Hafner et al. 2005a, b) and the immune system (Luo et al. 2002). In the intestinal epithelium, for example, Eph/ephrin signaling is essential for the correct formation of crypts and villuses (Batlle et al. 2002).

In addition, Eph receptors and ephrins have been recognized to be differentially expressed in various human cancers such as breast cancer, prostate cancer, small-cell lung cancer, endometrial cancer, malignant melanoma, neuroblastoma, esophageal cancer, gastric cancer, and colorectal cancer (Nakamoto and Bergemann 2002; Surawska et al. 2004). Differential expression

of Eph receptors and ephrins correlated with an altered tumor behaviour such as increased invasiveness, enhanced metastatic potential, neo-vascularization, and thus affected the outcome of the patients.

In vitro stimulation of EphA receptors caused morphologic changes such as rounding and detachment in tumor cells (Lawrenson et al. 2002), while ligand signaling enhanced integrin dependent attachment and migration potential (Davy and Robbins 2000). It is supposed that altered contact guidance and attachment may determine expansion and metastatic spread of cancer cells. Eph receptors and ephrins are also involved in tumor angiogenesis in concert with VEGF and Angiopoetins (Brantley et al. 2002).

EPH/EPHRIN EXPRESSION IN HUMAN SKIN

The skin represents the largest organ from which the most frequent cancers, basal cell carcinoma and squamous cell carcinoma, in mankind arise. Recently, the expression of Eph receptors and ephrin ligands in human skin has been systematically investigated (Hafner et al. 2006). The quantitative mRNA gene expression profile of all known Eph receptors and ephrin ligands (except EphA10) in the skin was analyzed by quantitative real-time RT-PCR, and the results were compared to 13 different adult human tissues published previously (Hafner et al. 2004).

In this study, all investigated Eph receptors and ephrin ligands were present in normal adult human skin on the mRNA level. However, the expression levels varied considerably. Compared to other tissues, EphA1 displays its highest relative mRNA expression in the skin (Hafner et al. 2006). In contrast, EphA8 and ephrin-A2 are expressed

at very low levels in human skin, while EphA2 and EphA4 show a strong expression. Ephrin-A3 displays its highest expression in the skin. Further ephrin-A ligands are also expressed at relatively high levels. Among B-receptors, EphB3 is the member which is most prominently expressed in human skin. EphB1 and particularly EphB2 show lower mRNA expression levels in the skin. Interestingly, human skin had the second highest EphB6 mRNA expression after thymus tissue. The ephrin-B ligands were found to be also quite prominently

expressed in skin. In fact, the skin was the organ with the third highest ephrin-B3 mRNA expression after brain and uterus.

Immunohistochemistry revealed that EphA1 protein is almost exclusively expressed in the keratinocytes of the epidermis and the hair follicles, whereas dermal cells (fibroblasts, vascular cells, and inflammatory cells) did not show a relevant EphA1 expression (Figure 28.2a). Furthermore, the staining of EphA1 was accentuated at the outer membranes of keratinocytes rather than in the cytoplasm compatible

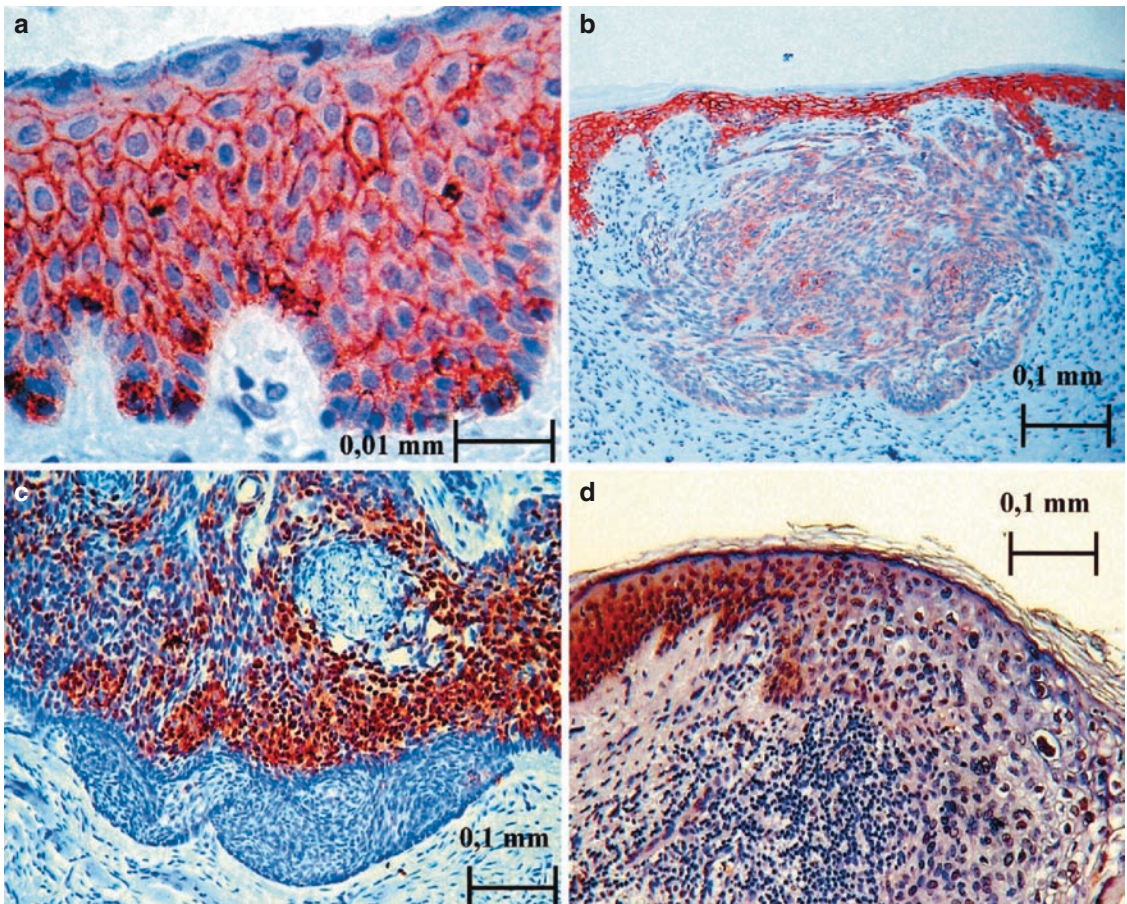


FIGURE 28.2. Immunohistochemistry of EphA1 (originally published in Hafner et al. 2006). (a) EphA1 shows strong protein expression in the epidermis with an accentuation at the outer membrane of the keratinocytes. (b) Basal cell carcinoma shows a reduced EphA1 protein expression. (c) This basal cell carcinoma reveals two components: the typical basaloid component with EphA1 expression, and a more poorly differentiated component with complete loss of EphA1 expression. (d) EphA1 protein expression is lost in squamous cell carcinoma in situ compared to adjacent normal epidermis

with its function as a membrane bound receptor tyrosine kinase.

EPHA1 AND NONMELANOMA SKIN CANCER

In the past, Eph receptors and ephrin ligands were reported to be involved in the tumorigenesis of a variety of cancers (Nakamoto and Bergemann 2002; Surawska et al. 2004). Because EphA1 particularly seems to be an important Eph member in human epidermis according to the Eph receptor mRNA expression profile and the prominent protein expression, this member was investigated in basal cell carcinoma and squamous cell carcinoma. Nonmelanoma skin cancer represents the most frequent cancer in men with an increasing incidence in the last decades (Diepgen and Mahler 2002). Immunohistochemistry for EphA1 was performed in 56 basal cell carcinomas and 32 squamous cell carcinomas.

In brief, sections (1.5 μm) of the paraffin embedded tissues were deparaffinized and rehydrated. The sections were incubated in citrate buffer (pH = 6) at 90°C for 40 min. After washing with H₂O and PBS, the sections were blocked with 2% H₂O₂/CH₃OH at 4°C for 30 min. The slides were washed again with H₂O and incubated with horse serum for 20 min to suppress non-specific binding. Then the samples were incubated with the primary antibody dilution at 4°C overnight (EphA1 antibody 1:50 from R&D Systems, Minneapolis, USA). Staining was performed using the Zyto Chem Plus HRP Broad Spectrum Kit (Zytomed, Berlin, Germany) according to the manufacturer's protocol. Leaving out the primary antibody served as a negative control (Hafner et al. 2006).

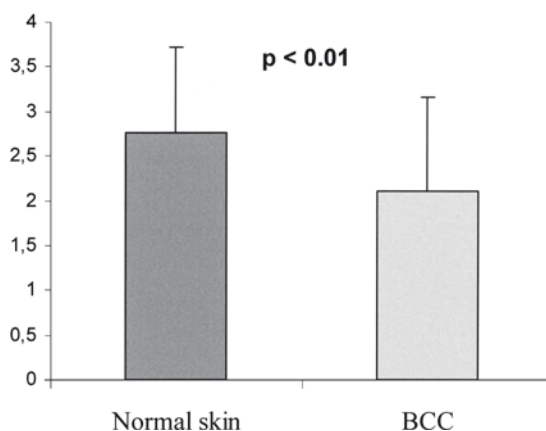


FIGURE 28.3. EphA1 protein expression assessed by immunohistochemistry was significantly reduced in basal cell carcinoma compared to adjacent normal epidermis (n = 56; expression was scored from 0 to 4) (originally published in Hafner et al. 2006)

The staining intensity of EphA1 in the tumor lesions was correlated to that of the adjacent normal epidermis (Figure 28.2b). Interestingly, the basal cell carcinomas showed a significant lower EphA1 expression than the corresponding normal epidermis (Figure 28.3), suggesting that EphA1 may be a marker for normal differentiation of the epidermis which is reduced or lost in basaloid tumors like basal cell carcinomas. This hypothesis is further substantiated by the fact that typical basal cell carcinoma cells retain some EphA1 expression, while more poorly differentiated spindle cells of a basal cell carcinoma completely lacked EphA1 protein expression (Figure 28.2c).

Similar observations were made in squamous cell carcinomas (Figure 28.2d). EphA1 expression was also significantly reduced in this cancer (Figure 28.4). In contrast to basal cell carcinoma, in squamous cell carcinoma metastatic spread of tumor cells can occur in lymph nodes and distant organs. It is well established that the vertical diameter of squamous cell carcinoma is an important prognostic

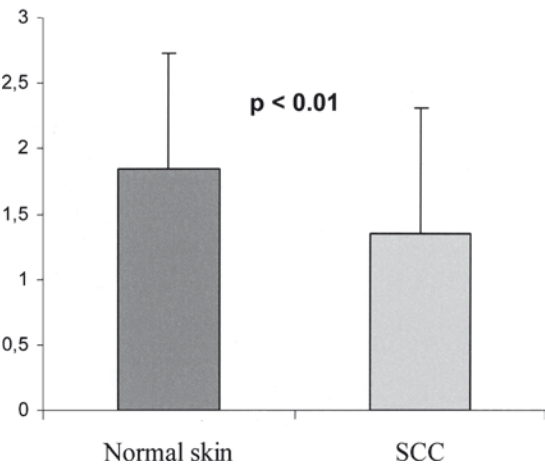


FIGURE 28.4. EphA1 protein expression assessed by immunohistochemistry was significantly reduced in squamous cell carcinoma compared to adjacent normal epidermis (n = 32; expression was scored from 0 to 4) (originally published in Hafner et al. 2006)

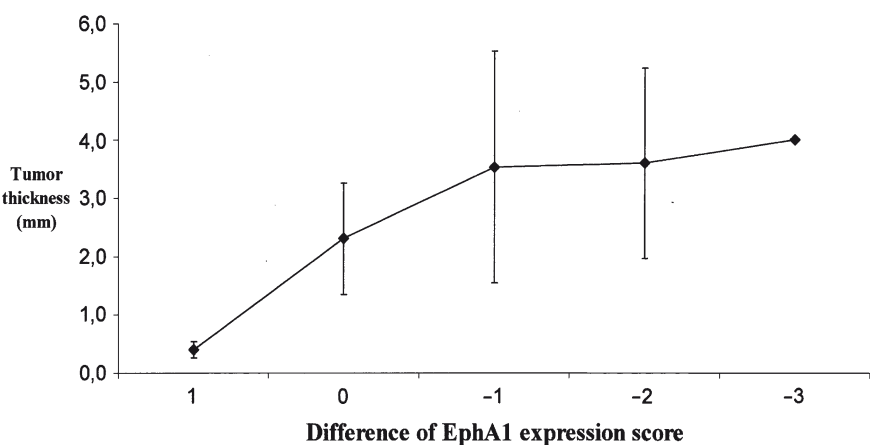


FIGURE 28.5. Comparison of the reduction of EphA1 expression and the tumor thickness of squamous cell carcinomas (n = 32). The reduction of EphA1 protein expression in squamous cell carcinoma increases with tumor thickness, but this correlation was not significant (difference of EphA1 expression score = expression score of the squamous cell carcinoma – expression score of the corresponding normal epidermis). The EphA1 expression assessed by immunohistochemistry was scored on a scale ranging from 0 to 4 (originally published in Hafner et al. 2006)

parameter for the risk of metastasis. Therefore, the thickness of the squamous cell carcinomas was compared to the reduction of EphA1 expression (Figure 28.5). A tendency toward a reduction of EphA1 protein expression in squamous cell carcinomas with an increasing vertical diameter was observed. However, this correlation was not significant, which may be due to the limited number of samples (Hafner

et al. 2006). Moreover, immunohistochemistry of skin ulcers suggested that EphA1 may also be involved in epidermal wound repair because some sections revealed a distinct staining pattern of EphA1 close to the rim of the ulcer compared to the normal epidermis (Hafner et al. 2006).

In summary, both basal cell carcinoma and squamous cell carcinoma showed a significant reduction of EphA1 protein

expression, while normal epidermis revealed a strong expression of this receptor. These results point to a potential role of EphA1 as a marker of differentiation in human epidermis. Reduced expression of Eph receptors is less frequently reported in human cancer than overexpression, which can correlate with cancer progression and poor prognosis of the patients (Nakamoto and Bergemann 2002; Miyazaki et al. 2003). Loss of expression of EphB4 has been reported for some tumors such as breast cancer (Berclaz et al. 2002) and EphB6 in metastatic melanoma (Hafner et al. 2003). In neuroblastoma, re-expression of EphB6 in tumor cells could suppress the tumorigenicity (Tang et al. 2000). Furthermore, down-regulation of EphA1 was also observed in human glioblastoma (Hafner et al. 2004) and human breast carcinoma cell lines (Fox and Kandpal 2004). In the latter cells, the down-regulation was associated with a dysregulation of other Eph/ephrins and an enhanced invasiveness of the cells. In squamous cell carcinoma of the skin, tumors with a stronger down-regulation of EphA1 tended to show an increased thickness (Hafner et al. 2006).

The functional consequences of reduced EphA1 expression in the keratinocytes of skin tumors are unknown. However, another study found that knockout of the EphA2 receptor in mice resulted in an increased susceptibility to carcinogen-induced skin tumors, and the latency of the tumors was reduced (Guo et al. 2006). The skin tumors in EphA2 knock-out mice also showed faster growth and increased invasive progression. In wild-type keratinocytes, EphA2/ephrin-A1 interaction resulted in the inhibition of ERK1/2. The results of this study suggest that EphA2 represents a tumor suppressor gene in mammalian cells

(Guo et al. 2006). EphA1 and EphA2 share structural homologies, and both receptors were found to be strongly expressed in human epidermis (Hafner et al. 2006). Therefore, EphA1 may represent an additional tumor suppressor gene in human skin, and both EphA1 and EphA2 may be involved in nonmelanoma skin carcinogenesis, but this has to be confirmed in further studies. In contrast to EphA1 protein expression which is reduced in the tumors, EphA2 was overexpressed in skin cancer of EphA2 wild-type mice despite its role as a tumor suppressor, which may be explained as a compensatory mechanism (Guo et al. 2006). The current data suggest that EphA1 and possibly other members of the Eph/ephrin family represent potential new diagnostic and prognostic parameters, and also therapeutic targets for nonmelanoma skin cancer.

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Part VII

Leukemia

Pretreated Chronic Lymphocytic Leukemia: Use of Alemtuzumab

Michael Fiegl and Jiri Mayer

INTRODUCTION

Alemtuzumab is a geno-technologically produced humanized monoclonal IgG1-kappa antibody, which binds specifically to the 21–28 kD glycoprotein CD52 on the surface of lymphocytes. The CD52 antigen consists of 12 amino acid residues and an N-terminal bound oligosaccharid complex which is responsible for membrane anchoring. The function of CD52 in the various expressing cell types is not fully known. CD52 is found on normal and neoplastic T- and B-cells. It is located very densely on the cell surface, with an estimated 450,000 copies of the CD52 molecule per cell (Hale 2001). CD52 expression is found in B-cell lymphoproliferative diseases (all cases of chronic lymphocytic leukemia [B-CLL], in most cases of low grade lymphomas, hairy cell leukemia, diffuse large B-cell lymphomas, large-cell anaplastic and lymphoblastic lymphomas). Furthermore, it is found in many T-cell lymphomas, such as, of highest clinical relevance, on T-cell prolymphocytic leukemia (T-PLL) and peripheral T-cell lymphomas (PTCL). Hematopoietic stem cells or progenitor cells do not express CD52 on their surface, and cells of the myeloid lineage are generally CD52 negative, with the exception of eosinophilic granulocytes

and mature monocytic cells/macrophages (Dyer 2005). Finally, CD52 expression is found on epithelial cells of epidymis, vas deferens and vesiculi seminales. It is also stated in this connection that alemtuzumab therapy appears not to compromise fertility. To mention the mechanisms of efficacy of therapeutically used alemtuzumab, there are three actions leading to the destruction of the target cells:

- Antibody-dependent cellular cytotoxicity
- Direct, complement-dependent cytotoxicity
- Direct induction of apoptosis

The approved standard application of alemtuzumab as monotherapy in the treatment of B-CLL are:

- Dose escalation in the first week (3 mg IV on day 1, 10 mg on day 2, and 30 mg on day 3).
- Subsequently, alemtuzumab is administered IV in a dose of 30 mg thrice weekly up to 12 weeks.

The steady state of distribution and plasma levels of alemtuzumab is reached (assuming that standard dosing as described above is feasible) after ~6 weeks. In the beginning of therapy, alemtuzumab is eliminated by nonlinear kinetics. The initially rapid elimination of lymphocytes binding

alemtuzumab is partially responsible for this observation. Finally, the clearance of alemtuzumab is carried out by phagocytosis of the reticulo-histiocytic system. With enhanced plasma levels, the elimination approximates a kinetics of null-order (Hale et al. 2004; Montillo et al. 2006).

EVOLUTION OF TREATMENTS FOR CHRONIC LYMPHO- CYTIC LEUKEMIA

Until approximately the year 2000, there were only minor therapy advances in the treatment of B-CLL for decades. Standard therapies were, as mono- or combination therapy, the alkylating substances chlorambucil and cyclophosphamide, and, as a second line therapy, fludarabine. In a series of phase III studies, alkylating substances were compared with purine antagonists such as fludarabine and cladribine in first line treatment, because superiority for purine antagonists was supposed. However, superiority of purine analogs was observed only for the parameters “response rate” (ORR) and “progression-free survival” (PFS), but not for “overall survival” (OS), as was demonstrated in a recent metaanalysis by Steurer et al. (2006). Although it was demonstrated in monocentric phase II studies that modern chemo-immunotherapies such as the FCR regimen (fludarabine, cyclophosphamide, rituximab) are highly efficacious both in first line therapy and after (multiple) pretreatments, prospective studies proving superiority are still underway to substantiate superiority also with respect to OS. For example, the German CLL Study Group (GCLLSG) prospectively tested FCR vs FC (CLL-8 trial). The investigators reported a marked superiority of FCR

with respect to ORR and PFS, but not (yet) OS (Hallek et al., 2008).

Regarding the molecular definition of prognostically relevant CLL sub-entities, enormous progress has been achieved in recent years. Thus, a number of risk factors for unfavourable prognosis could be defined and validated in large patients’ series reviewed by Montserrat (2006). For clinical practice, the definition of recurrent chromosomal changes, analysed by simple FISH analysis aiming at demonstrating four common recurrent anomalies, is feasible. The prognostic impact of such anomalies, documented at first diagnosis of B-CLL, was demonstrated by Döhner et al. (2000). Meanwhile, it is increasingly recognized that the FISH-karyotype is not only of value in estimation of prognosis, but also predictive for response to various therapies. In this context, it is important to know that during the course of disease, potentially accelerated by therapies, cytogenetic anomalies may accumulate in B-CLL, possibly resulting in a more aggressive disease (Shanafelt et al. 2006).

ALEMTUZUMAB AS MONOTHERAPY IN PRETREATED CHRONIC LYMPHOCYTIC LEUKEMIA

Since the mid-1990s, alemtuzumab has been tested in several phase II studies and case series in massively pretreated, often fludarabine-refractory patients (Table 29.1). Overall response rates (ORR) between 23% and 75% were observed. This large spectrum of ORRs is partly explainable by the fact that the 1996 National Cancer Institute (NCI) criteria for evaluation of response are often difficult to interpret in certain cases, e.g., in the

TABLE 29.1. Phase-II, and retrospective studies* in pretreated patients with B-CLL (in the order of ORR)

	No. of patients	Age (median, years)	Rai-stages III/IV (%)	Median number prior therapies (range)	Number fludarabine-refractory (%)	Median therapy duration/median dose	ORR CR + PR in (%)	OS median/ in fludarabine-refractory (months)	CMV (%)	Grad-III/IV-infection/ death during alemtuzumab therapy (%)
Karlsson et al. (2009)	20	65	60	2 (1–9)	84	12 weeks/1083 mg	75	n.r.	30	35/10
Moreton et al. (2005)	91	58	60	3 (1–8)	49	9 weeks	53	19 (MRD+)	8	36/8
Wickramanayake et al. (2005)*	51	66	n.r.	3 (1–13)	n.r.	8 weeks/613 mg	53	n.r.	10	12/n.r.
Hui et al. (2008)*	42	63	79	4 (1–8)	72	8.5 weeks	50	15.1	7	60/12
Rai et al. (2002a)*	187	65	n.r.	4 (1–13)	n.r.	8 weeks	43	n.r.	n.r.	35/7
Osterborg et al. (1997)	29	57	76	2 (1–4+)	n.r.	812 mg	42	n.r.	n.r.	n.r.
Rai et al. (2002b)	24	n.r.	71	3 (1–8)	71	n.r.	33	27.5	n.r.	42/21
Stilgenbauer et al. (2007)	108	63	>80	3 (1–9)	100	722 mg	33	19.1	30	25/n.r.
Keating et al. (2002)	93	66	76	3 (2–7)	99	673 mg	33	16	8	27/10
Ferrajoli et al. (2003)	42	61	76	3 (1–9)	55	n.r.	31	12	20	n.r.
Lozanski et al. (2004)	36	61	75	3 (1–12)	81	n.r.	31	n.r.	n.r.	n.r.
Fiegl et al. (2006)*	115	66	72	3 (1–11)	59	8 weeks/390 mg	23	20.2/14.6	13	37/12

CMV – cytomegalovirus, CR – complete remission; MRD – minimal residual disease; n.r. – not reported; PR – partial remission; ORR – overall response rate; OS – overall survival.

definition of complete response (CR), partial response (PR), and stabilization of disease (SD). For example, at least a 50% decline of lymph node or spleen size, prerequisite for response, was determined in many of the above cited studies only by clinical means, i.e., palpation. However, in the NCI criteria it is stated that within the context of clinical studies, documentation of shrinkage or disappearance of involved spleen and lymph nodes should be made by appropriate radiographic studies. This problem was addressed in systematic studies, in which clinical (by palpation) and radiographic (by CT) documentation of response in CLL were compared (Bosch et al. 2006; Eichhorst et al. 2006). It can be deduced from these studies that the rate of ORR (CR and PR) is approximately a third lower if CT scans were used for evaluation. As compensation, more patients will be categorized into the group of SD if CT scans are used for response evaluation (e.g., persisting size of abdominal or thoracic lymph nodes); however, such SD patients often achieve a clinical benefit, such as improvement of B-symptoms, independence of transfusion, etc. In order to harmonize response criteria and ease comparison in future studies, new response criteria have recently been proposed (Hallek et al. 2008a).

After feasibility with respect to dosing and response was demonstrated in previous phase I and II studies, Keating et al. (2002) prospectively investigated the impact of alemtuzumab in 93 fludarabine refractory CLL patients, recruited into the pivotal study CAM211. In this cohort of pretreated patients, an ORR of 33% and an OS of 16 months was observed. In patients with objective response (CR or PR), a median OS of even 32 months was evident. In this study, unlike in most

other studies, the median administered cumulative dose of alemtuzumab was also reported. The dose was 673 mg, instead of the planned ~1,100 mg (dose escalation of 1 week, then 30 mg thrice per week for 12 weeks). This smaller amount was to be explained by early disease progression and complications, which were reasons for early termination of therapy. These, as ASH abstract presented data, led to the fast track approval of alemtuzumab in fludarabine refractory CLL in 2001.

In Austria, we started to document patterns of routine use of alemtuzumab in CLL, aiming at analyzing treatment results as a means of quality control, and correlating the results with published study data. It was the stated objective to receive a complete data set on an unselected patients' cohort in a predefined area. Thus, an alemtuzumab registry was launched in 2001, and as many CLL patients as possible who received at least a single dose of alemtuzumab, were documented. Twenty-five Austrian cancer centres contributed to this registry (*The Austrian Collaborative Study Group on Alemtuzumab in Chronic Lymphocytic Leukemia*). In the data evaluation of 115 patients in 2006, we demonstrated high efficacy of therapy, with an ORR and SD rate of 23% and 36%, respectively (Fiegl et al. 2006). At first glimpse, an ORR of 23% appears relatively low; however, this is explained by strict application of NCI criteria in response evaluation, as we always evaluated response on the lymph node and spleen level with adequate radiographic studies. Thus, a significant amount of patients who had normalization of peripheral blood values were assigned to the SD group, for example, with insufficient shrinkage of abdominal lymphadenopathy. In historical comparison with prospective

studies, the efficacy of alemtuzumab in this retrospective study correlated quite well to other studies with respect to OS, especially including the subgroup of fludarabine refractory patients (Table 29.1).

Data from the Austrian–Czech Registry Meanwhile, the data base of our registry was enlarged by the contribution of patients from the Medical University of Brno, Czech Republic. At present, the registry surveys a complete data set of 196 patients with B-CLL having received alemtuzumab. In Table 29.2, clinical characteristics, response rates, and toxicities are summarized. In Figures 29.1 and 29.2, survival rates, stratified according to the number of prior therapies (PFS) and the response toward alemtuzumab therapy (OS), are shown. Briefly, we observed encouraging clinical outcome of alemtuzumab treatment, applied in a mostly difficult, far advanced clinical setting. With respect to toxicities, we obtained interesting results, well in line with published studies (Table 29.1). Firstly, we observed that if alemtuzumab was given SC, systemic hypersensitivity reactions rarely occurred (in 19%, of grades II–IV), whereas with IV application, these occurred significantly more often (38%; $p = 0.044$). Secondly, grade III/IV infection, CMV reactivation, and death under therapy were observed in 33%, 21% and 10% of treated patients, respectively. Thirdly, overall 17 secondary malignancies were documented after alemtuzumab therapy. However, the question of whether the rate of secondary malignomas is increased by alemtuzumab therapy, via deepening of immunosuppression, cannot be answered yet conclusively. In our registry, we observed a relatively small cumulative dose of 406 mg administered, and only 6% of patients achieved a target dose

of >1,000 mg. Another interesting result is the fact that median OS after initiation of alemtuzumab was not different in centers with ≥ 6 patients in comparison with those with <6 patients treated. Thus, it seems that efficacy of therapy is not dependent on the case load volume of the center; but rather, on the personal experience of the treating physician. Meanwhile, a similar project of establishing a nationwide registry on routine use of alemtuzumab in CLL was initiated in British Columbia, Canada. Hui et al. (2008) recently reported results in 42 patients (39 with pretreated CLL), and compared them to our results in Austria (Fiegl et al. 2006). Hui et al. (2008) observed higher response but lower survival rates; this, again, may be partially explained by differences in baseline characteristics of patients, small patient numbers, and interpretation of response criteria; the complications during therapy which were observed in the registries of Austria and British Columbia were consistent with rates published in prospective studies.

Retreatment with Alemtuzumab Little is known regarding the efficacy of a second treatment course (“retreatment”) with alemtuzumab in CLL. The Austrian registry revealed that nearly two thirds of CLL patients were subjected to another systemic therapy for relapse upon alemtuzumab treatment. In many instances (39%), this was again alemtuzumab (Fiegl et al. 2006). Those patients having been retreated by alemtuzumab were, of course, preselected because most of them had the benefit of a previous alemtuzumab course. Thus, we observed an ORR of 53% in the 26 patients with alemtuzumab retreatment evaluated to date, with an OS of 16.7 months, calculated from initiation of retreatment (Fiegl et al. 2007).

TABLE 29.2. Austrian-Czech Alemtuzumab Registry: characteristics of baseline disease and results of alemtuzumab therapy in pretreated CLL (n = 196)

	No. of patients (%)
Patients' characteristics	
Median age at alemtuzumab initiation (range) (years)	66 (46–92)
Male	134 (68)
Rai stage	
Stage I	20 (10)
Stage II	38 (19)
Stage III	39 (20)
Stage IV	99 (51)
Response to prior fludarabine	
Refractory	111 (57)
Sensitive	43 (22)
Unknown	12 (6)
No prior fludarabine	30 (15)
Median number of prior therapy lines (range)	2 (0–11)
Median levels of β 2-microglobuline (range) (mg/L)	3.7 (1–13.6)
Median WBC (range) (g/L)	55.1
Median haemoglobin (range) (g/dL)	10.7 (4–17.3)
Median platelet count (range), g/L	99 (2–768)
Lymphadenopathy	
<5 cm	119 (61)
$\geq$ 5 cm	67 (34)
Unknown	10 (5)
Alemtuzumab therapy	
Alemtuzumab dose (range) (mg)	406 (11–1333)
Duration of therapy (range) (weeks)	8 (1–29)
Mode of application	
IV	60 (31)
SC ^a	130 (66)
Unknown	6 (3)
Response rate	
ORR	63 (32)
CR	6 (3)
PR	57 (29)
SD	58 (30)
PD	61 (31)
NE	14 (7)
Toxicities	
Neutropenia grade IV	64 (33)
Thrombocytopenia grade IV	38 (19)
Infectious events	
Any grade	93 (47)
Grades III/IV	65 (33)
CMV reactivation ^b	41 (21)
Hypersensitivity reactions	
Any grade	93 (47)
Grades III/IV	20 (10)
Death under therapy ^c	20 (10)
Secondary malignancies ^d	17 (9)

^aInclusive cases with IV dose escalation and subsequent SC administration (n = 92).

^bInclusive CMV-pneumonia (n = 2).

^cDuring alemtuzumab therapy or within 2 months after last dose.

^dIn 14 patients, within 342 patient-years: melanoma (n = 2); basaloma (3); squamous cell carcinoma of the skin (6); myelodysplasia (2); myeloma (1); acute myeloid leukemia (1); lung cancer (1).

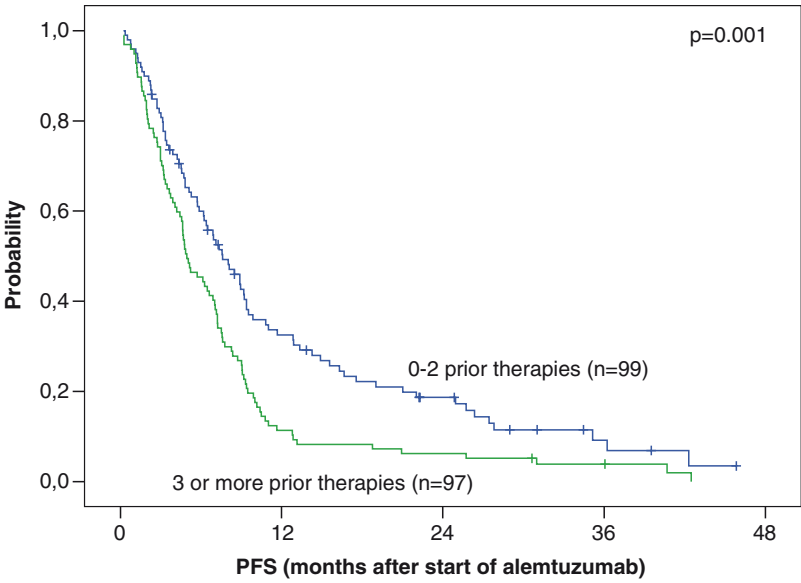


FIGURE 29.1. Kaplan–Meier plot for progression-free survival (PFS) in patients with CLL receiving alemtuzumab monotherapy: more lines of prior therapy (≥ 3) were significantly predictive of shorter PFS (5.0 months vs 7.6 months; $P = 0.001$)

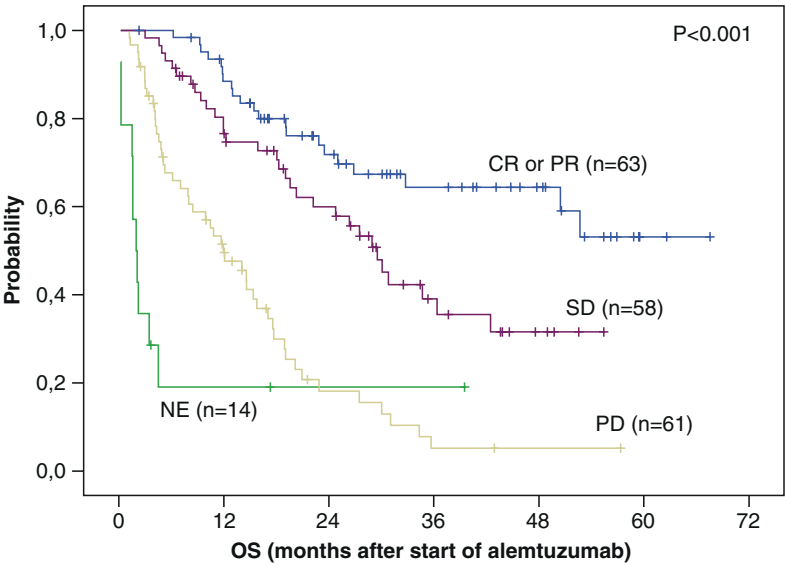


FIGURE 29.2. Kaplan–Meier plot for overall survival (OS) in patients with CLL receiving alemtuzumab monotherapy: non-response to therapy was significantly predictive of shorter OS ($P < 0.001$). Median OS for patients achieving a CR or PR, not reached; patients with SD, 29.5 months; patients with PD, 12.0 months; patients not evaluable for response (NE), 2.0 months

Cytogenetic Stratification of Alemtuzumab Use Another important question is the rational application of alemtuzumab in CLL defined by risk factors, of which

cytogenetics seem to be the most clinically relevant to date. It is well documented that conventional chemotherapy is relatively ineffective in 17p/p53 deleted CLL patients

(Flinn et al. 2007). In small case series (Cortelezzi et al. 2005; Osuji et al. 2005; Pettitt et al. 2006) and two prospective studies with 15 and 31 patients with 17p deletion (Lozanski et al. 2004; Stilgenbauer et al. 2007), encouraging ORRs on alemtuzumab were observed (40% and 39%, respectively). In the Austrian–Czech Registry, ORRs in the different cytogenetic subgroups compared well with the results published by Stilgenbauer et al. (2007) and Lozanski et al. (2004) (Fiegl et al., manuscript submitted). In the German CLL2H study, Stilgenbauer et al. (2007) found comparable outcome of alemtuzumab therapy across the cytogenetic subgroups, and this points to an improvement of prognosis in the cytogenetically adverse subgroups by administration of alemtuzumab.

On the basis of these data and recent results in alemtuzumab first line therapy in 17p deleted patients reported by Hillmen et al. (2007), the early (even first line) application of alemtuzumab in 17p-deleted CLL patients should be considered. In fact, various Medical Societies, among those the American Society of Hematology and the German Society of Haematology and Oncology, are currently recommending the administration of alemtuzumab as first line therapy in 17p-deleted CLL.

COMBINATION THERAPY

A number of studies are listed in Table 29.3, which document an additive or synergistic efficacy of combined alemtuzumab and chemotherapy and/or rituximab therapy, at acceptable tolerability. In a pilot study, Kennedy et al. (2002) demonstrated the combined potential of alemtuzumab and fludarabine in six patients who were refractory to

both fludarabine and alemtuzumab. Five of six patients indeed achieved a response, one of whom even demonstrated CR. Additional data on the combination fludarabine/alemtuzumab were delivered by Elter et al. (2005) in a phase II study. Here, alemtuzumab was not delivered continuously, but parallel with fludarabine for 3 days every 4 weeks, the aim being to deliver 6 cycles of the “FluCam” regimen. In 36 patients, an ORR of 83% (CR 30%) was achieved. As an extraordinary result, a median OS of 36 months in this unfavourable cohort was observed.

In the Phase II study UK-CLL02, fludarabine refractory patients were treated sequentially with the combination alemtuzumab/fludarabine (Sayala et al. 2006). Alemtuzumab was given either as SC monotherapy or, in non-responders, in combination with PO fludarabine. Obviously, this was a prognostically relatively favourable cohort (median two previous therapies), because a very high cumulative alemtuzumab dose of median 1,371 mg could be applied. Overall, 17 non-responding patients received fludarabine in addition. In 49 evaluable patients, an ORR of 49% was achieved. In the whole group, a median OS of 20.8 months was observed; in responding patients, the median OS was 25.5 months. It was concluded that by SC application, probably due to improved tolerability, superior results in comparison with previous studies as historical controls were seen.

In a recent US study, alemtuzumab was given simultaneously with fludarabine for 4–6 cycles in massively pretreated CLL patients. Here, fludarabine was administered in its conventional dosing (25 mg/m² days 1–5, repeated on day 29), supplemented with alemtuzumab in a very dense, pulsed way of 30 mg SC each on days 1–5 per cycle.

TABLE 29.3. Studies on combination therapies including alemtuzumab in patients refractory to or relapsing after fludarabine containing therapy (in the order of ORR)

	Regimen	No. of patients	Age (median, years)	Median number prior therapies (range)	ORR (CR + PR in %)	PFS/TTP (months)	OS (months)	CMV (%)	Grad-III/IV-infection (%)
Mauro et al. (2006) ^a	FAND-Cam	18	55	2 (1–6)	89	n.r.	n.r.	39	>50
Egle et al. (2007) ^a	FLUSALEM	7	n.r.	1	85	n.r.	n.r.	37	n.r.
Elter et al. (2005)	A + F, up to 6 cycles	36	61	2 (1–8)	83	13	35.6	5.6	5
Elter et al. (2008) ^a	FC-A	24	63	1	83	n.r.	n.r.	12.5	n.r.
Kennedy et al. (2002)	A + F	6	53	3 (3–4)	83	n.r.	n.r.	0	17
Montillo et al. (2007) ^a	FCC	19	57	2 (1–4)	79	n.r.	n.r.	32	21
Wierda et al. (2006) ^a	CFAR	78	58	3 (1–14)	65	26 (Responder)	19	15	n.r.
Flowers et al. (2007) ^a	A + F, 4–6 cycles	28	62	3 (1–14)	64	n.r.	n.r.	n.r.	n.r.
Faderl et al. (2003)	A + R	48	62	4 (1–9)	52	6	11	27	n.r.
Faderl et al. (2006) ^a	A + R	32	59	3 (1–8)	50	n.r.	n.r.	22	n.r.
Sayala et al. (2006) ^a	A; selected cases A + F	49	64	2 (1–8)	45	n.r.	20.8	43	49

^aASH – abstracts; A – alemtuzumab; CFAR – cyclophosphamide, fludarabine, alemtuzumab, and rituximab; CMV – Cytomegalovirus; CR – complete remission; F – fludarabine; FAND-Cam – fludarabine–cytarabine–mitoxantrone–dexamethasone + alemtuzumab; FCC – fludarabine–cyclophosphamide–alemtuzumab; n.r. – not reported; PFS – progression-free survival; PR – partial remission; ORR – overall response rate; OS – overall survival; TTP – time to progression.

In 28 evaluable patients, an ORR of 64% was observed, and eight patients achieved MRD-negativity (Flowers et al. 2007).

Even triple and quadruple combinations including alemtuzumab are being tested in CLL. In a recent study from Italy in pretreated CLL, the FCC-regimen (fludarabine (40 mg/m² PO), cyclophosphamide (250 mg/m² PO) and alemtuzumab (10 mg SC, potentially to be increased to 20 mg; days 1–3)) was given. ORR in 19 evaluable patients was 79%, and MRD negativity was 27% in 15 tested patients (Montillo et al. 2007). There are arguments for a combination of alemtuzumab and rituximab, such as targeting of different antigens, different mechanism of cell eradication, and non-overlapping toxicity profile. For the first time, this combination was given during a 4 weeks' period in Houston/Texas (Alemtuzumab, after dose escalation, weekly 30 mg IV on days 3 and 5; rituximab weekly on day 1 375 mg/m²). ORR in these massively pretreated patients was 52%; however, a differentiated response according to the disease compartments was observed, with subtotal clearance of circulating CLL cells and frequently persisting lymphadenopathy and bone marrow infiltration (Faderl et al. 2003).

These encouraging results were stimulating for complementing this with cytostatic drugs. Thus, the combination of cyclophosphamide, fludarabine, alemtuzumab, and rituximab was studied (CFAR regimen). In a recent evaluation in 78 patients, who had received median three prior therapies (range, 1–14), Wierda et al. (2006) achieved an ORR of 65% (CR 24%), with MRD-negativity (2-color FACS) in 18 of the 19 CR-patients. Most impressive was the response in high risk patients (e.g., in high risk cytogenetics 65%; in fludarabine refractory patients

51%). In fact, risk factors such as unfavourable cytogenetics, unmutated IgVH status, ZAP 70 positivity, and others were not predictive for response and PFS and, thus, seemed to be neutralized by efficacy of this four drug combination. On the other hand, OS after this regimen (median of 19 months) was not superior compared to two-drug alemtuzumab-containing regimens, pointing to the fact that a kind of therapeutic plateau seemed to be reached.

CONSOLIDATION THERAPY WITH ALEMTUZUMAB

It is well documented that, in B-CLL, the quality of achieved remission is closely correlated with OS duration (Moreton et al. 2005). The quality of remission is usually determined by flow cytometry (2- or 4-color FACS) or molecular methods; in the best case, elimination of minimal residual disease (MRD negativity) is documented. Moreton et al. (2005) nicely demonstrated, if clinical remission is achieved, MRD negativity in bone marrow is the most prognostically relevant factor. It was shown that patients with MRD-negative CR had dramatically superior OS. Efficacy of alemtuzumab is seen particularly at the level of peripheral blood and bone marrow; it is less pronounced, however, in the case of lymphadenopathy and spleen involvement (Rai et al. 2002b). Thus, it is the aim of the so-called consolidation therapy to raise response to chemotherapy by subsequent complementary alemtuzumab therapy.

In an early study of 41 B-CLL patients, in whom remission by conventional chemotherapy was achieved, subsequent alemtuzumab consolidation therapy over 4 weeks

led to an improvement in response quality (CR, nPR, even molecular MRD-negative CR) in 46% (O'Brien et al., 2003). The CLL4B Study of the GCLLSG was the only randomized study to date, examining alemtuzumab in the consolidation setting (Wendtner et al. 2004). For this, patients from the CLL4 study who achieved CR or PR after fludarabine or fludarabine-cyclophosphamide (FC-regimen) were randomized a second time for evaluation of alemtuzumab consolidation versus observation only. This trial had to be terminated prematurely due to toxicities, mainly severe opportunistic infections, which were fortunately not fatal. Recently, the long term results of the CLL4B study were presented, after a median follow-up of 48 months (Schweighofer et al. 2008). Eleven patients received a 12 weeks' alemtuzumab consolidation after chemotherapy (arm A), and ten patients were in the control arm (arm B, without therapy). At the time of analysis, only three patients had PD after consolidation with alemtuzumab, in contrast to eight patients with PD in the control arm. Despite the low patient number, there was a profoundly significant benefit in alemtuzumab consolidation, with a PFS of 20.6 months in the observation arm vs "not reached" in the alemtuzumab arm ($p = .004$). Since then it has become clear that, firstly, the time interval between the final dose of fludarabine containing therapy and the initiation of alemtuzumab consolidation should be at least 3 months, and, secondly, the dose in consolidation should be much lower than that in induction therapy. Turning to the importance of the latter point, in the recent CALGB10101 study, in which alemtuzumab was administered as consolidation in full dose over 6 weeks after fludarabine-rituximab (FR-) induction,

"inacceptable toxicity" with overall six treatment-associated fatalities in 51 treated patients was observed. These complications included one case each of EBV-associated lymphoproliferative disease; viral and Listeria-meningitis; Legionella-, CMV- and Pneumocystis carinii pneumonia (Lin et al. 2007).

Currently, studies of alemtuzumab consolidation are examining these problems. The German CLL2i study of the GCLLSG is currently testing consolidation after second line chemotherapy, with alemtuzumab administered after an interval of at least 90 days, in low doses. In an interim analysis, they reported that in IV administration the maximum tolerated dose of alemtuzumab was 10 mg per week when given during 8 weeks in this setting (Fischer et al. 2007).

Another interesting concept of maximum consolidation is administration of alemtuzumab after chemotherapy before stem cell harvesting and autologous transplantation, in the sense of in-vivo-purging (Montillo et al. 2006). In 34 patients, alemtuzumab was given at least 8 weeks after the end of fludarabine chemotherapy, SC for 6 weeks in a dose of 10 mg thrice per week. In this study, a CR rate of 35% after fludarabine and nearly 80% after alemtuzumab was observed, and 19 patients (56%) were finally MRD negative. Stem cell harvest was successful in 24 of 26 patients (92%) in whom this procedure was aimed. A similar concept was followed by the French CLL-study group, with comparable results (Delmer et al. 2006).

An even more aggressive multimodal concept was tested by a group from Naples, Italy (Gonnella et al. 2007). In 12 younger (aged between 35 and 52 years), thus far untreated patients they administered 6 cycles of FCR and after an

interval of 2 months, alemtuzumab consolidation with 10 mg SC thrice weekly during 8 weeks. As recently reported, they observed molecular CR in all patients without relapse at the time of analysis. The question of whether a low dose alemtuzumab maintenance therapy directly after alemtuzumab induction is beneficial has not been tested in a randomized way. In a French phase II-study, this sequential alemtuzumab therapy seemed to be feasible and efficient, with prolongation of PFS and OS when compared to a historical control (Thieblemont et al. 2004).

In conclusion, at present, the approved indication of alemtuzumab in Europe and the United States is the therapy of B-CLL (1) in first line therapy, if fludarabine therapy seems unfeasible, and (2) in B-CLL pretreated by alkylating substances and refractory towards fludarabine. In this review, the efficacy and tolerability of alemtuzumab therapy in different settings has been described. Generally, it is increasingly possible to give alemtuzumab in individualized ways based on evidence of published studies, e.g., in first line therapy in 17p deleted CLL. Not described in this review, is the well documented studies that alemtuzumab is also the central drug in the treatment of T-PLL and, in combination with chemotherapy, of PTCL. Furthermore, it aids in the treatment of autoimmune disease such as refractory autoimmune thrombocytopenia and haemolytic anemia, and is of great promise, in multiple sclerosis. Finally, it is used in immunosuppression in the context of stem cell and solid organ transplantation (reviewed by Morris and Russell 2006). In line with data from our Austrian–Czech registry, other investigators have also made the observation that in the routine use of alemtuzumab in CLL,

the dosing regimen frequently needs to be individualized in order to control cytopenias and other toxicities, a procedure which obviously does not compromise efficacy (Rieger et al. 2004; Hui et al. 2008).

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Part VIII

Multiple Myeloma

Immunotherapeutic Strategies, Radiotherapy, and Targeted Radionuclide Therapy Approaches for the Treatment of Multiple Myeloma

Malay Chatterjee, Rangasamy Manivannan, Amalendu Pande, Tridib Chakraborty, and Ajay Rana

INTRODUCTION

Multiple myeloma (MM) is the second most common hematologic B-cell malignancy, affecting >16,000 individuals each year in the United States with a median life expectancy of 3–5 years and a prevalence accounting for ~50,000 patients alive annually, and contributing to ~12,000 cancer related deaths. MM is characterized by accumulation of clonal malignant plasma cells predominantly within the bone marrow and a heterogenous clinical course, which can be often preceded by a premalignant condition, defined as monoclonal gammopathy of undetermined significance (MGUS), and may progress to extramedullary and/or disseminated disease. MM is associated with a constellation of disease manifestations, including osteolytic lesions due to uncoupled bone metabolism, anemia and immunosuppression due to loss of normal hematopoietic stem cell function, and end-organ damage due to monoclonal immunoglobulin secretion (Barlogie et al. 2004).

Treatment for MM has changed beyond recognition in the past decades. While until

the early 1980s, MM caused a slow progressive decline in quality of life (QOL) until death after ~2 years. Today's patients can expect a 50% chance of achieving a complete remission, and a 20% chance of surviving longer than 10 years. However, MM is still an incurable, lethal disease for the vast majority of patients. Improved understanding of myeloma biology has led to the development of promising molecular therapies and novel anti-MM agents such as thalidomide, lenalidomide, bortezomib, and bisphosphonates (Barlogie et al. 2004). Numerous immunotherapy-based approaches, including monoclonal antibodies (MoAbs), immunoconjugates and myeloma vaccines, targeted at cellular mechanisms and interactions, e.g., with the bone marrow microenvironment are currently being evaluated in clinical trials (Multiple Myeloma Research Foundation, URL 2007). These strategies are incrementally integrated into modern MM care. This chapter reviews the clinical and preclinical advancements in immunotherapeutic strategies and radiotherapy modalities including targeted radionuclide therapy and aims to define their perspectives in multimodality treatment of MM.

THERAPEUTIC STRATEGIES IN MULTIPLE MYELOMA

Immunotherapy

Immunotherapy is the manipulation of the immune system in order to prevent or treat a disease, and it is an experimental treatment strategy for MM (Barlogie et al. 2004). Strategies to harness the powerful immune system are mainly aimed at the pre-clinical stage of development for MM, but they are moving toward clinical testing. The goal of using immunotherapy is to either help the body elicit an “active” immune response to attack MM cells (active immunotherapy) or to substitute an alternative “passive” response elicited outside the body and administered to the patient (passive immunotherapy). Immunotherapy is likely to be most effective when the burden of disease has been minimized, such as after high-dose chemotherapy and stem cell transplantation (SCT). It seeks to destroy the remaining cells, which are thought to be responsible for relapse after therapy. In addition to MoAbs, various types of immunotherapies being investigated in MM include myeloma vaccines, cytokines/growth factors, as well as manipulation of immune cells. Researchers are investigating new molecular targets and strategies for immunotherapy, as well as working toward a better understanding of the immune defects in patients with MM. The following sections discuss various types of immunotherapeutic strategies under investigation in MM (Chatterjee et al. 2006).

Monoclonal Antibodies

Monoclonal antibodies (MoAbs) target specific molecules and are used as passive immunotherapy to treat various diseases, including certain types of cancers. The recent approval

of MoAbs for clinical use (Multiple Myeloma Research Foundation, URL) have renewed interest in MoAb-based targeted serotherapies in MM. The MRA or atlizumab (humanized anti-interleukin-6 [IL-6] antibody) (Chugai Pharmaceuticals) is being evaluated in open-label phase I and II trials to assess the safety and efficacy in MM. MRA is directed against the human IL-6 receptor (IL-6R). Blockade of IL-6R may prove effective in limiting MM cell growth. It is given as a monotherapy to patients with MM who are not candidates for, or who have relapsed after SCT. The TRM-1 (Fully Human TRAIL-R1 MoAb) (Human Genome Sciences) is directed against a specific receptor on MM cells known as TRAIL (TNF-related apoptosis-inducing ligand). Binding of the MoAb to TRAIL contributes to MM cell death. TRM-1 is being evaluated in a phase I, open-label, dose-escalation trial to assess the safety, tolerability, immunogenicity, and pharmacokinetics in subjects with MM. The AHM (anti-HM1.24 MoAb) is presently in phase I clinical trials (Chugai). HM1.24 is a marker expressed on MM cells. MoAbs to HM1.24 have been shown to induce killing of MM cells in vitro and in vivo. The mKap (PacMab Ltd.) is a murine MoAb that is specific for free human kappa light chains (the small arms of antibody molecules) and a marker on the surface of plasma cells. This antibody induces apoptosis in MM cell lines that expresses the surface antigen KMA in vitro and exhibits antitumor activity in a murine model in vivo.

The SGN-40 (anti-huCD40 MoAb) (Seattle Genetics) is a humanized anti-CD40 MoAb directed against the CD40 receptor on MM cells. Currently, it is being investigated in a phase I, multi-dose trial in patients with refractory or recurrent MM. Tai et al. (2004)

examined the potential therapeutic utility of SGN-40 for treating human MM using MM cell lines and patient MM cells (CD138⁺⁺, CD40⁺). Results showed that SGN-40 induced modest cytotoxicity in the presence of de novo protein synthesis inhibitor cycloheximide, significantly induced apoptosis in dexamethasone (Dex)-sensitive and Dex-resistant MM cells and in patient MM cells. Pretreatment of MM cells with SGN-40 blocked sCD40L-mediated phosphatidylinositol 3'-kinase/akt and nuclear factor- κ B activation. Moreover, SGN-40 suppressed IL-6R expression at both mRNA and protein levels, and importantly, pretreatment of MM cells with SGN-40 inhibited proliferation triggered by IL-6 but not by insulin-like growth factor-1 (IGF-1). SGN-40 had direct anti-MM effects that were unrelated to antibody-dependent cell cytotoxicity (ADCC), highlighting the preclinical rationale for the evaluation of SGN-40 as a potential new therapy.

HCD122 (formerly CHIR-12.12) is a novel, fully human, IgG₁ antagonistic MoAb targeting the CD40 receptor. CHIR-12.12 triggered lysis of MM cells via ADCC, but did not induce ADCC against CD40-negative MM cells, confirming specificity against CD40-expressing MM cells (Tai et al. 2005). The study thus provided the preclinical rationale for clinical trials of CHIR-12.12 in MM. The ongoing phase 1 study will determine the maximum tolerated dose (MTD) of CHIR-12.12 in MM patients who are relapsed or refractory after at least one prior therapy. In preliminary studies, HCD122 appears to be safe and well tolerated to date at doses of 1 and 3 mg/kg weekly for 4 doses and shows promising anti-MM activity. A MoAb to integrin very late antigen-4 (VLA-4) has been reported to affect tumor burden in a

highly aggressive murine model of MM (Olson et al. 2005). MoAb therapy, initiated after the myeloma had homed to and begun to establish itself within the bone marrow compartment, led to statistically significant reductions in (1) circulating levels of IgG2b; (2) percentage of IgG2b-positive MM cells circulating in blood; (3) spleen MM cell burden and spleen weight; and (4) bone marrow MM cell burden. The data suggest that blockade of VLA-4, alone or in combination with other modalities, may have a significant effect in the new treatment strategies for MM and other B-cell malignancies. Carlo-Stella et al. (2007) recently investigated the therapeutic efficacy of the fully human anti-human leukocyte antigen (HLA)-DR MoAb 1D09C3 in MM. Data showed that (1) one fourth of MM patients express HLA-DR on CD138⁺ cells; and (2) IFN- γ -induced up-regulation of HLA-DR and results in a potent enhancement of the in vivo anti-MM activity of 1D09C3.

Anti-IL-6 Therapy

Experimental and clinical findings support the role of IL-6 in cancer and provide a rationale for targeted therapeutic investigations that include IL-6-conjugated MoAbs directed against IL-6 and IL-6R. Bataille et al. (1995) reported the results of MoAb targeting to IL-6 in treating MM patients. Of the ten patients who had advanced and progressive MM with mainly primary or secondary plasma cell leukemia, the seven remaining patients received i.v., MoAb therapy for at least 1 week, with three exhibiting an objective antiproliferative effect as measured by myeloma cell labeling in the bone marrow. One of the three patients showed a 30% regression of tumor mass suggesting that a

MoAb to IL-6 therapy could help patients with early-stage disease. van Zaanen et al. (1998) conducted a phase I/II study based on a dose-escalation analysis of i.v., infusions of a chimerized mouse MoAb to IL-6 (human-mouse cMoAb) with the investigational name CNTO 328 (CCLB8) in patients with end-stage, progressive MM resistant to second-line chemotherapy. Myeloma Institute for Research and Therapy at the University of Arkansas for Medical Sciences (UAMS) and M.D. Anderson Cancer Center (MDACC) at the University of Texas have already initiated phase I trials involving multiple i.v., infusions of CNTO 328 in MM patients for evaluating five different dosing regimens of CNTO 328 to determine which dose/schedule can best be given safely.

Moreau et al. (2000) investigated the potentials of combination therapy, including a murine MoAb to IL-6 (BE-8; 250 mg), Dex (49 mg/day), and high-dose melphalan (220 mg/m² [HDM220]), followed by autologous SCT (ASCT) in the treatment of 16 patients with advanced MM. IL-6 activity was strongly inhibited, as indicated by reduced CRP levels. Overall, 13 of 16 patients (81.3%) exhibited a response, with a complete response seen in 6 patients (37.5%) without any toxic or allergic reactions. The results of another study by Rossi et al. (2005) further showed that (1) BE-8 was able to fully neutralize IL-6 activity in vivo before and after melphalan (HDM: 140 mg/m²) as shown by inhibition of CRP production; (2) there was no occurrence of hematological toxicity; (3) there was a significant reduction of mucositis and fever; along with (4) a median event-free survival (EFS) of 35 months and an overall survival of 68.2% at 5 years with a median follow-up of

72 months; and (5) the overall daily IL-6 production progressively increased on and after 7 days post-HDM, with the increased serum CRP levels. In 5/24 patients with uncontrolled CRP production, a large IL-6 production was detected (320 µg/day) that could not possibly be neutralized by BE-8. These data showed the feasibility to neutralize IL-6 in vivo with BE-8 in the context of HDM.

Another study by Lu et al. (1995) demonstrated that BE-8 could not efficiently block daily production of IL-6 at a level >18 µg/day. This particular study observed an inverse correlation between clinical response and daily production of IL-6 during treatment if production exceeded 18 µg/day. This confirms the importance of calculating this particular parameter for optimizing an anti-IL-6 dosing strategy. It was therefore suggested that the cMoAb CNTO 328 with an 18-day half-life might be beneficial for chronic administration. Moreau et al. (2006) recently reported that, in high-risk MM patients, the dose intensity of melphalan at 420 mg/m² led to encouraging results, but the addition of BE-8 to the second conditioning regimen, however, did not improve progression-free survival (PFS)/EFS or overall survival.

Alternative approaches have been developed by using humanized anti-IL-6R MoAb (rhPM-1, IgG1 class). PM1 is currently tested in phase I/II trials in patients with MM. Other approaches include the combination of anti-IL-6 or anti-IL-6R MoAbs that shorten the half-life of the IL-6/IL-6R complexes (from 4 days to >20 min) in vivo, in addition to the formation of polymeric complexes instead of monomeric complexes, a situation compatible with increased clearance of these IL-6/IL-6R complexes. Administration of

anti-IL-6 MoAbs resulted in inhibition of CRP production below detection limits as well as neutralization of IL-6 production in vivo transiently inhibiting MM cell proliferation. However, many patients had severe disease and produced huge amounts of IL-6, making anti-IL-6 MoAb unable to completely and/or very efficiently neutralize them. Considering the risk of immunization against the murine anti-IL-6 MoAb, humanized anti-IL-6 MoAb might prove useful, especially for treatment of patients with earlier stage disease (Tripathi et al. 2003). Because IL-6 is mainly a survival factor rather than a proliferation factor for human MM cells and blocks Dex-induced apoptosis, a main issue in MM will be to use anti-IL-6 MoAb therapy to potentiate tumor killing by various drugs, including Dex or high-dose chemotherapy.

It appears trivial that in a complex disease, such as MM, growth cannot be sufficiently reduced by blocking a single cytokine signal, which suggests that multidrug regimens may be useful as alternative therapies to improve the poor outcome of MM patients. Honemann et al. (2001) analyzed the effects of the IL-6R antagonist SANT-7 on growth and survival of IL-6-dependent MM cell lines as well as primary MM cells from seven patients. In particular, the study was aimed to find out whether SANT-7 enhances the growth-inhibitory effects of Dex and all-trans retinoic acid (ATRA). When Dex and ATRA were given in combination with SANT-7, strong growth-inhibition was achieved in cell lines and primary MM cells owing to cell-cycle arrest and induction of apoptosis. Thus, combining SANT-7 with additional drugs could be a useful approach to the treatment of MM.

Tassone et al. (2002) have shown that SANT-7 significantly enhanced growth inhibition and apoptosis in both MM cell lines and primary MM cells. The study indicated that overcoming IL-6-mediated cell resistance by SANT-7 potentiates the effect of glucocorticoids and bisphosphonates on MM cell growth and survival, providing a rationale for therapies including IL-6 antagonists in MM. Another study by the same group (Tassone et al. 2005) demonstrated that in a novel murine model of human MM in which IL-6-dependent INA-6 MM cells were directly injected into human bone marrow implants in severe combined immunodeficient (SCID) mice (SCID-hu), inhibition of IL-6 signaling by SANT-7 significantly potentiates the therapeutic action of Dex against MM cells. This provides the preclinical rationale for clinical trials of SANT-7 in combination with Dex to improve patient outcome in MM. Yoshio-Hoshino et al. (2007) reported the potentials of a new receptor inhibitor of IL-6 (NRI) by genetically engineered tocilizumab, a humanized anti-IL-6R MoAb that specifically blocks IL-6 signaling. An adequate amount of the serum NRI level to exert anti-IL-6 action could be obtained by the NRI gene introduction combined with adenovirus gene delivery, and this treatment significantly inhibited the growth of S6B45, an IL-6-dependent MM cell line in vivo. These findings indicate that NRI is a promising agent applicable to the therapeutic gene delivery approach for IL-6-driven diseases.

Strategies for Targeting IGF-1

Besides IL-6, the IGF-1 signaling pathway is also implicated in cellular mitogenesis, angiogenesis, tumor cell survival, and

tumorigenesis. Inhibition of this pathway results in decreased cell growth, inhibition of tumor formation in animal models, and increased apoptosis in cells treated with cytotoxic chemotherapy. Strategies targeting IGF-1 and IGF-1 receptor (IGF-1R) may therefore be important to the development of efficient anti-MM agents. Studies have shown the functional association of IGF-1R and β 1 integrin in mediating MM cell homing, providing the preclinical rationale for novel treatment strategies targeting IGF-1/IGF-1R in MM. In one study, synergistic combination of rapamycin (Rapamune), a specific mTOR inhibitor with Revlimid (CC-5013) was able to overcome drug resistance as well as the growth advantage conferred on MM cells by IL-6, IGF-1, or adherence to bone marrow stromal cells (BMSCs) and also induced apoptosis of MM cells (Raje et al. 2004). Yasui et al. (2005) have further demonstrated the potential cytotoxicity and apoptosis-inducing effects of the novel immunomodulator FTY720 on MM cells and MM patient samples. Neither IL-6 nor IGF-1 (both of which induce MM cell growth and abrogate dex-induced apoptosis) protected against FTY720-induced growth inhibition. FTY720 further down-regulated IL-6-induced phosphorylation of Akt, signal transducers and activators of transcription 3 (STAT), and p42/44 mitogen-activated protein kinase (MAPK); IGF-1-triggered Akt phosphorylation; and TNF- α -induced I κ B α and NF- κ B p65 phosphorylation. These studies, thus, provide the rationale for clinical evaluations of mTOR inhibitors combined with IMiDs as well as FTY720 in MM.

Recent studies of Menu et al. (2006) showed that, IGF-1R tyrosine kinase (IGF-1RTK) inhibitor picropodophyllin (PPP)

possesses a marked antitumor activity and strongly points to the possibility of using IGF-1R inhibitors in the treatment of MM. CP-751,871, a fully human anti-type 1 IGF-R IgG2 antibody (Cohen et al. 2005), both as a single agent and in combination with adriamycin, 5-fluorouracil, or tamoxifen showed significant antitumor activity in vivo. CP-751,871 further blocked the binding of IGF-1 to its receptor, IGF-1-induced receptor autophosphorylation and induced down-regulation of IGF-1R in vitro and in tumor xenografts. It is currently being investigated in phase I trials in patients with relapsed and refractory MM.

Immunotoxins and Immunoconjugates

The direct linking of MoAbs with drugs, toxins, or radionuclides to specifically target cancer cells has been widely studied. The use of these chimeric and humanized MoAb-based immunoconjugates, that are relatively nonimmunogenic with high affinity for tumor-associated antigens and are efficiently internalized into cells once they bind to the target antigen, have the potential to both improve antitumour efficacy and reduce the systemic toxicity of therapy. Immunotoxins are bifunctional protein molecules consisting of a MoAb chemically conjugated or genetically fused to a protein toxin. MoAbs are selected for immunotoxins based on their reactivity towards cell surface receptors or antigens that are preferentially expressed on malignant cells. After MoAb-mediated internalization, the toxin portion of immunotoxin traffics into the cytosol, where the enzymatic activity innate to the toxin catalytically inhibits protein synthesis resulting in cell death. Immunoconjugates that deliver doxorubicin, maytansine or calicheamicin are currently being evaluated in clinical trials

for targeted treatment of cancer. Several immunoconjugates, particularly those that incorporate internalizing antibodies and tumor-selective linkers have demonstrated impressive activity in preclinical models, MM cell lines, and MM patients' sample (Francisco et al. 1997; Sapra et al. 2005).

Maytansine derivatives represent a new class of highly cytotoxic agents suitable for conjugation with MoAbs. Maytansine is a natural product, originally derived from the Ethiopian shrub *Maytenus serrata*, which inhibits tubulin polymerization, thereby resulting in mitotic block and cell death. The activity of maytansine is ~200–1,000-fold greater than that of the *Vinca* alkaloids, which exert their cytotoxic potential by a similar mechanism. Because of their extremely high potency, maytansinoid derivatives are presently of great interest. Several other related conjugates progressing toward (or undergoing) clinical trials are bivatuzumab mertansine (DM1-Anti CD44V6 MoAb), huN9-01-DM1 (BB-10901), trastuzumab-DM1, MLN591-DM1, MLN2704-DM1, and My9-6-DM1 targeted against the CD33 antigen on myeloid cells.

HuN901 is a humanized MoAb that binds with high affinity to CD56, the neuronal cell adhesion molecule. This antibody covalently conjugated with the novel, cytotoxic maytansinoid N²-deacetyl-N²-(3-mercapto-1-oxopropyl)-maytansine (DM1) (a potent antimicrotubular cytotoxic agent) may provide targeted delivery of the drug to CD56 expressing tumors. Based on the preliminary results of a study by Tassone et al. (2004b) BB-10901 has significant in vitro and in vivo anti-MM activity against CD56(+) MM cells. Chanan-Khan et al. (2006) have currently initiated a phase I clinical study to determine

the MTD, dose-limiting toxicities, and pharmacokinetics of BB-10901 given on a weekly schedule in CD56-expressing relapsed or relapsed and refractory MM patients who have failed at least one prior therapy. This phase I study provides preliminary evidence of safety and clinical activity of BB-10901 in patients with CD56-positive MM who have failed with established MM treatments. Tassone et al. (2004a) have further showed the in vitro and in vivo antitumor activity of the immunoconjugate B-B4-DM1 against a panel of CD138(+) and CD138(–) cell lines, as well as CD138(+) patient MM cells. Based on the data obtained, the authors concluded that B-B4-DM1 is a potent anti-MM agent that kills cells in an antigen-dependent manner in vitro, and mediates in vivo antitumor activity at doses that are well tolerated, providing the rationale for clinical trials of this immunoconjugate in MM.

Radioimmunoconjugates

Targeted radioimmunotherapy (RIT) has proved efficient in the treatment of radiosensitive tumors, such as lymphomas and MM, but it is less suitable for more radio-resistant solid tumors. Among several methodologic approaches intended to improve RIT efficacy under evaluation, one involves optimizing the use of radionuclides, for instance, by replacing iodine 131 (¹³¹I), which has been widely used until 2002, with a more suitable radioisotope, such as yttrium 90. This isotope is particularly useful for lymphomas that are often treated only when the tumor mass has become large. In the case of MM, cells are found either isolated in bone marrow or in small clusters. Among the different beta-emitters available, it would be logical to choose ¹³¹I for the treatment of MM, because the energy of beta particles

emitted is distributed within a range of ~1 mm, whereas more energetic beta emitters (yttrium-90, rhenium-186, and -188) deliver their energy over a greater range. However, the notion of energy distribution is not the only parameter to consider; high gamma emission, physical half-life, or the coupling technique used can make the choice of a suitable radionuclide more complicated. For instance, in MM, alpha particles have a theoretic advantage over beta particles because of their high linear energy transfer and shorter range of action. Presumably, cell destruction would be more selective and irradiation less harmful to adjacent tissues.

RIT in MM causes intense, prolonged blockade of the cell-cycle that maintains the cells in a more radiosensitive state, which accounts for greater cell destruction. Using a specific MoAb, B-B4 coupled to bismuth-213 ((213)Bi) by a chelating agent (CITC-DTPA), the feasibility of alpha-RIT for MM has been demonstrated. The two MoAbs tested, MA5 and B-B4, targeted the epithelial antigens Muc-1 and syndecan-1, respectively, which are both expressed by MM cell lines. Supiot et al. (2002) assessed in vitro that MA5 stained all MM cells in only 50% of patients, whereas B-B4 recognized all MM cells in all the patients. The study indicated that B-B4 might be the more effective therapeutic tool for MM and further suggested that alpha-RIT might be more suitable than beta-RIT for treating single-cell tumor models. Thus, these findings set the stage for the beginning of phase I/II clinical trials using alpha-emitter-radiolabeled B-B4, with special attention paid to hepatic, pulmonary and intestinal side effects. Furthermore, the continued over-expression of syndecan-1, the target of

B-B4 in progressive MM, suggests that B-B4-alpha-RIT would be effective even in the treatment of refractory forms of the disease. Furthermore, in a recent report, the same group (Supiot et al. 2005) analyzed various cellular mechanisms that might underlie potential synergy between chemotherapy and RIT with alpha radionuclides. The authors concluded that, both cell cycle arrest in the G2-M phase and an increase in DNA double-strand breaks could lead to radiosensitization of MM cells by doxorubicin or paclitaxel, but apoptosis would not be involved in radiosensitization mechanisms.

Myeloma Vaccines

Vaccines are being developed to treat MM rather than prevent it. Several of the vaccines being investigated in MM are active vaccines (Multiple Myeloma Research Foundation, URL). These vaccines are developed using the patient's own MM cells or proteins unique to the MM cell. They are usually combined with a stimulus or adjuvant that increases the body's own immune response. The patient is then immunized with this myeloma vaccine to stimulate his or her immune system to fight the disease. Because vaccines are targeted to the tumor cells, they have the potential to be more specific and less toxic to the patient than conventional therapies. Idiotypes, the unique portions of a patient's monoclonal protein, offer a unique target for vaccination because each idotype is unique to a patient's MM. A number of studies have investigated the administration of idotype protein vaccines to patients with minimal disease following ASCT. Because immune responses against idiotypes are often weak, patient-specific idiotypes are often administered with an adjuvant.

Dendritic cell vaccines utilize dendritic cells that play an important role in initiating and regulating immune responses. These vaccines are combined with myeloma idiotypes, other myeloma proteins, or stimulatory compounds, or are fused with entire MM cells. It is thought that these cells can help elicit strong anti-MM responses. Cellular vaccines utilize the patient's MM cells to stimulate an immune response. DNA vaccines are composed of fragments of DNA that encode specific myeloma proteins or markers and substances to help further activate the immune system. The result is that the expressed protein is then presented in a more natural and effective way. One type of DNA vaccine being developed at the Tenovus Laboratory, Southampton, UK utilizes the specific idotype of a patient's MM. To enhance the ability of the patient's immune system to respond to the idotype, a sequence from a bacterial product is added. The bacterial product selected is derived from the safe vaccine already used against tetanus toxin. The result of this fusion is a dramatic enhancement of the immune response against the idotype. Viral vector vaccines use a virus to transfer specific genes into a patient's cell. The viral genes stimulate the production of a variety of growth factors that enhance the immune response against the tumor cell. Delivery via bacterial vectors can also help to activate immunity, especially in the gut or lung. A further possibility is to combine the various gene-based vaccines by first injecting a DNA vaccine, followed by a viral vector delivery of the same tumor-associated antigen.

Growth Factors

A variety of hematopoietic growth factors are used therapeutically in MM (Multiple Myeloma Research Foundation, URL). In

some cases they are used as part of general supportive care as adjuncts to other therapies. In other cases they are a critical component of the SCT process. Growth factors used in MM include erythropoietin, a substance that stimulates the production of red blood cells, and CSFs, substances that stimulate the production of white blood cells. One of the most common and debilitating effects of MM is fatigue, which often occurs as a result of anemia. Erythropoietic agents are used to treat anemia because they stimulate erythropoiesis in the bone marrow. Besides erythropoietin, there are several commercially available erythropoietic agents, viz., Procrit, Epogen, and Eprex are the same substance (epoetin alfa) while NeoRecormon is slightly different (epoetin beta). Aranesp® (darbepoetin alfa, Amgen, Inc.) has the same molecular make-up as natural erythropoietin and acts in the same way, but has two additional carbohydrate chains to give it a longer half-life. It is referred to as erythropoiesis-stimulating protein.

MM patients may have low numbers of white blood cells as a result of chemotherapy or as a result of MM cells crowding out the normal blood-producing cells in the bone marrow. CSFs stimulate production of infection-fighting white blood cells. Examples of G-CSFs available in the United States include filgrastim (Neupogen®) and pegfilgrastim (Neulasta®). An example of GM-CSF is sargramostim (Leukine®). Each varies slightly in its effect in the body and in the indications in which they are marketed for usage. Mozobil™ (plerixafor, AMD3100, AnorMED) is an investigational agent that increases the number of stem cells that can be collected prior to transplant. By blocking CXCR4, a specific cellular receptor, Mozobil triggers

the rapid movement of stem cells out of the bone marrow and into the circulating blood. The efficacy of a mobilization regimen of Mozobil in combination with G-CSF is currently being compared with G-CSF alone in phase III trials in MM patients undergoing ASCT.

Manipulation of Immune Cells (Cell Therapy)

Another example of immunotherapy is the manipulation of immune cells to help improve outcome and reduce the rate of relapse. A number of diverse strategies involving immune cells are being investigated in MM (Multiple Myeloma Research Foundation, URL). One experimental technique that harnesses the beneficial effects of immune cells is known as donor lymphocyte infusion (DLI). This technique is used following high-dose chemotherapy and allogeneic SCT, a type of transplant whereby a patient receives stem cells from a matched donor. During the process of allogeneic SCT, donor immune cells present in the transplant help attack the patient's MM cells, a phenomenon known as graft-versus-myeloma effect. With a DLI, the patient receives additional immune cells from the same donor some time after the SCT. It is thought that this additional infusion of immune cells can help continue to control the disease.

Another technique for manipulating immune cells in MM is being investigated by Xcyte Therapies, Inc. (Seattle). They have developed a process of collecting, stimulating, and growing patient T cells referred to as Xcellerated T Cells™, which mimic host immunology. Xcellerated T cells are being investigated in MM in several ways: as a single therapy in combination with chemotherapy or following high-dose chemotherapy and SCT. A phase II study is underway to evaluate the efficacy and safety

of Xcellerated T Cells with or without fludarabine (a chemotherapy drug) in treating patients who have relapsed or refractory MM. Another technique under investigation in a National Institutes of Health clinical research study is active immunization of the donor who will be providing stem cells for a certain type of allogeneic SCT. The donor in this case is the patient's tissue-matched sibling, who is immunized with the patient's purified myeloma protein along with GM-CSF. It is hoped that the donor will develop tumor-specific immunity. The patient, who is also immunized, then receives a non-myeloablative (mini-allogeneic) transplant, which contains stem cells from the immunized donor. Preliminary results reported at the American Society of Hematology (ASH) in 2004 confirm that such vaccines can be safely and effectively given to normal stem cell donors and suggest that tumor-specific immunity can be induced and passively transferred. Another means of attacking MM is to generate an immune response outside the body and infuse it back into the patient. One experimental technique being investigated at Johns Hopkins involves harvesting immune cells from a patient's bone marrow. These immune cells, known as marrow infiltrating lymphocytes (MILS), appear to be more effective in killing MM cells than immune cells circulating in the blood. MILS are stimulated and cultured in the laboratory and then returned to the patient. Because it involves the transfer of cells, this type of passive immunotherapy is also referred to as adoptive immunotherapy.

Thalidomide and Immunomodulatory Drugs

Thalidomide, initially introduced for treatment of MM because of its anti-angiogenic properties, has shown remarkable activity

alone and in combination with other drugs in patients across all stages of the disease. The precise mechanism of anti-MM activity of thalidomide is not clear, but studies suggest that besides its potent anti-angiogenic activity, it appears to exert multiple effects which inhibit the growth and survival of MM cells (Kumar and Rajkumar 2006). These include (1) inhibition of the growth and survival of stromal cells, tumor cells and cells in the bone marrow; (2) altering production/activity of cytokines through inhibition of cyclooxygenase-2 and TNF- α , downregulation of IL-6, increased production of IL-10, IL-4, IL-5, and IL-12, and inhibition of TNF- α -induced IL-8; (3) altering the expression of cell adhesion molecules; and (4) stimulation of T-cells, which help the immune system to directly attack MM cells. Thalidomide has been investigated in numerous clinical trials (Kumar and Rajkumar 2006) as a single-agent or as part of combination therapy with potent anti-MM chemotherapeutic agents, such as Dex, melphalan, prednisone, or in combination with multiple other drugs, steroids, and alkylators in patients with relapsed and refractory MM. It has also been evaluated in patients with newly diagnosed MM/smoldering MM, and as maintenance/consolidation therapy.

Revlimid® (lenalidomide, formerly known as Revimid), is the first of Celgene's new class of oral cancer drugs called "immunomodulatory drugs" (IMiDs). These immunomodulatory derivatives are chemically similar to thalidomide but are more potent in the laboratory and have a different side effect profile than thalidomide. Lenalidomide (CC-5013) and Actimid (CC-4047) are second generation analogues of thalidomide that share a similar chemical structure. They have multiple mechanisms of action that

affect both the cancer cell and its microenvironment. In 2006, Revlimid was approved for use in combination with Dex as a treatment for patients with MM who have received at least one prior therapy. Revlimid-Dex is now listed as a recommended option for relapsed and refractory MM in the National Comprehensive Cancer Network® (NCCN) Clinical Practice Guidelines for MM. Revlimid-dex has also been examined in a number of newly diagnosed patients and, as a result, is also listed in the NCCN guidelines as a recommended option for initial therapy in patients who are candidates for SCT. Revlimid has also been evaluated in combination with other novel agents, including bortezomib (Velcade®, Millennium) in relapsed, refractory MM.

Radiotherapy

Radiotherapy has been a major component of anti-MM therapy and palliative care in MM patients. Pain symptoms caused by bone lesions can be relieved by a local irradiation treatment. Fifty-nine patients with MM referred for treatment of painful bony lesions received irradiation to 95 local areas, and 16 of the 59 were irradiated using hemibody techniques (Bosch and Frias 1988). Pain relief was obtained in practically all of the irradiated regions. Most local areas were treated to doses of 3,000 cGy in 10 to 15 fractions. Patients with generalized pain due to multiple site involvement were treated with single dose hemibody irradiation to doses of 600 cGy to the upper hemibody and of 800 cGy to the lower hemibody. This treatment was well tolerated and side effects were minimal. Median survival from diagnosis was 30 months and the survival at 1, 3, and 5 years was 80%, 42%, and 12%, respectively.

Adamietz et al. (1991) reviewed the records of 70 MM patients treated with chemotherapy combined with or followed by local irradiation to estimate the influence of systemic treatment on the palliative effect of local radiotherapy. The local response rate, defined as complete pain relief at the irradiated site, was 80% in patients receiving irradiation during chemotherapy (melphalan and prednisone) and this palliative effect endured 31.8 ± 3.6 months. If irradiation was started in the period without systemic treatment, the local response rate was 39.6% and lasted 24.8 ± 17.9 months. In sites treated with more than one radiotherapy course, 94% response rate after the first treatment, 56% after the second treatment, and no response after the third course was achieved. The duration of local pain control was positively related to the applied radiation dose. It was concluded that irradiation during concomitant chemotherapy is superior to radiotherapy performed in a period without systemic treatment. Local long-term palliation can only be achieved by a sufficient high radiation dose. Occasional patients with more extensive bone pain may benefit from more extensive hemibody irradiation.

In another study, immediately before first hemi-body irradiation, 59 patients with relapsed MM were randomized to receive or not to receive subsequent interferon alpha-2b maintenance (Giles et al. 1992). Thirteen patients (22%) received single hemi-body irradiation alone due to progressive disease and/or persistent cytopenias following the initial procedure. Of 23 patients randomized to receive interferon and completing double hemi-body irradiation, 15 (65%) achieved peripheral blood counts adequate to allow interferon administration as per study criteria commencing

at a mean 116 days (61–241) from time of study entry. The mean period of interferon therapy, starting at a mean 65 days (26–160) post second hemi-body irradiation, was 16.4 months (2–33.5). There was no significant difference in median survival durations (10 months) from time of initial radiotherapy between control and interferon patients. In patients with refractory disease, sequential hemi-body radiation can be used to temporarily control the disease. This is rarely successful for very long, particularly in patients with aggressive, active MM. Other indications for radiotherapy include treatment of impending or actual pathologic fractures, spinal cord compression, tumor causing local neurologic problems, and large soft tissue plasma cell tumors.

Radiotherapy has also been shown to prevent the development of new vertebral fractures in MM patients (Lecouvet et al. 1997). In this small nonrandomized study, there was a suggestion that less vertebral fractures occurred in irradiated vertebrae than in nonirradiated ones as assessed by MRI. However, MM is known to be very susceptible to radiation treatment. The major disadvantage is that radiation therapy permanently damages normal BMSCs in the area of treatment. The importance of this point cannot be overemphasized in a patient whose overall clinical status depends upon the use of agents that cause loss of bone marrow function such as chemotherapy. Wide field radiation encompassing large amounts of normal bone marrow should therefore be avoided, because this type of destruction makes it difficult, if not impossible, to use other treatment options following this approach. A general strategy is to rely on systemic chemotherapy to achieve overall disease control, limiting the use of local radiation therapy to areas with particular

problems. Therefore, it is advisable to radiate the minimal portion of the shaft of a long bone affected by MM to preserve as much functional marrow as possible.

Total body irradiation (TBI) is an important part of bone marrow transplantation (BMT) conditioning regimens. TBI has played an important role in conditioning regimens for patients undergoing hematopoietic SCT. The primary reasons for using TBI include tumor cell eradication and immunosuppression to allow for engraftment of donor marrow. In addition, TBI, acting as a form of “systemic radiotherapy,” potentially complements high-dose systemic chemotherapy, providing therapy to sanctuary sites not easily reached by chemotherapy drugs, and provides another mechanism of tumor cell kill against chemotherapy-resistant cell clones. TBI or sequential radiation of half of the body followed by hematopoietic system rescue by BMT has been used as part of an overall strategy in Intergroupe Francophone du Myelome (IFM) trials to improve the response of patients with MM to high-dose therapy with melphalan and/or in the management of relapsing/refractory disease (Moreau et al. 2002). Although TBI was used in the past as a preparatory regimen for transplant as a standard therapy, treatment-related morbidity and mortality have increased, negating any potential advantage for survival. Recent studies have also shown no added benefit and, unfortunately, with additive toxicity of the two modalities. Therefore, TBI is no longer recommended as part of preparatory regimens.

Targeted Radiotherapy

It has been known for at least 100 years that radiation can kill cancer cells, but its original mode of therapeutic delivery which uses a beam via an external source

requires knowing the anatomic location of the tumor. Metastases can occur at multiple sites, and even when the location of a tumor is known, healthy tissues in the line of fire can suffer collateral damage. In contrast to this conventional radiotherapy, an outside-in approach – targeted radiotherapy-systemic administration of radioactive agents that home in on a particular tissue, antigen, or receptor type proceeds from the inside out (Multiple Myeloma Research Foundation, URL). Several radiolabelled compounds have been shown to be effective in early phase clinical trials as well as in preclinical studies. To be effective by themselves, therapeutic agents have to be potent and penetrating, and they need to kill only those cells that express large amounts of the target molecule. As a component of targeted radiotherapy, however, a MoAb is merely a delivery vehicle. The attached radioisotopes do the killing, and their emissions penetrate tumors reaching diseased cells that may or may not express the target antigen. But antibodies are large; whereas smaller molecules travel far away, penetrate tumor tissue better and are excreted much more swiftly when released into circulation. Research efforts are now shifting to these smaller molecules to better exploit the effects of radioisotopes. Some of these newer radiopharmaceuticals often conjugated with newly tamed radioisotopes/radionuclides are working satisfactorily than their older counterparts for targeting cancer cells and providing pain relief, and some are even showing promise as potential therapeutic agents alone or in combination with other chemotherapeutic agents.

Bone Seeking Radiopharmaceuticals

Representatives of the bone-seeking radiopharmaceuticals readily bind to sites where

new bone is being deposited, a frequent reaction to the presence of a metastatic lesion. Phosphonate chelates are taken up in the skeleton and can be combined with radioactive isotopes to deliver high levels of radiation to bone and bone marrow while sparing normal tissues. In adults, pronounced bone formation occurs almost exclusively at sites of metastases. In the 1980s, strontium-89 (MetaStron; Albersham), a radioactive calcium analog was approved for pain palliation. Indeed, like chemotherapy, strontium-89 is itself myelosuppressive. It suppresses bone marrow proliferation.

In the late 1990s, the FDA approved Quadramet ($^{153}\text{Sm-EDTMP}$; lexidronam), a short-range, beta-emitting, radioisotopic pharmaceutical with avid skeletal uptake with the aim of targeting potentially ablative radiation dose to bone marrow. This radiopharmaceutical consists of 1:1 radioactive samarium-153 chelated by and forming a stable complex with a bone-seeking ethylenediaminetetramethylene phosphonate (EDTMP). $^{153}\text{Sm-EDTMP}$ concentrates by chemi-absorption and it associates with the hydroxyapatite crystal in areas of enhanced metabolic and increased osteoblastic activities, with the tumor-to-normal bone ratio being 4:1–7:1. Clearance is biexponential after i.v., administration, comprising rapid bone uptake (half-life = 5.5 min) and plasma renal clearance (half-life = 65 min). Fifty percent of injected activity is excreted renally within 8 h of administration. Total skeletal uptake ranges between 55% and 75%, depending on the skeletal tumor burden (Lewington 2005). The rapid depletion in residual radioactivity or very rapid clearance from nonosseous tissues means that patients can move quickly into chemotherapy. It also means that those with

diminished blood cell counts can, in short order, be given hematopoietic agents, such as recombinant erythropoietin or GM-CSF without risking high mutation rates in newly burgeoning blood cells. Several preclinical and clinical investigations and phase trials have demonstrated the potential therapeutic efficacy of Quadramet in MM (Iuliano et al. 2003; Dispenzieri et al. 2005).

Skeletal Targeted Radiotherapy

Skeletal Targeted Radiotherapy (STRTM) is a novel experimental approach (Multiple Myeloma Research Foundation, URL) based on the use of Holmium-166 1, 4, 7, 10-tetraazacyclododecane-1, 4, 7, 10-ethramethylenephosphonate ($^{166}\text{Ho-DOTMP}$) which is a radioactive bone-seeking drug. Because most MM cells are found in the bone marrow and are sensitive to radiation, STR may be effective in the treatment of MM with high-dose chemotherapy and peripheral blood SCT (PBSCT).

Mechanism of Action of STR

$^{166}\text{Ho-DOTMP}$ consists of two components that are bound together. The first component is DOTMP, a phosphate group that attaches to bone, particularly in areas of rapid bone turnover that occur in MM and various bone cancers. The other component of $^{166}\text{Ho-DOTMP}$ is the radioactive holmium (^{166}Ho). The high energy and long path length of radionuclide ^{166}Ho -beta particles provide deep penetration and uniform irradiation of disease sites in the bone and its relatively short half-life of 26.8 h permits reinfusion of stem cells within 6 to 8 days. It also has a minor gamma component suitable for imaging and dosimetry. These properties make $^{166}\text{Ho-DOTMP}$ a potentially useful drug for delivering a bone-based dose of targeted radiation for

the treatment of cancers in the bone and bone marrow. By selectively targeting the radiation to the site of disease, the exposure to normal organs can be reduced compared to conventional TBI.

Skeletal targeted radiotherapy, in combination with high-dose chemotherapy and ASCT is designed to produce both a direct therapeutic effect on tumor sites in the bone, plus a general bone marrow-ablative effect to help destroy tumor cells in the bone marrow. Because STR causes marrow ablation, it can be used as a preparative regimen for PBSCT. For this reason, before receiving STR therapy, patient's PBSCs are collected and stored for later reinfusion. Then a small dose of ^{166}Ho -DOTMP (a "tracer") is injected intravenously during 1 to 17 min, and tests are done to determine if enough ^{166}Ho -DOTMP reaches the bone. If so, the patient is treated with a larger dose of STR, along with high-dose chemotherapy (melphalan). ^{166}Ho -DOTMP that is not localized in the bone is rapidly excreted from the kidneys and bladder. Patients are treated in shielded rooms and remained shielded until their activity decreases to institutional radiation safety standards. The time in a shielded room generally lasts from 6 to 24 h depending upon the amount of radioactivity delivered and the safety requirements of the institution.

Skeletal targeted radiotherapy is followed by high-dose chemotherapy with the drug melphalan. Most of the potential side effects of transplantation are the result of high-dose chemotherapy. The more common side effects include nausea, vomiting, fatigue, diarrhea, mouth sores, skin rash, and hair loss. Because high-dose chemotherapy destroys normal blood-producing cells in the bone marrow, patients are susceptible to infection, anemia, and bleeding until engraftment of the new

stem cells has occurred. Stem cell transplantation is not appropriate for all patients due to the toxicities and risks involved. Patients over the age of 65–70 years, or those with a poor performance status or even minimally impaired kidney function may not be eligible for this type of therapy within a clinical trial.

Clinical Trials of ^{166}Ho -DOTMP

Phase I/II Dosimetry Study

The two phase I/II trials (Multiple Myeloma Research Foundation, URL; Giralt et al. 2003) were conducted at the MDACC, Fred Hutchinson Cancer Research Center, University of Miami Medical Center, Miami, FL, University of Washington Medical Center, and NeoRx, Seattle, WA to evaluate the MTD (escalating doses) of ^{166}Ho -DOTMP in combination with various melphalan transplant-conditioning regimens (as part of a myeloablative preparative regimen) for ASCT in MM. Melphalan was selected because it is one of the most active agents in MM; it is well-tolerated and is frequently used either alone or in combination with TBI as a preparative regimen for ASCT. A subset of patients, enrolled in a phase I/II dose escalation myeloablative trial received a 30 mCi (1.110 Gbq) tracer dose of ^{166}Ho -DOTMP to assess skeletal uptake and to calculate a patient-specific therapeutic dose to deliver a nominal radiation dose of 20, 30, or 40 Gy to the bone marrow. The average amount of administered radioactivity was 74.3 GBq (2,007 mCi) (range, 21.5–147.5 GBq [581–3,987 mCi]) of ^{166}Ho -DOTMP. Administered activity $<27.8 \text{ GBq/m}^2$ (750 mCi/m²) or not exceeding 55.5 GBq (1,500 mCi) was found to be safe, based on retrospective analysis of those dose escalation phase I/II trials, following patients for more than 4 years.

A total of 83 MM patients were treated with STR in combination with high-dose chemotherapy (melphalan) followed by ASCT 6 to 10 days later in the two phase I/II clinical trials. Positive clinical activity of STR was noted. Of the patients, 81 had rapid and sustained hematologic recovery, and 2 died from infection before day 60. Many (64%) of these patients had substantial response rates after the treatment, and 35% of them had a complete response (no detectable disease as determined by absence of monoclonal protein or M component in two consecutive immunofixation electrophoresis assays performed at least 6 weeks apart) as well as 91% achieved disease stabilization or better. With a minimum follow-up of 23 months, the median survival had not been reached and the median EFS was 22 months. The complete response and EFS rates compared favorably to other published transplantation regimens.

Long-term safety and efficacy data from this study were presented at the American Society of Hematology (ASH) meeting in 2003 (Giralt et al. 2003). Analysis of the data showed that a STR dose of 750 millicuries of radiation per square meter of body surface area (mCi/m^2) provided optimal patient safety and tumor response (this is the dose being evaluated in the current phase III trial). Of the 83 evaluable patients in the study, 10 had received this dose. Among these ten patients, disease control (i.e., disease stabilization or better) was achieved in eight patients, including four who achieved a complete response, and the 3-year survival rate was 90%. At this dose, there were no reported cases of TTP/hemolytic uremic syndrome (TTP/HUS) or Grade 4 renal toxicity. In 2004, the 4-year survival rate for these ten patients was reported to be 70%. These

trials demonstrated the feasibility of STR with ^{166}Ho -DOTMP as part of a standard preparative regimen with either $200 \text{ mg}/\text{m}^2$ melphalan or $140 \text{ mg}/\text{m}^2$ melphalan and 800 cGy of TBI. As TBI has been associated with higher rates of regimen-related toxicity, future trials are being designed to determine the safety and efficacy of ^{166}Ho -DOTMP without TBI. The data from this study suggested that ^{166}Ho -DOTMP in addition to a standard preparative regimen can result in a high rate of complete remissions in MM patients undergoing autografts.

Although no engraftment delays were seen even at the highest doses in phase I/II studies, higher absolute exposure to critical nontarget organs, such as the kidney and the bladder, was observed and likely contributed to the late, nonhematologic toxicities that developed. Follow-up of these patients revealed 3 main late toxicities: grades 2 to 3 hemorrhagic cystitis (defined by the National Cancer Institute [NCI] Common Toxicity Criteria as greater than grade 2 haematuria with or without associated symptoms) with onset from 1 to 45 months after ^{166}Ho -DOTMP therapy, thrombotic microangiopathy (TMA) of the kidney and late renal dysfunction, and myelodysplastic syndrome (Giralt et al. 2003). Some patients experienced serious bladder problems, that included grade 1 or higher hematuria, hemorrhagic cystitis, diffuse erythema (particularly around the ureteral outlets), bladder neck obstruction, radiation-induced nephritis, and relapses in the bladder after receiving the drug. Almost all of the patients who experienced bladder toxicities did not have a procedure called "continuous bladder irrigation" (CBI) that helps flush extra radioactivity from the body. In all current and future STR trials, patients will undergo CBI and receive i.v.,

fluid infusions to assist in clearing excess radiation from the kidneys and bladder.

A few patients who experienced bladder problems also experienced renal dysfunctions including renal toxicity of grade 3 or higher at a dose of more than 40 Gy targeting the marrow. Moreover, a severe form of sustained renal impairment associated with microangiopathic hemolytic anemia, thrombocytopenia, uncontrolled hypertension, elevated lactate dehydrogenase, elevated creatinine levels and TMA consistent with a radiation nephropathy were also observed. A small number of these patients died of kidney failure. Most of these problems occurred in patients who received the highest doses of STR, which are no longer used. However, no grade 4 kidney toxicity was observed at activity levels less than 55.5 MBq, and only one case of grade 3 toxicity was observed in a patient with progressive MM. TTP/HUS occurred in patients receiving doses of STR that are higher than the dose being evaluated in the phase III trial and was possibly caused by a high rate of radiation exposure to the kidney immediately after ^{166}Ho -DOTMP infusion. No patients who received this dose in the earlier trials experienced TTP/HUS. Other possible side effects of this regimen include nausea, vomiting, diarrhea, fatigue, fever, increased risk of infection, and organ damage.

Phase III Pivotal Trial

A phase III trial of STR was initiated in 2000 but was put on hold after some patients in the phase I/II trials developed delayed renal toxicity. In 2001, NeoRx convened an expert panel to conduct a comprehensive scientific review of the STR development program and data from the phase I/II program. The expert review of the data confirmed the positive clinical

activity of STR in patients with MM. The expert panel also looked at the safety profile of STR and specifically evaluated the delayed renal toxicity and identified strategies for its management. In 2002, a small pivotal study (Multiple Myeloma Research Foundation, URL) initiated by the NeoRx evaluated the method to be used for calculating radiation dose in the planned phase III study. This dosimetry study included 12 patients at 5 transplant centers in the United States. In the study, tracer doses of STR were first administered to determine whether there was sufficient uptake by the bone and to determine the appropriate treatment dose. Eligible patients then received a treatment dose of STR, followed by high-dose chemotherapy and PBSCT to restore bone marrow function. Enrollment in this study was completed in December 2002.

The safety data were encouraging, with no reported cases of TTP/HUS or bladder toxicities. One grade 4 adverse event (severe weight loss) was reported. Seven serious adverse events (fever) were reported but were considered to be related to the high-dose chemotherapy rather than to STR. Two episodes of grade 3 mucositis (mouth sores) were reported. NeoRx also submitted a proposal for further clinical development of STR in patients with primary refractory or relapsed MM, using a revised dosing method. Based on the results of the dosimetry study and having received FDA approval to reinitiate clinical studies, NeoRx opened a multi-center, randomized pivotal phase III trial in March 2004.

Ongoing Phase III Trials

- (1) A multi-center, randomized, phase III study (Multiple Myeloma Research Foundation, URL), which expects to enroll ~240 patients with primary

refractory MM (patients with newly diagnosed disease who do not respond to standard chemotherapy) is presently ongoing. This trial is designed to evaluate the safety and efficacy of STR in patients (age >18 and <70 years) with primary refractory MM who are <18 months from the beginning of induction therapy to enrollment on study with adequate pulmonary, cardiac, liver, and renal function being defined in the trial protocol are eligible. Patients on the experimental arm receive STR at a dose of 750 mCi/m², plus the chemotherapy drug melphalan at 200 mg/m², followed by ASCT. Patients on the control arm receive melphalan only, followed by transplantation.

All subjects first receive a tracer dose of STR to determine whether skeletal uptake is adequate and tissue distribution is normal. If this is the case, patients then undergo randomization to receive STR plus melphalan or melphalan alone. Patients on both study arms are evaluated for response to treatment 6 months after transplantation, using an immunofixation assay to detect M protein in patient samples. Analysis of patient samples is conducted in a central laboratory, and blinded results are reviewed by an independent panel of experts. The study's primary endpoint is complete response, as determined by the complete disappearance of the M protein at 6 months post-transplant. The trial opens at up to 40 clinical sites in the United States and Canada.

- (2) Phase I/II trials showed feasibility and tolerability when combined with high-dose melphalan with or without TBI in patients with MM undergoing ASCT. Retrospective review (Christoforidou

et al. 2007) of transplant outcomes was undertaken at the MDACC among patients who received an ASCT between 1998 and 2001 with either melphalan 200 mg/m² or a STR containing regimen as part of their initial therapy. The purpose of this current study was to define the potential impact of STR on outcomes in patients with MM undergoing ASCT. The study indicated that, (166)Holmium-DOTMP, in combination with high-dose melphalan, can result in higher complete response rates when given in optimal doses (<2,400 mCi) when compared to melphalan alone, and should be further tested in phase III trials in patients with MM undergoing ASCT.

CONCLUSION AND PERSPECTIVES

The major clinical problems that arise in MM patients relate to the enhanced bone loss that commonly occurs in these patients. Recent developments and technological advancements in the field of radiotherapy and targeted radionuclide therapy approaches have made enormous improvements in the palliation of metastatic bone disease. As our understanding of the biology of myeloma bone disease continues to develop, increasing numbers of new potential therapies are emerging. Skeletal targeted radiotherapy may prove to be an effective strategy for the treatment of MM. It is vital that future clinical trial designs incorporate prospective whole-body and tumor dosimetry. The goal of patient-specific activity prescription offers the only realistic prospect of enhancing response rates within predictable toxicity limits

and of progressing beyond symptom palliation to earlier intervention for survival gain. Induction of specific anti-MM immune responses has been demonstrated in a multitude of studies and confirmed in preclinical models. Considering the continued progress in optimization of gene transfer techniques and understanding of MM immuno- and pathobiology, recent advances in molecular therapies are extraordinarily promising to target specific pathways in MM. New agents are being developed to block adherence of MM cells to stromal cells in the bone marrow, which may prevent multiplication of the MM cells, development of drug resistance, and occurrence of lytic bone lesions. Advancements in molecular diagnostics, genomic and proteomic technologies at present have become powerful tools in predicting disease outcome of MM patients. These should offer a comprehensive source of novel therapeutic targets for specific anti-MM strategies in the future, and may facilitate inclusion into innovative treatment strategies and clinical trials.

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Part IX

Sarcoma

Diagnosis

Low Grade Fibromyxoid Sarcoma: Diagnosis by Detecting FUS-CREB3L2 Fusion Gene Using Reverse Transcription–Polymerase Chain Reaction

Atsuji Matsuyama, Masanori Hisaoka, and Hiroshi Hashimoto

INTRODUCTION

Low grade fibromyxoid sarcoma (LGFMS) is a rare soft tissue sarcoma first described by Evans (1987) to have bland-looking fibromyxoid features that had been diagnosed as “fibroma” or “fibromatosis” but metastasize. Hyalinizing spindle cell tumor with giant rosettes (HSCT), which was initially reported by Lane et al. (1997), has been considered a histologic variant of LGFMS. Although LGFMS has a metastatic potential, the morphologic distinction of LGFMSs from other benign or low grade fibromyxoid lesions, such as desmoid-type fibromatoses, soft tissue perineurioma and low grade myxofibrosarcoma, is often difficult and problematic. Besides, LGFMS has no specific or characteristic immunohistochemical marker.

It was shown by Storlazzi et al. (2003) that the gene fusion between the *FUS* on chromosome 16p11.1 and the *CREB3L2*, also known as the *BBF2H7*, on chromosome 7q33 was detected by fluorescence

in situ hybridization (FISH) and reverse transcription–polymerase chain reaction (RT-PCR) in a classic LGFMS and a HSCT. Subsequently, two larger series by Panagopoulos et al. (2004) and Mertens et al. (2005) also demonstrated the *FUS-CREB3L2* fusion gene and a rare variant of the *FUS-CREB3L1* to be specific in LGFMSs. The molecular detection of the fusion gene transcripts is thus considered to be useful in diagnosing LGFMSs as used in other translocation-associated soft tissue sarcomas.

The cytogenetic analysis or molecular assay of soft tissue sarcomas usually requires cultured tumor cells or fresh or snap frozen tumor tissue. Previous analyses for detecting *FUS-CREB3L2* fusion gene were also based on the fresh or frozen tumor samples. However, such samples of tumor tissues are not always available. Although the length of the extracted RNA from formalin-fixed, paraffin-embedded (FFPE) tumor specimens is usually limited, molecular approaches using mRNA extracted

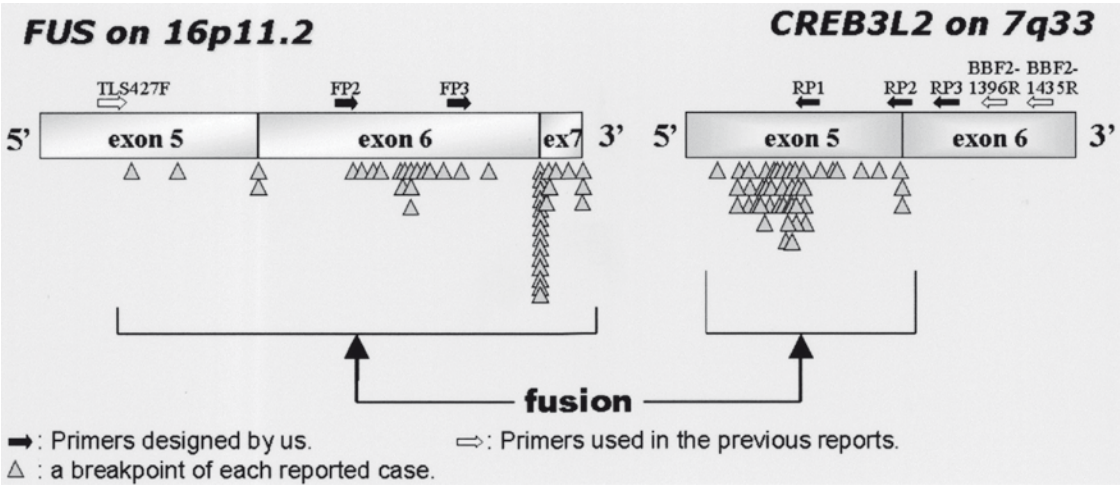


FIGURE 31.1. A schematic representation of variable portions of the *FUS* and the *CREB3L2* breakpoints in the *FUS*-*CREB3L2* fusion gene transcripts in the 46 cases of LGFMSs reported by Storlazzi et al. (2003), Panagopoulos et al. (2004), Mertens et al. (2005), and Matsuyama et al. (2006), and the primers used in the present study. Each arrowhead indicates a breakpoint of a LGFMS. The arrows represent primers. The forward primer TLS165F located on the exon 3 of the *FUS* gene is not described in this schematic drawing

from FFPE tissues have been carried out to identify fusion genes in a variety of soft tissue sarcomas such as Ewing’s sarcoma family, alveolar rhabdomyosarcoma, synovial sarcoma, myxoid liposarcoma, desmoplastic small round cell tumor, dermatofibrosarcoma protuberans, and extraskeletal myxoid chondrosarcoma. It seems to be more difficult to detect the *FUS*-*CREB3L2* fusion transcripts using FFPE tumor tissue, because of variable fusion patterns in the *FUS*-*CREB3L2* fusions due to variable breakpoints, which may be present within exons (Figure. 31.1). An RT-PCR was conducted by Matsuyama et al. (2006) to assess its feasibility for the *FUS*-*CREB3L2* fusion transcripts in LGFMSs using archival FFPE tumor specimens, and successfully detected them. This method can thus help in accurately diagnosing LGFMS.

DETECTION OF THE
FUS-CREB3L2 FUSION
TRANSCRIPTS USING
FORMALIN-FIXED,
PARAFFIN-EMBEDDED
TUMOR TISSUE

Primers

The primers were designed to specifically amplify most of the junctional regions of the *FUS*-*CREB3L2* fusion gene transcripts previously reported (Figure 31.1). Although we designed many primers, only two forward and three reverse primers, by which we have confirmed to successfully detect the *FUS*-*CREB3L2* fusion gene transcripts, are described to simplify the procedures: FP2, 5’-CAG TGG TGG CGG TTA TGG CAA-3’; FP3, 5’-TGG TGG TTA CAA CCG CAG CA-3’; RP1, 5’-CTG

GAG GGG CTG TGG GTC TGA-3'; RP2, 5'-AGT TTA TGA GGA GCC GTG AGG-3'; RP3, 5'-TCT TCT CCT CCT CTG TCA GGA C-3'. The primers designed by Storlazzi et al. (2003) may also be useful to detect the FUS-CREB3L2 in some cases or using snap frozen tumor samples: TLS165F, 5'-AGC CAG TCC ACG GAC ACT TCA GGC-3'; TLS427F, 5'-GCT ACA GCC AGC AGC CTA GCT ATG-3'; BBF2-1396R, 5'-CAA TTT GGT GGG GAT GGG ATA-3'; BBF2-1435R, GAA TTT TCT TCA GGG CCT TCT CC-3'.

RNA Extraction

The isolation of RNA from FFPE tumor tissues including LGFMS is based on the method described by Hisaoka et al. (1998) and Tsuji et al. (1998) for detecting other tumor type-specific fusion transcripts.

1. Collect five FFPE tumor sections of 5 μ m-thickness in a sterile microtube, and deparaffinize with xylene and ethanol.
2. Mince in 200 μ L of lysis buffer (20 mmol/L Tris-HCl, pH8.0; 20 mmol/L ethylenediaminetetraacetic acid; and 2% sodium dodecyl sulfate).
3. Add 10 μ L of proteinase K (100 mg/mL) and incubate at 55°C overnight.
4. Add 1.0 mL of Trizol reagent (Invitrogen, San Diego, CA) and incubate for 5 min.
5. Add 200 μ L of chloroform, incubate for 3 min and centrifuge (13,000 rpm, 4°C for 15 min).
6. Transfer an aqueous phase into a 2.0 mL sterile microtube, precipitate with 0.75 mL of 2-propanol and centrifuge (13,000 rpm, 4°C for 10 min).
7. Wash the precipitated total RNA with 75% ethanol and centrifuge (11,000 rpm, 4°C for 5 min).

8. Resuspend the RNA pellet in 11 μ L of water which is free of DNase and RNase.
9. RNA extracts were treated with DNase I (Invitrogen) for 15 min at 37°C.

RT-PCR

1. Reverse transcription : 11 μ L of extracted RNA solution is reverse transcribed into complementary DNA (cDNA) using 1 μ L of random primers (Invitrogen) and 200 U of reverse transcriptase (SuperScript III; Invitrogen). The cDNA is then incubated with 60 U RNase H (Takara, Ohtsu, Japan) for 20 min at 37°C.
2. Evaluation of the extracted mRNA: To assess the quality and integrity of mRNA, PCR for a 247-base pair fragment of the ubiquitously expressed phosphoglycerate kinase (PGK) gene and a 127-base pair fragment of the porphobilinogen deaminase (PBGD) mRNA is carried out using the following primers: PGK-forward, 5'-CAG TTT GGA GCT CCT GGA AG-3' and PGK-reverse, 5'-TGC AAA TCC AGG GTG CAG TG-3'; PBGD-S, 5'-TGT CTG GTA ACG GCA ATG CGG CTG CAA C-3' and PBGD-A, 5'-TCA ATG TTG CCA CCA CAC TGT CCG TCT-3' with the amplification consisted of after an initial denaturation at 94°C for 9 min, 45 cycles of denaturation at 94°C for 30 s and annealing and elongation at 68°C for 60 s, followed by a final extension at 72°C for 10 min.
3. PCR for detecting the FUS-CREB3L2 fusion transcripts: The PCR should be performed with each primer pair. The mixture of more than two of each forward and reverse primers, i.e., nested RT-PCR, may demonstrate several non-specific amplification products.

4. The preferable practical procedures of the primer selection are as follows:
 - a. At first, PCR using two primer sets, FP2 and RP1, and FP3 and RP1, is performed separately, which would detect the major fusion transcripts.
 - b. In negative cases, each remaining primer pair is tested to amplify any other forms.
 - c. The primer combination used in the previous reports, TLS165F and BBF2-1435R, and TLS427F and BBF2-1396R, which were designed for detecting the FUS-CREB3L2 fusion transcripts from the snap frozen tumor samples, may detect a rare fusion pattern, and worth to be tried with the described amplification profile composed of after an initial denaturation at 94°C for 5 min, 30 to 40 cycles of denaturation at 94°C for 60 s, 60°C for 60 s, and 72°C for 60 s, followed by a final extension at 72°C for 10 min (RT-PCR using these primers could not successfully amplify the FUS-CREB3L2 fusion transcripts in any of our cases).

Sequence Analysis

For detailed techniques to confirm the sequences of the amplified PCR products, other textbooks should be consulted. In our examination, the PCR products were cloned into a pCR2.1 vector by a TA cloning kit (Invitrogen) and sequence analysis was performed using an automated sequencing system, ALFexpress DNA sequencer (Pharmacia Biotech, Uppsala, Sweden).

RESULTS

Here we describe the results of our examination of the RT-PCR using FFPE tumor tissues of 16 LGFMSs. The FUS-CREB3L2

gene transcripts were detected in 14 (88%) of the 16 cases of LGFMSs including a case with giant rosettes. Of the 14 examples, 11 (79%) were detected by primer sets of the FP2 and RP1 (5 cases) or the FP3 and RP1 (6 cases). Using the FP2 and RP3 or the FP3 and RP2, the fusion gene transcripts were detected in another two cases and one case, respectively. A nucleotide sequence analysis of all the 14 PCR products confirmed that fusion had formed between the FUS and the CREB3L2 in an in-frame manner. The size of the amplified products ranged from 72 to 203 bp. The breakpoints of both the *FUS* and the *CREB3L2* were distributed at various sites, thus resulting in 12 patterns of different nucleotide sequences. The breakpoints of the *FUS* gene were located within exon 6 in seven cases and exon 7 in one case, respectively. A precise determination of the breakpoints was not possible due to the existence of identical nucleotide(s) at the junction in the remaining six cases, and the breakpoints of the *FUS* gene were located within exon 6 or intron 6 in two cases, within exon 6, intron 6 or exon 7 in three cases, and intron 6 or exon 7 in one case, respectively. On the other hand, all the breakpoints of the *CREB3L2* gene were positioned within exon 5, but they also varied among the cases. In the two negative cases, the PCR using other primers we designed to detect the FUS-CREB3L2 or the FUS-CREB3L1 fusion transcript, or used in the previous reports, namely TLS165F, TLS427F, BBF-1435R and BBF2-1396R, were also performed, but no positive results were obtained. The results of the RT-PCR and sequences of the representative cases are shown in Fig. 31 2.

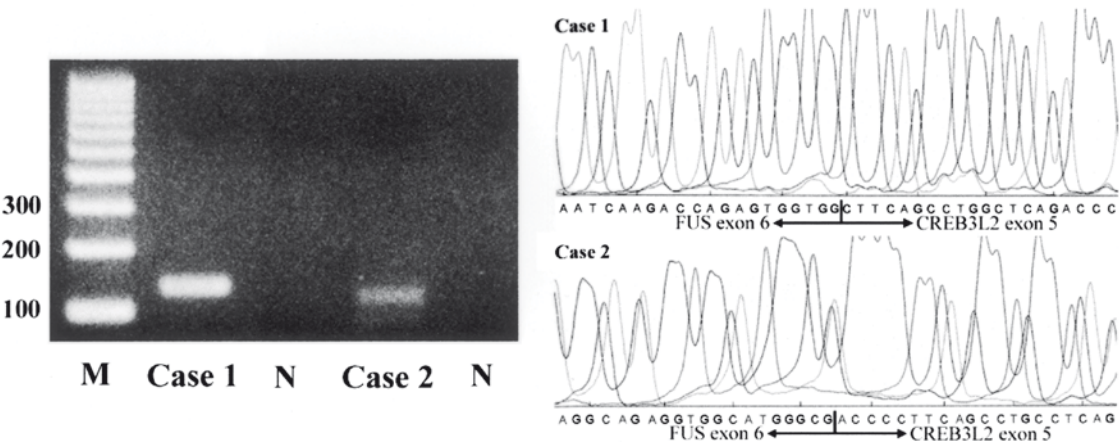


FIGURE 31.2. (Left) Identification of the FUS-CREB3L2 fusion transcripts by RT-PCR using each primer pair in two cases of LGFMS. M, 100-bp DNA ladder; N, negative control. The fusion gene transcripts were detected using the FP2 and RP1 primer pair in Case 1 and the FP3 and RP1 primer pair in Case 2. (Right) Junctional sequences of the FUS-CREB3L2 fusion transcripts and the predicted breakpoints of the both cases. Note the different breakpoints in both the *FUS* and the *CREB3L2* genes

EVALUATION OF THE RT-PCR RESULTS

1. In most of the fusion gene of other translocation-associated soft tissue sarcomas than LGFMS, the breakpoints are located introns. Therefore, the validity of the RT-PCR result can be evaluated based on the molecular size of the PCR product, which is specifically determined by the each primer pair used on the PCR procedures. However, as a result of a greater variety of the breakpoint localization in both the *FUS* and the *CREB3L2* segments, the size of the RT-PCR products of the FUS-CREB3L2 fusion transcripts amplified even by the same primer pair is different from case to case. Sequence analysis is necessary to confirm the successfully amplified fusion transcripts.
2. The variation of the breakpoints, which were detected from FFPE tissue specimens, may indicate that our detection

of the fusion transcripts using FFPE samples is reliable and not caused by contamination. If the same sequences are shown in two or more LGFMSs conversely, the reproducibility should be confirmed to rule out contamination.

3. The negative results in this assay do not necessarily mean the absence of the fusion transcript. The variation of the fusion transcripts of the FUS-CREB3L2 or the FUS-CREB3L1 or unknown fusion partners, which cannot be detected by the primers we used, might also have occurred.

In conclusion, the RT-PCR assay to detect the FUS-CREB3L2 fusion gene transcripts characteristic of LGFMSs using archival FFPE tumor tissue specimens is described. This method is not only useful as a diagnostic adjunct for LGFMS, but also allows us to retrospectively assess a possible relationship between the molecular findings and the clinicopathologic features using a large series of tumors.

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Synovial Sarcoma: Role of TLE1 as a Diagnostic Immunohistochemical Marker

Jefferson Terry and Torsten O. Nielsen

INTRODUCTION

The diagnosis of synovial sarcoma, the fourth most common soft tissue tumor, is accompanied by an often poor prognosis with high chance of metastasis despite surgical resection (Fisher 1998). Local control requires wide local excision and radiation therapy, causing significant morbidity in relatively young patients (peak age 15–35). Attempts to control locally advanced or metastatic synovial sarcoma with conventional chemotherapy have met with little success, and the main role of such agents is in a palliative setting (Lewis et al. 2000; Spurrell et al. 2005; Eilber et al. 2007; Moreno Martin-Retortillo et al. 2007).

The diagnostic gold standard for synovial sarcoma is demonstration of the characteristic translocation between the *SS18* (*SYT*) gene on chromosome 18 and one of three *SSX* genes (*SSX1*, *SSX2*, or rarely *SSX4*) on chromosome X (t(X;18)(p11.2;q11.2)). Detection of t(X;18) can be accomplished by cytogenetic karyotyping, fluorescence in situ hybridization (FISH) or reverse-transcriptase polymerase chain reaction (RT-PCR), although use of these techniques is limited by many practical issues (cost, specialized equipment, personnel,

special tissue handling procedures). Thus, in practice the diagnosis of synovial sarcoma is usually based on histological examination and immunohistochemistry. When first described >100 years ago, synovial sarcoma was defined by biphasic histology (admixed spindle and glandular areas) and it took decades for the more common monophasic subtype (nonpleomorphic spindle cells; ~75% of cases) to be recognized, primarily because monophasic synovial sarcoma bears a strong resemblance to many other spindle cell tumors (including malignant peripheral nerve sheath tumor (MPNST), fibrosarcoma and hemangiopericytoma) (Fisher 1998). Further adding to the confusion, the uncommon poorly differentiated subtype of synovial sarcoma (comprised of small blue cells) can be mistaken for other small blue round cell malignancies, such as Ewing family tumors (which are treated with neoadjuvant chemotherapy). The correct diagnosis of synovial sarcoma based entirely on histology can be very difficult, particularly in core needle biopsies which are now the main diagnostic approach in many centres.

Many immunohistochemical markers have been used adjunctively to help confirm a suspected diagnosis of synovial sarcoma;

however, there is considerable overlap between synovial sarcoma and the other entities in its histological differential diagnosis (Folpe et al. 1998; Mezzelani et al. 1998; Cole et al. 1999; Michal et al. 2006). The partially epithelial nature of many synovial sarcomas can help differentiate them from other spindle cell neoplasms by epithelial markers such as cytokeratins and epithelial membrane antigen (EMA). However, the biphasic cases where these are most strongly expressed do not present diagnostic difficulty. In monophasic cases, these markers are expressed focally if at all, making them unsuitable for small tissue samples like core biopsies (Folpe et al. 1998; Remotti et al. 2001; Llombart-Bosch et al. 2002; Pelmus et al. 2002; Okamoto et al. 2004; Suster and Moran 2005; Michal et al. 2006). Furthermore, focal expression of such markers is not specific, and has been reported in MPNST, angiosarcoma, and spindle cell carcinomas, among other entities. S100 is detectable in some synovial sarcomas but is also expressed in MPNST, as well as Ewing family tumors, clear cell sarcomas, some histiocytic neoplasms and many soft tissue tumors with adipocytic or chondroid areas (Pelmus et al. 2002; Michal et al. 2006). BCL2 is a very sensitive marker for synovial sarcomas with good negative predictive value (Hirakawa et al. 1996; Kawauchi et al. 2000; Barbashina et al. 2002; Olsen et al. 2006), but its expression is found in some MPNSTs and almost all cellular solitary fibrous tumors, spindle cell lipomas, gastrointestinal stromal tumors, and many fibroblastic neoplasms (Suster et al. 1998), diminishing its specificity. Thus, there remains a considerable need for an immunohistochemical marker that is both highly sensitive and specific for synovial sarcoma.

Using cDNA microarrays to generate expression profiles of synovial sarcoma is an efficient, high-throughput means for discovering consistent changes in expression of genes that may encode sensitive immunohistochemical markers. Moreover, comparing expression of these genes to that in other tumors in the differential diagnosis can establish the potential specificity of the gene product as a marker. In this manner, the *TLE1* gene (Transducin-like enhancer of split 1, a homologue of *Drosophila* gene *groucho*) was found to be consistently overexpressed in synovial sarcoma in a number of expression profiling studies employing different platforms and methodologies. Comparison of *TLE1* expression in synovial sarcoma to other soft tissue tumors (including MPNST) identifies *TLE1* as among the five best discriminators for synovial sarcoma out of 12,600–46,000 genes investigated (Nielsen et al. 2003; Segal et al. 2003; Baird et al. 2005). The only other genes in the genome which appear as strongly correlated with synovial sarcoma, such as the *SSX* genes, lack available antibodies that work for immunohistochemistry on paraffin-embedded tissues.

The specificity of *TLE1* expression for synovial sarcoma, particularly when compared to other sarcomas in the differential diagnosis, suggests that immunohistochemical detection of TLE1 protein may be a clinically useful diagnostic marker. Antibodies suitable for formalin-fixed, paraffin-embedded tissue that recognize TLE1 are available and can be used in combination with large numbers of synovial sarcoma tumor samples in tissue microarrays (TMA) for high-throughput validation of TLE1 overexpression. Moreover, TMAs allow comparison of TLE1 expression to

other tumors in the differential diagnosis. The small size of the tumor cores in TMAs simulate small biopsy samples (core tissue biopsies) and offer an additional stringent validation of TLE1 for use in these circumstances. To investigate the protein expression of TLE1 in synovial sarcoma and compare it to broad range of mesenchymal neoplasms, we investigated multiple TMAs with two anti-TLE1 antibodies using both manual and automated immunostaining protocols.

MATERIALS

1. Tissue: 4 μm thick sections of standard formalin-fixed, paraffin-embedded tissue were used for immunohistochemistry. Tissue microarray studies were used to validate the sensitivity and specificity of this methodology representing, in total, > 90 molecularly-confirmed synovial sarcomas and > 600 other mesenchymal and spindle cell tumors (Terry et al. 2007).
2. Chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise specified.
3. Fisherbrand Superfrost Plus glass slides (cat# 12-550-15) and cover slips (cat# S175221) (Fisher Scientific, Pittsburgh, PA).
4. Acrodisc 0.2 μm syringe filters (cat# 4602, Pall, East Hills, NY).
5. Ten milliliter (cat# 309604) and 20 mL (cat# 309661) luer lock syringes (BD, Franklin Lakes, NJ).
6. Filter paper for gravity filtration (cat# 1001-090, Whatman, Florham Park, NJ).
7. Citrisolve (Amity International Inc., Anderson, SC).
8. Cytoseal XYL (cat# 8312-4, Richard-Allan Scientific, Kalamazoo, MI).
9. Phosphate buffered saline (PBS): To make 10 $\times$ stock, dissolve 81.86 g sodium chloride, 12.46 g potassium phosphate dibasic and 4.14 g of potassium phosphate monobasic in 0.9 L double distilled water (ddH₂O), adjust pH to 7.4 and make volume up to 1.0 L with ddH₂O. Dilute 1/10 with ddH₂O to make 1 $\times$ PBS.
10. PBS with 0.1% Tween 20 buffer (PBS/Tween): add 100 mL 10 $\times$ PBS and 1 mL Tween 20 to 0.8 L ddH₂O. Stir until Tween 20 is dissolved and make up volume to 1.0 L.
11. Citrate buffer (10 mM Sodium Citrate, 0.05% Tween 20, pH 6.0): dissolve 2.94 g sodium citrate (dihydrate) in 1.0 L ddH₂O. Adjust pH to 6.0 then add 0.5 mL Tween 20.
12. Three percent and 0.3% hydrogen peroxide solutions: for 3% hydrogen peroxide, add 2 mL of 30% hydrogen peroxide to 18 mL PBS. For 0.3% hydrogen peroxide, add 0.5 mL of 3% hydrogen peroxide to 4.5 mL PBS. These solutions can be stored for up to 1 month at 4°C.
13. Diaminobenzidine (DAB) substrate solution: make a 20 $\times$ stock solution of DAB by adding 100 mg of 3,3'-diaminobenzidine tetrahydrochloride to 10 mL of ddH₂O followed by two drops of concentrated hydrochloric acid. Shake to fully dissolve the DAB (typically ≥ 10 min). Syringe filter (0.2 μm) and store at -20°C. Make a 1 $\times$ DAB solution (DAB) just prior to use by adding 250 μL 20 $\times$ DAB to 5 mL PBS followed by 250 μL 0.3% hydrogen peroxide. Mix well.
14. Hematoxylin solution (Gill 1): add 250 mL ethylene glycol to 750 mL ddH₂O followed by 2 g hematoxylin, 0.2 g

sodium iodate, 20 g aluminum sulfate (hexadecahydrate) and 20 mL glacial acetic acid. Mix for at least 1 h at room temperature and gravity filter.

15. Two percent sodium bicarbonate: add 2 g sodium bicarbonate to 100 mL ddH₂O, mix to dissolve and syringe or gravity filter.
16. Antibody diluent (cat# S0809) and ready to use serum-free protein blocking solution (cat# X0909) (DAKO, Carpinteria, CA).
17. Polyclonal rabbit anti-TLE1 (clone M101, cat# sc-9121, Santa Cruz Biotechnology, Santa Cruz, CA).
18. Monoclonal rat anti-human pan-TLE antibody was provided by S. Stifani (Montreal Neurological Institute, Montréal, Canada) (Stifani et al. 1992).
19. Secondary horseradish peroxidase (HRP) conjugated anti-rat antibody (cat# ab7090, Abcam, Cambridge, UK).
20. Secondary HRP-conjugated anti-rabbit antibody (cat# ab6721, Abcam).
21. Ventana Discovery XT automated immunostainer, cell conditioner #1 (CC1, cat# 950-124), EZPrep automated immunostainer deparaffinization solution (cat# 950-100), LCS reagent (cat# 650-010), reaction buffer (cat# 950-300), DABMap Kit (Includes Inhibitor D, Blocker D, SA-HRP D, DAB D, DAB H₂O₂ and Copper D; cat# 760-124), hematoxylin counterstain (cat# 760-2021), and bluing reagent (cat# 760-2067) were purchased from Ventana (Tucson, AZ).

Additional materials required include various glass and plastic ware such as Coplin jars, slide racks, water bath, pipettors, pipettor tips, incubation chambers, and Parafilm M (Pechiney Plastic Packaging, Menasha, WI).

METHODS

We present a detailed description of optimized protocols for manual and automated immunohistochemical detection of TLE1 in formalin-fixed, paraffin-embedded tumor tissue samples. Ideally, a clinically-applicable anti-TLE1 antibody should be monoclonal (to reduce the potential for cross-reaction with other epitopes), specific for TLE1 (affinity for other members of the TLE family may reduce specificity), easily obtainable (i.e., from a commercial source) and not require atypical immunohistochemical reagents or protocols that may restrict use. No single antibody is presently available that satisfies all these requirements, so we tested and compared a research grade monoclonal antibody that does not discriminate between TLE1 and the closely related TLE2,3, and 4 proteins, as well as a commercially available polyclonal antibody that preferentially recognizes TLE1. In working up these antibodies using tissue microarrays, large numbers of samples are represented in parallel on single slides, reducing the potential for technical variance and ensuring samples representing the full range of TLE1 antigen expression are present on each slide, greatly facilitating consistent interpretation. Inclusion of proper control tissue samples and matched isotype nonspecific antibody and mock (i.e., secondary antibody only) staining controls are required for proper interpretation of results.

Manual Immunostaining

1. Four μ m TMA sections are transferred to glass slides, including a control section for secondary antibody background (no primary antibody) and a control for endogenous peroxidase activity (no primary or secondary antibody).

2. Formalin-fixed, paraffin-embedded sections are deparaffinized by sequential incubation in three Citrisolve baths for 10, 5, and 5 min. Citrisolve is removed and the tissues rehydrated with sequential ethanol baths of 100%, 100%, 95%, and 80% for 3 min each followed by ddH₂O for more than 2 min.
3. Antigen retrieval is performed by immersing the rehydrated slides in citrate buffer at 95°C for 30 min followed by cooling to room temperature and three sequential 5 min rinses in PBS.
4. Endogenous peroxidase activity is quenched by incubating slides in 3% hydrogen peroxide for 10 min followed by three sequential 5 min rinses in PBS.
5. Nonspecific primary antibody hybridization is blocked by application of blocking reagent for 30 min at room temperature.
6. Primary antibody is hybridized by draining the blocking reagent, applying 200 µL of a 1:200 dilution of the M101 anti-TLE antibody per slide and incubating at 4°C overnight in a sealed chamber. The slides are then washed three times in PBS/Tween buffer (5 min each).
7. Two hundred microliters of 1:2000 dilution of HRP-labelled anti-rat secondary antibody is then applied and the slides incubated at room temperature for 30 min in a sealed chamber followed by three washes in PBS/Tween (5 min each).
8. Hybridized antibody is visualized by applying DAB for exactly 10 min followed by two sequential rinses in ddH₂O of 5 min each.
9. Nuclei are counterstained with hematoxylin for 30 to 60 s, rinsed in ddH₂O, exposed to 2% sodium bicarbonate for 30 s and rinsed again in ddH₂O.
10. Slides are then dehydrated through sequential 5 min washes of 80%, 95%, 100%, and 100% ethanol followed by two 5 min washes in xylene and mounting in permanent mounting medium.

Automated Immunostaining

The following is a description of a proprietary protocol for automated immunostaining on the Ventana Discovery XT immunostainer with specifications for use with either the polyclonal rabbit anti-TLE1 antibody or the monoclonal rat anti-pan-TLE antibody.

1. Slides are liquid coverslipped with LCS reagent for all incubations after deparaffinization.
2. Incubate slide at 75°C for 8 min, apply EZprep and incubate for 8 min. Rinse slide and incubate at 37°C for 4 min followed by another rinse and jet drain.
3. Apply cell conditioner #1 (CC1) and repeat application of CC1.
4. Heat slide to 95°C for 8 min, apply CC1 and heat to 100°C for 4 min.
5. Apply CC1 and incubate for 4 min, repeat, and then reapply CC1, remove heat and incubate for 8 min.
6. Rinse slide with reaction buffer and adjust slide volume with reaction buffer and then heat slide to 37°C and incubate for 4 min followed by a rinse with reaction buffer.
7. Apply one drop of inhibitor D and incubate for 4 min (one drop equals one reagent dispense), followed by two rinses with reaction buffer.
8. Primary antibody hybridization: apply either a drop of 1:20 dilution of M-101 polyclonal anti-TLE1 antibody or a 1:2 dilution of monoclonal anti-pan-TLE

antibody and incubate for 32 min followed by a rinse with reaction buffer.

9. Secondary antibody: for M-101 polyclonal anti-TLE1 antibody, add one drop of universal secondary antibody; for monoclonal anti-pan-TLE antibody, add one drop of 1:1000 dilution of HRP-conjugated anti-rat IgG antibody. Incubate 32 min and then rinse slide with reaction buffer.
 10. Apply one drop of Blocker D and incubate for 4 min, then apply one drop of SA-HRP D and incubate for 16 min. Rinse slide with reaction buffer three times.
 11. Apply one drop of DAB D and one drop of DAB H₂O₂ and incubate for 8 min, rinse slide and apply one drop of Copper D and incubate for 4 min. Rinse slide twice with reaction buffer, let stand for 8 min and then rinse again.
 12. Apply one drop of hematoxylin as counterstain and incubate 8 min, rinse twice with reaction buffer and then apply one drop of bluing reagent and incubate for 8 min. Rinse slide with reaction buffer and apply coverslip.
- 10% of cells exhibit intense nuclear staining obvious at low power, and “0” (negative) for no visible nuclear staining. Examples of each staining grade are presented in Figure. 32.1.
2. Tumors with a score of 2+ or 3+ on at least one examined tissue microarray core were considered positive for TLE. By these criteria, focal staining (i.e., intense but in <10% of tumor cells) does not count as positive.
 3. In tissue microarray studies, where scores of duplicate cores were discrepant, the higher score was used. Uninterpretable sets of duplicate cores (i.e., no tumor cells, absence of viable cells or folded/lost tissue core) were excluded from analysis.
 4. Sections of each microarray were also stained with hematoxylin and eosin (H&E) using standard methods as an additional histologic reference.

Interpretation of TLE Staining

1. TLE immunostaining is graded as “3+” (strong) if >50% of tumor cells exhibit intense nuclear staining visible with a 4× low power objective lens, “2+” (moderate) if 10–50% exhibit intense nuclear staining obvious at low power and/or greater than 50% of cells display nuclear staining well above background (endothelial cells within the tumor can serve as a reference) when assessed with 10× objective magnification, “1+” (weak) if <50% of cells exhibit weak to moderate nuclear staining and less than

RESULTS AND DISCUSSION

To determine the sensitivity and specificity of TLE1 as an immunohistochemical marker for synovial sarcoma, we tested two antibodies against TLE1: (1) a commercially-available polyclonal antibody raised against TLE1, and (2) a monoclonal antibody recognizing an epitope in its C-terminal WD-40 domain, previously shown to work for immunohistochemical applications (Stifani et al. 1992). These have been validated against more than 700 cases of adult soft tissue tumors including 100 molecularly-validated synovial sarcomas, in tissue microarray and core biopsy formats.

Comparison of staining characteristics between the anti-TLE1 antibodies on

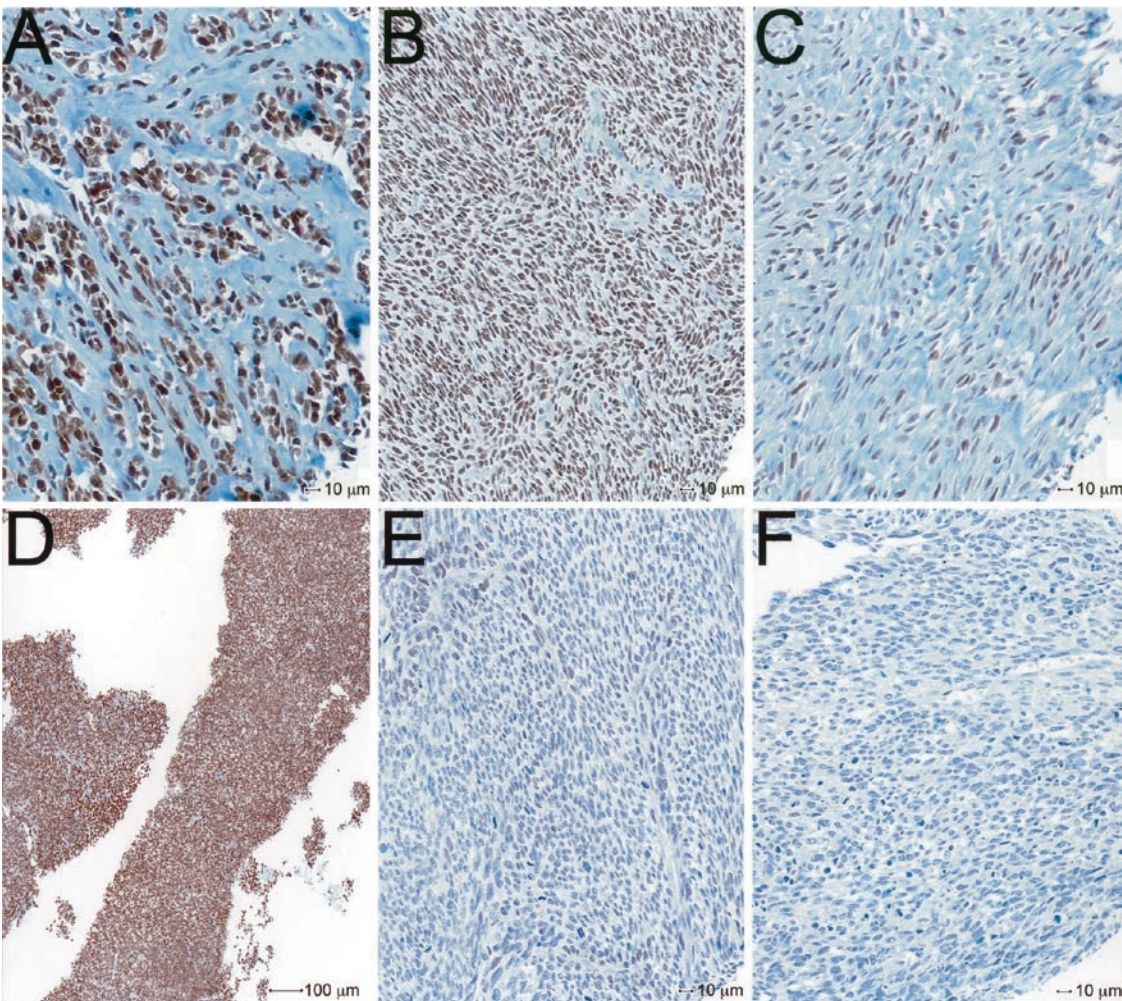


FIGURE. 32.1. TLE1 immunostaining in representative synovial sarcoma and non-synovial sarcoma cores. (a) Scanning power view of 3+ staining with pan-TLE antibody in a biphasic synovial sarcoma. (b) Detail of 3+ staining with M101 TLE1 antibody in a monophasic synovial sarcoma. (c) 2+ staining with pan-TLE antibody in a monophasic synovial sarcoma. (d) 3+ staining with pan-TLE antibody in a monophasic synovial sarcoma core biopsy. (e) 1+ staining with M101 TLE1 antibody in an MPNST. (f) Absent (i.e. 0+) staining with M101 TLE1 antibody in an MPNST. Scale bars are provided for reference in the lower right corner of each micrograph

sequential sections from the same TMA revealed that, in general, the intensity of M101 antibody staining under the optimized conditions described was slightly less than with the monoclonal anti-pan-TLE antibody, with very little to no background for either antibody. The specific staining grade between antibodies for each tumor core was almost identical (eight discrepancies among 177 cases, Kappa statistic 0.78, $p < 10^{-25}$). All discrepancies in scoring were cases staining positive by pan-TLE

and negative by M101, suggesting that the M101 antibody is marginally less sensitive. The combined results of TLE1 staining for all tumor types examined in both TMA

and core biopsy format are summarized in Table 32.1.

In almost all synovial sarcoma tumor samples, both antibodies produced intense

TABLE 32.1. Summary of tissue microarray and core biopsy TLE immunohistochemistry results.

Tumor type	0	1+	2+	3+	n	Total positive	% Positive
Synovial Sarcoma	0	3	24	73	100	97	97
Angiosarcoma	13	0	0	0	13	0	0
Breast carcinoma	2	0	0	0	2	0	0
Carcinosarcoma	5	2	1	0	8	1	13
Chondroblastoma	3	1	0	0	4	0	0
Chondromyxoid fibroma	2	0	0	0	2	0	0
Chondrosarcoma	39	2	1	0	42	1	2
Clear cell sarcoma	5	1	0	1	7	1	14
Dermatofibrosarcoma protuberans ^a	16	1	0	0	17	0	0
Desmoplastic small round cell tumor	3	0	0	0	3	0	0
Enchondroma	4	0	0	0	4	0	0
Endometrial stromal sarcoma	10	1	0	0	11	0	0
Epithelioid sarcoma	2	0	0	0	2	0	0
Ewing sarcoma	11	1	0	1	13	1	8
Fibromatosis	17	0	0	0	17	0	0
Fibrosarcoma	3	0	0	0	3	0	0
Fibroanthoma	3	0	1	0	4	1	25
Gastrointestinal stromal tumor	26	7	2	0	35	2	6
Giant cell tumor, tenosynovial	9	0	0	0	9	0	0
Glomous tumor	6	1	0	0	7	0	0
Granular cell tumor	8	0	0	0	8	0	0
Hemangiopericytoma	2	2	1	1	6	2	33
Inflammatory myofibroblastic tumor	3	0	0	0	3	0	0
Kaposi sarcoma	2	0	0	0	2	0	0
Leiomyoma	8	0	0	0	8	0	0
Leiomyosarcoma	36	4	0	1	41	1	2
Lipoblastomatosis	2	0	0	0	2	0	0
Liposarcoma	28	3	0	0	31	0	0
Pleomorphic sarcomas (MFH) ^b	51	5	0	0	56	0	0
Malignant peripheral nerve sheath tumor	74	13	3	1	91	4	4
Melanoma	7	4	0	0	11	0	0
Mesothelioma	2	0	0	0	2	0	0
Myxofibrosarcoma, low grade	5	0	0	0	5	0	0
Myxoma	8	0	0	0	8	0	0
Myxosarcoma	3	1	0	0	4	0	0
Neurofibroma	34	10	0	0	44	0	0
Osteosarcoma	15	1	0	0	16	0	0
Perineurioma	4	0	0	0	4	0	0
Rhabdomyosarcoma	11	2	0	0	13	0	0
Sarcoma, not otherwise specified	6	1	0	0	7	0	0
Schwannoma	6	11	6	0	23	6	26
Solitary fibrous tumor	8	3	4	0	15	4	27
Vascular tumor ^c	9	0	0	0	9	0	0

^a Eight of the DFSP cases contained fibrosarcomatous change.
^b MFH: Malignant fibrous histiocytoma, includes pleomorphic variants of myxofibrosarcomas, myofibroblastic sarcomas, fibrosarcomas and undifferentiated sarcomas.
^c Includes two cases of epithelioid hemangiioendothelioma, four capillary hemangiomas and three intramuscular hemangiomas.

nuclear staining that was very easy to interpret (Figure. 32.1a–c). The vast majority (97/100) of synovial sarcomas cases exhibited positive TLE1 staining; the remaining three exhibited weak (1+) TLE1 immunoreactivity (Table 32.1). All included biphasic synovial sarcomas exhibited staining in the spindle cell component and equivalent or stronger intensity in the glandular areas (Figure. 32.1a). Moreover, all four of the included poorly-differentiated synovial sarcoma cases were positive for TLE1. For those synovial sarcomas in which the fusion subtype had been determined, no significant difference in TLE1 staining was found between tumors harboring the *SS18-SSX1* as opposed to the *SS18-SSX2* fusion subtype. In the subset of examined core biopsies, all the synovial sarcomas were TLE1 positive (Figure. 32.1d), whereas all the non-synovial sarcoma tumors were TLE1 negative and these results were entirely consistent with a FISH-based *SS18* gene breakapart assay for t(X;18). Thus, TLE1 is a particularly sensitive immunohistochemical marker for synovial sarcoma that is applicable to core biopsies.

Positive TLE1 staining was rare to absent in most other bone and soft tissue tumors (Table 32.1). A particular effort was made to test for specificity against MPNST, the most difficult histological entity in the differential diagnosis of synovial sarcoma. The vast majority of MPNSTs were TLE1 negative (Figure. 32.1e,f), with only 4 of 91 (4.3%) exhibiting positive staining. Other non-synovial sarcoma tumors exhibiting positive TLE1 staining included hemangiopericytomas (2/6), solitary fibrous tumor (4/15), schwannoma (6/23), fibroxanthoma (1/4), clear cell sarcoma (1/7), carcinosarcoma (1/8), Ewing sarcoma (1/13 cases), gastrointestinal stromal tumor (2/35), leiomyosarcoma

(1/41), and chondrosarcoma (1/42). TLE1 staining was mostly absent (0+) or occasionally minimal (1+) in most of the other bone and soft tissue tumors, as well as the breast carcinomas, melanomas, and mesotheliomas (Table 32.1). Overall, the lack of TLE1 immunoreactivity in most other soft tissue tumors, including all major entities in the histologic differential diagnosis for synovial sarcoma, indicates that TLE1 would be a specific marker for synovial sarcoma in clinical practice, under the staining and interpretation conditions described.

Thus, TLE1 expression is a consistent and prominent feature of synovial sarcoma by both gene expression and immunohistochemistry, whereas it is mostly absent in other tumors in the differential diagnosis. Our group has now assessed >35 established and novel immunohistochemical markers of synovial sarcoma in TMA format and none are as sensitive and specific as TLE1 (Nielsen et al. 2003). Presently, TLE1 is being used clinically as an immunohistochemical marker for diagnosis of synovial sarcoma at the British Columbia Cancer Agency (Vancouver, BC, Canada) and the Cleveland Clinic (Cleveland, OH). Our experience to date has been that positive TLE1 staining correlates perfectly with positive FISH detection of t(X;18) molecular changes, but has a significantly quicker turnaround time (i.e., 1–2 days compared to 1–2 weeks).

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The Immunohistochemistry of Kaposi's Sarcoma

Liron Pantanowitz, Gabriel Caponetti, and Bruce J. Dezube

INTRODUCTION

Kaposi's sarcoma (KS) is an angioproliferative, multifocal tumor that depends upon infection with Kaposi's Sarcoma Herpesvirus/Human Herpesvirus-8 (KSHV/HHV8). KS lesions are comprised of spindle lesional cells, abnormal vessels, and a variable chronic inflammatory infiltrate. There is an emerging body of literature pertaining to the immunohistochemistry (IHC) of this captivating vascular neoplasm. This chapter deals with the IHC of KS related to (1) its histogenesis (cell of origin), (2) pathogenesis, (3) diagnosis, and (4) the identification of novel therapeutic targets.

Kaposi's sarcoma was first described by Moritz Kaposi in the late nineteenth century. Today, we recognize four different epidemiological forms of KS: Classic (sporadic), African (endemic), Acquired immune deficiency syndrome (AIDS)-associated (epidemic), and immunosuppression-associated (iatrogenic) KS. KS may involve the skin, mucosa, and less frequently the viscera. While Classic KS typically manifests on the extremities as violaceous lesions, patients with this form of sarcoma may infrequently present with KS lesions involving the mucosa, genitalia,

and gastrointestinal tract. As a result of the widespread AIDS epidemic in Africa, it has become difficult to differentiate true endemic from AIDS-associated KS. AIDS-related KS is an aggressive tumor that may cause minimal disease or widespread lesions. KS skin lesions may vary from small papules to fungating nodules, often with concomitant ulceration and lymphedema. Extracutaneous disease in the setting of human immunodeficiency virus (HIV) infection is common. In transplant recipients, HHV8 infection can be associated with fatal KS. KS may undergo regression spontaneously, or more often in patients receiving therapy. KS exacerbation (flare) can occur following therapy with corticosteroids, after rituximab, and as part of the immune reconstitution syndrome seen with antiretroviral therapy in HIV-infected persons.

The histology of KS is identical in the different epidemiologic KS types. KS lesions evolve through patch, plaque, and nodular stages (Figure 33.1). Early patch lesions are morphologically characterized by a proliferation of irregular, thin-walled vascular channels. These dissecting vessels are lined by a single layer of endothelial cells. As the cellularity increases due to tumor cell proliferation, the lesion

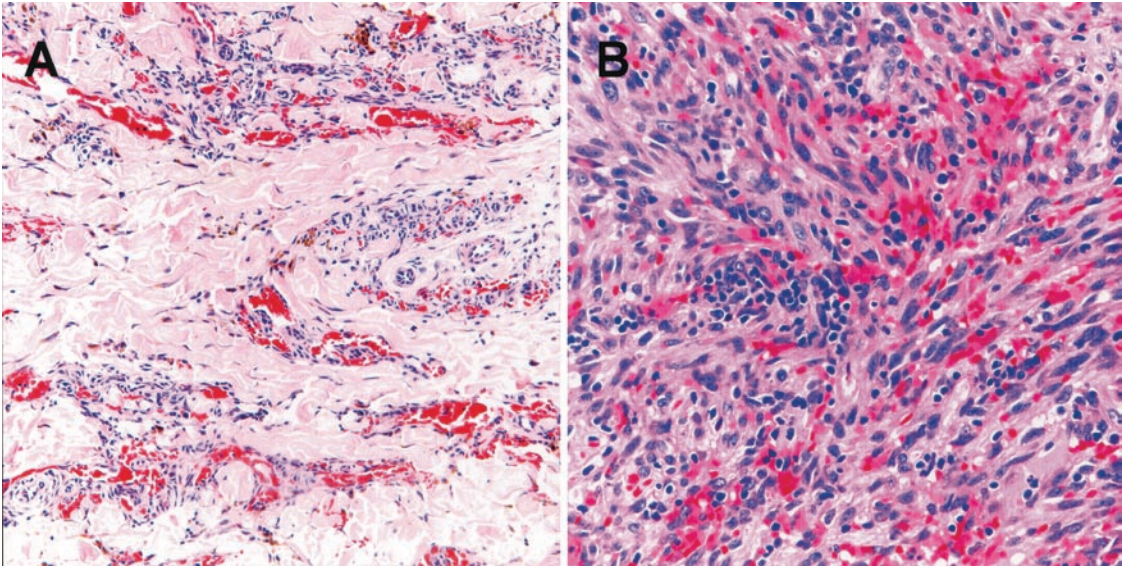


FIGURE 33.1. Kaposi's sarcoma histopathology. (A) Early patch stage showing abnormal, thin-walled blood channels dissecting through dermal collagen (H&E stain). Note the abundant intravascular erythrocytes that harbor endogenous peroxidases, as well as the hemosiderin pigment deposition. (B) Spindled tumor cells admixed with scattered chronic inflammatory cells (H&E stain)

begins to involve the full dermal thickness, developing into a palpable plaque. The proliferating spindled shaped tumor cells form interlacing bundles or fascicles juxtaposed with newly-formed vascular spaces containing erythrocytes. Lesional spindled cells may exhibit variable nuclear pleomorphism, and occasionally mitotic figures are evident. Extracellular hemosiderin deposition and intracytoplasmic so-called hyaline globules are typically seen at this stage. Also notable at this stage is a predominantly perivascular mononuclear inflammatory infiltrate comprised of lymphocytes, plasma cells, macrophages, and admixed dendritic cells. Continued proliferation of tumor cells eventually results in a nodular tumor that may ulcerate. Various histological variants (e.g., pyogenic-granuloma like KS, lymphangioma-like KS, intravascular KS, keloidal KS, and pleomorphic KS) exist, that may mimic other

vascular lesions. The differential diagnosis of KS includes other vascular lesions such as bacillary angiomatosis, pyogenic granuloma, lymphangioma, kaposiform heman-gioendothelioma, and angiosarcoma.

Investigators have studied KS tumors using patient procured material (in vivo) as well as several in vitro methods (e.g., explant derived tissue cultures, infected dermal microvascular endothelial cells or DMVEC, and animal models). These researchers have employed several methods including histochemistry (chemistry of microscopically visible structures), electron microscopy, and more recently in situ hybridization and molecular studies (e.g., microarray analysis) to study KS. Immunohistochemistry (IHC), introduced in its rudimentary form in the 1940s and later as a routine diagnostic procedure in surgical pathology, merges the fields of immunology and histology (Hunt et al. 2006).

IHC is used not only to determine whether a specific tissue expresses a particular antigen, but also allows one to determine the microanatomic cellular location of the antigen (Elias 2003). IHC is more specific and sensitive than histochemistry and less cumbersome than electron microscopy. With the ever expanding menu of diagnostic immunomarkers available for IHC, researchers have begun looking at antibodies not just for diagnostic purposes, but also to help provide prognostic information and perhaps identify novel therapeutic markers. The impact of IHC on our understanding, ability to confidently diagnose, and treat KS has grown steadily in the past few decades. The aim of this chapter is to review the body of literature pertaining to the use of IHC in the *in vitro* and *in vivo* study of KS. This chapter also addresses specimen handling, processing, IHC methodology, and highlights potential technological and interpretive pitfalls. Immunohistochemical methodology, including antigen retrieval, has also been recently discussed by Hayat (2002, 2004–2006).

MATERIALS

The type of KS sample, fixation, and subsequent tissue processing require special consideration prior to IHC investigation, irrespective of whether the specimen is obtained from a patient or *in vitro* source. KS samples may include mucocutaneous or visceral KS from any of the epidemiological forms. Moreover, KS specimens can include patch, plaque, nodular, and regressed lesions for the purpose of comparative study. Previous investigators have successfully performed IHC on cell supernatants and cell lysates

prepared from KS cell cultures (Samaniego et al. 1998). IHC has also been carried out on small amounts of KS specimen, such as cytopins (Simonart et al. 1998) and tissue microarrays (Higgins et al. 2003). For antibodies available to be used only with frozen tissue, KS specimens will need to be frozen from which cryostat sections can be prepared. Some researchers have noted that immunoreactivity with certain biomarkers (e.g., factor VIII-related antigen) is consistently positive only in frozen section material (Facchetti et al. 1988). Exposure to harsh solutions (e.g., used for bone decalcification) is best avoided because they can potentially impair immunoreactivity. Appropriate positive and negative controls, internal (e.g., non-neoplastic blood vessels within the KS specimen) or non-KS specimens, are essential for any good quality IHC study.

Samples to be studied by IHC may need to be fixed. Several researchers have opted to fix *in vitro* specimens with acetone at 4°C (Simonart et al. 2000). However, formalin fixation is the most common method used for IHC study. Fixed tissue requires subsequent processing with paraffin, commonly referred to as formalin-fixed paraffin-embedded (FFPE) samples. Occasionally, fixation may have little or no bearing on IHC. Traweek et al. (1991) showed that CD34 immunoreactivity was equally intense in frozen sections, alcohol-fixed tissue, or formalin-fixed specimens. However, fixation can certainly adversely influence IHC. When working with fixed specimens, methods for antigen retrieval or enhancement may be needed, including proteolytic enzyme digestion or heat-induced epitope retrieval (HIER). This will greatly improve the sensitivity of

immunostaining of fixed tissue. Formalin fixation, by preserving the native conformation of proteins, can mask tissue antigenicity, thereby reducing IHC staining intensity (Elias 2003). The extent of this masking is related to the duration of fixation, temperature, and fixative concentration. HIER (e.g., microwave irradiation or steam pressure cooker) prior to the application of immunoreagents is effective at unmasking (restoring) a wide range of antigens and/or epitopes (Leong et al. 1996; Hayat 2004–2006). A number of KS related studies utilized HIER by either microwave (Timar et al. 2005) or pressure cooking/steaming (Pantanowitz et al. 2004a). Other investigators performed antigen retrieval using pronase (Thewes et al. 2000). Flamini et al. (1997) studied p53 by IHC with or without microwave antigen retrieval pretreatment. They found that p53 immunoreactivity had slightly increased intensity in the antigen retrieval series.

METHODS

IHC is the application of antigen–antibody interactions to histochemical techniques (Leong et al. 1996). Antibodies bind to a specific portion (epitope) of an antigen. To date, many different investigational and commercial primary antibodies have been used by investigators to study KS by IHC (Table 33.1). They include polyclonal and monoclonal antibodies. Monoclonal antibodies usually exhibit greater specificity, and do not show much ‘batch to batch’ variation (Leong et al. 1996). Visualization of an antibody–antigen interaction can be achieved in several ways. Most often, an antibody is conjugated to

an enzyme (e.g., horseradish peroxidase, alkaline phosphatase, or glucose oxidase) that can catalyze a color-producing reaction (immunoperoxidase staining). The visible color change occurs at the site of the antibody–enzyme complex within the tissue/sample. Different chromogens are available, such as 3,3-Diaminobenzidine (DAB) that produces a brown reaction product. Ordinary bright-field microscopy (i.e., light microscope) is the method frequently used for viewing this color reaction. Alternatively, an antibody can be tagged to a fluorophore (e.g., FITC, rhodamine) to label tissue epitopes (immunofluorescence). IHC is often chosen over immunofluorescence because of the need for fresh frozen tissue and poor morphological preservation with the latter. In a number of studies investigators chose to demonstrate more than one antigen (double or even triple immunostaining) in KS lesional cells; e.g., Bcl-2 and factor VIII (Morris et al. 1996). This can be achieved by using double or multiple IHC staining techniques.

Two strategies can be used for the immunohistochemical detection of antigens in tissue; the (1) direct and (2) indirect method (Figure 33.2). Both methods appear to have been used for studying KS. The direct method is a one-step staining method, in which a labeled (conjugated) antibody reacts directly with the antigen in tissue sections. This technique is relatively simple and rapid because it utilizes only one antibody. However, it can suffer problems with sensitivity because there is minimal signal amplification. The indirect method uses one (primary) unlabelled antibody against the antigen being probed for, and a second (labeled) antibody against the first. The secondary antibody must

TABLE 33.1. Published data on immunohistochemical studies of Kaposi's sarcoma (listed alphabetically)

Antibody	Antigen/epitope	Biomarker details	Specimen source	Proportion positive cases (%)	Immunoreactive cell type	Reference
1B10	Fibroblast Surface Protein	Mouse monoclonal antibody	In vitro	45–90	Spindled cells	Simonart et al. (2000)
AKT	Serine/threonine kinase family	Phosphorylated antibody	In vivo	Not reported	Spindled cells	Koon et al. (2005)
Alpha-smooth muscle actin	Smooth muscle actin	Clone HHF35	In vitro	25–60	Spindled cells	Simonart et al. (2000)
		Not reported	In vivo	100	Spindled cells	Massarelli et al. (1989)
		14A	In vivo	100	Vascular channels	Rudolph et al. (1997)
AR	Androgen receptor	Not reported	In vivo	0	Not applicable	Pantanowitz et al. (2004b)
bFGF	Basic fibroblast growth factor	Monoclonal	In vivo	100	Spindled and endothelial cells	Samaniego et al. (1998)
Bax	Bax protein	Polyclonal I-19	In vitro	100	Spindled	Simonart et al. (1998)
		Not reported	In vivo	81–100	Spindled and endothelial cells	Morris et al. (1996)
		Not reported	In vivo	100	Spindled cells	Dada et al. (1996)
		N-19, rabbit polyclonal	In vitro	100	Not reported	Simonart et al. (1998)
		Clone Ab-1	In vitro	30–60	Spindled cells	Simonart et al. (2000)
		Not reported	In vivo	100	Spindled and endothelial cells	Pantanowitz et al. (2004a)
Bcl-X	Bcl-X protein	Rabbit polyclonal	In vivo	100	Spindled and endothelial cells	Foreman et al. (1996)
Bcl-xS/L	Bcl-2 homolog	Goat polyclonal	In vivo	100	Spindled cells	Fernandez-Figueras et al. (2000)
BMA 120	Endothelial marker	Not reported	In vivo	Most	Spindled and endothelial cells	Hashimoto et al. (1987)
Calcitonin receptor-like receptor	G protein-associated receptor	Polyclonal	In vivo	100	Predominantly spindled cells	Hagner et al. (2006)
Cathepsin B	Cathepsin	Sheep antiserum	In vivo	0	Not applicable	Thewes et al. (1997)
Chemokines	MIP-1alpha, MIP-1beta, MCP-1, MCP-2, and RANTES	Not reported	In vivo	100	Macrophages and dendritic cells	Uccini et al. (2003)
Chemokine receptors	CCR5, CCR1, and CCR2	Not reported	In vitro	100	Spindled cells	Uccini et al. (2003)
CD1a	Antigen presenting cells	Clone SK9	In vitro	<1	Spindled cells	Simonart et al. (2000)
CD3	T lymphocytes	Not reported	In vitro	100	Peripheral lymphocytes	Uccini et al. (2003)
CD4	T helper cells	Clone SK3	In vitro	<1	Spindled cells	Simonart et al. (2000)
CD8	T cytotoxic cells	Clone SK1	In vitro	<1	Spindled cells	Simonart et al. (2000)

(continued)

TABLE 33.1. (continued)

Antibody	Antigen/epitope	Biomarker details	Specimen source	Proportion positive cases (%)	Immunoreactive cell type	Reference
CD11	Leukocytes	CD11c and CD11b	In vivo	100	Peripheral macrophages	Uccini et al. (2003)
CD13	Monocytes and granulocytes	Clone L138	In vitro	25–80	Spindled cells	Simonart et al. (2000)
CD14	Lipopolysaccharide dependent signal transduction	Not reported	In vivo and vitro	75	Spindled cells	Huang et al. (1993a)
CD16	NK cells and granulocytes	Clone Mφ-P9	In vitro	<1	Spindled cells	Simonart et al. (2000)
		Clone B73-1	In vitro	<1	Spindled cells	Simonart et al. (2000)
		Not reported	In vivo	100	Macrophages	Uccini et al. (2003)
CD19	B lymphocytes	Clone 4G7	In vitro	<1	Spindled cells	Simonart et al. (2000)
CD21	Dendritic cell marker	Not reported	In vivo	100	Dendritic cells	Pantanowitz et al. (2003)
CD23	B lymphocytes and dendritic cells	Not reported	In vivo	100	Dendritic cells	Pantanowitz et al. (2003)
		Clone EBVCS-5	In vitro	<1	Spindled cells	Simonart et al. (2000)
CD25	IL2 receptor component and leukocytes	Clone 2A3	In vitro	<1	Spindled cells	Simonart et al. (2000)
		Not reported	In vivo	100	Spindled cells	Uccini et al. (2003)
		Clone JC/70A	In vivo	70	Spindled and endothelial cells	El-Sayed and Ramadan (2004)
		Clone JC/70A	In vivo	89	Not reported	Higgins et al. (2003)
		Clone JC/70A	In vivo	24	Spindled and endothelial cells	Russell Jones et al. (1995)
		Clone JC/70A	In vivo	90	Not reported	Fukunaga (2005)
		Clone JC/70A	In vivo	Most	Spindled and ovoid cells	Xu et al. (2004)
		Not reported	In vivo	100	Spindled and endothelial cells	Pantanowitz et al. (2004a)
CD34	Endothelial cells and stem cells	Clone L133.1	In vitro	<2	Spindled cells	Simonart et al. (2000)
		Not reported	In vivo	100	Spindled cells	Uccini et al. (2003)
		QBEND/10	In vivo	100	Spindled cells	El-Sayed and Ramadan (2004)
		My10	In vivo	100	Not reported	Higgins et al. (2003)
		Not reported	In vivo	100	Spindled and endothelial cells	Timar et al. (2005)
		Not reported	In vivo	70–100	Spindled and endothelial cells	Lee et al. (2003)
		QBEND/10	In vivo	Most	Spindled and ovoid cells	Xu et al. (2004)

	QBEND/10	In vivo	Most	Spindled and ovoid cells	Russell Jones et al. (1995)
	Not reported	In vivo	100	Not reported	Traweek et al. (1991)
	Not reported	In vivo	75	Spindled cells	Pyakurel et al. (2006)
	My10	In vitro	<3	Spindled cells	Simonart et al. (2000)
	Not reported	In vitro	0	Not applicable	Huang et al. (1993a)
CD35	Not reported	In vivo	100	Dendritic cells	Pantanowitz et al. (2003)
CD36	Clone FA6-152	In vitro	<3	Spindled cells	Simonart et al. (2000)
CD40	Clone EA-5	In vitro	<2	Spindled cells	Simonart et al. (2000)
CD45	Clone 2D1	In vitro	<1	Spindled cells	Simonart et al. (2000)
	Not reported	In vivo	100	Mononuclear inflammatory cells	Blasig et al. (1997)
CD54	Not reported	In vitro	100	Not reported	Huang et al. (1993a)
	Clone LB-2	In vitro	35–80	Spindled cells	Simonart et al. (2000)
	Not reported	In vitro	100	Spindled cells	Simonart et al. (1998)
CD62E	Clone 1.2B6	In vitro	<4	Spindled cells	Simonart et al. (2000)
CD68	Not reported	In vivo	100	Macrophages	Uccini et al. (2003)
	Clone KP1	In vitro	0	Not applicable	Simonart et al. (2000)
	Clone M734	In vitro	2–7	Spindled cells	Simonart et al. (2000)
CD71	Not reported	In vivo	100	Spindled cells and macrophages	Pantanowitz et al. (2005d)
CD79a	Not reported	In vivo	100	Lymphocytes	Uccini et al. (2003)
CD86	Clone IT2-2	In vitro	<1	Spindled cells	Simonart et al. (2000)
CD95	Clone DX2	In vitro	30–85	Spindled cells	Simonart et al. (2000)
CD102	Clone CBR-IC2/2	In vitro	<1	Spindled cells	Simonart et al. (2000)
CD106	Clone 1.G11B1	In vitro	<1	Spindled cells	Simonart et al. (2000)
CDK4	Clone N-19	In vitro	35–90	Spindled cells	Simonart et al. (2000)
CEA	Clone TF3H8-1	In vitro	0	Not applicable	Simonart et al. (2000)
c-erbB-2 (Her-2neu)	Not reported	In vivo	50	Not reported	Spandidos et al. (1990)
CIV (anti-E92)	IgG1 non-commercial	In vivo	100	Spindled and endothelial cells	Rutgers et al. (1986)
c-kit (CD117)	Not reported	In vivo	100	Spindled cells	Pantanowitz et al. (2004a)
	Clone A4502	In vivo	43	Spindled cells	Pantanowitz et al. (2005a)

(continued)

TABLE 33.1. (continued)

Antibody	Antigen/epitope	Biomarker details	Specimen source	Proportion positive cases (%)	Immunoreactive cell type	Reference
c-myc Collagen IV	Myc oncogene protein	Not reported	In vivo	50	Not reported	Spandidos et al. (1990)
	Basal lamina	Not reported	In vivo	0	Not applicable	Sturzl et al. (1992)
		Not reported	In vivo	100	Basement membrane material between cells	Bendelac et al. (1985)
CXCR3 and CXCR4 cyclin-D1		Not reported	In vitro	100	Spindled cells	Simonart et al. (1998)
	Chemokine receptors	Not reported	In vitro	90–100	Spindled cells	Simonart et al. (2000)
	Cell cycle cyclin D1 oncoprotein	Rabbit monoclonal	In vivo	100	Spindled cells	Uccini et al. (2003)
Cytokeratin D2-40		Not reported	In vivo	100	Spindled cells	Pantanowitz et al. (2004a)
	Keratin intermediate filaments	Clone AE1/AE3	In vivo	33.3	Spindled cells	Horenstein et al. (1997)
	Lymphatic endothelial marker	Monoclonal IgG1	In vivo	Not applicable	Not applicable	Simonart et al. (2000)
			In vivo	100	Spindled and endothelial cells	Kahn et al. (2002)
Desmin EBM11 ELAM-1		Monoclonal	In vivo	100	Spindled and endothelial cells	Pantanowitz et al. (2003)
	Smooth muscle desmin	Monoclonal	In vivo	90	Not reported	Fukumaga (2005)
	Macrophage antigen	Not reported	In vivo	0	Not applicable	Massarelli et al. (1989)
	Endothelial leukocyte adhesion molecule 1	Not reported	In vivo	Not reported	Spindled cells	Hashimoto et al. (1987)
		Not reported	In vitro	0	Not applicable	Simonart et al. (2000)
ER	Estrogen receptor	Not reported	In vitro	0	Not applicable	Huang et al. (1993a)
ERK (phospho-ERK)	Serine/threonine kinase	Not reported	In vivo	>1	Spindled cells	Pantanowitz et al. (2004b)
			In vivo	100	Not reported	Koon et al. (2005)
		Polyclonal and monoclonal	In vivo	100	Endothelial cells	Rutgers et al. (1986)
Factor XIIIa Fascin		Monoclonal	In vivo	100	Endothelial cells	Lee et al. (2003)
		Not reported	In vivo	0	Not applicable	Massarelli et al. (1989)
		Not reported	In vivo	Most	Spindled and endothelial cells	Facchetti et al. (1988)
		Not reported	In vivo	Most	Ovoid and spindled cells	Xu et al. (2004)
		Clone BGX016A	In vitro	0	Not applicable	Simonart et al. (2000)
Dermal dendrocyte marker Dendritic and endothelial cells		Not reported	In vitro	100	Spindled cells	Huang et al. (1993a)
		Not reported	In vivo	100	Dendritic cells	Pantanowitz et al. (2003)
		Not reported	In vivo	100	Spindled and endothelial cells	Pantanowitz et al. (2003)

Ferritin	Iron storage protein	Murine monoclonal	In vivo	100	Macrophages	Yuen et al. (2003)
Fibronectin	Non-collagenous glycoprotein	Not reported	In vivo	0	Not applicable	Sturzl et al. (1992)
		Not reported	In vivo	100	Pericellular connective tissue	Massarelli et al. (1989)
FKBP12	FK506 binding protein to calcineurin	Polyclonal rabbit	In vivo	90	Not specified	Higgins et al. (2003)
FLI-1	ETS family protooncogene	Clone Sc356	In vivo	100	Endothelial cells	Folpe et al. (2001)
		Monoclonal	In vivo	100	Not reported	Rossi et al. (2004)
Glucocorticoid receptor	Glucocorticoid signalling	Rabbit monoclonal	In vivo	100	Spindled cells	Guo et al. (1996)
HCG	Human chorionic gonadotropin receptor	Not reported	In vivo	100	Spindled cells	Pantanowitz et al. (2005c)
HLA class II	Major histocompatibility complex	Clone L243	In vitro	<1	Spindled cells	Simonart et al. (2000)
HLA-DR	CD4 binding	Non-commercial IgG1	In vivo	100	Spindled and endothelial cells	Rutgers et al. (1986)
Int-2 protein	Fibroblast growth factor-3	Not reported	In vivo	Most	Spindled cells	Modin et al. (1983)
Interleukin 4 receptor	IL4 cytokine signaling	Sheep polyclonal	In vivo	33	Spindled cells	Huang et al. (1993b)
Interleukin 6	IL-6 cytokine signaling	Monoclonal	In vivo	100	Endothelial cells	Varricchio et al. (1997)
Interleukin 6, viral (vIL-6)	HHV8 homolog of cellular IL-6	Polyclonal	In vivo	0	Not applicable	Oxholm et al. (1989)
Ki-67	Proliferation index	Rabbit antibody	In vivo	Most	Spindled cells	Brousset et al. (2001)
		Mib-1	In vitro	19-70	Spindled cells	Simonart et al. (2000)
		Mib-1	In vitro	100	Spindled cells	Dada et al. (1996)
Ki-M1p	CD68 pan-monocytic marker	Monoclonal	In vivo	100	Dendritic cells	Rudolph et al. (1997)
Laminin	Basement membrane constituent	Not reported	In vivo	100	Pericellular	Bendelac et al. (1985)
		Clone L-8971	In vitro	90-100	Spindled cells	Simonart et al. (2000)
Latent Nuclear Antigen-1 (LNA-1)	Anti-ORF73 of Human Herpesvirus 8	Not reported	In vivo	100	Spindled cells	Uccini et al. (2003)
		Clone 13B10	In vivo	78	Spindled and endothelial cells	Hong et al. (2003)
		Clone 13B10	In vivo	63	Spindled and endothelial cells	Schwartz et al. (2003)
		Rat IgG2c	In vivo	100	Spindled cells	Schmid and Zietz (2005)
		Clone 13B10	In vivo	80	Spindled cells	Pantanowitz et al. (2005a)
		Murine antibody	In vivo	100	Spindled cells	Pantanowitz et al. (2004a)
		Clone 13B10	In vivo	100	Spindled cells	Urquhart et al. (2006)
		LN53	In vivo	100	Spindled cells	Brousset et al. (2001)

(continued)

TABLE 33.1. (continued)

Antibody	Antigen/epitope	Biomarker details	Specimen source	Proportion positive cases (%)	Immunoreactive cell type	Reference
Lysozyme LYVE-1	Muramidase enzyme Hyaluronan receptor on lymphatic endothelium	Not reported	In vivo	100	Spindled cells	Patel et al. (2004)
		Not reported	In vivo	100	Spindled cells	Cheuk et al. (2004)
		Not reported	In vivo	80	Spindled cells	Pak et al. (2007)
MAC 387	Monocyte/macrophage-associated antigens	Clone 13B10	In vivo	92	Spindled cells	Hammock et al. (2005)
		LN53	In vivo	100	Spindled and endothelial cells	Dupin et al. (1999)
Mannose receptor	Macrophages and lymphatic endothelium	Not reported	In vivo	0	Not applicable	Massarelli et al. (1989)
		Mouse monoclonal	In vivo	Most	Ovoid and spindled cells	Xu et al. (2004)
Mcl-1 MDM2	Bcl-2 homolog P53 inhibitor	Not reported	In vivo	100	Spindled cells	Pyakurel et al. (2006)
		Not reported	In vivo	100	Monocytes	Biasig et al. (1997)
MMP-1 (collagenase)	Matrix metalloproteinase	Not reported	In vivo	100	Spindled cells and peripheral macrophages	Uccini et al. (2003)
		Rabbit polyclonal IF2 antibody	In vivo	100	Spindled cells	Fernandez-Figueras et al. (2000)
		Clone 41-1E5	In vivo	100	Spindled and inflammatory cells	Li et al. (1997) Pantanowitz et al. (2006)
MMP-2 (gelatinase)	Matrix metalloproteinase	Clone 42-5D11	In vivo	89	Spindled and inflammatory cells	Pantanowitz et al. (2006)
		IM33L	In vivo	82	Peri-lesional fibroblasts	Impola et al. (2003)
MMP-3 (stromelysin-1)	Matrix metalloproteinase	Clone 55-2A4	In vivo	33	Spindled and inflammatory cells	Pantanowitz et al. (2006)
		Clone 141-7B2	In vivo	78	Spindled and inflammatory cells	Pantanowitz et al. (2006)
MMP-7 (matrilysin)	Matrix metalloproteinase	Clone 56-2A4	In vivo	56	Spindled and inflammatory cells	Pantanowitz et al. (2006)
		MMP9abm-213	In vivo	67	Endothelial and inflammatory cells	Impola et al. (2003)

MMP-13 (collagenase 3)	Matrix metalloproteinase	Clone 181-15A12	In vivo	89	Spindled and inflammatory cells	Pantanowitz et al. (2006)
MMP-14	Matrix metalloproteinase	Clone 114-IF2	In vivo	0	Not applicable	Pantanowitz et al. (2006)
NGF receptor	Nerve growth factor (p75 neurotrophin receptor)	ME 20.4 antibody	In vivo	0	Not applicable	Rudolph et al. (1997)
OKM1	Monocytes and granulocytes	IgG2b antibody	In vivo	15	Spindled cells	Rutgers et al. (1986)
OKM5	Endothelial cells	IgG1 antibody	In vivo	100	Spindled and endothelial cells	Rutgers et al. (1986)
P16	G1-S cell cycle gene product	Rabbit polyclonal	In vivo	100	Not reported	Cohen and Geradts (1997)
P53	Cell proliferation tumor suppressor oncoprotein	Monoclonal BP53-12	In vivo	25	Spindled cells	Bergman et al. (1996)
		Not reported	In vivo	65	Spindled cells	Dada et al. (1996)
		Clone DO7	In vitro	<2	Spindled cells	Simonart et al. (2000)
		PAb180 and DO7	In vivo	27	Spindled cells	Li et al. (1997)
		Monoclonal D07	In vivo	75	Not reported	Flamini et al. (1997)
		PAb 240	In vivo	13	Not reported	Flamini et al. (1997)
PAI-1	Inhibitor of urokinase plasminogen activator	Not reported	In vivo	10	Endothelial cells	Thewes et al. (2000)
PAI-2	Plasminogen activator inhibitor	Monoclonal antibody	In vivo	6	Endothelial cells	Thewes et al. (2000)
PCNA	Proliferating cell nuclear antigen	Clone PC10	In vitro	3-80	Spindled cells	Simonart et al. (2000)
PDGF-B	Platelet-derived growth factor-B	Not reported	In vitro	100	Endothelial cells	Sturzl et al. (1992)
		Phosphorylated antibody	In vivo	100	Not reported	Koon et al. (2005)
Peroxiredoxin II (Prx II)	Endothelial and red blood cells	Not reported	In vivo	100	Endothelial cells	Lee et al. (2003)
Platelet glycoprotein IIIa	Platelet-collagen adhesion molecule	Monoclonal	In vivo	100	Platelets	Sturzl et al. (1992)
PR	Progesterone receptor	Not reported	In vivo	0	Not applicable	Pantanowitz et al. (2004b)
Procathepsin L	Proteinase	Rabbit polyclonal	In vivo	0	Not applicable	Thewes et al. (2000)
Prox-1	Lymphatic endothelial cells	Not reported	In vivo	100	Spindled cells	Reis et al. (2005)
Ras p21	Ras encoded protein with GTPase activity	Not reported	In vivo	100	Spindled cells	Spandidos et al. (1990)
Retino-blastoma protein (Rb)	Tumor suppressor protein	Not reported	In vivo	100	Spindled cells	Horenstein et al. (1997)
S-100	Neural cells, cartilage, melanocytes, fat and histiocytes	3C8, QED	In vivo	100	Spindled cells	Cohen and Geradts (1997)
		Not reported	In vivo	0	Not applicable	Massarelli et al. (1989)
		Not reported	In vivo	0	Not applicable	Sturzl et al. (1992)
		Not reported	In vivo	0	Not applicable	Rudolph et al. (1997)
SV40	Simian Virus 40 T antigen related p53 interference	Monoclonal antibody	In vivo	0	Not applicable	Li et al. (1997)

(continued)

TABLE 33.1. (continued)

Antibody	Antigen/epitope	Biomarker details	Specimen source	Proportion positive cases (%)	Immunoreactive cell type	Reference
TGF-beta 1	Transforming growth factor	Rabbit LC(1-30), CC(1-30), and Pre(266-278)	In vivo	100	Vascular smooth muscle cells, pericytes and connective tissue	Williams et al. (1995)
TNF alpha UEA-I (Ulex europaeus -I)	Tumor necrosis factor Endothelial cells	Polyclonal	In vivo	0	Not applicable	Oxholm et al. (1989)
		Not reported	In vivo	0	Not applicable	Massarelli et al. (1989)
		Not reported	In vivo	Rare	Spindled and endothelial cells	Russell Jones et al. (1995)
uPA	Urokinase plasminogen activator system	Not reported	In vivo	Most	Spindled and endothelial cells	Hashimoto et al. (1987)
		Not reported	In vivo	68-83	Spindled and endothelial cells	Beckstead et al. (1985)
		Not reported	In vivo	36	Endothelial cells	Thewes et al. (2000)
uPA-R	Urokinase plasminogen receptor	Not reported	In vivo	52	Endothelial cells	Thewes et al. (2000)
		Not reported	In vivo and vitro	75	Spindled cells	Huang et al. (1993a)
Vascular cell adhesion molecule 1 (VCAM-1)	Leukocyte-endothelial cell adhesion	Not reported	In vitro	0	Not applicable	Simonart et al. (1998)
Vascular endothelial growth factor receptor 3 (VEGFR-3)	Endothelial cell growth and angiogenesis	Not reported	In vitro	100	Spindled and mononuclear cells	Samaniego et al. (1998)
		Not reported	In vivo and vitro	100	Spindled cells	Dupin et al. (1999)
Vimentin	Intermediate filament	Not reported	In vivo	100	Spindled cells	Massarelli et al. (1989)
Wilms Tumor-1 (WT-1)	Zinc finger transcription factor	Clone V9	In vivo	100	Not reported	Rudolph et al. (1997)
		Clone V9	In vitro	90-100	Spindled cells	Simonart et al. (2000)
		Clones 6F-H2wt and 2C12.WT17AA	In vivo	17-100	Spindled cells	Timar et al. (2005)

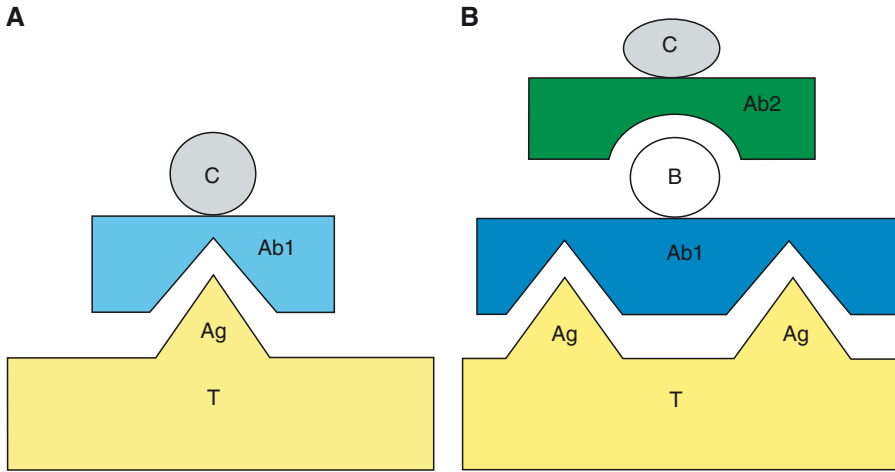


FIGURE 33.2. (A) Direct and (B) indirect immunohistochemical staining methods. The primary antibody is labelled in the direct method. In the indirect method, the primary antibody is unlabelled whereas the secondary antibody is labeled. Ab1 = primary antibody, Ab2 = secondary antibody (anti-Ig), Ag = antigen, B = biotin, C = chromogen marker (enzyme), T = tissue section

be against the IgG of the animal species (murine, rabbit, goat, and sheep have all been used to date) in which the primary antibody has been raised. The indirect method is more sensitive due to signal amplification because of the ability of the polyvalent secondary antibody to detect multiple sites on the Fc and Fab portions of the primary antibody (Elias 2003). More recent KS studies have employed commercially available detection kits and autostainers (Patel et al. 2004). These contemporary, and often more sensitive detection systems, are able to amplify the signal by increasing the number of reporter molecules even while using highly diluted immunoreagents.

The most frequent IHC detection system reported in published KS studies involved a biotinylated secondary antibody coupled with streptavidin i.e., avidin-biotin system (Uccini et al. 2003). Other researches used enzyme–anti-enzyme detection methods, such as the alkaline phosphatase anti-alkaline

phosphatase technique (Rudolph et al. 1997). In specific circumstances, pretreatment regimens may be required. The use of proteolytic enzymes before IHC staining greatly reduces nonspecific staining while simultaneously enhancing immunoreactivity of many tissue antigens (Elias 2003). Researchers should be aware that KS lesions frequently harbor many red blood cells. Enzyme activity normally present in erythrocytes (pseudoperoxidase) can react with DAB to produce nonspecific brown product that is indistinguishable from specific immune staining. Therefore, such endogenous enzyme reactivity needs to be ablated.

INTERPRETATION

Once a slide containing KS material (e.g., 4 or 5 μm thick tissue section) has been successfully immunostained, there are several factors to take into consideration

when examining the stained slide. These include the presence (positive staining) or absence (negative) of immunoreactivity, intensity of the color reaction, staining pattern (e.g. diffuse or granular), cellular component (e.g., spindled, endothelial and/or inflammatory cells) that is immunoreactive, as well as the localization (e.g., cytoplasmic, membranous, luminal surface, perinuclear and/or nuclear) of this immunoreactivity. For example, a granular perinuclear disposition may suggest an organelle related localization (Fernandez-Figueras et al. 2000). Most publications in this field have relied on subjective grading to provide a semi-quantitative assessment of immunostains. We are not aware of any KS study in which computer-assisted image analysis was used to standardize (quantify) the intensity of immunoreactivity. Evaluation of the proportion of positive cells may also be of value (e.g., early versus advanced lesions). However, because KS patch lesions contain little or no spindled cells and KS tumors harbor the most number of spindled cells, caution is required when comparing these different stages and/or evaluating the staining of spindled tumor cells.

It is imperative to interpret both intensity and the degree of background (i.e., non-specific) staining in relation to controls. Immunostaining of nearby (internal control) structures (e.g. native blood vessels and epidermis) is often very informative (Kahn et al. 2002). Care must be taken to avoid false positive staining from hemosiderin pigment in KS lesions, non-specific staining of necrotic tissue and debris from ulcers associated with large KS tumors, and with phagocytosed antigens within macrophages. The stratum granulosum

of the epidermis and free edges of tissue sections often shows non-specific reactivity with many antibodies (Leong et al. 1996). False positive reactions may be caused by cross-reactions with antigens other than the target, as well as from endogenous peroxidase or biotin. False-negative staining may be due to inappropriate titre or dilution of reagents, denatured antibody, low antigen levels, masking or loss of antigens, as well as inappropriate incubation and temperature.

HISTOGENESIS

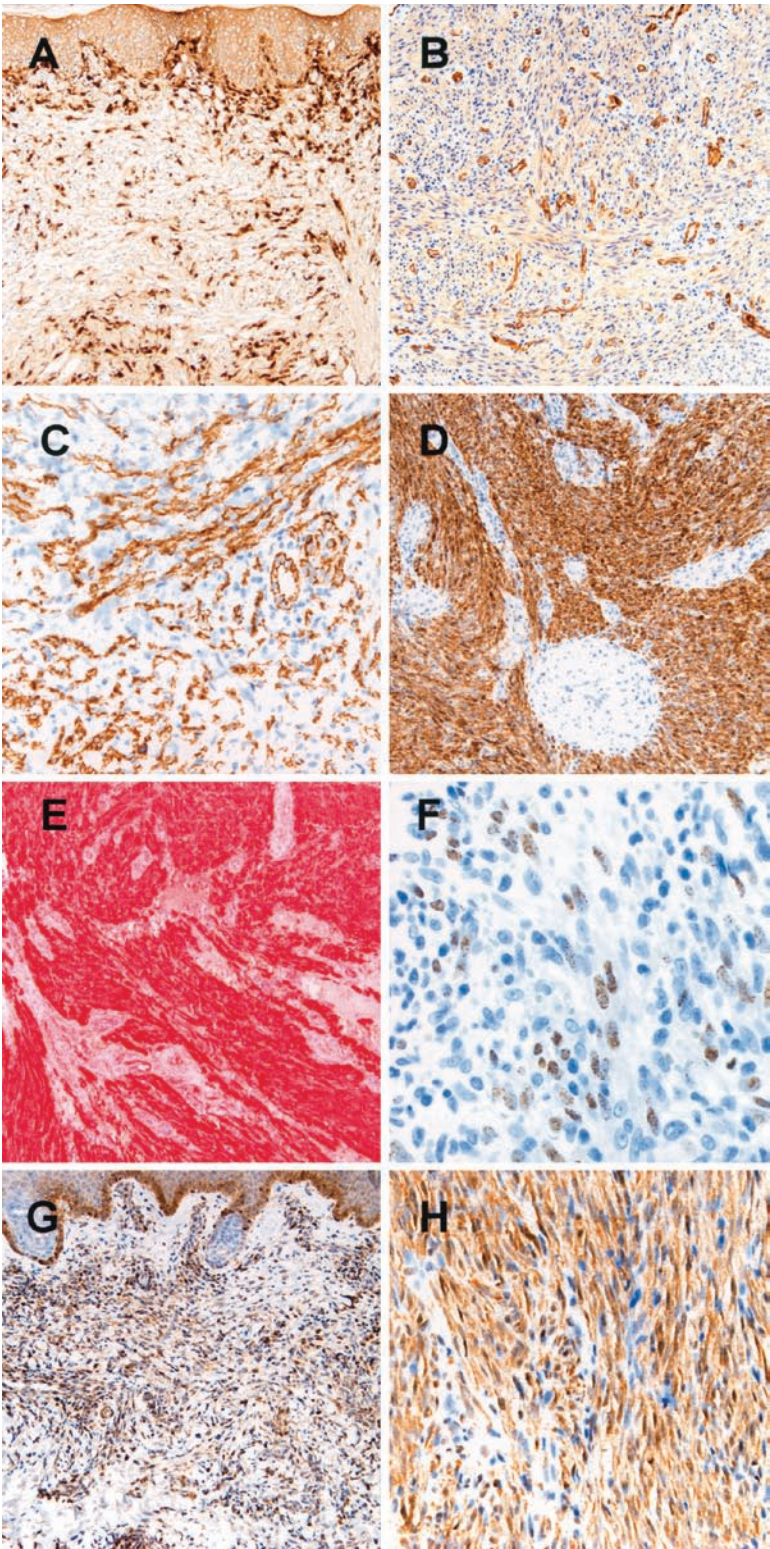
The cellular origin of KS lesional cells is controversial. The exact origin of the tumor cell (usually a spindled cell), which is generally agreed to be a type of endothelial cell, remains elusive. A plethora of articles have been published reporting that these cells may be derived from vascular endothelium, lymphatics, dendritic cells, macrophages, fibroblasts and smooth muscle cells. Immunohistochemical evaluation, along with enzyme histochemistry and ultrastructural studies, has been used in several studies to address this exact issue. Although some investigators speculated a derivation from macrophages and/or dendrocytes (dendritic cells normally present in the dermis), this no longer appears to be the case. Rather, hyperplasia of dendritic cells included within and at the edges of growing KS lesions (Figure 33.3a) more than likely only contributes to the angioproliferative process (Pantanowitz et al. 2003).

The majority of IHC studies have indicated that KS lesional cells express endothelial antigens, based on their

immunoreactivity for factor VIII-related antigen (Huang et al. 1993a), CD31 (Figure 33.3b) (Russell Jones et al. 1995), CD34 (Figure 33.3c) (Russell Jones et al. 1995), and UEA-1 (Massarelli et al. 1989). Factor VIII-related antigen (von Willebrand factor) is present in the vascular endothelium of all organs except renal glomerular capillaries. It is also present in megakaryocytes and platelets. Factor VIII-related antigen is fixation resistant and enhanced by proteolytic digestion or HIER (Leong et al. 1999). The endothelium of blood and lymphatic vessels share many common antigens such as von Willebrand factor, CD31, CD34 and UEA-1 (Leong et al. 1999). Antibodies to CD31 are immunoreactive in FFPE and frozen tissue sections. CD31, or platelet endothelial cell adhesion molecule-1 (PECAM-1), is a 130 kD glycoprotein normally expressed on endothelial cells and hematopoietic cells (platelets, monocytes, macrophages, granulocytes, and B lymphocytes). The CD34 antigen is a 110 kD transmembrane protein found on bone marrow stem cells, dendritic cells, and normal and malignant endothelium. *Ulex Europaeus* Agglutinin 1 Lectin (UEA-1) appears to be only variably immunoreactive in KS.

More recent IHC studies have implicated a lymphatic endothelial cell origin for KS tumor cells. This is because the majority of KS spindled cells have been shown to express lymphatic specific markers such as D2-40 (Figure 33.3d), LYVE-1, VEGFR-3, and Prox-1 (Reis et al. 2005). D2-40 is a novel monoclonal antibody directed against a sialoglycoprotein epitope present on lymphatic endothelium (Kahn et al. 2002). Kahn

et al. (2002) showed that D2-40 stained 100% of the endothelial cells lining the irregular spaces in patch- and plaque-stage KS lesions, as well as the spindled cells in the plaque- and nodular-stage tumors. LYVE-1 is a recently discovered homologue of the CD44 glycoprotein receptor for hyaluronan that enters the lymphatic system and is degraded in lymph nodes. It is expressed by endothelial cells of normal lymphatic vessels, but not blood vessels. The demonstration of LYVE-1 immunoreactivity in KS lesions by Xu et al. (2004) strongly favors derivation of this vascular tumor from lymphatics. VEGFR-3 is the receptor for VEGF-C. During early embryogenesis, VEGFR-3 mRNA is detected in most endothelial cells, but later becomes restricted to lymphatic endothelium of adult tissues. VEGF-C stimulates the proliferation of lymphatic endothelium, and VEGF-C transgenic mice exhibit lymphatic endothelium proliferation. In KS lesions, VEGFR-3 was shown by IHC to be expressed in spindled tumor cells, but not well formed vascular endothelium (Dupin et al. 1999). Costaining experiments by these investigators confirm that VEGFR-3 colocalizes with HHV8 (LNA-1) in KS tumors. Prox-1 found in KS lesions (Reis et al. 2005) is a nuclear transcription factor required during embryonic lymphangiogenesis, and is deemed to be a useful marker for differentiating lymphatic endothelial cells from other blood vessel endothelial cells. However, more work in this area is still required to better explain why KS tumor cells also coexpress other antigens such as fibroblast, dendritic (Figure 33.3e), and smooth muscle cell markers.



PATHOGENESIS

HHV8 Infection

We have learned that HHV8 infection is necessary for KS development. In fact, HHV8 is associated with all forms of KS. Initial studies that detected HHV8 in KS lesions relied on polymerase chain reaction (PCR) and in situ hybridization (ISH). Today, many studies have been able to localize HHV8 using IHC within KS spindle cells and endothelial cells of neoangiogenic vessels. HHV8 infected monocytes in KS lesions are also often immunoreactive, and serve as an important reservoir for transmission of this virus into KS tumor cells (Blasig et al. 1997). The commercial monoclonal antibody most often used for IHC is directed against the C-terminus of the latency-associated nuclear antigen (LNA-1 or LANA-1) encoded by ORF-73. HHV8 is a gamma-herpesvirus from the Rhadinovirus genus that encodes ~87 open reading frames (ORFs). ORF-73 exerts its transforming ability via several different mechanisms, such as decreasing the transcriptional activation of the tumor suppressor p53 (which prevents p53-induced apoptosis), or inactivating the retinoblastoma protein (pRB). There are many other HHV8-related gene products that induce cellular proliferation, transformation, cell signaling, cytokine production, immune

evasion, anti-apoptosis, and angiogenesis (Douglas et al. 2007).

HHV8 has a lytic and latent phase of replication. Both phases of the viral life cycle play significant roles in the pathogenesis of KS. In the lytic phase, HHV8 enters the host cell, uncoats, and undergoes viral transcription and DNA replication within the nucleus. Eventually viral particles get released from the infected cell. However, during their latent life cycle, multiple copies of their viral DNA are maintained as episomes, attached to the host chromosome with the help of the HHV8-encoded LNA-1. This probably explains why LNA-1 immunoreactivity in KS cells appears as stippled intranuclear staining (Figure 33.3f). Rare cases of cytoplasmic LNA-1 immunoreactivity have been observed in HHV8-infected dermal microvascular endothelial cells (A. Moses, personal communication). The significance of this observation is unclear. Most of the cells found in both KS lesions and HHV8-infected cultures are latently infected, and in only a small percentage of infected cells is lytic replication observed (Douglas et al. 2007). With regard to IHC, this is very helpful because most KS lesional cells are therefore likely to be LNA-1 immunoreactive. Mature endothelial cells of normal vascular blood vessels present in or adjacent to KS lesions have

FIGURE 33.3. Immunoreactivity of Kaposi's sarcoma lesional cells. (A) Factor XIIIa highlights dendritic cells within and around a cutaneous KS lesion. (B) CD31 stains the endothelial cells of abnormal vessels much more strongly than the KS spindle tumor cells. (C) CD34 demonstrates immunoreactivity in spindle and endothelial cells. (D) D2-40 stains KS lesional cells, but not the normal blood vessels. (E) Fascin, an endothelial and dendritic cell marker, strongly stains KS spindle cells. The red color is due to alkaline phosphatase labelled polymer. (F) LNA-1 immunoreactivity is identified as intranuclear stippled staining within KS tumor cells. (G) KS spindle cells and associated inflammatory cells are bcl-2 positive. Note that the overlying stratum basale is also immunoreactive in this case. (H) KS tumor cells exhibit strong c-kit (CD117) immunoreactivity

been shown to be consistently negative for LNA-1 in all cases studied (Dupin et al. 1999).

The exact role of the HHV8 lytic cycle in KS pathogenesis is uncertain. Initially, it was assumed that because only a small number of tumor cells express lytic proteins and that the viral lytic cycle results in cell death, it was likely that only the latent viral proteins would be responsible for cellular transformation. This no longer appears to be the case, as there are several lytic proteins that have been found to have intrinsic immortalizing or transforming ability (Douglas et al. 2007). HHV8 encodes two modulators of immune recognition (ubiquitin ligases), MIR-1 (K3) and MIR-2 (K5), during lytic replication that downregulate major histocompatibility complex (MHC) class I and/or T-cell co-stimulatory molecules from the surface of infected cells (Tomescu et al. 2003). This likely helps with evasion from the host's immune system. K3 downregulates all classes of MHC-1, HLA-A, B, C and E, while K5 is specific for HLA-A and B. K5 has also been shown to downregulate several cell surface molecules including ICAM-1 and CD31 (Tomescu et al. 2003). In fact, K5 targets both newly synthesized and preexisting CD31 molecules, resulting in their endocytosis and lysosomal degradation (Mansouri et al. 2006). The loss of CD31 from infected endothelial cells appears to affect their adhesion and migration. This phenomenon may also explain why CD31 immunoreactivity in various KS tumors is often weak and less easy to define compared to other endothelial markers such as CD34 (El-Sayed and Ramadan 2004). Moreover, CD31 immunoreactivity may also be patchy and less consistent than that related to CD34 (Russell Jones

et al. 1995). However, this observation is variable, and may be related to the fact that in vivo HHV8 exists in a predominantly latent state, with less than 5% of infected cells expressing discernible lytic gene products (Tomescu et al. 2003). Finally, CD31 positivity is greater in patch and plaque KS lesions compared to the nodular stage (Pantanowitz et al. 2004a), perhaps related to the increased number of LNA-1 positive cells in more advanced lesions.

Angiogenesis

The formation of new blood vessels, often from preexisting vessels, is a fundamental step in the pathogenesis of malignant tumors. Angiogenesis is key to the tumorigenesis of KS tumors. Some of the major cellular regulators involved in KS angiogenesis include vascular endothelial growth factor (VEGF), interleukin-6 (IL-6), the angiopoietins (Ang1 and Ang2), matrix metalloproteinases (MMPs), cathepsins, platelet derived growth factor (PDGF), and fibroblast growth factor (FGF). Using IHC, researchers have been able to study the contribution of most of these factors in KS. VEGF is a potent stimulator of angiogenesis. In the presence of VEGF, endothelial cells will proliferate and migrate. VEGF also causes a massive signaling cascade in endothelial cells. IL-6 has been shown to indirectly induce angiogenesis by upregulating VEGF. While early studies failed to demonstrate IL-6 in KS with IHC (Oxholm et al. 1989), later investigations using antibodies directed against the HHV8 homolog of cellular IL-6 (vIL-6) noted immunoreactivity in most in vivo KS spindled cells (Brousset et al. 2001). This is surprising given that using different

methods of detection the levels of cellular IL-6 are high in KS tumors, whereas those of vIL-6 expression are quite low (Moore et al. 1996). IL-6 mediates its inflammatory response via the JAK/STAT, MAPK, and PI3K/AKT signaling pathways. In addition to encoding its own IL-6 homolog, HHV8 encodes several other proteins (e.g., HHV8-encoded chemokines or vCCLs) that induce VEGF.

MMPs are zinc-dependent proteinases involved in remodeling the extracellular matrix during tumor invasion, metastasis, and angiogenesis. MMP-2, -3, -7, -9, and -13 have been shown with IHC to be expressed in KS tumors (Pantanowitz et al. 2006). Various HHV8-related proteins (e.g., K1 and endothelin-1) have been shown to induce the expression and activation of MMPs often critical for the angiogenic switch in tumor progression. In addition to these virally induced factors, there are a large variety of cytokines, chemokines, and MMPs secreted by admixed leukocytes that also add considerably to the progression of KS (Douglas et al. 2007).

Chemokines

It is well established that inflammatory cytokines are an important component in KS development (Dezube 2000, 2002). Cytokines, acting in synergy with HIV-1 Tat protein in AIDS-related KS, induce endothelial cells to acquire a KS cell phenotype and produce angiogenic factors. Using IHC techniques, Uccini et al. (2003) were able to prove that macrophages and dendritic cells in vitro expressed several chemokines (MIP-1alpha, MIP-1beta, MCP-1, MCP-2, and RANTES), and that spindle tumor cells expressed chemokine receptors CCR5, CCR1,

and CCR2. This supports a paracrine loop in KS tumorigenesis. Chemokine receptors usually signal through coupled G proteins. Indeed, HHV8 encodes a chemokine-like G protein-coupled receptor (vGPCR), a homolog of the human IL-8 receptor (CXCR2), that promotes endothelial cell proliferation through activation of the VEGF receptor-2/KDR and NF-kappa B (Douglas et al. 2007). This, in turn, induces the expression of a number of other proinflammatory and angiogenic factors including IL-1 β , TNF α , IL-6, IL-2, IL-4, IL-8, and GM-CSF, as well as the adhesion molecules VCAM-1, ICAM-1, and E-selection.

Apoptosis

Apoptosis is another mechanism that the host has evolved to fight viral infection. Not surprisingly, HHV8 has evolved several mechanisms to counteract apoptosis (Douglas et al. 2007). For example, HHV8 expresses several proteins (e.g., LNA-1, vFLIP, vIRF-1, vIRF-3, RTA, K1, K7, and K8) that can block the extrinsic (FAS and TNFR-1 activated) and intrinsic (p53) apoptosis pathways. HHV8 also expresses a Bcl-2 homolog, vBcl-2 (ORF16), which inhibits p53-mediated apoptosis by blocking Bax. By means of IHC, quite a few studies have successfully identified the expression of Bcl-2 (Figure 33.3g), Bax, Bcl-X, and the Bcl-2 homolog Bcl-xS/L in KS cells both in vitro and in vivo (Fernandez-Figueras et al. 2000). This method of immune evasion also leads to some of the immortalizing and transforming properties of HHV8. For example, inactivation of the tumor suppressor gene *p53* promotes cell immortalization, and inactivation of pRb promotes host cell cycle progression into S phase.

DIAGNOSIS

The histopathological diagnosis of KS may be problematic because of its similar morphologic, and often clinical, appearance to other benign and malignant vascular lesions. Most of these other vascular entities will stain positively with immunomarkers that are generically associated with endothelial differentiation, including factor VIII-related antigen, CD31, CD34, and UEA-1 (Wick et al. 2006). Presently, IHC is generally available and routinely used to localize HHV8 in KS lesions. Given the strong association between HHV8 and KS, positive immunoreactivity for LNA-1 has proved to be a useful diagnostic tool to help differentiate KS from its mimics (Cheuk et al. 2004). LNA-1 is a highly sensitive and specific marker of KS in both the general population and in HIV-positive patients (Hammock et al. 2005). Patel et al. (2004) suggest that strong, diffuse, nuclear staining in at least >10% of the tumor cells should be considered a positive result. HHV8 immunoreactivity appears not to be affected by HIV status, patient age, gender, tumor recurrence, or the site of the KS lesion (Hong et al. 2003).

Immunostaining of KS with LNA-1 is particularly helpful in difficult to diagnose early patch stage lesions, those KS lesions that may mimic other entities such as lymphangioma-like KS (Audard et al. 2003) and pyogenic granuloma-like KS (Urquhart et al. 2006), or for KS lesions that present in unusual locations (Huang et al. 2005). However, the presence of HHV8 appears not to be fully restricted to KS, as HHV8 has been detected (albeit rare) in some angiosarcomas, hemangiomas, and dermatofibromas (Pantanowitz et al. 2005b). HHV8 detection by PCR

has been reported to be positive in a small number of HIV-associated non-KS vascular tumors (e.g., hemangioma), most likely due to the detection of HHV8 within intratumoral blood mononuclear cells by this highly sensitive real-time PCR technique (Hammock et al. 2005). For this reason, LNA-1 IHC is preferable to PCR (for HHV8) for the evaluation of problematic vascular proliferations in HIV-infected individuals. HHV8 has also been reported to be identified in primary effusion lymphoma, other HHV8-associated lymphomas, Castleman's disease, and in the cells of the plexiform lesions of primary pulmonary hypertension. Finally, because other entities and spindle cell neoplasms (e.g., carcinoma and sarcoma) may need to be excluded, it is often important to include additional biomarkers (e.g., cytokeratin) in one's diagnostic workup.

THERAPY

Treatment of KS is aimed at symptom palliation, tumor shrinkage, prevention of disease progression, and possibly cure (Dezube et al. 2004). Therapeutic options include local therapy, immunotherapy (e.g., interferon alpha), highly active antiretroviral therapy (HAART) in the setting of HIV infection, systemic chemotherapy (typically with liposomal anthracyclines and taxanes), and the administration of pathogenesis-related investigational agents. The advent of HAART has led to a marked reduction in the incidence, morbidity and mortality associated with AIDS-related KS (Di Lorenzo et al. 2007). Systemic chemotherapy is usually given to patients with more advanced or rapidly progressive KS. With the expanding menu of diagnostic

markers available, including those for use with IHC, researchers have begun looking at antigens that can provide targeted therapy. The identification of such molecular targets has provided us with several new potential therapeutic strategies to treat KS (Koon et al. 2007). Thalidomide, for example, which exhibits antiangiogenic activity partly through inhibition of FGF-induced angiogenesis, has been shown to provide a partial response in treating KS.

Other novel agents used to treat KS include tyrosine kinase inhibitors and MMP inhibitors. Clinical trials have shown that the chemically modified tetracycline COL-3 (6-demethyl-6-deoxy-4-dedimethylaminotetracycline), a MMP inhibitor, is beneficial in the treatment of AIDS-related KS (Dezube et al. 2006). In vitro studies have shown that HHV8 induces the tyrosine receptor kinase c-kit in infected cells. The expression of c-kit (CD117) in vivo has also been systematically studied in KS lesions with IHC in FFPE tissue specimens (Pantanowitz et al. 2005a). In this study, KS lesions of all epidemiologic forms at various histologic stages demonstrated c-kit immunoreactivity of equal intensity (Figure 33.3h). The exact role of c-kit is unclear, but may contribute to the spindle morphology of tumor cells. Platelet-derived growth factor (PDGF) is important in cell proliferation, cell migration, and plays a significant role in angiogenesis. Therefore, it is not surprising that Sturzl et al. (1992) were able to show in vivo PDGF-B immunoreactivity within KS tumors. Like c-kit, the receptor for PDGF (PDGF-R) is also classified as a tyrosine kinase. Imatinib mesylate, a PDGF-R and c-kit inhibitor, has been found to result in clinical and histologic regression of KS (Koon et al. 2005).

CONCLUSION

Immunohistochemistry is an excellent technique to detect the cellular expression of specific antigens and has the advantage of being able to demonstrate exactly where a given protein is located within the tissue or cells being examined. However, unlike other techniques (e.g., immunoblotting method where staining is checked against a molecular weight ladder), it is usually not possible to prove that immunostaining corresponds exactly with the protein of interest. To date, many varied antigens have been detected using IHC in KS lesions that have contributed to our understanding of KS histogenesis and pathogenesis. However, differences in these IHC results can be confusing, particularly the inconsistent staining sometimes reported by different researchers. For example, investigators have reported strong positive staining of some KS tissue, moderate staining, and no staining at all for the common endothelial markers Factor VIII, CD34, and UEA-1. This may be related to several factors including the choice of antibodies (e.g., monoclonal versus polyclonal, commercial versus experimental) and/or technical differences (e.g., tissue fixation, pretreatment, antigen retrieval).

Frozen section remains the gold standard for antigen preservation. However, many investigators dealing with KS samples often shy away from working with unfixed specimens, mainly because of the potential for exposure to HHV8 and possibly HIV. Unfortunately fixation, along with routine tissue processing and paraffin-embedding, has the potential to reduce the amount of immunohistochemically detectable antigen (Leong et al. 1996). In research laboratories, it is often common practice to

store precut unstained tissue sections on a glass slide. This is best avoided, as there is frequently loss of antigenicity in stored paraffin sections, and rarely an increase of antigenicity (Elias 2003). In one study, loss of immunoreactivity for polyclonal factor VIII was reported in slides stored for as little as 12 weeks (Jacobs et al. 1996). Moreover, LNA-1 immunoreactivity was shown by one group of investigators to be markedly diminished in archival tissue (of ~40 years) of endemic KS because of poor antigen preservation (Schwartz et al. 2003).

It is uncertain how much of the variability and heterogeneous marker expression reported in previous studies is related to subjective interpretation of immunoreactivity. A plausible explanation for these discrepancies is that KS lesional cells do not represent a homogeneous cell population, but rather a mixture of different cell types. In addition to neoplastic spindled and endothelial cells, KS lesions also contain dendritic cells, macrophages, plasma cells, lymphocytes, and erythrocytes. Moreover, it appears that as KS lesions develop from patch through to plaque and eventually a nodular stage, different cell types predominate (Roth et al. 1992) and with such evolution there is recruitment of tumor precursor cells with a continuous phenotype switch (Pyakurel et al. 2006). Many of the findings reported in Table 1 show that in vitro findings occasionally differ from those reported in vivo. This is likely related to the fact that different explanted cell types may survive in vitro processing, that in turn may influence which cellular phenotype predominates in transformed tissue culture.

Finally, while current evidence overwhelmingly supports the derivation of KS

lesional cells from lymphatic endothelium, a number of IHC studies have shown that KS cells coexpress both blood vessel and lymphatic endothelial markers. This may be related to the fact that HHV8 has the ability to reprogram its target host cell, thereby masking the true origin of the cell (Douglas et al. 2007). The identification of various biomarkers in KS has provided us with superior diagnostic tools and potential therapeutic targets. As our knowledge regarding KS pathogenesis increases, so to have the opportunities to design therapeutic agents to target these pathways. Additional potential therapeutic agents may be derived from the roles of p53, viral cyclin, NF-kappaB, VEGF, and insulin-like growth factor, as well as the MAPK, JAK/STAT, and AKT/mammalian target of rapamycin (mTOR) pathways. If conducted appropriately, IHC is an excellent technique to study novel disease mechanisms, evaluate emerging prognostic markers, and discover therapeutic targets.

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Synovial Sarcoma: Role of Immunohistochemistry and Molecular Genetics in Diagnosis and Prognosis

Andrew E. Horvai

INTRODUCTION

Synovial sarcoma (SS) was described some 80 years ago by Smith (1927) as *synovioma*, receiving this designation because of histologic similarities to immature synovium, and perhaps because the soft tissue around the knee joint, albeit extra-articular, is a common location. However, modern techniques have demonstrated that this sarcoma lacks true synovial differentiation. Furthermore, SS presents in sites such as the upper aerodigestive tract, pleura, and heart which are devoid of native synovium. Practically speaking, SS can be thought of as a malignant mesenchymal tumor with at least focal epithelial differentiation and very frequently possessing a translocation involving chromosomes X and 18 (described in detail below). Synovial sarcoma is rare, representing ~8% of soft tissue sarcomas (Weiss and Goldblum 2008).

Although the histogenesis of SS remains somewhat uncertain, our knowledge of the clinical manifestations, pathology, and genetics of this sarcoma is expanding. In general, SS affects adolescents and young adults, with the vast majority of patients under 50 although examples at both extremes of age have been reported (Chan

et al. 2003; Ferrari et al. 1999). This sarcoma most commonly presents as a slowly growing tender mass in the mid to distal extremities. Radiographically, the presence of stippled calcification can be suggestive of the diagnosis. Overall 5 year survival for SS has been reported between 51% and 82% depending on the patient's age, tumor size and resectability (Weiss and Goldblum 2008). Mortality is largely a consequence of metastatic rather than local disease. The treatment, based on National Comprehensive Cancer Network guidelines, consists of gross total surgical resection with 1 cm or intact fascial margins with preoperative or postoperative radiation and chemotherapy (www.nccn.org 2007). Therapy is, of course, tailored to the location and extent of the disease. First line chemotherapy is ifosfamide based, analogous to a wide variety of high-grade sarcomas. Consequently, one might argue that distinguishing SS from other spindle cell sarcomas does not affect drug choice. However, clinical evidence suggests that SS responds better than other sarcomas to a high dose of ifosfamide (Rosen et al. 1994); therefore, distinguishing SS from other sarcomas is clinically relevant. Targeted therapy,

while not in routine use today, is a focus of several ongoing trials, and will further necessitate accurate diagnosis of SS in the future (Albritton and Randall 2005).

DIAGNOSIS

While it is impossible to devise a universal protocol for the diagnosis of SS that incorporates every possible clinical setting, Figure 34.1 presents a practical outline for diagnosis. The diagnosis is based on a synthesis of the clinical setting, histology and, if necessary, special studies such as immunohistochemistry or genetic analysis. Based on this scheme, ancillary studies prove most helpful, or necessary, when the differential diagnosis includes other spindle cell sarcomas (in the case of monophasic tumors) or round cell sarcomas and carcinomas (in the case of biphasic tumors).

Histology

Routine histologic features can be adequate for diagnosis in classic, biphasic, SS. Unfortunately, only ~30% of SS fall into this category with the remainder composed of monophasic fibrous, poorly differentiated, and the very rare monophasic epithelial subtypes (Weiss and Goldblum 2008). Biphasic synovial sarcoma demonstrates an admixture of cuboidal to columnar cells forming gland-like or papillary structures alternating with a monomorphic population of spindle cells. The transition between the two components is gradual. The epithelial and spindle cells often share similar nuclear features (Figure 34.2a). Often, the gland-like structures contain dense eosinophilic material but true mucin production is absent. These findings are sufficiently unusual in mesenchymal tumors that the diagnosis can be made by routine histology alone.

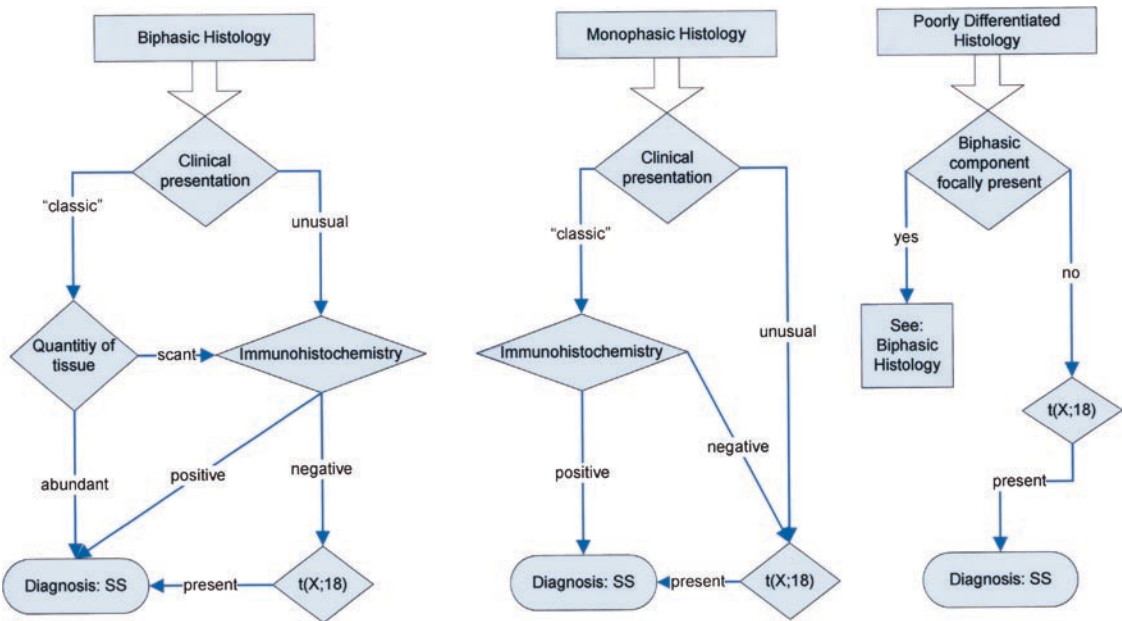


FIGURE 34.1. Outline of diagnostic approach to synovial sarcoma. The findings with routine H&E histology, the adequacy of biopsy material, and clinical presentation guide the need for additional supporting data including immunohistochemistry or genetic/molecular studies

However, glands and gland-like structures have been described in rare cases of malignant peripheral nerve sheath tumor (MPNST) (Wong et al. 1991), whereas several other malignancies can demonstrate a biphasic phenotype (uterine carcinosarcoma, mesothelioma). Thus, unusual presentations, for example, intraneural or visceral location, should prompt confirmation of the diagnosis with additional studies, outlined in the following sections.

Monophasic fibrous SS may be considered less of a subtype and instead one extreme

of a spectrum in which the spindle cells predominate. In these examples, the histology is dominated by a highly cellular spindle cell population with short intersecting fascicles frequently with a so-called “hemangiopericytomatous” vasculature. Individual tumor cells have scant cytoplasm and ill-defined borders with nuclear overlap a common feature (Figure 34.2b). However, scattered plumper, more eosinophilic cells, sometimes in irregular cords, are often present in the monophasic type even in the absence of distinct gland-like structures.

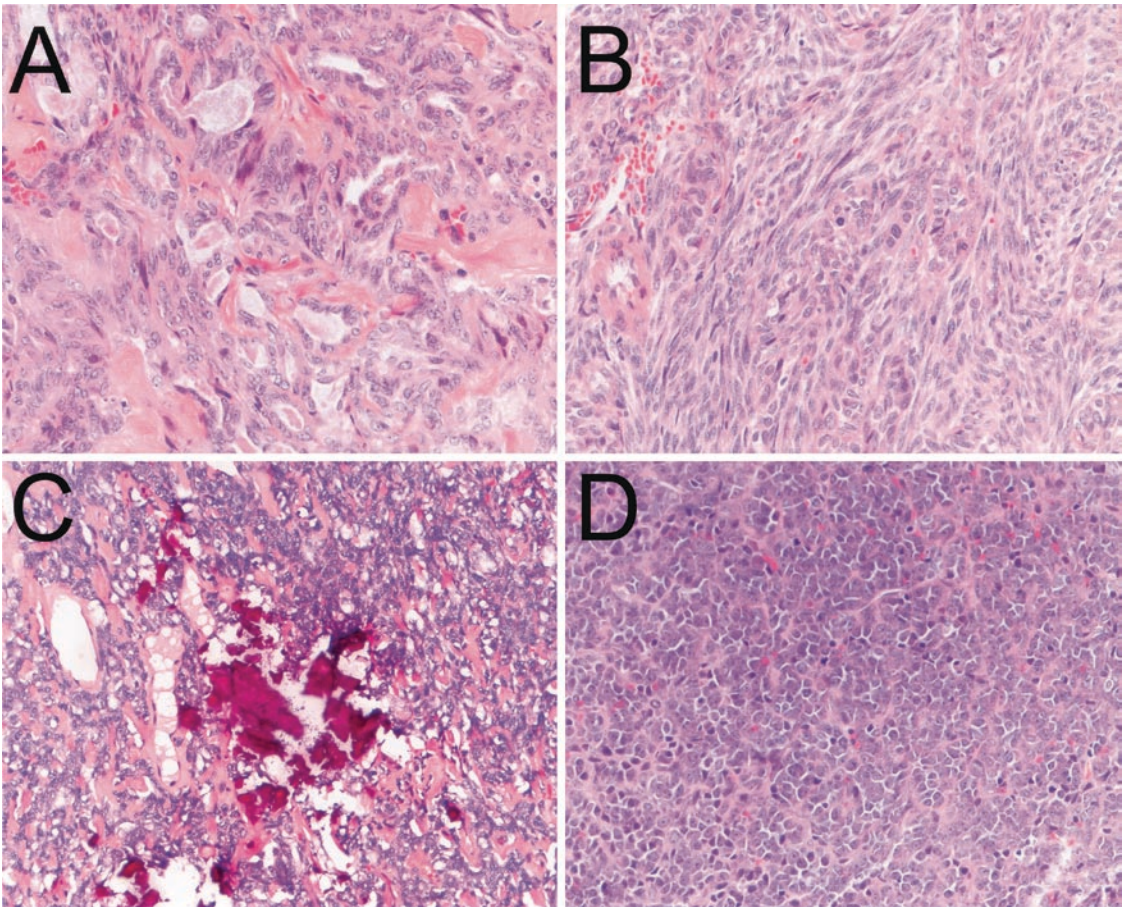


FIGURE 34.2. Histologic findings in synovial sarcoma. (A) The spindled and epithelioid cells in biphasic synovial sarcoma share similar nuclear features, (B) monophasic (fibrous) synovial sarcoma, (C) calcifications are a common feature of synovial sarcoma, and (D) poorly differentiated synovial sarcoma composed of round cells with higher grade nuclear features than monophasic and biphasic types

Features that are present to varying degrees in a subset of cases include myxoid change, cystic change, hyalinized stroma, calcification (Figure 34.2c), or ossification. Given the rarity of the monophasic epithelial variant (discussed below) the term “monophasic SS” typically refers to the fibrous type.

Conceptually, monophasic epithelioid SS exists at the other end of the histologic spectrum from the fibrous subtype. However, the monophasic epithelioid type is exceedingly rare; the subject of a few series and case reports (reviewed in Weiss and Goldblum 2008). If adequate histologic sampling of a suspected SS fails to reveal a spindle cell component, a diagnosis of metastatic carcinoma should be excluded. A diagnosis of pure epithelioid SS essentially requires genetic or molecular evidence of t(X;18), a somatic location and absence of a primary carcinoma based on clinical and radiographic findings.

Poorly differentiated SS can present either as a morphologically high grade round cell or spindle cell sarcoma. In either case, the nuclei are larger with vesicular chromatin, macronucleoli and the cells have scant cytoplasm (Figure 34.2d). Unlike conventional SS, the mitotic rate is high (>10 mf/10 hpf). The round cell morphology raises the differential of Ewing sarcoma and other malignant small round cell neoplasms. Frankly anaplastic, bizarre giant tumor cells such as those often seen in the so-called malignant fibrous histiocytoma are not typically present. Poorly differentiated SS often occurs in association with a component of biphasic or monophasic fibrous SS, suggesting that some form of tumor progression gives rise to this morphology (Weiss and Goldblum 2008). The presence of focal areas of

conventional biphasic SS is sufficient in the correct clinical context for a diagnosis based on histology alone. Adequate sampling is essential under such circumstances and since the finding is usually very focal, most cases will require ancillary techniques.

Ultrastructure

In most practices, immunohistochemistry has largely replaced electron microscopy in the diagnosis of SS, so ultrastructure is discussed briefly. The advantages of cost, speed, access to technology and ease of interpretation all tend to favor immunohistochemistry. Nevertheless, in select instances (inadequate tissue for immunohistochemistry or equivocal immunohistochemical results when molecular or genetic studies are unavailable), ultrastructure can be a useful adjunct to diagnose SS. Briefly, the epithelioid areas demonstrate small microvilli on the luminal surface and a complete basal lamina surrounding the gland-like structures. The spindle cell component also demonstrates epithelial features including intercellular junctions or small microvilli (Weiss and Goldblum 2008).

Immunohistochemistry

A variety of antigens, most notably markers of epithelial differentiation, has proven useful in confirming the diagnosis of SS and is summarized in Table 34.1. Antibodies to epithelial markers (low molecular weight keratins, EMA, CK19 and CK7) stain most strongly the gland-like areas in biphasic tumors (Figure 34.3a). As previously discussed, the H&E morphology of biphasic SS is often sufficient for diagnosis, and immunohistochemistry is not always

TABLE 34.1. Immunohistochemical stains of diagnostic utility in synovial sarcoma

Antigen	% Positive	References
Keratin	70	Fisher (1986)
EMA	80	Fisher (1986)
CK19	80	Olsen et al. (2006), Smith et al. (1999)
CK7	80	Olsen et al. (2006), Smith et al. (1999)
S100	30	Olsen et al. (2006)
CD34	<5	Olsen et al. (2006), Weiss and Nickoloff (1993)
HER2	30	(Begueret et al. (2005), Nuciforo et al. (2003)
EGFR	50	Begueret et al. (2005), Dobashi et al. (2007)
SYT	87	He et al. (2007)
Fascin	100	Grogg et al. (2005)

required. However, immunostains are useful confirmatory tests in small biopsies or unusual clinical presentations (site, age) even with biphasic morphology.

In contrast, the morphologic overlap between monophasic SS and other spindle cell sarcomas, especially on small biopsies, most often requires immunohistochemistry. Stains for keratin and EMA highlight the plumper, eosinophilic cells in monophasic SS that may be otherwise difficult to discern with H&E (Figure 34.3b). However, monophasic and poorly differentiated SS

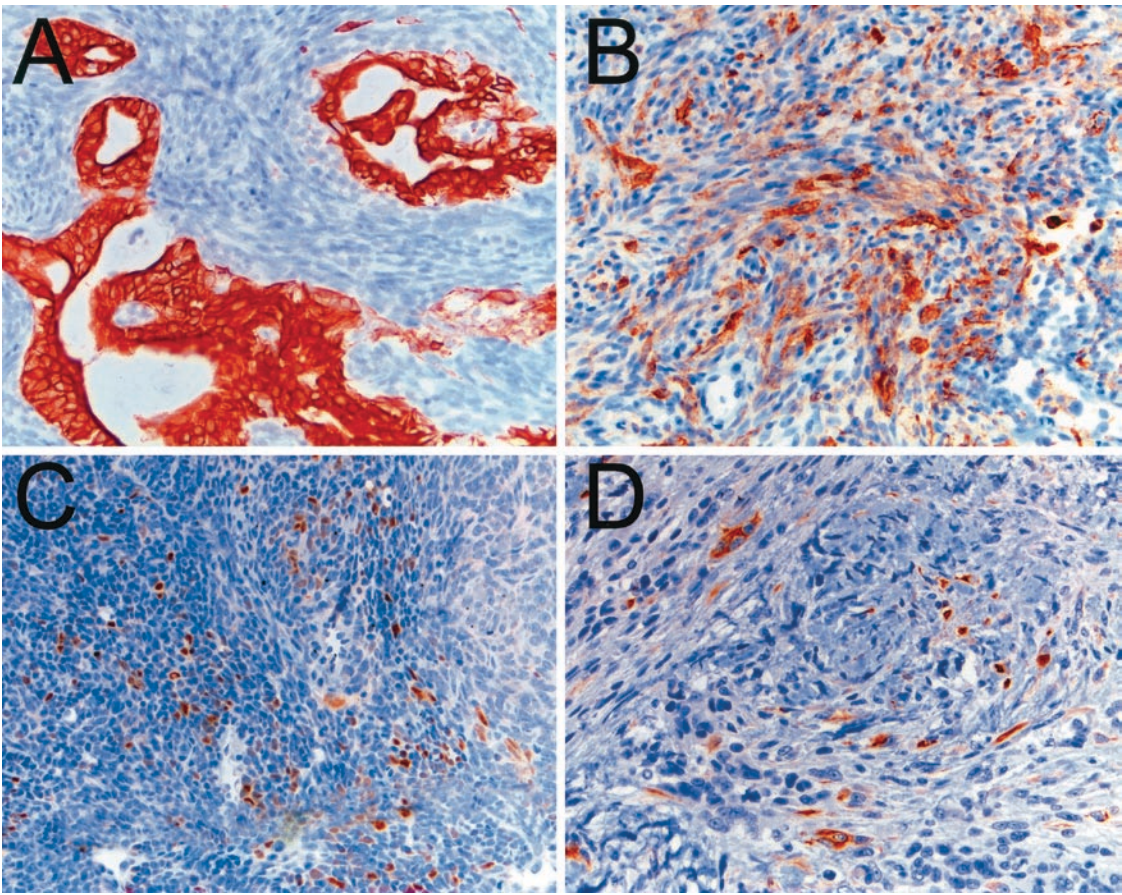


FIGURE 34.3. Immunohistochemical studies useful in the diagnosis of synovial sarcoma. (A) Antibodies to keratins (AE1/AE3 and CAM5.2 cocktail) stain gland-like areas in biphasic synovial sarcoma, (B) antibody to EMA in monophasic synovial sarcoma shows weaker, more focal staining, and highlights plumper spindle cells, (C) S100 staining is present in ~30% of synovial sarcomas, usually in a patchy distribution, and (D) CK7 staining is focally present in the spindle cell component in a subset of cases

stain weakly and/or focally for keratin and EMA, and in our and others' experience, require staining multiple sections for both of these markers (Folpe et al. 1998). The limited intensity and extent of staining interpreted as "positive" in some cases underscores one of the limitations of immunohistochemistry, such as variations in protocols between laboratories and interobserver variability in interpretation. Nevertheless, analysis of the following four markers: broad spectrum keratin, EMA, CK7, and CK19 is recommended to diagnose SS. The combination of positive EMA and CK7 (Figure 34.3d) demonstrate the highest specificity for SS among morphologic mimics (Smith et al. 1999; Olsen et al. 2006).

The S-100 protein is expressed in approximately one third of SS (Figure 34.3c; Smith et al. 1999; Olsen et al. 2006). Given that positive S-100 immunostaining is often used in the diagnosis of MPNST, S-100 staining in monophasic SS can cause confusion between these morphologically similar tumors. It is concluded that S-100 is neither sensitive nor specific for SS. CK7 and CK19, and possibly fascin, are the most useful markers in this differential diagnosis. More specifically, CK7, CK19, and fascin are negative in MPNST (Smith et al. 1999; Grogg et al. 2005). CD34 is positive in a wide variety of mesenchymal neoplasms, most notably the solitary fibrous tumor/hemangiopericytoma family of tumors but is negative in SS (Weiss and Nickoloff 1993; van de Rijn et al. 1994; Weiss and Goldblum 2008). Thus, positive staining for CD34 is useful to exclude the diagnosis of SS.

Although CD99 and bcl2 are positive in some cases of SS, these antigens probably lack sufficient specificity to separate SS

from morphologic mimics, especially if the epithelial markers are negative (Folpe et al. 1998). Similarly, cell surface markers E-cadherin, EGFR, and HER2 probably lack sufficient sensitivity in monophasic and poorly differentiated SS where they would be of greatest use for routine diagnosis (Nuciforo et al. 2003; Begueret et al. 2005; Dobashi et al. 2007).

Intriguingly, a recent report by He et al. (2007) demonstrated good sensitivity and specificity (>80%) for SS using an antibody against SYT. SYT is the N-terminal part of the fusion gene produced by t(X;18). In general, the authors reported strong, diffuse staining in most cases, including monophasic subtypes. SYT is a nuclear protein, a feature that may offer an advantage over cytoplasmic markers as the latter are hampered by background staining. At least on a theoretical basis, increased expression of SYT driven by the translocation may provide a highly specific target for immunohistochemical staining. The results, thus far, are encouraging, and the utility of this antigen in routine practice remains to be seen. Finally, the transcription factor TLE-1 has been proposed as a marker of SS although its diagnostic utility remains controversial (Terry et al. 2007, Kosemehmetoglu et al. 2009)

Cytogenetics and Molecular Genetics

A balanced translocation involving t(X;18) (p11.2;q11.2) has been identified in >90% of synovial sarcomas, regardless of histologic subtype (van de Rijn et al. 1999). The translocation joins the C-terminus of the SSX1, SSX2 or SSX4 gene on the X chromosome fused to the N-terminus of the SYT gene on chromosome 18 (dos Santos et al. 1997). The resulting fusion protein localizes to the nucleus where it

may regulate cyclin D1 (Xie et al. 2002a, b) or chromatin remodeling. Most importantly, t(X;18) appears to be highly specific for SS. Some controversy exists in the literature whether a small subset of malignant peripheral nerve sheath tumors also harbor t(X;18) or whether presence of this translocation can be used as an absolute “gold standard” for the diagnosis of SS (O’Sullivan et al. 2000; Ladanyi et al. 2001). The controversy raised questions regarding the validity of the RT-PCR technique that is prone to contamination and false-positive results. The experts involved also expressed concerns as to how one defines MPNST in the absence of von Recklinghausen’s disease or a preexisting benign nerve sheath tumor. Furthermore, rare examples of morphologically classic biphasic SS have been reported that lack t(X;18) (Yang et al. 1998). Nevertheless, the presence of t(X;18), especially with cytogenetics or FISH, can be used as validation of the histologic differential when the immunophenotype is equivocal (Antonescu 2006). However, as recognized by most experts, molecular and genetic approaches should serve as an *aid* in diagnosis rather than a *replacement* for routine histology.

Cytogenetics

Cytogenetics involves the analysis of the complete karyotype of dividing tumor cells. Consequently, this technique requires a viable, fresh tumor sample. The cells are allowed to proliferate in tissue culture, and using colchicine, arrested at metaphase. The chromatin is stained to generate reproducible banding patterns in chromosomes. A trained cytotechnologist examines the stained metaphase chromosomes compared to a known standard

human karyotype. The most important advantage of cytogenetics over the other molecular techniques described below is that it screens the entire genome for any large chromosomal rearrangements without *a priori* suspicion of a specific abnormality. More specifically, a cytogenetic analysis will not only reveal the presence of t(X;18) in SS, but may detect variant rearrangements and additional abnormalities. However, as it requires viable tissue, the decision to obtain cytogenetics on a specimen must be made based on limited information; usually at the time of intraoperative consultation (frozen section or cytologic material). Further disadvantages of cytogenetics include the long turn around time, the lack of resolution to detect small chromosomal abnormalities and the expertise required to culture tumor cells and to interpret the karyotype. Cytogenetics is particularly useful in the workup of poorly differentiated SS since a genome-wide screening for chromosomal aberrations may help diagnose other round cell sarcomas (e.g., Ewing sarcoma, alveolar rhabdomyosarcoma) that show characteristic translocations.

In Situ hybridization

In situ hybridization (ISH) detects chromosomal rearrangements or repetitive sequences at higher resolution than does cytogenetics using fluorescent (FISH) or chromogenic (CISH) probes targeted at specific sequences of the genome. Unlike cytogenetics, the cells can be fixed, as either paraffin sections or cytologic preparations, and the technique is applied to cells in interphase so that they can be examined histologically. This allows correlation of the genetic abnormality to the histologic findings. To screen for a

translocation with FISH, a pair of probes labeled with different fluorochromes are generated that hybridize to opposite sides of the breakpoint(s) involved. The probes consist of hundreds of kilobase length sequences of the genome and use bacterial artificial chromosomes as vectors. Dual color probe pairs detect the presence of t(X;18) by either the “break apart” or

“bring together” technique (Figure 34.4a). Commercially available (Vysis, Zymed) “break-apart” probes flank the breakpoint in the SYT gene on 18q11.2, and demonstrate the presence of a break at this chromosomal region (Figure 34.4b). However, break-apart probes do not detect the specific translocation, only the presence of a break. To date, data indicate that SYT

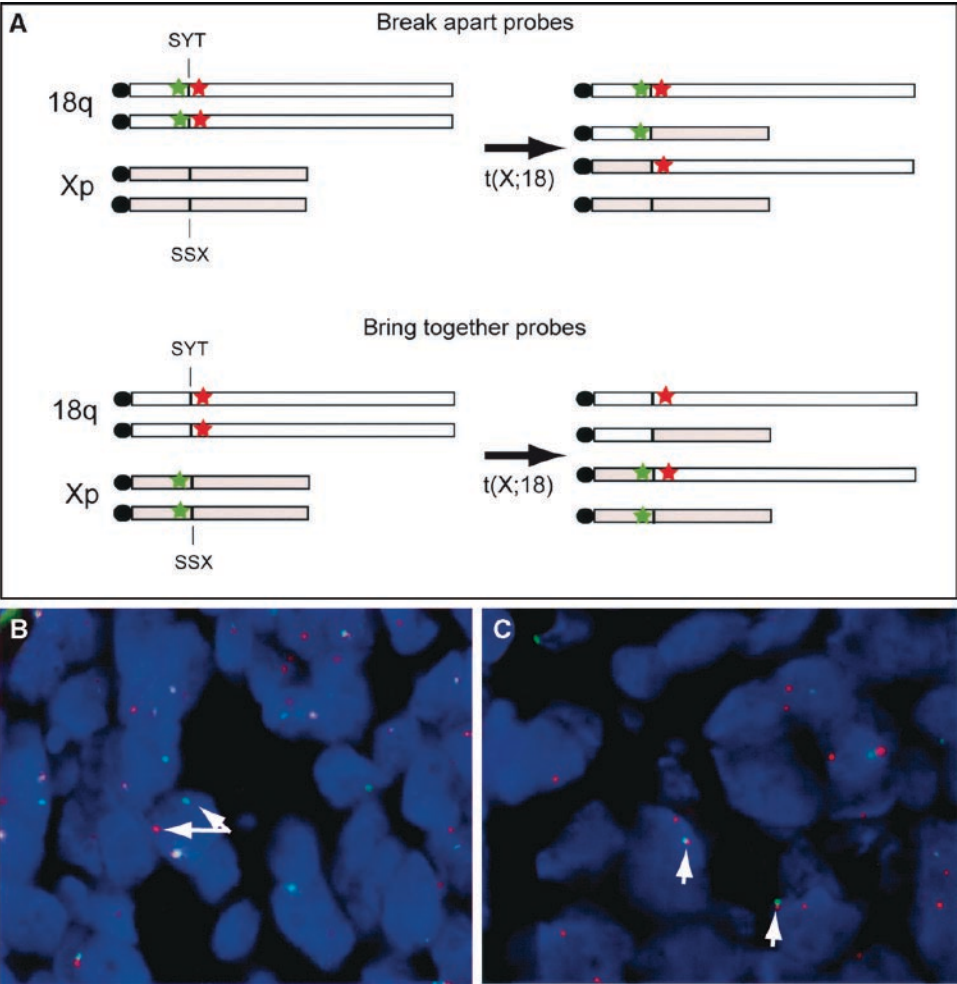


FIGURE 34.4. Fluorescence in-situ hybridization to detect t(X;18) in synovial sarcoma. (A) Top panel demonstrates dual color “break-apart” probes (green and red stars) that hybridize to DNA sequences flanking the break in the SYT gene; bottom panel shows dual color “bring-together” probes that are brought in close approximation on the chromosome by the translocation event, (B) positive break-apart signal (arrows) using Vysis probe pair, and (C) positive bring-together signal using custom probe pair. This is a male patient, with only one X chromosome, and thus only one green SSX signal

breaks are highly specific for synovial sarcoma, so a break at this locus is good confirmatory evidence of the diagnosis. It should be recognized, however, that divergent types of sarcomas have been documented to share breakpoints in a given proto-oncogene. For example, breaks in the EWS gene are found in Ewing sarcoma, extraskeletal myxoid chondrosarcoma and clear cell sarcoma (Antonescu 2006). To address this ambiguity and obtain increased specificity, one can use a “bring-together” probe pair to confirm the diagnosis of SS (Figure 34.4c). In this technique, one probe hybridizes 5′ of the SYT gene at 18q11.2 while the other hybridizes 3′ of the SSX gene(s) at Xp11.2. The translocation brings the two signals in close approximation. Currently, no commercially available probes of this type are available, but custom probes have been successfully used in the research setting (Horvai et al. 2006). The disadvantages of FISH include the relatively long turnaround time, cost, and labor involved, especially if custom probes need to be constructed. The SSX probe can be targeted downstream of the entire SSX gene cluster, thus detecting a break in any of the SSX genes, or be specific for only a subset of the SSX genes.

Polymerase Chain Reaction (PCR)

This technique relies on the specificity of short (~20 base length) probes that flank the SYT-SSX fusion point. These probes are used as primers for a PCR reaction that will only generate a product if a translocation is present that allows the probes to hybridize in close proximity. In theory, the technique could be applied to genomic DNA, but because only a single copy of t(X;18) is present per genome, the sensitivity of this approach

tends to be too low for routine use. In contrast, the mRNA product of the SYT-SSX fusion gene is expressed as many (perhaps thousands) copies per SS cell, in essence serving as an amplification step. The RNA is reverse transcribed and the product is then used as the template for the PCR reaction. RNA is inherently less stable than DNA, so frozen tissue is preferred as the RNA source. However, formalin-fixed, paraffin-embedded tissue has been used successfully (Guillou et al. 2001). In some cases, a second round of PCR (nested RT-PCR) is used to increase sensitivity in paraffin embedded tissue. RT-PCR is rapid, relatively inexpensive, and requires less specialized equipment than the above described methods. It has a further advantage that probes can be constructed to distinguish between the SSX gene fusion partner (SSX1, 2 or 4) involved in t(X;18), which may have prognostic significance as described below.

Reverse transcriptase polymerase chain reaction is not without disadvantages, however. First, suitable RNA cannot always be obtained from fixed tissue. Second, since the probes are very specific for short regions flanking the breakpoints in SYT and SSX, a variant breakpoint that does not fall between the probe pair will go undetected and result in a false-negative. Finally, and perhaps most importantly, the tremendous amplification potential of PCR makes the risk of cross-contamination between samples very high requiring multiple, rigorous, controls, and DNA sequencing. Some authors have suggested that the controversy in t(X;18) detected in MPNST may reflect this limitation of the RT-PCR technique (Ladanyi et al. 2001; Antonescu 2006).

PROGNOSTIC MARKERS FOR SYNOVIAL SARCOMA

Several large multicenter studies of prognostic features of SS have been reported. From multivariate analysis of 128 patients, the parameters that correlated with outcome (both disease free survival and metastasis free survival) were histologic grade and stage (Trassard et al. 2001). The Fédération Nationale des Centres de Lutte Contre le Cancer (FNCLCC) system used in the above study assigns a score based on three variables. One of these, differentiation score, is constant among all SS. Thus, the FNCLCC grade for SS is based on mitotic activity and presence of necrosis. The prognostic significance of histologic subtype is controversial. Some studies have suggested a survival advantage of biphasic over monophasic SS (Roth et al. 1975; Schmidt et al. 1991; Bergh et al. 1999), while others did not demonstrate a significant difference (Evans 1980; el-Naggar et al. 1990; Kawai et al. 1998; Trassard et al. 2001). Nevertheless, the poorly differentiated variant of SS shows a trend for poor survival if at least 20% of the tumor shows this pattern (Folpe et al. 1998). As this type is very rare, multivariate statistical analysis is not available. Calcifications, especially when extensive, demonstrate a more favorable prognosis although more limited calcifications do not impact behavior (Weiss and Goldblum 2008).

The type of SSX fusion appears to be a prognostic factor, at least for localized disease. Specifically, patients with SYT-SSX2 fusions demonstrate longer metastasis free survival than those with SYT-SSX1 fusions (Ladanyi et al. 2002). A correlation between the fusion variant

and histologic subtype (monophasic or biphasic), though not exclusive, has also been reported. Nearly all SSX2 fusions are found in monophasic SS (Kawai et al. 1998; Antonescu et al. 2000a; Ladanyi et al. 2002) while SSX1 fusions are identified in both biphasic and monophasic SS (Kawai et al. 1998). These data suggest that the SSX1 fusion is more permissive to epithelial differentiation. The type of fusion correlates with both clinical course and histologic subtype. However, histologic subtype, by itself, does not predict survival suggesting that the mechanisms of these effects of fusion type may be divergent. The mechanistic relationship between the specific SSX gene involved in the translocation and epithelial differentiation or biologic behavior is not well understood. Gene expression analysis using cDNA microarrays suggests that expression of ERBB2, IGFBP2, and ELF3 are associated with epithelial differentiation (Allander et al. 2002). Rare cases of SS (<2%) demonstrate a translocation between SYT and SSX4 or between SS18L1 and SSX1 (dos Santos et al. 2001; Storlazzi et al. 2003). Little or no data exist on prognostic implication of these rare gene fusions.

Alterations in signaling molecules and cell-cycle regulators are thought to be important in the pathogenesis of sarcomas. In sarcomas such as SS that show recurrent genetic abnormalities, alterations in the genes of signaling molecules may be secondary changes (Antonescu et al. 2000b). Alternatively, the products of the recurrent genetic abnormalities (such as SYT-SSX) may regulate the expression or function of signaling molecules. Thus, cell cycle regulators and signaling molecules have been evaluated as prognostic markers for SS

at the immunohistochemical level. The limitations of such studies include variability in antibodies and conditions used between laboratories, threshold of a positive result and the interobserver variability in interpreting immunostaining. Nevertheless, the immunophenotype of SS for some cell cycle regulators has been shown to have prognostic significance.

β -Catenin plays a role in cell-adhesion by complexing with the cadherins and may have phenotypic and prognostic relevance in synovial sarcoma. The wnt signaling pathway activates β -catenin and ultimately leads to an active transcription complex involving TCF/LEF (Nelson and Nusse 2004). By this mechanism, β -catenin promotes transcription from the cyclin D1 promoter and contributes to elevated cyclin D1 expression in carcinoma and possibly some soft tissue tumors (Tetsu and McCormick 1999; Saito et al. 2001). Although cyclin D1 expression does not appear to be an independent prognostic marker in SS, cell cycle control may be important in SS transformation and progression (Antonescu et al. 2000b). In our own studies, we noted a trend of poorer survival in those patients with nuclear β -catenin immunohistochemistry similar to previous reports (Saito et al. 2000; Horvai et al. 2006) (Figure 34.5). Furthermore, Cyclin D1 overexpression requires either abnormal β -catenin expression or the presence of p-Akt (Horvai et al. 2006). Intriguingly, the SYT-SSX fusion gene, resulting from the t(X;18) translocation, was recently reported to regulate cyclin D1, possibly at the degradation level, and thus may be critical in cyclin D1 expression in synovial sarcoma (Xie et al. 2002a, b). However, the SYT-SSX mediated increase in cyclin D1, at least

in cultured SS cells, is not dependent on the PI3K-Akt-GSK3 α pathway, unlike other tumor types (Xie et al. 2002a). The results suggest that abnormally expressed β -catenin may upregulate cyclin D1 in SS but is independent of pAkt. Whether cyclin D1 regulation mediated by SYT-SSX takes place through pathways that are distinct from, or overlapping with, β -catenin is unknown.

E-cadherin is a membrane protein that mediates cell–cell adhesion, is linked to the cytoplasmic catenins and is generally present only in biphasic SS (Saito et al. 2004). Recent work by Saito et al. (2006) suggests that the products of the fusion genes SYT-SSX1 and SYT-SSX2 prevent transcriptional repressors from binding to the E-cadherin promoter; thus, allowing increased expression of E-cadherin. However, SYT-SSX1 interferes with a stronger repressor (the repressor SNAIL) than SYT-SSX2 (the repressor SLUG). In theory, relief of a stronger repressor permits more e-cadherin expression and gives a potential explanation for the more common presence of SYT-SSX1 in biphasic over monophasic SS. The preserved expression of E-cadherin, with immunohistochemistry is correlated with increased survival (Saito et al. 2000).

The tumor suppressor p53 regulates genes involved in cell cycle arrest and mediates apoptosis. p53 overexpression, measured by immunohistochemistry is rare in SS (6–16% of cases), but overexpression correlates with decreased disease-free survival and with larger, more aggressive tumors (Antonescu et al. 2000b). In one reported case of paired primary and metastatic SS, only the metastasis demonstrated p53 inactivation suggesting a role in tumor progression (Pollock et al. 1996).

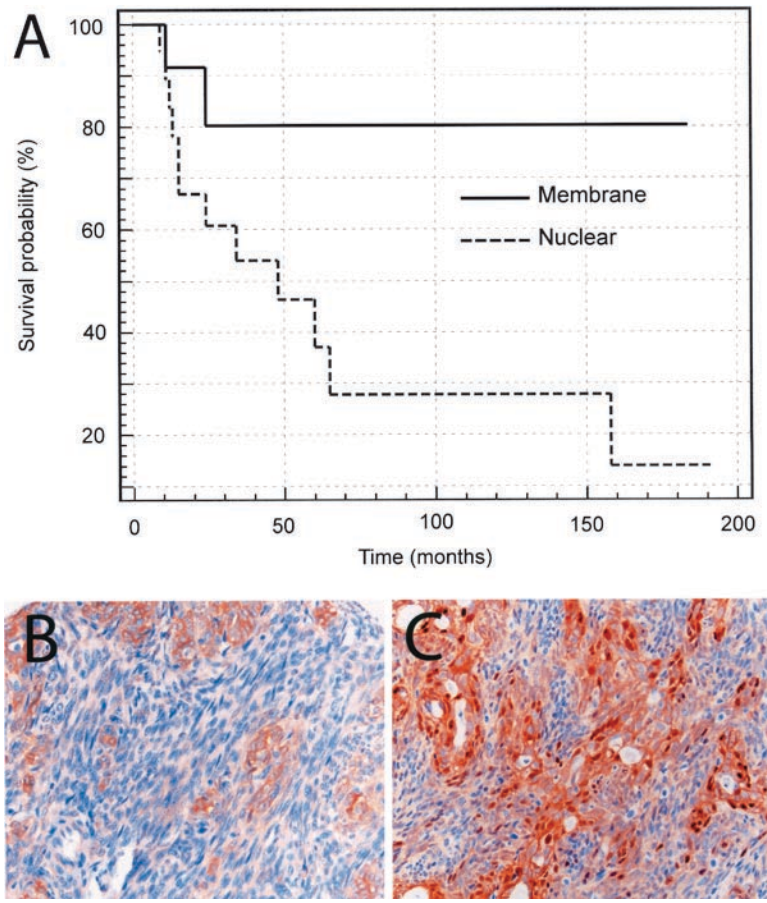


FIGURE 34.5. Aberrant β -catenin expression as a prognostic indicator in synovial sarcoma. (A) Aberrant (nuclear) β -catenin staining correlates with poorer outcome in both biphasic and monophasic synovial sarcoma (see text for references), (B) normal β -catenin expression in synovial sarcoma is largely restricted to the membranes of the epithelioid cells, and (C) aberrant staining of β -catenin consists of increased nuclear and cytoplasmic signal

C-myc nuclear expression correlates with an increased relapse rate in SS (Shen et al. 2000). The same study also demonstrated a trend toward worse overall outcome, but this finding was not statistically significant. Finally, p53, myc, and mdm2 nuclear positivity correlate with a high proliferative index (Ki-67 staining in >20% of the nuclei) (Antonescu et al. 2000b). Proliferation index, as measured with Ki-67 is also an independent prognostic indicator of survival, in a sense serving as an immunohistochemical analog to mitotic count, which

is a part of the prognostically significant FNCLCC grade.

In summary, disease stage and FNCLCC grade are the most consistent discriminating factors of survival and recurrence. The prognostic significance of histologic subtype is unclear, beyond a generally worse outcome for the poorly differentiated SS. However, the detection of some markers, such as SYT-SSX1 and a high Ki-67 proliferation index, correlate with a worse prognosis and may help identify high-risk patients who might benefit from more aggressive treatment.

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Treatment

Sarcoma: Treatment with Ecteinascidin-743

Jérôme Fayette, Philippe Cassier, Laura Brousseau, Armelle Dufresne, Isabelle Ray Coquard, Laurent Alberti, Pierre Méeus, Anne-Valérie Decouvelaere, Marie-Pierre Sunyach, Dominique Ranchère, and Jean-Yves Blay

INTRODUCTION

Thirty to 50% of patients with adult type soft tissue sarcoma will develop metastasis. At this stage, systemic chemotherapy is then generally proposed when a complete resection of all metastasis is not feasible (Clark et al. 2005). The two most active therapeutic drugs for advanced soft tissue sarcomas are doxorubicin and ifosfamide; dacarbazine although considered as the third active agent, actually shows a marginal activity. Initial response rates to doxorubicin single agent regimens are 10–30% with 5–8% long term survivors (Blay et al. 2003, 2004). The combination of doxorubicin with ifosfamide has yielded higher response rate (25–70%), but with no survival improvement. After failure of doxorubicin and ifosfamide, few therapeutic options are available. The combination of gemcitabine and docetaxel was reported to induce high response rates and progression free survival (PFS) in leiomyosarcomas (Bay et al. 2006). ET-743, trabectedin, Yondelis®, is a

tetrahydro-isoquinoline alkaloid isolated from *Ectenascidia turbinata*, a tunicate that grows on mangrove roots throughout the Caribbean sea. ET-743 interferes with the activity of several transcription factors and traps protein from the nucleotide excision repair (NER) system, thus resulting in DNA damage, modulation of expression of various genes, and blockade of cells in G2-M phase. After failure of doxorubicin and/or ifosfamide, as well as in first line treatment, ET-743 at 1.5 mg/m² in 24 h CI every 21 days yielded an overall response rate close to 8% and 30–40% stabilisation rates. Leiomyosarcomas, liposarcomas (in particular myxoid liposarcomas), and synovial sarcomas may be the more sensitive histotypes. The major toxicities of ET-743 are hepatic, through biliary duct destruction, and hematologic. They are not cumulative. In vitro, ET-743 exerts synergistic cytotoxic activity with doxorubicin or cisplatin, and in vivo with irinotecan. Phase I combination studies are in currently progress. We detail all these aspects of ET-743.

MECHANISMS OF ACTION AND RESISTANCE

Binding to DNA

ET-743 binds to the minor groove of DNA and alkylates N2 of guanines located either in the sequence 5'-PuGC-3' or 5'-PyrGG-3'; thus, bending DNA toward the major groove. Other alkylations are formed but they are less stable and reversible. Two subunits of ET-743 bind to DNA while the third does not have contact with DNA, and protrudes out from the minor groove interfering with several DNA-binding factors. Using chemical analogs of this third domain of ET-743, alterations were found to alter the DNA binding capacity of this compound (David-Cordonnier et al. 2005). Such analog deficient for DNA-binding shows, however, a decreased but persistent biological activity on tumor cell lines, suggesting the existence of a non-DNA target for antitumor response.

Inhibition of Transcription Factors

The biological activity of ET-743 occurs through a modulation of transcription factors and interactions with protein of the DNA repair machinery. ET-743 inhibits the binding of NF-Y, a factor that activates the CCAAT element present in 25% of eukaryotic promoters, including many promoters that regulate genes controlling the cell cycle. In vivo studies showed that the HSP70 promoter containing two CCAAT boxes activated by NF-Y is rapidly inhibited by ET-743 while promoters lacking CCAAT boxes were not affected. Yet, ET-743 does not inhibit the DNA binding capacity of NF-Y (Minuzzo et al. 2005), and may act downstream from transcription factor binding. Importantly, the

promoter of the MDR1 gene (multidrug resistance) encoding for the P glycoprotein (P-gp) also contains CCAAT boxes, a phenomenon which may account for the activity of ET-743 on cells over-expressing MDR1. Of note, ET-743 does not select P-gp expressing cells phenotype in ET-743 resistant cell lines, and does not exhibit cross-resistance with other cytotoxic agents. This may however vary among different cell lines. Recent data showed that ET-743 is a substrate for the P-gp (Beumer et al. 2007). But ET-743 displayed the typical multidrug resistance phenotype only in highly P-gp expressing cell lines, but not in cell lines with expression levels more closely conforming to clinical samples, suggesting that P-gp will not confer resistance to ET-743 in cancer patients.

ET-743 also acts through the inhibition of cancer-activated transcription. Induction of the Sp1-regulated p21 gene by Trichostatin A (TSA, a promoter activator) was blocked by ET-743 at concentrations that had minimal effect on uninduced constitutive expression. Using microarray analysis, it was shown that activation of TSA-responsive promoters was blocked by ET-743 with little effect on non-responsive promoters. In inducible gene expression experiments using the metallothionein and CYP3A4 promoters, ET-743 was not found to be a general inhibitor of cancer-activated transcription (Minuzzo et al. 2005). Indeed, ET-743 did not affect either the basal or the activated levels of transcription. Actually, ET-743 can activate some inducible promoters, and, at higher concentrations, can alter the interaction between several DNA binding proteins and DNA (two subunits of NF-Y exhibit homology with histones 2A and 2B).

Inhibition of DNA Repair Machinery

ET-743 activity may also involve the DNA repair machinery. ET-743 targets topoisomerase I resulting in DNA breaks, although this effect seems not to be relevant *in vivo* because it is observed only at high concentration, while the drug remains active in cells deficient for the topoisomerase I. Defects in the mismatch repair pathway, usually associated with increased resistance to alkylating agents and cisplatin, do not affect the cytotoxic activity of ET-743. Whereas all known DNA-interacting cytotoxic drugs are either more or equally active in nucleotide excision repair (NER)-deficient cells, ET-743 exerts decreased cytotoxic activity in NER-deficient cell lines. Indeed, the DNA bending induced by the binding of ET-743 to the minor groove is detected by the NER machinery, which in the repair process makes single strand breaks (SSBs) on each side of the lesion (David-Cordonnier et al. 2005). These breaks are then made irreversible by the DNA-protein crosslinking capacities of ET-743. In the absence of an intact NER nuclease complex, these toxic lesions do not occur, and ET-743-DNA adducts, although not repaired by the NER pathway, are less toxic to cells. In the yeast model *Schizosaccharomyces pombe*, cells deficient for Rad13 (the yeast equivalent to the human XPG, which is an endonuclease of the NER system) were resistant to ET-743. In the same time, those with an inactive Rad51 (a protein of the homologous recombination repair (HRR) pathway involved in the repair of double strand breaks) were more sensitive to ET-743 than the wild-type cells. Based on these observations the following sequence was suggested: ET-743 binds covalently to the DNA minor groove and

the resulting adduct is recognized by the NER machinery, then the recruited Rad13 (XPG) protein binds to DNA and interacts with the minor groove-bound drug by means of an arginine residue located in the COOH terminus. Other proteins of the NER machinery trying to repair the damage are then hijacked, forming larger, more toxic complexes. Lastly, during the S phase, the aforementioned complexes give rise to double strand DNA breaks, explaining the sensitivity of cells deficient for HRR pathway proteins (e.g., Rad51) (Herrero et al. 2006).

There is a clinical relevance as demonstrated in a retrospective analysis of the NER and HRR status in the tumor of 92 patients treated with ET-743 using RT-PCR (Schöffski et al. 2006). Patients with higher ERCC1 expression (part of the NER machinery) had improved 6-month PFS rate and median overall survival compared with patients whose tumor expressed lower levels of ERCC1 (32 versus 15% and 12 versus 7 months, respectively). Furthermore patients whose tumor expressed lower levels of BRCA1 (part of the HRR system) also had improved 6-month PFS rate and median overall survival compared with patients whose tumor expressed high levels of BRCA1. BRCA2 (HRR system) and XPD (NER system) expressions had no significant impact on PFS and survival. A combined analysis of ERCC1 and BRCA1 expression identified a subgroup of patients with high ERCC1 and low BRCA1 expression who had 50% 6-month PFS rate and a 20.4 months median overall survival. Multivariate analysis showed that both ERCC1 and BRCA1 expression levels were independent predictors of PFS and that only BRCA1 independently predicted overall survival.

p53 status is ambiguous. Such studies showed a correlation and others did not. But in an *in vitro* study, p53 deletions and mutations correlated with high sensitivity of cells to ET-743 (Moneo et al. 2007). Finally, the telomerase activity decreases the efficiency of ET-743 repairing the induced damages (Biroccio et al. 2003).

Other Effects

ET-743 blocks cells in the G2-M phase. Indeed, cell lines exposed for 1 h to ET-743 progress through S phase more slowly than control cells and then accumulate in the G2-M phase. The sensitivity to ET-743 of G1 synchronized cells was much higher than that of cells synchronized in S phase, and even higher than that of cells synchronized in G2-M. Gene expression profile was used to identify genes involved in sensitivity or resistance to ET-743. The first study determined a set of 70 genes whose expression was modulated in drug-resistant cells (Marchini et al. 2005). Another study with cDNA microarray containing 6,700 cancer-related genes showed upregulation of 86 genes and down-regulation of 244 genes in response to ET-743 (Martinez et al. 2005).

A recent study reported an increase in expression of insulin-like growth factor receptor-I (IGF-IR) and insulin receptor substrate-1 in ET-743-resistant cells (Manara et al. 2005). Functional studies using a neutralizing antibody anti-IGF-IR induced partial reversion, and confirmed involvement of IGFR pathway in the resistance to ET-743. This observation could have therapeutic implications because inhibitors of IGF-IR pathways are in development. Immunohistochemistry revealed marked differences in the cytoskeleton architecture and collagen I

expression between ET-743-sensitive and ET-743-resistant cells. ET-743 exerts its cytotoxic role through an original mode of action involving DNA repair machinery. Its potent cytotoxic activity on sarcoma cell lines prompted then investigation of its activity in clinic.

METABOLISM AND TOXICITY OF ET-743

Thus far the metabolism of ET-743 has not been elucidated. It has a large volume of distribution ranging from 1,000 to 4,000 L. Intravenous administration of [(14)C]-ET-743 to 8 cancer patients showed excretion mainly in faeces (mean 55.5% of the dose) and less importantly in urine (5.9%) (Beumer et al. 2005a). The excretion of unchanged ET-743 is very low both in faeces and in urine (<1% of dose). Using liquid chromatography with tandem quadrupole mass spectrometric detection (LC-MS/MS) and radiochromatography with off-line radioactivity detection by liquid scintillation counting (LC-LSC), a large number of ET-743 metabolites were detected (ET-745, ET-759A, ETM-259, ETM-217 and ET-731) (Beumer et al. 2007). ET-743 is mainly metabolized by cytochrome P450 (CYP) 3A4, but also by 2C9, 2C19, 2D6, and 2E1, and the phase II enzymes uridine diphosphoglucuronosyl transferase and glutathione-S-transferase (Brandon et al. 2005). The cytotoxic activity of ET-743 (and thus global toxicity) is increased with CYP inhibitors, but not modified by phase II enzyme inhibitors and CYP inducers even if CYP metabolites of ET-743 are less toxic as compared to the parent compound. Thus, combination therapy of ET-743 with other Anticancer. Drugs.

is able to inhibit CYP, and may increase the hepatotoxicity of ET-743 (Scripture et al. 2005).

The most prominent toxicities observed in the different phase II studies were grade 3 or 4 transaminase increase (26% to 59%) and neutropenia (33% to 52%). Regarding liver toxicity, studies in rat showed a predominant biliary toxicity: 24 h after ET-743 liver degeneration and patchy focal necrosis of bile duct epithelial cells are observed and associated with mild inflammation followed by fibrosis. Sporadic and focal zones of hepatic necrosis and hemorrhage were observed, although the majority of hepatocytes appeared normal. Pathological alterations persisted up to 3 months. However, it must be noted that metabolic profile and hepatotoxicity of ET-743 are different in rats and humans: liver damages are reversible in humans but not in rats (Beumer et al. 2005b).

Human patients with any baseline liver function test exceeding the upper limit of the normal ranges have a significantly greater incidence of severe hepatic toxicity. Levels of plasma liver enzymes (transaminases, bilirubin, alkaline phosphatase, and 5'-nucleotidase) should be checked before each course. Pretreatment with dexamethasone may decrease this toxicity. Pretreatment, but not concomitant treatment of rats with high dose of dexamethasone 24 h before ET-743, improved or prevented ET-743-induced liver damages, probably through the induction of the cytochrome p450. However, CYP inducers were not able to decrease liver toxicity of ET-743 (Brandon et al. 2005). In humans the use of steroid premedication prevents biochemical hepatic and hematological toxicity (Grosso et al. 2006a). Indeed, 54 patients with sarcoma were

treated with ET-743, 23 without premedication, and 31 received dexamethasone 4 mg twice daily beginning the day before administration of ET-743. In the group receiving dexamethasone, the incidence of grade 3–4 liver enzyme elevation, neutropenia, and thrombocytopenia fell to 3%, 10% and 0%, respectively, compared with 70%, 39% and 35%, respectively, in the group without premedication. The progression-free survival was comparable in both groups, and was in the range of that reported in phase II trials. Though these data are derived from a nonprospective study, given the major impact of steroid premedication on the main side effects of ET-743 argues for its use in standard clinical practice. Beta-naphthoflavone and indole-3-carbinol were reported to protect liver in *in vitro* experiments. Granulocytic (CFU-GM) and megakaryocytic progenitors are sensitive to the drug, but the dose of ET-743 that inhibit 90% of CFU-GM only inhibited 45% survival of stem cells resulting in the lack of long-term myelosuppression.

Interestingly, ET-743 exerts present anti-inflammatory properties through a toxic effect on monocyte macrophages. This specific effect may be clinically useful in cancer patients with paraneoplastic inflammatory syndrome through the activity on monocytes/macrophages. Human monocytes are highly susceptible *in vitro* to its cytotoxic effect and underwent apoptosis at pharmacologically relevant concentrations (5 nmol/L), whereas lymphocytes were up to fivefold less sensitive. Moreover, ET-743 inhibits the *in vitro* differentiation of monocytes to macrophages and decreases the production of CCL2, a major chemokine for monocyte recruitment at tumor sites.

CLINICAL ACTIVITY OF ET-743 IN PATIENTS WITH SARCOMA

Several phase I studies have enabled delineation of the schedule of ET-743 administration. The recommended schedule for ET-743 is delivered by continuous intravenous 24 h-infusion at the dose of 1,500 µg/m², every 3 weeks, until progression or toxicity. Table 35.1 gives a synthetic overview of the management of ET-743 in sarcoma.

First multicentric phase II study enrolled 54 heavily pretreated patients. Before ET-743, 48% had received one or two drugs and 52% three or more; 41% had leiomyosarcoma (8 of 22 of uterine origin). Two partial responses (PR) were observed, for an overall response rate (OR) of 4% (95% CI, 0.5–12.8), with four minor responses and 9 disease stabilizations (SD) lasting more than 6 months. Twenty-four percent of patients were free from progression at 6 months. The median survival was 12.8 months, with 30% of patients alive at 2 years. Two treatment-related deaths occurred (Yovine et al. 2004).

The second study included 36 patients, and reported one complete response (CR) and two PR for a response rate of 8% (95% CI, 2–23%) with a clinical benefit rate of 14%. Prolonged responses were observed (up to 20 months) and the 1-year overall survival was 53% (95% CI, 39–73%), an unusual observation in this population of patients (Garcia-Carbonero et al. 2004, 2005).

These results were confirmed by a large phase II study of the EORTC STBSG in 104 pretreated patients. Eight PR were observed for an OR of 8%, along with 45

TABLE 35.1. ET-743 synthetic data.

ET-743 in sarcoma	
Indication	• Sarcoma after failure of anthracyclin-based chemotherapy • Best results with synovialosarcoma, leiomyosarcoma and liposarcoma
Schedule	• Continuous intravenous 24 h-infusion at the dose of 1,500 µg/m², every 3 weeks • Dexamethasone 4 mg twice daily, 3 days (the first day before ET-743)
Pretherapeutic exams	• Neutrophils ≥1,500/mm³, and platelets ≥100,000/mm³ • Normal creatinin • Normal bilirubin • Normal alkaline phosphatases: if elevation, normal 5'-nucleotidase is needed • Transaminases <2.5 ULN (upper limit of normal)
Surveillance	• Hemogram and ionogram each week • Transaminases, bilirubin and alkaline phosphatases twice a week
Toxicity (grade 3–4)	• Neutropenia: 34–61% • Febrile neutropenia: 6–9.1% • Thrombopenia: 17–19% • Anemia: 7–22% • Nausea: 4–7.1% • Transaminasitis: 20–57%
Dose adaptation according to toxicity	• Toxicity • Bilirubin: grade ≥1: decrease dose of 1 level • Transaminasitis: grade 3–4, and normalization at grade 0–1 the first day of the next cycle: decrease dose of 1 level • Alkaline phosphatases: Grade ≥1: decrease dose of 1 level • Persistence or reappearance of a grade 1 toxicity: the same dose • Apparition of a grade ≥ 2 after first reduction of dose: decrease dose of again 1 level • Other toxicity: grade 3–4: decrease of 1 level • Dose adaptation • First reduction: 1,200 µg/m² • Second reduction: 1,000 µg/m²
Evaluation	• CT-scan every 3 cycles • CR: continue for 2 more cycles • PR or SD: continue treatment • PD: stop treatment
Results	• Overall response: 4–8% • Clinical benefit (OR + SD): 29–54% • Overall survival: 9.2–12.8 months • 6-month progression-free survival: 24–29%

SD (longer than 6 months in 26% patients). Overall, a clinical benefit was observed for 56% of leiomyosarcoma and 61% of synovial sarcoma. The progression-free survival at 6 months was 29% (Le Cesne et al. 2005). The median duration of survival of 9.2 months was again unusually high in this cohort of heavily pretreated patients, especially considering the low response rate. Of note, the rates of objective regression and stable disease were found to be similar in doxorubicin/ifosfamide chemosensitive and chemoresistant sarcomas. However, in this study, patients exhibiting a prolonged stable disease had an outcome similar to that of responding patients, supporting the relevance of the progression arrest rate (i.e., objective response + stable disease) as a prognostic parameter instead of objective response (Van Glabbeke et al. 2002). In a single institution experience, 89 patients (82 assessable) with advanced refractory sarcoma (leiomyosarcoma 33%, liposarcoma 18% and osteosarcoma 16%) were treated with ET-743. The objective response rate was 7% (1 CR, 5 PR), and the clinical benefit rate at 3 and 6 months was 38% and 23% (Huygh et al. 2006).

ET-743 is highly efficient in myxoid liposarcoma. This histological subtype contains a characteristic chromosomal translocation, t(12;16), resulting in the fusion of a transcription factor (CHOP) and a RNA binding protein (TLS/FUS). At least three different fusion products have been identified, based on the TLS/FUS breakpoint, but were not shown to have prognostic significance. In a retrospective analysis of 44 patients with myxoid liposarcoma treated in five different European

and American institutions, the overall response rate was 43% (CR = 2%, PR = 41%), with MR and SD seen in 18% and 25%, respectively. The 6 months PFS rate was 80%, and the median PFS was 17 months (Grosso et al. 2006b).

In order to decrease toxicity, a randomized phase II study of ET-743 given by two different dosing schedules (3 h infusion weekly $\times$ 3 every 4 weeks versus 24 h infusion every 3 weeks, standard arm) in 270 patients with leiomyosarcoma or liposarcoma (due to the observed better activity of ET-743 in these subtypes with respect to the other) refractory to conventional doxorubicin and ifosfamide chemotherapy was carried out (Morgan et al. 2007). The median PFS was 3.3 months versus 2.3 ($p = 0.0418$) and median survival was 13.8 months versus 11.8 (not significant, but only 175 patients analyzed) in favor of the standard arm (despite more but clinically irrelevant toxicity).

In view of its activity in pretreated patients, ET-743 was tested in first line of treatment for unresectable advanced sarcoma. Thirty-six patients (35 assessable) were treated with the standard schedule: 1 CR and 5 PR were achieved for an overall response rate of 17.1% (95% CI, 6.6–33.6%). In addition, one patient had a minor response. The estimated 1-year progression-free and overall survival rates were 21% (95% CI, 11–41%) and 72% (95% CI, 59–88%), respectively, (Garcia-Carbonero et al. 2005). ET-743 was unable to induce responses in GIST (Blay et al. 2004) and in osteosarcoma (Laverdiere et al. 2003), although prolonged stabilization was observed. Responses were reported in Ewing sarcoma.

ET-743 IN COMBINATION WITH OTHER DRUGS

Because of its original mode of action, ET-743 may act synergistically in combination with other cytotoxic agents. Several preclinical or phase I studies explored combination chemotherapy regimens. Preclinical studies had shown that ET-743 and cisplatin are synergic, without additive toxicity (D'Incalci et al. 2003). Interestingly, ET-743 could be used at lower, relatively nontoxic doses to potentiate the cytotoxicity of cisplatin. A phase I study has been performed to determine ET-743 and cisplatin doses in combination regimens.

The combination with doxorubicin may be effective for tumors displaying low sensitivity to each drug given alone (Meco et al. 2003). For tumor cells sensitive to both agents, additive effects are observed, whereas another study showed synergy. The most favorable synergy in vitro was observed using a sequence with ET-743 first followed by doxorubicin. Based on these findings, a phase I study on the combination of both drugs were initiated.

Synergy between ET-743 and irinotecan in human rhabdomyosarcoma cell line has been shown (Riccardi et al. 2005). In vitro studies indicate an overall additive effect. Neither transcription nor expression of DNA topoisomerase I is affected by ET-743 treatment. In vivo, the therapeutic results of the combination are more impressive with a strong and long-lasting effect on tumor growth, whereas each drug given as a single agent was only marginally active. The discrepancy between the in vitro and in vivo results suggests possible mechanisms involving host cells and/or stromal cells, other than tumor cells, anti-inflammatory effects of ET-743

could be important. Other combinations were tested, with paclitaxel (showing a limited schedule dependent synergy) with plasminogen-related protein B (an antiangiogenic factor) in preclinical models of chondrosarcoma. No synergy was observed between ET-743 and radiotherapy.

In conclusion, ET-743 has an original mode of action, involving enzymes of the DNA repair machinery and inhibition of transcription factors. It could have indirect effects on tumor growth through anti-inflammatory activity. ET-743 has a demonstrated activity as first or second treatment for advanced sarcomas, following doxorubicin, ifosfamide, and dacarbazine. Some patients achieved very prolonged long term survival. Responses and clinical benefit are important for myxoid liposarcomas and leiomyosarcomas.

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Index

- ABCF2, 6
 - Absolute excess risk (AER), 182
 - Actimid, 371
 - ADCC. *See* Antibody-dependent cell cytotoxicity
 - Adenocarcinoma
 - cervical, 244
 - clear cell
 - differential gene expression in, 9–10
 - TP53 mutations in, 7–8
 - endocervical, 231–234, 237–241
 - endometrial, 232
 - MUC expression in, 238, 240–241
 - treatment of, 287
 - FDG-PET appearance of, 276–277
 - gene expression in, 43
 - metastatic, 161, 163
- Adenocarcinoma in situ (AIS), 234, 237, 240
- Adenoma, 161
- Adenomatoid tumors (AT)
 - differential diagnosis of, 162–164
 - general features of, 161
 - histology of, 162
 - immunophenotype of, 163–164
- Adenosquamous carcinoma, 275
- Adnexal masses
 - characterization of, 93
 - differentiating, 23, 27–29
 - ultrasonography of, 25–31
- Adrenal adenomatoid tumors
 - differential diagnosis of, 162–164
 - general features of, 161
 - histology of, 162
 - immunophenotype of, 163–164
- Adrenal cortical tumor, 163
- Adriamycin, 366
- AER. *See* Absolute excess risk
- AHM, 362
- AIDS-related Kaposi's sarcoma, 423–425
- AIS. *See* Adenocarcinoma in situ
- AKT, 426
- Alemtuzumab, 343
 - autoimmune disease treatment with, 354
 - combination therapy with, 350–352
 - consolidation therapy with, 352–354
 - maintenance therapy with, 354
 - monotherapy with, 344
 - cytogenic stratification of, 349–350
 - retreatment with, 346
 - studies of, 345–347
- Alkaline phosphatase, 408, 417
- All-trans retinoic acid (ATRA), 365
- Alveolar rhabdomyosarcoma, 388, 439
- AMD473, 49
- AMD3100, 369
- American Joint Committee on Cancer Staging System, 167, 311
- American Society of Hematology, 350, 370, 376
- Anemia
 - chemotherapy and, 375
 - haemolytic, 354
 - microangiopathy hemolytic, 377
 - multiple myeloma and, 361
 - treatment of, 369
- Angina pectoris, 116
- Angiogenesis
 - KS and, 422–423
 - Wilms tumor and, 150–152

- Angiogenesis inhibitors, 133
 - quantification of, 148–150
 - Wilms tumor and, 150–152
- Angiogenic growth factors, 151
- Angiopoietin, 422
- Angiosarcoma, 394, 424
- Ankylosing spondylitis, 188
- AnorMED, 369
- Antiangiogenic agents, 151
- Antibody-dependent cell cytotoxicity (ADCC), 363
- Anti-human leukocyte antigen (HLA-DR), 363
- Anti-IL-6 therapy, 363–365
- Antiretroviral therapy, 405
- Apoptosis
 - Fas-driven, 138–139
 - KS and, 423
 - tumor cells and machinery of, 138
- Aranesp, 369
- Array comparative genomic hybridization, 35
- Arthritis, inflammatory, 116
- ASCT. *See* Autologous stem cell transplantation
- AT. *See* Adenomatoid tumors
- Atlizumab, 362
- ATRA. *See* All-trans retinoic acid
- Autoimmune diseases
 - alemtuzumab and, 354
 - interleukin-2 and, 116
- Autologous stem cell transplantation (ASCT), 353, 364, 370, 375
- Basal cell carcinoma, EphA and, 337–338
- BB-10901, 367
- B cell lymphocytic leukemia, 138, 343
- B cell lymphoma, 138
 - diffuse large, 343
- B-cell lymphoproliferative disease, 343
- Bcl-2, 137, 394
 - expression of, 141–142
 - Fas-driven apoptosis and, 138–139
 - RCC response to immunotherapy and, 140–142
- Bcl12, 438
- B-CLL. *See* Chronic lymphocytic leukemia
- BE-8, 364
- Benign nerve sheath tumor, 439
- Benign tumors, 5. *See also specific tumors*
 - differentiating, 23
 - gene expression in, 43
- Benzidine, 198
- Beta-naphthylamine, 198
- Bevacizumab, 143
- Bismuth-213, 368
- Bisphosphonates, 361
- Bivatuzumab mertansine, 367
- Bladder cancer
 - multifocality in, 223
 - OCT-4 in, 221–222
 - screening for
 - biases/pitfalls in, 206–207
 - BTA test, 203
 - cost-effectiveness of, 205–206
 - future trends in, 207
 - hematuria screening, 199–200
 - ImmunoCyt/uCyt, 204–205
 - marker evaluation methods, 201–202
 - nuclear matrix protein-22 test, 203–204, 205
 - occupational exposure and, 198
 - previous programs for, 198
 - urine-based tumor markers, 200–201, 203–205
 - Urovysion, 204
 - tumor recurrence in, 223
 - urothelial
 - natural history of, 197
 - prevalence of, 197
- Bladder tumor associated antigen (BTA), 203
- Bleomycin, 168, 170
 - interstitial fibrosis from, 176
- B-mode ultrasound, 23, 25–26, 27
 - tumor differentiation with, 28–29
- BMT. *See* Bone marrow transplantation
- Bone marrow transplantation (BMT), 373
- Bone scintigraphy, 311
- Bone seeking radiopharmaceuticals, 373–374
- Borderline tumors (BOT), 5, 36
 - gene expression in, 43, 45
- Bortezomib, 361, 371
- BOT. *See* Borderline tumors
- Brachytherapy
 - classification of, 257
 - image-based, 260–261
- Brain tumors, CSCs for, 211
- BRCA1, 453
- BRCA2, 453
- Breast cancer
 - CSCs for, 211
 - EphB and, 338
- Brenner (transitional) ovarian cancer, 14
- BTA. *See* Bladder tumor associated antigen

- CA-125. *See* Cancer antigen-125
- Café-au-lain macule (CALM), 301, 304–305
- Calicheamicin, 366
- CALM. *See* Café-au-lain macule
- Calretinin
- adenomatoid tumor and, 163, 164
 - inhibin v., 64–65
 - SEC and, 63–64
- Cancer antigen-125 (CA-125), 15–16, 24
- EOC and, 71–72
 - surgical outcome prediction and, 99–100, 101
- Cancer genes
- biological functions of, 304
 - defining, 302
 - inactivation of, 303
 - mutation frequencies of, 303
 - statistical significance of, 303
- Cancer screening
- bladder cancer
 - biases/pitfalls in, 206–207
 - BTA test for, 203
 - cost-effectiveness of, 205–206
 - future trends in, 207
 - hematuria screening, 199–200
 - ImmunoCyt/uCyt, 204–205
 - marker evaluation methods, 201–202
 - nuclear matrix protein-22 test for, 203–204, 205
 - occupational exposures and, 198
 - previous programs for, 198
 - urine-based tumor markers, 200–201, 203–205
 - Urovision, 204
 - National Cancer Institute requirements for, 197–198
- Cancer stem cell hypothesis (CSC), 211–212, 223
- Carboplatin, 49
- Carcinoembryonic antigen (CEA)
- colon carcinoma and, 99
 - mesothelioma diagnosis and, 163
- Carcinosarcoma, 401
- Cardiac arrhythmia, 116
- Cardiovascular disease, 116
- Caretaker genes, 302
- Carrier proteins, 101
- Castleman's disease, 424
- β -Catenin, 443
- CBC. *See* Complete blood count
- CBI. *See* Continuous bladder irrigation
- CD31, 419, 424
- CD34, 419, 424, 425
- CD44, 74, 419
- CD52, 343
- CD56. *See* Neural cell adhesion molecule
- CD99, 438
- CDI. *See* Color Doppler imaging
- CEA. *See* Carcinoembryonic antigen
- Cellular solitary fibrous tumors, 394
- Cerebrovascular accident, 116
- Cervical cancer
- adenocarcinoma, 244
 - dissemination of, 275
 - EORTC module for, 269–271
 - gross appearance of, 244
 - imaging of, 275
 - incidence of, 275
 - metastasis of, 275–276
 - MRI of
 - CT v., 250–252
 - findings with, 246–247
 - staging with, 247–250
 - technique for, 245–246
 - mucins and, 232
 - parametrial involvement of, 245, 250
 - patterns of spread of, 244
 - posttherapy monitoring for, 281–282
 - prevalence of, 243
 - prognosis of, 244–245
 - FDG-PET for, 280–281
 - QoL measurement module for, 269–271
 - regional lymph node evaluation in, 251–252
 - sarcoma, 275
 - staging of, 243, 275
 - CT v. MRI in, 250–252
 - MRI for, 247–250
 - PET/CT in, 278–279
 - treatment of, 257
 - brachytherapy, 258–261
 - changes in, 263
 - chemoradiotherapy for, 260, 263
 - radiotherapy, 257, 263
 - types of, 244, 275
- Chalkley grid, 148–149
- Chemokines, 423
- Chemoradiotherapy
- for cervical cancer, 260, 263
 - FDG-PET monitoring of, 281
- Chemotherapy
- anemia from, 375
 - for chronic lymphocytic leukemia

Chemotherapy (*continued*)

- alemtuzumab monotherapy, 344–350
 - combined alemtuzumab-fludarabine, 350–352
 - consolidation therapy, 352–354
 - evolution of, 344
 - cytoreduction and, 94–95
 - FDG-PET evaluation of, 280
 - gene expression profiles and, 48–51
 - Kaposi's sarcoma and, 424
 - for multiple myeloma, 372
 - for myeloid leukemia, 188
 - PC-RPLND and, 170
 - platinum-based, 48, 167, 168, 185, 317
 - resistance to
 - in clear cell cancer, 5, 6, 87
 - IDO and, 292
 - in ovarian cancer, 293
 - second primary tumor risk and, 186, 188–189
 - for synovial sarcoma, 433
 - testicular cancer and, 168, 185
 - tumor vascularization and, 94
- Chemotherapy Response Profile (CRP), 50
- CHIR-12.12, 363
- Chlorambucil, 344
- Chondrosarcoma, 401, 458
- Chromogenic in situ hybridization (CISH), 439
- Chronic lymphocytic leukemia (B-CLL), 138, 343
- bone marrow infiltration by, 352
 - remission quality determination for, 352
 - treatment of
 - alemtuzumab monotherapy for, 344–350
 - combined alemtuzumab-fludarabinefor, 350–352
 - consolidation therapy, 352–354
 - evolution of, 344
 - maintenance therapy, 354
- Chylous ascites, 176
- Cimetidine, 118, 131
- CISH. *See* Chromogenic in situ hybridization
- Cisplatin, 48, 49, 168, 170, 287, 451
- CK7, 436, 438
- CK19, 436, 438
- C-kit, 425
- Cladribine, 344
- Clear cell adenocarcinoma, differential gene expression in, 9–10
- Clear cell ovarian cancer, 14
- chemotherapy resistance of, 5, 6, 87
 - clinical characteristics of
 - early stage presentation, 85–86
 - endometriosis and, 86
 - clinical outcomes of
 - platinum chemotherapy resistance, 87
 - prognosis, 5, 87–89
 - retroperitoneal involvement, 87
 - genetic alterations in, 6–8
 - molecular characteristics of, 86–87
 - transcription profiles of, 8–9
 - treatment of, 5–6
 - tumor suppressor genes and, 7
- Clear cell sarcoma, 394, 401, 441
- Clinical impression, 23
- C-myc, 444
- CNTO 328, 364
- Cohort studies, 181–183
- Colchicine, 439
- Collagen, 329
- Colon carcinoma, CEA and, 99
- Color Doppler imaging (CDI), 24
- Colorectal cancer
- Eph/ephrins and, 334
 - IDO expression in, 291, 293
 - IFN- α and, 130
 - IL-2 and, 127
 - MUC1 and, 231
 - RCC metastasis to, 111
 - testicular cancer and risk of, 186
- Combination therapy
- alemtuzumab-fludarabine, 350–352
 - chemoradiotherapy, 263, 281
 - ET-743 and, 458
 - fludarabine-rituximab, 353
- Complete blood count (CBC), 116
- Computed tomography (CT)
- advantages of, 93
 - cervical cancer assessment with,
 - MRI v., 250–252
 - cervical cancer staging with, 278–279
 - cytoreduction outcome prediction with, 96–99, 101
 - lymph node evaluation with, 251–252
 - malignant melanoma management and, 311
 - metastasis detection with, 314
 - PET integrated with, 314–317
 - post-nephrectomy surveillance with, 109
 - RCC appearance in, 111
- Consolidation therapy, 352–354
- Continuous bladder irrigation (CBI), 376

- Contrast-enhanced ultrasonography, 30
Conventional spin echo (CSE), 245–246
Corticosteroids, 116, 405
CRP. *See* Chemotherapy Response Profile
CSC. *See* Cancer stem cell hypothesis
CSE. *See* Conventional spin echo
CT. *See* Computed tomography
Cumulative incidence, 183
Cyclin D1, 443
Cycloheximide, 363
Cyclophosphamide, 287, 344, 352
CYP3A4 promoters, 452
CYP inducers, 454–455
CYP inhibitors, 454–455
Cytokeratins
 KS diagnosis and, 424
 SEC and, 62–63
Cytokines, 133
 for immunomodulation, 122–123
 KS and, 423
Cytolysis, Interleukin-2 and, 115
Cytopenia, 372
Cytoreductive surgery
 chemotherapy effectiveness and, 94–95
 for clear cell ovarian cancer, prognosis after, 87–89
 CT for planning of, 96–99
 peritoneal thickening and, 97–98
 predicting outcomes of
 CA-125 and, 99–100, 101
 CT and, 96–99, 101
 MRI and, 100, 101
 new technologies for, 101
 PET and, 100–101
 value of, 93–96
Cytostatic drugs, 352

D2-40, 419
DAB. *See* 3,3-Diaminobenzidine
Dacarbazine, 317, 451, 458
Darbepoetin alfa, 369
Death inducing signaling complex (DISC), 138
Death receptors, 138
Deep penetrating nevus (DPN), 323–324, 327, 329
Dermatofibroma, 424
Dermatofibrosarcoma protuberans, 388
Desmoid-type fibromatosis, 387
Desmoplastic small round cell tumor, 388
Dex. *See* Dexamethasone
Dexamethasone, 363–365, 366, 371, 455
3,3-Diaminobenzidine (DAB), 19, 233, 288, 395, 408
Diffuse large B-cell lymphoma, 343
Dihydroxyphenylalanine (DOPA), 319
Dipeptidyl peptidase IV
 antigen retrieval for, 325
 distinguishing MM from DPN with, 324, 328
 evaluation of, 325–327
 immunohistochemistry for, 325
 mediation of, 329
DISC. *See* Death inducing signaling complex
DLI. *See* Donor lymphocyte infusion
Docetaxel, 451
Donor lymphocyte infusion (DLI), 370
DOPA. *See* Dihydroxyphenylalanine
Doxorubicin, 287, 366, 451, 457, 458
DPN. *See* Deep penetrating nevus
Dysplastic nevus, 323

Eastern Cooperative Oncology Group (ECOG), 116
E-cadherin, 329, 438, 443
ECOG. *See* Eastern Cooperative Oncology Group
Ecteinascidin-743 (ET-743)
 anti-inflammatory properties, 455
 clinical activity of, 456–457
 drug combinations with, 458
 fibrosis from, 455
 IGF and, 454
 mechanisms of action/resistance
 DNA binding with, 452
 DNA repair machinery inhibition, 453–454
 other effects, 454
 transcription factor inhibition, 452
 metabolism of, 454–455
 response rate to, 451
 sarcoma and, 456–457
 toxicities of, 451, 454–455
Ectocervix, MUC genes in, 236
EGF. *See* Epidermal growth factor
EGFR, 438
Eisen clustering, 38
Ejaculatory disorders
 antegrade ejaculation, 170
 PC-RPLND and, 171, 173
ELISA. *See* Enzyme-linked immunosorbent assay
EMA. *See* Epithelial membrane antigen
Embryonic stem cells (ESC), 212
Endocervical adenocarcinoma, MUC expression in, 231, 237–238

- Endocervical adenocarcinoma in situ,
237, 240
- Endocervical epithelium, MUC genes in, 236, 238
- Endocervical glandular dysplasia,
MUC expression in, 239
- Endometrial cancer
adenocarcinoma, 232
MUC expression in, 238, 240–241
treatment for, 287
IDO and progression of, 292–293
IDO expression in, 289–290, 291
metastasis of, 285, 291
prevalence of, 285
prognosis for, IDO and, 290, 291–292
sertoliform endometrioid carcinoma
clinical features of, 59–60
differential diagnosis of, 61–62
gross findings in, 60
immunohistochemistry of, 62–65
microscopic findings in, 60–61
overview of, 59
prognosis for, 66
survival rates for, 290, 293
- Endometriosis
CA-125 and, 71
clear cell ovarian carcinoma and, 85–86
- Endometrium, mucins in, 241
- Endothelial cell markers, 149–150
- Enzyme-linked immunosorbent assay
(ELISA), 151
bladder cancer marker detection with, 200
immunomonitoring with, 124, 126–127
- EOC. *See* Epithelial ovarian cancer
- EORTC. *See* European Organisation for Research
and Treatment of Cancer
- EORTC QLQ-30. *See* European Organisation
for Research and Treatment of Cancer
Quality of Life Questionnaire
- Eph receptors, 333
in adult human tissues, 334
breast cancer and, 338
colorectal cancer and, 334
in human skin, 334–336
melanoma and, 338
neuroblastoma and, 338
nonmelanoma skin cancer and, 336–338
- Ephrin ligands, 333
in adult human tissues, 334
in human skin, 334–336
- Epidermal growth factor (EGF), 16
- Epithelial membrane antigen (EMA), 436
SEC and, 63
- Epithelial ovarian cancer (EOC), 14
classification of, 5
gene expression in, 45, 53
mortality rate of, 71
NK cell protection layers in, 82
- Epoetin alfa, 369
- Epoetin beta, 369
- Epogen, 369
- Eprex, 369
- ERCC1, 453
- Erythropoietin, 369, 374
- ESC. *See* Embryonic stem cells
- EST. *See* Expression sequence tags
- Estrogen receptors, SEC and, 64
- ESWL. *See* Extracorporeal shock wave lithotripsy
without anesthesia
- ET-743. *See* Ecteinascidin-743
- Ethylenediaminetetramethylene phosphonate, 374
- Etoposide, 168, 185, 287
- Eukaryotic promoters, 452
- European Organisation for Research and
Treatment of Cancer (EORTC),
267–271
- European Organisation for Research and
Treatment of Cancer Quality of Life
Questionnaire (EORTC QLQ-30),
267, 269–271
- Everolimus, 143
- Ewing sarcoma, 388, 393, 394, 401, 439, 457
- Expression sequence tags (EST), 39
- External beam radiation therapy, 257
MRI for planning, 259
- Extracorporeal shock wave lithotripsy without
anesthesia (ESWL), 128
- Extraskelatal myxoid chondrosarcoma, 388, 441
- FACT. *See* Functional Assessment of Cancer
Therapy
- Factor VIII-related antigen, 419, 424, 425
- FADD. *See* Fas-Associated protein with Death
Domain
- Famotidine, 115, 118
- Fas, 137
apoptosis driven by, 138–139
expression of, 142
RCC response to immunotherapy and, 140–142
- Fas-Associated protein with Death Domain
(FADD), 138

- Fast recovery fast spin echo (FRFSE), 245
Fast spin echo (FSE), 245–246
n-3 fatty acids, 131
FCM. *See* Flow cytometry
FDG-PET. *See* ^{18}F fluorodeoxyglucose-positron emission tomography
FDOPA. *See* Fluorodihydroxyphenylalanine
Federation Internationale de Gynecologie et d'Obstetrique (FIGO), 275, 280, 287
Fédération Nationale des Centres de Lutte Contre le Cancer (FNCLCC), 442, 444
FGF. *See* Fibroblast growth factor
Fibroblast growth factor (FGF), 422
Fibromatosis. *See also* Neurofibromatosis type 1 desmoid-type, 387
Fibronectin, 329
Fibrosarcoma, 393
Fibrosis
 bleomycin-induced, 176
 ET-743 toxicity and, 455
 PC-RPLND and, 168, 174
Fibroxsanthoma, 401
FIGO. *See* Federation Internationale de Gynecologie et d'Obstetrique
Filgrastim, 369
FISH. *See* Fluorescence in situ hybridization
FITC, 408
Flow cytometry (FCM), 41
 immunomonitoring with, 124–126
 leukemia remission quality determination with, 352
FLT. *See* Fluorothymidine
Fludarabine, 344, 346
 alemtuzumab combined with, 350–352
 rituximab combined with, 353
Fluorescence in situ hybridization (FISH)
 bladder cancer screening with, 204, 207
 chronic lymphocytic leukemia analysis with, 344
 disadvantages of, 441
 FUS-CREB3L2 fusion detection with, 387
 synovial sarcoma marker detection with, 393, 401, 440, 441, 446, 447
Fluorescent dyes, 101
 ^{18}F fluorodeoxyglucose-positron emission tomography (FDG-PET), 93
 chemotherapy evaluation with, 280
 for malignant melanoma, 317–318
 directing therapy with, 279–280
 false-negative results in, 277–278
 lymph node metastasis evaluation with, 276–279
 sensitivity/specificity of, 313
malignant melanoma management and indications for, 312–313
 locoregional lymph node invasion detection, 313–314
metastasis detection with, 277
 brain, 315
 distant, 314
 patient management and, 318
 posttherapy monitoring with, 281–282
 prognostic use of, 280–281
 uptake mechanism for, 312
Fluorodihydroxyphenylalanine (FDOPA), 319
Fluorodopa, 319
11C-fluoromethyluracil, 319
Fluorophores, 408
Fluorothymidine (FLT), 319
5-fluorouracil, 366
FNCLCC. *See* Fédération Nationale des Centres de Lutte Contre le Cancer
Folate receptors, 101
FRFSE. *See* Fast recovery fast spin echo
FSE. *See* Fast spin echo
Functional Assessment of Cancer Therapy (FACT), 267
FUS-CREB3L2 fusion gene
 detection of
 primers for, 388–389
 reverse transcription polymerase chain reaction for, 389–390
 RNA extraction for, 389
 sequence analysis for, 390
 fusion patterns in, 388
 HSCT and, 387
 LGFMS and, 387, 390
G2-M, 454
Gadolinium-diethylenetriaminepenta-acetic acid (Gd-DTPA), 246
Gatekeeper genes, 302
GCN. *See* Giant congenital melanocytic nevus
G-CSF. *See* Granulocyte colony stimulating factor
Gd-DTPA. *See* Gadolinium-diethylenetriaminepenta-acetic acid
GEC-ESTRO. *See* Group Européen de Curiethérapie-European Society for Therapeutic Radiology and Oncology

- Gemcitabine, 451
- Gene expression
- analysis techniques for, 35
 - ovarian cancer classification and, 54
 - profiles of
 - contamination of, 37–38
 - number of samples for, 38
- Gene expression microarray analysis, 35
- cancer subgroup determination with, 41–43
 - chemotherapeutic response correlation with, 48–51
 - clinical correlations of, 39
 - data analysis for, 40
 - goals for, 41
 - metastasis profiles in, 46–48
 - number of samples for, 38
 - ovarian cancer staging/grading with, 43–46
 - pathological quality control and, 38–39
 - patient survival correlation with, 52–54
 - platforms for, 39
 - profile contamination in, 37–38
 - RNA isolation for, 39–40
 - sample selection for, 36–37
 - secondary validation of, 40–41
 - surgical debulking correlation with, 51–52
 - tissue processing protocols for, 38
- Genetic alterations, in clear cell ovarian cancer, 6–8
- German Society of Hematology and Oncology, 350
- Germ-cell testicular cancer
- classification of, 185
 - incidence of, 182
 - mortality rates for, 182
 - OCT-4 and, 223
 - second primary cancers after, 181
 - investigation methods for, 181–185
 - survival rates for, 181
- Germ-cell tumors, OCT-4 and, 223
- Giant congenital melanocytic nevus (GCN), 304
- Glioblastoma, 338
- Glucose oxidase, 408
- Glutamine, 131
- GOG. *See* Gynecologic Oncology Group
- Granulocyte colony stimulating factor (G-CSF), 132
- Group Européen de Curiethérapie-European Society for Therapeutic Radiology and Oncology (GEC-ESTRO), 260–261
- Growing teratoma syndrome (GTS), 168
- Growth factors, multiple myeloma immunotherapy with, 369–370
- GTS. *See* Growing teratoma syndrome
- Gynecologic Oncology Group (GOG), 88, 280
- HAART. *See* Highly active antiretroviral therapy
- Haemolytic anemia, 354
- Hairy cell leukemia, 343
- HCD122, 363
- Hemangioma, 163, 424
- Hemangiopericytoma, 393, 401, 438
- Hematuria testing, bladder cancer screening and, 199–200
- Hemolytic anemia, microangiopathic, 377
- Hemolytic uremic syndrome, 376
- Hepatocyte nuclear factor (HNF), 8, 9
- HER2, 438
- HHV8. *See* Human herpesvirus-8
- HHV8-associated lymphoma, 424
- HIER, 419
- High density cDNA hybridization filter, 35
- High-grade tumors, 36
- Highly active antiretroviral therapy (HAART), 424
- Histiocytic neoplasms, 394
- HIV. *See* Human immunodeficiency virus
- HLA-DR. *See* Anti-human leukocyte antigen
- HNF. *See* Hepatocyte nuclear factor
- ¹⁶⁶Ho-DOTMP. *See* Holmium-166 1, 4, 7, 10-tetraazacyclododecane-1, 4, 7, 10-etramethylenephosphonate
- Holmium-166 1, 4, 7, 10-tetraazacyclododecane-1, 4, 7, 10-etramethylenephosphonate (¹⁶⁶Ho-DOTMP), 374–378
- Homologous recombination repair (HRR), 453
- Horseradish peroxidase, 408
- HRR. *See* Homologous recombination repair
- HSCT. *See* Hyalinizing spindle cell tumor
- hTERT. *See* Human telomerase reverse transcriptase
- Human herpesvirus-8 (HHV8), 405, 419, 421–422, 424. *See also* Kaposi's sarcoma
- Human immunodeficiency virus (HIV), 405
- KS and, 423, 424
 - vascular tumors associated with, 424
- Human telomerase reverse transcriptase (hTERT), 200
- HuN901, 367
- Hyalinizing spindle cell tumor (HSCT), 387
- Hyperchromasia, 324

- ICBT. *See* Intracavitary brachytherapy
- ICRU. *See* International Commission for
Radiation Units and Measurements
- IDO. *See* Indoleamine 2,3-dioxygenase
- IDO inhibitors, 287
- IFN- α . *See* Interferon- α
- IFN- γ . *See* Interferon- γ
- Ifosfamide, 433, 451, 457, 458
- IGF. *See* Insulin-like growth factor
- IL-1. *See* Interleukin-1
- IL-2. *See* Interleukin-2
- IL-6. *See* Interleukin-6
- Image-based brachytherapy, 260–261
- Imatinib, 303
- Imatinib mesylate, 425
- Immune cell therapy, 370
- Immunoconjugates, 361
- Immunocytochemistry, 41
- Immunofluorescence, 408
- Immunohistochemistry, 41
- of adenomatoid tumors, 163–164
 - defining, 18
 - for EphA1, 336
 - IDO expression evaluation with, 287–288
 - immunofluorescence v., 408
 - of Kaposi's sarcoma, 406, 425
 - diagnosis with, 424
 - interpretation of, 417–418
 - materials for, 407–408
 - methods of, 408, 417
 - studies of, 409–416, 418–419
 - MM v. DPN evaluation with, 325
 - MUC4 methodology for
 - immunolabeling, 18–19
 - staining assessment, 19
 - tissue sectioning, 18
 - of OCT-4, 224
 - of sertoliform endometrioid carcinoma, 62–65
 - of synovial sarcoma, 393–394, 436–438
 - TLE1 as marker for, 398–401
- Immunolocalization, 327, 329
- Immunomodulation, 370–371
- cytokines for, 122–123
 - perioperative, 131–132
 - with IFN- α , 129–131
 - with IL-2, 127–129
 - preoperative, 132
- Immunomonitoring, perioperative, 123
- with ELISA, 124, 126–127
 - with FCM, 124–126
- Immunoperoxidase staining, 408
- Immunoprecipitation, 41
- Immunoreactivity, 327, 329
- of Kaposi's sarcoma, 420, 421
- Immunostaining, 325, 326
- Immunosuppression, 116
- from multiple myeloma, 361
 - in tumor microenvironment, 292
- Immunotherapy, 137
- for Kaposi's sarcoma, 424
 - multiple myeloma treatment and, 361, 379
 - anti-IL-6 therapy, 363–365
 - growth factors, 369–370
 - IGF-1 targeting, 365–366
 - immune cell therapy, 370
 - immunoconjugates, 366–367
 - immunomodulation, 370–371
 - immunotoxins, 366–367
 - monoclonal antibodies, 362–363
 - targeted radioimmunotherapy, 367–368
- IMRT. *See* Intensity-modulated radiotherapy
- Indoleamine 2,3-dioxygenase (IDO)
- in cancer tissues, 286–287
 - chemoresistance and, 292
 - colorectal cancer and, 291, 293
 - expression of, 291–293
 - function of, 285
 - immunohistochemical staining for
 - antibodies for, 287
 - expression evaluation with, 288, 289–290
 - patient samples for, 287–288
 - staining procedure for, 288
 - statistical analysis of, 288–289
 - immunosuppression by, 286, 292
 - metastasis and, 291
 - myometrial invasion and, 291
 - ovarian cancer and, 291
 - patient survival and, 290
 - as prognostic variable, 290, 291–292
 - survival rates and, 293
 - tryptophan catabolism and, 286, 291
 - tumor progression and, 292–293
- Inflammatory arthritis, 116
- Inhibin
- adrenal cortical tumors and, 164
 - calretinin v., 64–65
 - SEC and, 63
 - sex cord-stromal tumors and, 65

- In situ hybridization (ISH)
 FISH/CISH v., 439
 synovial sarcoma marker detection with, 439–441
- Insulin-like growth factor (IGF), 44, 426
 ET-743 and, 454
 treatments targeting, 365–366
- Integrated PET/CT, 314–317
 accuracy of, 316–317
 cancer staging with, 316
 malignancy staging with, 320–321
 metastasis detection with, 314–315
 single-modality imaging v., 317
- Integrin $\alpha_v\beta_3$ expression, 319–320
- Integrin β_3 , 324
- β integrins, 74, 75
- Intensity-modulated radiotherapy (IMRT), 279
- Interferon- α (IFN- α), 133, 137, 424
 colorectal cancer and, 130
 immunomodulation with, 122–123, 129–131
- Interferon-alpha-2b, 372
- Interferon- γ (IFN- γ), 131, 286
- Interleukin-1 (IL-1), 132
- Interleukin-2 (IL-2), 132–133, 137, 143
 cancer cell cytolysis and, 115
 immunomodulation with, 122, 127–129
 metastasis response to, 117
 type-2 histamine antagonists and, 118
- Interleukin-6 (IL-6), 131, 132, 422
 multiple myeloma treatment and, 363–365
- International Commission for Radiation Units and Measurements (ICRU), 257, 258
- International Federation of Gynecology and Obstetrics (FIGO). *See* Federation Internationale de Gynecologie et d'Obstetrique
- International Quality of Life Assessment (IQOLA), 268
- Intracavitary brachytherapy (ICBT), 257
 for cervical cancer
 applicator insertion for, 258
 dose specification for, 258–259
 image-based, 260–261
 MRI and, 259–260
- IQOLA. *See* International Quality of Life Assessment
- Irinotecan, 451, 458
- ISH. *See* In situ hybridization
- JAK/STAT, 426
- Kaposi, Moritz, 405
- Kaposi's sarcoma (KS)
 diagnosis of, 424
 etiology of, 405
 histogenesis of, 418–419
 histology of, 405–406
 HIV and, 423
 immunohistochemistry of, 406, 425
 diagnosis with, 424
 materials for, 407–408
 methods of, 408, 417
 studies of, 409–416, 418–419
 immunoreactivity of, 420, 421
 lesion development in, 426
 pathogenesis of
 angiogenesis, 422–423
 apoptosis and, 423
 chemokines in, 423
 HHV8 infection, 421–422
 treatment of, 424–425
 variants of, 406
- Kaposi's sarcoma herpesvirus (KSHV).
See Human herpesvirus-8
- Keratinocytes, EphA1 and, 335, 338
- Kidney cancer, metastatic, 115. *See also* Renal cell cancer
- KS. *See* Kaposi's sarcoma
- KSHV. *See* Kaposi's sarcoma herpesvirus
- LAK. *See* Lymphokine activated killer cells
- Large-cell anaplastic/lymphoblastic lymphoma, 343
- Laser ablation, 109
- LEF, 443
- Leiomyosarcoma, 401, 451, 456–457
- Lenalidomide, 361, 371
- Lentian, 132
- Leukemia
 B cell lymphocytic, 138, 343
 bone marrow infiltration of, 352
 chronic lymphocytic, 138, 343
 bone marrow infiltration by, 352
 remission quality determination for, 352
 treatment of, 344–354
 consolidation therapy for, 352–354
 CSCs for, 211
 evolution of treatment for, 344
 hairy cell, 343
 myeloid, 186
 chemotherapy and, 188

- radiotherapy and, 188–189
 - remission quality determination for, 352
 - T-cell prolymphocytic, 343
- Leukine, 369
- Lexidronam, 374
- LGFMS. *See* Low grade fibromyxoid sarcoma
- Lipopolysaccharide (LPS), 131, 286
- Liposarcoma, 451, 457
- Liposomal anthracyclines, 424
- LMP. *See* Low malignant potential tumors
- LNA-1, 419, 424, 426
- LOH. *See* Loss of heterozygosity
- Loss of heterozygosity (LOH), 7
 - cancer gene identification and, 303
 - NF1 inactivation and, 301
 - NF1 mutation and, 305
- Low grade fibromyxoid sarcoma (LGFMS), 387
- Low grade myxofibrosarcoma, 387
- Low malignant potential tumors (LMP), 5, 36
 - gene expression in, 43, 45–46
- Low molecular weight keratins, 436
- LPS. *See* Lipopolysaccharide
- Lymphadenopathy, 346, 352
- Lymphangiography, 251
- Lymphangioma, 163
- Lymphangioma-like Kaposi's sarcoma, 424
- Lymph node metastasis, 251
 - of cervical cancer, 275–276
 - imaging of, 276–277, 279
 - locoregional, detection of, 313–314
 - of malignant melanoma, 311
 - MRI of, 251–252, 279
- Lymphocytes
 - antitumor abilities of, 115
 - famotidine and, 115
- Lymphokine activated killer cells (LAK), 115, 118
- Lymphoscintigraphy, 251
- LYVE-1, 419
- Magnetic resonance imaging (MRI)
 - adnexal mass characterization with, 93
 - cervical cancer brachytherapy and, 259–260
 - contrast-enhanced, 110, 246
 - lymph node-specific, 251
 - cytoreductive surgery outcome prediction and, 100, 101
 - intracavitary brachytherapy and, 259–260
 - lymph node evaluation with, 251–252, 279
 - metastasis detection with, 314
 - normal uterine cervix anatomy in, 244
 - post-nephrectomy surveillance with, 110
 - radiation therapy evaluation with, 259–260
 - radiation therapy planning and, 259
 - radiofrequency thermal ablation zone
 - characteristics for, 110–112
 - of uterine cervical cancer
 - findings of, 246–247
 - staging with, 247–250
 - technique for, 245–246
 - of uterine cervix, 244
- Maintenance therapy, chronic leukemia and, 354
- Major histocompatibility complex (MHC), 422
- Malignant melanoma (MM)
 - alternative PET tracers for, 318–321
 - CT of, 311
 - DPN v., 327
 - FDG-PET imaging of, 312–313
 - workup for, 320
 - histological diversity of, 323
 - incidence of, 311
 - lymph node involvement by, 311
 - markers for, invasive/noninvasive failing of, 324
 - metastasis of, 311
 - brain, 315
 - distant, 314
 - full-body FDG-PET scans for, 315
 - small-lesion, 314–315
 - mimics of, 323
 - prognostic factors for, 311
 - staging of
 - FDG-PET for, 320
 - integrated PET/CT for, 316, 320–321
- Malignant peripheral nerve sheath tumor (MPNST), 393, 435
 - markers in, 394
 - SS v., 438, 439
- Malignant tumors, 5. *See also specific malignancies*
 - differentiating, 23
- Mammalian target of rapamycin (mTOR), 426
- MAPK. *See* Mitogen-activated protein kinase
- Marrow infiltrating lymphocytes (MILS), 370
- Massively parallel signature sequencing (MPSS), 49
- Mass spectrometry, 41
- Matrix metalloproteinase (MMP), 324, 325, 327, 329, 422
- Maytansine, 366–367
- MDR1, 452

- Megakaryocyte potentiating factor (MPF), 74
- Melanocytes
 density of, 304–305
 DPN v., 324
 invasive, non-metastisizing growths of, 323
 neurofibromin in, 304–305, 307
 NF1 and, 301
- Melanocytic borderline lesions, 323
- Melanoma, 64. *See also* Malignant melanoma
 dipeptidyl peptidase IV and, 324
 Eph receptors and, 336–338
 neurofibromin and, 305
 NF1-associated, 301
 clinical features of, 307
 incidence of, 304, 307
 NF1 gene in
 inactivation, 306–307
 mutation, 305–306
 oncogenesis of, 307
- Melphalan, 317, 364, 371–373, 375–376
- Mesothelin
 metastasis and, 73
 MUC16 binding with, 74
 kinetics of, 75
 n-linked oligosaccharides in, 76–79
 serum levels of, 74
- Mesothelioma, 63, 74
- Metallothionein promoters, 452
- Metastasis
 of adenocarcinoma, 161, 163
 assessing potential for, 320
 bone, 117
 brain, 116, 314–315
 of cervical cancer, 275–276
 colorectal cancer, 111
 detecting, 277, 314, 320
 of endometrial cancer, 285, 291
 gene expression profiles based on, 46–48
 IDO and, 291
 interleukin-2 and, 117
 kidney, 115
 to lymph nodes, 251
 by cervical cancer, 275–276
 imaging of, 276–277
 of malignant melanoma, 311
 brain, 315
 distant, 314
 full-body FDG-PET scans for, 315
 small-lesion, 314–315
 markers of, 327
 mesothelin and, 73
 of RCC, 111, 121–122, 137
 small-lesion, 314–315
 of soft tissue sarcoma, 451
 of testicular cancer, 167–168, 177
- Methotrexate, 116
- MGUS. *See* Monoclonal gammopathy of undetermined significance
- MHC. *See* Major histocompatibility complex
- Microangiopathic hemolytic anemia, 377
- Microglandular hyperplasia, 239
- Micro RNA profiling, 35
- Microsatellite analysis, 35
- Microvessel density (MVD), 148–152
- Millennium, 371
- MILS. *See* Marrow infiltrating lymphocytes
- Mitochondria, 138
- Mitogen-activated protein kinase (MAPK), 329, 426
- MM. *See* Malignant melanoma; Multiple myeloma
- MMP. *See* Matrix metalloproteinase
- MMP inhibitors, 425
- MoAbs. *See* Monoclonal antibodies
- Molecular targeting drugs, 143
- Monoclonal antibodies (MoAbs), 361, 362–363
- Monoclonal gammopathy of undetermined significance (MGUS), 361
- Mozobil, 369–370
- MPF. *See* Megakaryocyte potentiating factor
- MPNST. *See* Malignant peripheral nerve sheath tumor
- MPSS. *See* Massively parallel signature sequencing
- MRI. *See* Magnetic resonance imaging
- mTOR. *See* Mammalian target of rapamycin
- mTOR inhibitors, 133
- MUC4. *See* Mucin glycoprotein 4
- MUC16. *See* Mucin glycoprotein 16
- MUC genes, 231–232
 AIS and, 240
 benign lesions and, 237, 239
 endocervical adenocarcinoma and, 237–238
 endocervical epithelium expression of, 236, 238
 endocervical glandular dysplasia and, 239
 endometrial adenocarcinoma and, 238, 240–241
 evaluating expression of
 materials for, 232–233
 methods for, 233–236
 MUC genes in, 236–238
 squamous metaplasia and, 239

- Mucin glycoprotein 4 (MUC4)
 - endocervical adenocarcinoma and, 231, 237–238
 - immunohistochemistry methodology for
 - immunolabeling, 18–19
 - staining assessment, 19
 - tissue sectioning, 18
 - ovarian cancer and, 13–14, 16–18
 - structure/biology of, 16
- Mucin glycoprotein 16 (MUC16), 14, 17
 - domains of, 72
 - EOC and, 72–73
 - mesothelin binding with, 74
 - kinetics of, 75
 - n-linked oligosaccharides in, 76–79
 - NK cell binding with
 - immunosuppression due to, 79
 - NK cell differentiation and, 81–82
 - phenotypic shift in, 79–81
- Mucins
 - aberrant expression of, 15–18
 - cancer development and, 13
 - cervical cancer and, 232
 - defining, 231
 - malignancy and, 231
 - normal endometrium and, 241
- Multiple myeloma (MM)
 - bone loss from, 378
 - prevalence of, 361
 - treatment of, 361
 - anti-IL-6 therapy, 363–365
 - growth factors, 369–370
 - IGF-1 targeting, 365–366
 - immune cell therapy, 370
 - immunoconjugates, 366–367
 - immunomodulation, 370–371
 - immunotherapy, 361, 362–371, 379
 - immunotoxins, 366–367
 - monoclonal antibodies, 362–363
 - myeloma vaccines, 361, 368–369
 - radiotherapy, 371–373
 - targeted radioimmunotherapy, 367–368
 - targeted radiotherapy, 373–378
- Multiple regression analysis, 182–183
- Multiple sclerosis, 354
- MVD. *See* Microvessel density
- Myelodysplastic syndrome, 376
- Myeloid leukemia, 186
 - chemotherapy and, 188
 - radiotherapy and, 188–189
- Myeloma vaccines, 361, 368–369
- Myocardial infarction, 116
- Myxofibrosarcoma, 387
- Myxoid liposarcoma, 388, 451, 457
- Nabothian cysts, 243
- Nanoparticles, 101
- National Cancer Institute, 14, 344
 - screening program requirements of, 197–198
- National Comprehensive Cancer Network (NCCN), 371, 433
- National Institutes of Health, 370
- National Survey of Ovarian Cancer (NSOC), 14
- Natural killer cells (NK cells)
 - differentiation of, 81–82
 - interleukin-2 and, 115
 - MUC16 binding with
 - immunosuppression due to, 79
 - NK cell differentiation and, 81–82
 - phenotypic shift in, 79–81
 - tumor cell protection from, 82
 - type-2 histamine antagonists and, 118
- N-CAM. *See* Neural cell adhesion molecule
- NCCN. *See* National Comprehensive Cancer Network
- NeoRacormon, 369
- Nephrectomy
 - post-procedure surveillance for, 109–110
 - radical, 109
 - segmental, 109
- Nephropathy, radiation, 377
- NER. *See* Nucleotide excision repair system
- Nerve sheath tumors
 - benign, 439
 - malignant peripheral, 393, 435
 - markers in, 394
 - SS v., 438, 439
- Nested case-control studies, 183–184
- Neulasta, 369
- Neupogen, 369
- Neural cell adhesion molecule (N-CAM/CD56), 64
- Neuroblastoma, 338
- Neurofibroma, 301
- Neurofibromatosis type 1 (NF1)
 - etiology of, 301
 - incidence of, 301
 - melanocytes in, 304–305
 - melanoma in, 301
 - clinical features of, 307
 - incidence of, 304, 307
 - NF1 gene inactivation in, 306–307

- Neurofibromin
 - melanocyte abnormalities and, 304–305, 307
 - melanoma and, 305
- Neutropenia, 455
- Nevus
 - deep penetrating, 323–324, 327, 329
 - dysplastic, 323
 - giant congenital melanocytic, 304
 - plexiform spindle, 323
 - spitz, 323
- NF1. *See* Neurofibromatosis type 1
- NF1 gene
 - malignant melanoma and
 - inactivation, 306–307
 - mutations, 305–306
 - NF1 etiology and, 301
 - oncogenesis and, 307
- NF-kappaB, 426
- NF-Y, 452
- Nicotinamide adenine dinucleotide (NAD), 285–286
- Nitrosureas, 317
- NK cells. *See* Natural killer cells
- Nonmelanoma skin cancer, 336–338
- Non-seminomatous germ cell tumors (NSGCT), 167–168
- NSOC. *See* National Survey of Ovarian Cancer
- Nuclear karyometry, 200
- Nucleotide excision repair system (NER), 451, 453
- Occupational exposures, bladder cancer and, screening programs and, 198
- OCT-4, 212
 - in bladder tumors, 221–222
 - detecting expression of
 - agarose gel electrophoresis, 217
 - clinical samples for, 215
 - immunohistochemistry, 219
 - reverse transcriptase polymerase chain reaction, 216–217
 - RNA extraction, 215–216
 - SDS-Page, 217
 - statistical analyses, 219
 - Western blotting, 217
 - expression of, 220–221, 224
 - germ cell tumors and, 223
 - immunohistochemical analysis of, 224
 - marker sensitivity/specificity of, 223–224
- Oncogenes
 - clear cell ovarian cancer and, 7
 - defining, 302
 - identification of, 302–304
- Oncogenesis
 - genes implicated in
 - defining, 302
 - identification of, 302–304
 - NF1 gene mutations and, 307
- 1-MT, 287
- Osteolytic lesions, 361
- Osteosarcoma, 457
- Ovarian cancer
 - biomarkers for, 14–15
 - chemoresistance in, 293
 - classification of, 5, 14, 26–27, 36, 38, 85
 - gene expression and, 54
 - clear cell, 14
 - chemotherapy resistance of, 5, 6, 87
 - clinical characteristics of, 85–86
 - clinical outcomes of, 87–89
 - genetic alterations in, 6–8
 - molecular characteristics of, 86–87
 - prognosis for, 5, 87–89
 - transcription profiles of, 8–9
 - treatment of, 5–6
 - tumor suppressor genes and, 7
 - cytoreduction of, 93–96
 - epithelial, 14
 - classification of, 5
 - gene expression in, 45, 53
 - mortality rate of, 71
 - NK cell protection layers in, 82
 - gene expression profiles and, 52–54
 - glandular patterns in, 62
 - grading of, 39, 43–46, 66
 - heterogeneity of, 35–36
 - histopathology of, 14
 - IDO and, 291
 - incidence of, 5, 13
 - mesothelin levels and, 74
 - mortality/morbidity rates from, 13, 35
 - MUC4 and, 13–14, 16–18
 - MUC16-mesothelin binding in, 74–79
 - prognosis for, 14, 36, 52–54
 - screening for, 14–15
 - small cell, 14
 - stages of, 14, 26, 43–46, 66
 - subgroup determination for, 41–43
 - transcription profiles of, 8–9

- transitional (Brenner), 14
- tubular/pseudotubular patterns in, 62
- types of, 5, 35–36
- Oxaliplatin, 49
- p53, 426, 443, 454
- Paclitaxel, 293, 458
- Palliative radiotherapy, 371–372, 374
- Pathological quality control, importance of, 38–39
- PBSCT. *See* Peripheral stem cell transplantation
- PCA. *See* Principle component analysis
- PCNA. *See* Proliferating cell nuclear antigen
- PCR. *See* Polymerase chain reaction
- PC-RPLND. *See* Post-chemotherapy retroperitoneal lymph node dissection
- PCR-SSCP. *See* Polymerase chain reaction/single strand conformational polymorphism
- PDGF. *See* Platelet derived growth factor
- PECAM-1. *See* Platelet endothelial cell adhesion molecule-1
- Pegfilgrastim, 369
- Pelvic examination, 15, 24
- Perineurioma, soft tissue, 387
- Perioperative immunomonitoring, 123
 - with ELISA, 124, 126–127
 - with FCM, 124–126
- Peripheral stem cell transplantation (PBSCT), 374–375
- Peripheral T-cell lymphoma (PTCL), 343
- Peritoneal fluid (PF)
 - CA-125 levels in, 71
 - MUC16 in, 71
- Peritoneal thickening, cytoreduction and, 97–98
- PET. *See* Positron emission tomography
- PF. *See* Peritoneal fluid
- P glycoprotein, 452
- Phase II enzyme inhibitors, 454
- Pheochromocytoma
 - adrenal AT v., 161
 - NF1 gene and, 301
- Phosphonate chelates, 374
- PI. *See* Proliferation index; Pulsatility index
- Picropodophyllin (PPP), 366
- Plasminogen-related protein B, 458
- Platelet derived growth factor (PDGF), 422, 425
- Platelet endothelial cell adhesion molecule-1 (PECAM-1), 419
- Platinum-based chemotherapy, 48, 167, 168, 185, 317
- Plerixafor, 369
- Plexiform spindle nevus, 323
- Polymerase chain reaction (PCR), 220, 390. *See also* Reverse transcriptase-polymerase chain reaction
 - HHV8 detection with, 421, 424
 - Kaposi's sarcoma diagnosis and, 421, 424
 - NF1 analysis with, 307
 - SYT-SSX fusion detection with, 441
- Polymerase chain reaction/single strand conformational polymorphism (PCR-SSCP), 7
- Positron emission tomography (PET). *See also* ¹⁸F fluorodeoxyglucose-positron emission tomography
 - alternative tracers for, 318–321
 - of cervical cancer, 275
 - cervical cancer staging with, 278–279
 - CT integrated with, 314–317
 - cytoreductive surgery outcome prediction and, 100–101
 - of lymph node metastasis, 276–277
 - malignant melanoma management and, 311
 - resolution limitations of, 277
 - of testicular cancer, 169
- Post-chemotherapy retroperitoneal lymph node dissection (PC-RPLND)
 - complications of, 176
 - fibrosis and, 168, 174
 - full bilateral template dissection in, 171–173
 - indications for, 167–169
 - modifications to, 170–171
 - modified template dissection in, 170–173
 - morbidities associated with, 170
 - postoperative follow-up for, 176–177
 - preoperative considerations for, 169–170
 - surgical approaches for, 171
 - technical considerations for, 170–173
 - teratoma and, 168
 - treatment-related outcomes of, 173–175
- PPP. *See* Picropodophyllin
- Prednisone, 371, 372
- Principle component analysis (PCA), 38, 40
- Procrit, 369
- Progesterone receptors, SEC and, 64
- Proliferating cell nuclear antigen (PCNA), 324
- Proliferation index (PI), 137, 142
- Prostate cancer, CSCs for, 211
- Prox-1, 419
- PTCL. *See* Peripheral T-cell lymphoma
- Pulmonary hypertension, 424

- Pulsatility index (PI), 28
- Pulsed Doppler ultrasound, 24, 26
- Pyogenic granuloma-like Kaposi's sarcoma, 424
- QoL. *See* Quality of Life
- Quadramet, 374
- Quality of Life (QoL)
- defining, 263–264
 - measurements for
 - cancer site-specific, 267–268
 - cervical cancer module for, 269–271
 - development/cross-cultural validation of, 268
 - disease specific, 267
 - EORTC modular approach to, 268
 - generic, 266–267
 - psychometric properties of, 265–266
 - reliability of, 265
 - responsiveness to change of, 266
 - selecting, 264–265
 - types of, 266–268
 - validity of, 265–266
- Radiation nephropathy, 377
- Radiofrequency thermal ablation, 109
- tissue damage from, 110
 - zone of
 - involution of, 110
 - MR signal characteristics of, 110–112
- Radioimmunotherapy, targeted, 367–368
- Radiolabeled RGD peptide, 319–320
- Radiotherapy. *See also* Brachytherapy;
- Intracavitary brachytherapy
 - for cervical cancer, 257, 263
 - MRI evaluation of, 259–260
 - for multiple myeloma
 - chemotherapy combined with, 372
 - dosage, 371
 - effectiveness of, 372–373
 - palliative, 371–372, 374
 - targeted, 373–378
 - multiple myeloma treatment with, 371–373
 - myeloid leukemia and, 188–189
 - palliative, 371–372, 374
 - second primary tumor risk and, 186, 188–189
 - skeletal targeted
 - mechanism of action of, 374–375
 - ongoing phase III trials, 377–378
 - phase I/II dosimetry study, 375–377
 - phase III pivotal trial, 377
 - for synovial sarcoma, 433
 - systemic, 373
 - for testicular cancer, 185
 - total body, 373
 - types of, 257
- Ranitidin, 131
- Rapamune, 366
- Rapamycin, 366, 426
- RCC. *See* Renal cell cancer
- Refractory autoimmune thrombocytopenia, 354
- Reliability, of QoL instruments, 265
- Renal cell cancer (RCC)
- apoptosis mechanisms and, 138–139
 - CT appearance of, 111
 - immunodysfunction and, 132
 - immunotherapy for, 140–142
 - incidence of, 109
 - metastasis of, 111, 121–122, 137
 - prognosis for
 - Bcl-2/Fas and, 139, 140
 - variation in, 143
 - proliferation detection in, 142–143
 - residual/recurrent, 112
 - subgroups of, 143
 - treatment of, 121
- Retroperitoneal lymph nodes, clear cell carcinoma
- involvement in, 87
- Reverse transcriptase-polymerase chain reaction (RT-PCR), 389, 391
- ABCF2 analysis with, 6
 - antiangiogenic therapy tested with, 151
 - bladder cancer marker detection with, 200, 213, 216–217, 220
 - ET-743 testing and, 453
 - FUS-CREB3L2 fusion gene detection with, 387–390
 - LOH amplification with, 303
 - OCT-4 analysis with, 213, 215–217, 221
 - renal cell carcinoma analysis with, 121–122
 - RNA expression validation with, 41, 45
 - SYT-SSX fusion detection with, 441
 - TCF2 analysis with, 8
 - t(X;18) detection with, 393, 439, 441
- Revimid. *See* Revlimid
- Revlimid, 366, 371
- RGD-PET, 319–320
- Rhabdomyosarcoma, 458
- Rhadinovirus, 421
- Rhenium-186, 368
- Rhenium-188, 368
- Rhodamine, 408

- RIT. *See* Targeted radioimmunotherapy
- Rituximab, 344, 350, 352, 405
- fludarabine combined with, 353
- RNA isolation, gene expression data and, 39–40
- RT-PCR. *See* Reverse transcriptase-polymerase chain reaction
- S-100 protein, 438
- SANT-7, 365
- Sarcoma
- AIDS-related Kaposi's, 423–425
 - alveolar rhabdomyosarcoma, 388, 439
 - angiosarcoma, 394, 424
 - carcinosarcoma, 401
 - cervical, 275
 - chondrosarcoma, 401, 458
 - clear cell, 394, 401, 441
 - dermatofibrosarcoma protuberans, 388
 - ET-743 and, 456–457
 - Ewing, 388, 393, 394, 401, 439, 457
 - extraskeletal myxoid chondrosarcoma, 388, 441
 - fibrosarcoma, 393
 - Kaposi's
 - diagnosis of, 424
 - etiology of, 405
 - histogenesis of, 418–419
 - histology of, 405–406
 - HIV and, 423
 - immunohistochemistry of, 406–419, 424, 425
 - immunoreactivity of, 420, 421
 - lesion development in, 426
 - pathogenesis of, 421–423
 - treatment of, 424–425
 - variants of, 406
 - leiomyosarcoma, 401, 451, 456–457
 - liposarcoma, 451, 457
 - low grade fibromyxoid sarcoma, 387
 - low grade myxofibrosarcoma, 387
 - lymphangioma-like Kaposi's sarcoma, 424
 - myxoid liposarcoma, 388, 451, 457
 - osteosarcoma, 457
 - pyogenic granuloma-like Kaposi's sarcoma, 424
 - rhabdomyosarcoma, 458
 - soft tissue, 387–388
 - metastasis of, 451
 - treatment of, 451
 - spindle cell sarcoma, 424, 433
 - synovial, 388
 - biphasic, 434
 - diagnosis of, 393, 434–436, 438–441
 - ET-743 and, 457
 - grading of, 442–444
 - histogenesis of, 433
 - immunohistochemistry of, 393–394, 398–401, 436–438, 446, 447
 - incidence of, 433
 - monophasic epithelioid, 436
 - monophasic fibrous, 435–436
 - poorly differentiated, 436
 - prognosis for, 393, 442–444
 - radiographic appearance of, 433
 - round cell sarcomas v., 439
 - survival rates for, 433
 - treatment of, 433
- Sargramostim, 369
- SCC. *See* Squamous cell cancer
- Schwannoma, 401
- malignant, NF1 gene and, 301
- SCJ. *See* Squamocolumnar junction
- SEC. *See* Sertoliform endometrioid carcinoma
- SEER. *See* Surveillance, Epidemiology, and End Results
- Sentinel lymph node scintigraphy (SLN), 311, 313
- Serial analysis of gene expression, 35
- Serous tumors, 5, 14, 35
- Sertoli cell tumor, 59, 62, 65
- Sertoliform endometrioid carcinoma (SEC)
- clinical features of, 59–60
 - differential diagnosis of, 61–62
 - gross findings in, 60
 - immunohistochemistry of
 - calretinin, 63–64
 - cytokeratins, 62–63
 - epithelial membrane antigen, 63
 - estrogen/progesterone receptors, 64
 - inhibin, 63
 - neural cell adhesion molecule, 64
 - other markers, 64–65
 - reaction patterns, 65
 - microscopic findings in, 60–61
 - overview of, 59
 - prognosis for, 66
- Sertoli-Leydig cell tumor (SLCT), 59, 65
- Serum tumoral markers, 23
- Sex cord stromal tumors, 59
- SGN-40, 362–363
- Single nucleotide polymorphism analysis, 35
- Single photon emission computer tomography (SPECT), 311

- SIR. *See* Standardized incidence ratios
- Skeletal targeted radiotherapy (STR)
 mechanism of action of, 374–375
 ongoing phase III trials, 377–378
 phase I/II dosimetry study, 375–377
 phase III pivotal trial, 377
- Skin cancer, nonmelanoma, EphA1 and, 336–338
- Skin ulcers, 337
- SLCT. *See* Sertoli-Leydig cell tumor
- SLN. *See* Sentinel lymph node scintigraphy
- Small cell ovarian cancer, 14
- Soft tissue perineurioma, 387
- Soft tissue sarcomas, 387
 metastasis of, 451
 treatment of, 451
- Solitary fibrous tumor, 401, 438
- Sorafenib, 133, 143
- SPECT. *See* Single photon emission computer tomography
- Spindle cell carcinoma, 394, 424
- Spindle cell lipoma, 394
- Spindle cell sarcoma, 424, 433
- Spitz nevus, 323
- Squamocolumnar junction (SCJ), 243–244
- Squamous cell cancer (SCC)
 cervical cancer types and, 275
 detection of, 243
 EphA and, 336–338
 FDG-PET appearance of, 276
 SCJ and, 244
- Squamous cell carcinoma antigen, 282
- Squamous metaplasia, MUC expression in, 239
- SS. *See* Synovial sarcoma
- SSX fusion, 441, 442
- Standardized incidence ratios (SIR), 181–182
- Stem cells. *See also* Cancer stem cell hypothesis;
 Embryonic stem cells
 advances in understanding of, 211
 autologous transplantation of, 353, 364, 370, 375
 harvesting, 353
- STR. *See* Skeletal targeted radiotherapy
- Streptavidin, 417
- Strontium-89, 374
- Sunitinib, 133, 143
- Suppression subtractive hybridization, 35
- Surface epithelial tumors, 35
- Surveillance, Epidemiology, and End Results (SEER), 14
- Synovial sarcoma (SS), 388
 biphasic, 434
 diagnosis of, 393
 approach to, 434
 cytogenetics/molecular genetics, 438–441
 histology for, 434–436
 ultrastructure in, 436
 ET-743 and, 457
 grading of, 442–444
 histogenesis of, 433
 immunohistochemistry of, 393–394, 398–401, 436–438, 446, 447
 incidence of, 433
 monophasic epithelioid, 436
 monophasic fibrous, 435–436
 poorly differentiated, 436
 prognosis for, 393, 442–444
 radiographic appearance of, 433
 round cell sarcomas v., 439
 survival rates for, 433
 treatment of, 433
- Synovioma. *See* Synovial sarcoma
- SYT, 438
- SYT gene, 440
- SYT-SSX fusion gene, 441–444
- t(X;18), 393, 439, 440
- Tamoxifen, 366
- TAMXV. *See* Time-averaged maximum velocity
- Targeted radioimmunotherapy (RIT), 367–368
- Targeted radiotherapy, 373–374
- Taxanes, 424
- TBI. *See* Total body irradiation
- T-cell prolymphocytic leukemia (T-PLL), 343, 354
- TCF, 443
- TCF2, 8
- Telomeric repeat amplification protocol assay (TRAP), 200
- Temsirolimus, 143
- Teratoma, 26, 168, 174. *See also* Growing teratoma syndrome
- Testicular cancer. *See also* Post-chemotherapy retroperitoneal lymph node dissection
 classification of, 167
 germ-cell
 incidence of, 182
 mortality rates for, 182
 second primary cancers after, 181–185
 survival rates for, 181
 incidence of, 167

- metastasis of, 167–168, 177
- second primary cancers after
 - in all testicular cancers, 185–186, 187
 - by cancer type, 186
 - by treatment type, 186, 188–189
- treatment for, 185
- Tetracycline COL-3, 425
- Thalidomide, 361, 370–371, 425
- 3D-PD. *See* Three-dimensional Power-Doppler ultrasound
- Three-dimensional Power-Doppler ultrasound (3D-PD), 30–31
- Thrombocytopenia, 354, 455
- Thrombotic microangiopathy (TMA), 376
- Thymidine, 319
- Thymostimulin, 132
- Time-averaged maximum velocity (TAMXV), 28
- Tissue microarrays (TMA), 53
- TLE1, 394, 438
 - detecting expression of
 - method for, 396–398
 - results of, 398–401
 - as immunohistochemical marker, 398–401
- TMA. *See* Thrombotic microangiopathy; Tissue microarrays
- TNF- α . *See* Tumor necrosis factor-alpha
- TNF-related apoptosis-inducing ligand (TRAIL), 362
- Total body irradiation (TBI), 373
- TP53, 7–8
- T-PLL. *See* T-cell prolymphocytic leukemia
- Trabectedin, 451
- TRAIL. *See* TNF-related apoptosis-inducing ligand
- Transcription profiles, of clear cell ovarian cancer, 8–9
- Transitional (Brenner) ovarian cancer, 14
- TRAP. *See* Telomeric repeat amplification protocol assay
- Trastuzumab, 367
- Trichostatin A (TSA), 452
- TRM-1, 362
- Tryptophan
 - catabolism of, 286, 291
 - immune function and, 286
 - metabolism of, 285
- TSA. *See* Trichostatin A
- TSE. *See* Turbo spin echo
- Tubal metaplasia, 239
- Tumor cells
 - apoptotic machinery and, 138
 - natural killer cells and, 82
- Tumor differentiation, 27–29
 - with 2D-PD, 27–29
 - adnexal masses, 23, 27–279
 - benign tumors, 23
 - with B-mode ultrasound, 28–29
 - malignant tumors, 23
- Tumor markers, 15
 - immunolocalization/immunoreactivity of, 327, 329
 - metastasis detection and, 314
 - urine-based, 200–201
- Tumor necrosis factor-alpha (TNF- α), 286
- Tumor suppressor genes, 302, 443
 - clear cell ovarian cancer and, 7
- Tumor vascularization
 - chemotherapy effectiveness and, 94
 - microvessel density assessment for, 148–149
 - ultrasound assessment of, 24, 26, 31
- Turbo spin echo (TSE), 245
- 2D-PD. *See* 2D Power-Doppler ultrasound
- 2D Power-Doppler ultrasound (2D-PD), 24, 25
 - 3D-PD v., 30–31
 - tumor differentiation with, 27–29
 - tumor vascularization assessment with, 26
- Type-2 histamine antagonists, 118
- Tyrosine, 319
- Tyrosine kinase inhibitors, 133, 425
- UEA-1, 419, 424, 425
- Ultrasmall particles of iron oxide (USPIO), 251–252, 279
- Ultrasonography, 15
 - adnexal mass characterization with, 25–31
 - ascite detection with, 93
 - B-mode, 23, 25–27
 - color Doppler, 24
 - contrast-enhanced, 30
 - forms of, 23–24
 - metastasis detection with, 314
 - pulsed Doppler, 24, 26
 - three-dimensional Power-Doppler, 30–31
 - tumor differentiation with, 23–24, 27–29
 - tumor vascularization assessment with, 24, 26, 31
 - two-dimensional Power-Doppler, 24–31
- Undifferentiated carcinomas, 14

- Urothelial bladder cancer
 - natural history of, 197
 - prevalence of, 197
 - screening for
 - biases/pitfalls in, 206–207
 - BTA test, 203
 - cost-effectiveness of, 205–206
 - future trends in, 207
 - hematuria screening, 199–200
 - ImmunoCyt/uCyt, 204–205
 - marker evaluation methods, 201–202
 - nuclear matrix protein-22 test, 203–204, 205
 - occupational exposure and, 198
 - previous programs for, 198
 - urine-based tumor markers, 200–201, 203–205
 - Urovysion, 204
- Urovysion, 204
- USPIO. *See* Ultrasmall particles of iron oxide
- Uterine cervical carcinoma
 - gross appearance of, 244
 - MRI of
 - CT v., 250–252
 - findings of, 246–247
 - staging of, 247–250
 - technique for, 245–246
 - parametrial involvement of, 245, 250
 - patterns of spread of, 244
 - prevalence of, 243, 257
 - prognosis of, 244–245
 - regional lymph node evaluation in, 251–252
 - staging of, 243
 - CT v. MRI in, 250–252
 - MRI for, 247–250
 - treatment of, 257
 - types of, 244
- Uterine cervix
 - MRI of, 244
 - normal anatomy of, 243–244
 - Validity
 - of QoL instruments, 265–266
 - types of, 265–266
- Vascular endothelial growth factor (VEGF), 422, 426
- VEGF. *See* Vascular endothelial growth factor
- VEGF-C, 419
- VEGFR-3, 419
- Velcade, 371
- Vinca alkaloids, 317
- Viral cyclin, 426
- von Recklinghausen's disease, 439
- Western immunoblotting, 41
- WHO. *See* World Health Organization
- Wilms tumor
 - angiogenesis and, 150–152
 - incidence of, 147
 - prognostic factors for, 147–148
 - staging of, 147–148
- World Health Organization (WHO), 263, 287
- Xcellerated T Cells, 370
- Yondelis, 451
- Yttrium-90, 367–368