

RONALD M. BUKOWSKI

ROBERT A. FIGLIN

ROBERT J. MOTZER

EDITORS

Renal Cell Carcinoma

Molecular Targets
and Clinical Applications

SECOND EDITION

 Humana Press

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Ronald M. Bukowski • Robert A. Figlin
Robert J. Motzer
Editors

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Clinical Applications

Second Edition

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Editors

Ronald M. Bukowski
Cleveland Clinic Foundation
Cleveland Clinic Taussig Cancer Center
and
CCF Lerner College of Medicine of CWRU
Cleveland, OH

Robert J. Motzer
Memorial-Sloan Kettering Cancer Center
New York, NY

Robert A. Figlin
Division of Medical Oncology
& Experimental Therapeutics
City of Hope National Medical
Center/Beckman Research Institute
Duarte, CA

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Preface

Ronald M. Bukowski, Robert J. Motzer, and Robert A. Figlin

Renal cancer comprises 3% of all malignant tumors, with an estimated incidence of 39,000 new cases with 13,000 deaths in 2006 (1). A study comparing 43,685 cases of renal cancer from 1973–1985 with those diagnosed in 1986–1998 (SEER database) demonstrated a marginal increase in the proportion of localized cancers and a decrease in advanced cases in the latter group. During the next 10-year period, however, the increase in localized and smaller tumors appears real, but overall survival (OS) differences are not yet apparent (2). While increased imaging and laboratory testing may generally explain the increased incidence, other environmental factors may also play a role (2).

Historically, patients presented with the classic triad of symptoms including flank pain, hematuria, and a palpable abdominal mass; but recently, increasing numbers of individuals are being diagnosed when asymptomatic with an incidentally discovered renal mass. Advances in imaging and techniques have increased the percent of patients who are eligible for surgical intervention, but a significant percent of patients still present with surgically unresectable disease (3) or will subsequently develop metastatic disease.

Histology

The importance of histology in predicting the biologic characteristics and clinical behavior of renal cancers was recognized in the last decade. Renal cell carcinoma (RCC) represents a group of histologic subtypes with unique morphologic and genetic characteristics (4).

Clear-cell renal carcinoma is the most common type of renal cancer, accounting for ~70–85% of renal epithelial malignancies, and arises from the proximal convoluted tubule. Papillary renal cancer is the second most common type comprising 10–15% of renal tumors. Understanding histologic subtypes and associated gene alterations has provided the opportunity to develop targeted therapy, and has ultimately lead to the development of a new treatment paradigm.

von Hippel–Lindau (VHL) Syndrome

The von Hippel–Lindau (VHL) syndrome provided a unique opportunity to study the development of clear-cell tumors and delineate the genetic characteristics of this tumor. In sporadic renal cancer, both the maternal and paternal VHL alleles are inactivated by acquired mutations, whereas in the VHL syndrome the first mutation is inherited. Loss of VHL function may occur in ~60–80% cases of sporadic clear-cell renal carcinomas (5).

The VHL protein is the product of the VHL gene, functions as a tumor-suppressor gene, and is responsible for ubiquitination of hypoxia-inducible factor- α (HIF- α) and its subsequent degradation by the proteasome (5). Under hypoxic conditions or in the presence of abnormal VHL function, HIF- α accumulates and activates the transcription of a variety of hypoxia-inducible genes. These include vascular endothelial growth factor (VEGF), platelet-derived growth factor- β (PDGF- β), transforming growth factor- α (TGF- α), and erythropoietin (EPO). The VHL gene may control this process by suppressing angiogenesis, but loss of the VHL gene or its function allow increased secretion of factors such as VEGF and produces the vascular phenotype characteristic of clear-cell carcinoma. Blocking components of the VEGF pathway and/or the function of HIF- α is currently the major therapeutic strategy for treatment of this malignancy, replacing immunotherapy with cytokines.

Systemic Therapy: Metastatic Disease

Immunotherapy consisting of interleukin-2 (IL-2) and/or interferon alpha (IFN α) had been the standard approaches for treatment of metastatic RCC, in addition to clinical trials investigating new agents. Responses were best with high-dose intravenous IL-2 (21%) compared to low-dose intravenous IL-2 (11%) and subcutaneous IL-2 (10%), although no survival advantage was observed (6). Similar response rates were reported comparing high-dose IL-2 (23.2%) versus subcutaneous IL-2 plus IFN α (9.9%) and again, no improvement in time to progression (TTP) or survival (7) were seen.

IFN α has been established as the standard comparative treatment arm for Phase III clinical trials of new agents for the treatment of metastatic renal cancer. Several randomized trials have demonstrated improvement in median survival for treated patients (8), and in a retrospective review a median OS of 13.1 months and a median TTP of 4.7 months for IFN α patients were reported (9).

A major advance in the field during the past 10 years has been the recognition that a variety of clinical characteristics can be used to categorize patients into groups with differences in prognosis. For previously untreated patients a prognostic model was developed by investigators at Memorial Sloan Kettering Cancer Center (9) and then validated and expanded. Five clinical characteristics were identified (9)

and later validated at the Cleveland Clinic (10). These prognostic criteria have been utilized in Phase III clinical trials of the targeted agents, such as sorafenib, sunitinib, temsirolimus (CCI-779), and bevacizumab.

The cloning of the VHL tumor-suppressor gene and the elucidation of its role in up-regulating growth factors associated with angiogenesis have provided insights into RCC biology, as well as defining a series of potential targets for novel therapeutic approaches. The highly vascularized nature of this neoplasm has ultimately been utilized to control its growth and survival. VEGF and its receptors (VEGFR) are overexpressed in RCC compared to normal renal tissue, and VEGFR-2 is believed to be the major receptor mediating the angiogenic effects of VEGF (11). The binding of VEGF to the extracellular domain of the VEGFR induces tyrosine autophosphorylation and subsequent increases in tumor-associated angiogenesis, endothelial cell proliferation, migration, and enhanced survival. During the past 5 years a number of agents inhibiting the VEGF pathway have been investigated in advanced RCC patients, and a series of these have produced significant clinical benefit including increases in progression-free and OS.

This group of novel agents has formed the central part of the new treatment paradigm for this tumor. The purpose of the current textbook is to provide an overview of these developments, as well as provide insights into the other targeted approaches that may ultimately play a role in the treatment of patients with this tumor. Chapters include a discussion of the biologic rationale for each target, as well as potential clinical approaches to provide inhibition of the pathway. The clinical data supporting the current approaches utilizing agents, such as sunitinib, sorafenib, temsirolimus, and bevacizumab, are outlined. In addition, novel targets including tumor necrosis factor, EGFR, Smac/DIABLO, and Eph2A are discussed in detail. The approval of three new agents for treatment of advanced RCC in 2007, and the likelihood that two additional drugs will receive regulatory approval in 2008–2009, make RCC a disease where not only significant clinical progress has occurred, but also an area that will be exploited to increase our understanding of how angiogenesis inhibitors function biologically and clinically.

The treatment paradigm for patients with localized and advanced RCC has changed dramatically in the last 5–10 years. Surgical advances are now mirrored by the dramatic changes in therapy available for metastatic disease. The collection of chapters in this text provides an update for urologists, medical oncologists, and researchers interested in the biology and therapy of this tumor.

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Contributors

Michael B. Atkins

Department of Hematology and Oncology, Beth Israel Deaconess Medical Center, Boston, MA

Arie Beldegrun

Division of Urologic Oncology, Department of Urology, David Geffen School of Medicine at University of California, Los Angeles, CA

Vinay Bhaskar

PDL Biopharma, Inc., Redwood City, CA

Otto C. Boerman

Department of Nuclear Medicine, Radboud University Nijmegen Medical Center, Nijmegen, the Netherlands

Benjamin Bonavida

Department of Microbiology, Immunology, and Molecular Genetics, University of California at Los Angeles, Los Angeles, CA

Ernest C. Borden

Center for Hematologic and Oncologic Molecular Therapeutics, Cleveland Clinic Taussig Cancer Center; CCF Lerner College of Medicine of CWRU, Cleveland, OH

Donald P. Bottaro

Urologic Oncology Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD

Gennady Bratslavsky

Urologic Oncology Branch, National Cancer Institute, Bethesda, MD

Ronald M. Bukowski

Cleveland Clinic Foundation, Cleveland Clinic Taussig Cancer Center; CCF Lerner College of Medicine of CWRU, Cleveland, OH

John C. Cheville

Department of Laboratory Medicine and Pathology, Mayo Clinic College of Medicine, Rochester, MN

Daniel Cho

Department of Hematology and Oncology, Beth Israel Deaconess Medical Center, Boston, MA

Peter Cohen, MD, PhD

Department of Hematology and Oncology, Mayo Clinic, Scottsdale, AZ

Tanya Das

The Department of Animal Physiology, Bose Institute, Kolkata, India

Nadeem Dhanani

Urologic Oncology Branch, National Cancer Institute, Bethesda, MD

Haidong Dong

Department of Immunology, Mayo Clinic, Rochester, MN

Tim Eisen

Addenbrooke's Hospital, Cambridge, UK

Robert A. Figlin

Division of Medical Oncology and Experimental Therapeutics, City of Hope National Medical Center/Beckman Research Institute, Duarte, CA

Barbara A. Finck

Osprey Pharmaceuticals Ltd., Saint-Laurent, QC, Canada

James H. Finke

Department of Immunology, Lerner Research Institute; CCF Lerner College of Medicine of CWRU, Cleveland Clinic Foundation, Cleveland, OH

Melvin Fox

PDL Biopharma, Inc., Redwood City, CA

Xavier Frigola

Department of Immunology, Mayo Clinic, Rochester, MN

Ram Ganapathi

Cleveland Clinic Taussig Cancer Center; CCF Lerner College of Medicine of CWRU, Cleveland Clinic Foundation, Cleveland, OH

Jorge A. Garcia

Cleveland Clinic Taussig Cancer Center, Cleveland Clinic Foundation, CCF Lerner College of Medicine of CWRU, Cleveland, OH

Daniel J. George

Duke Clinical Research Institute, Durham, NC

Saby George

Experimental Therapeutics, Cleveland Clinic Taussig Cancer Center,
Cleveland Clinic Foundation, Cleveland, OH

Christopher J. Herrem

Department of Dermatology, University of Pittsburgh, Pittsburgh, PA

Thomas Hutson

Baylor-Sammons Cancer Center, Dallas, TX

Brant A. Inman

Department of Urology, Mayo Clinic, Rochester, MN

Eric Jonasch

MD Anderson Cancer Center, University of Texas, Houston, TX

William G. Kaelin Jr.

Dana Farber Cancer Institute, Boston, MA

A. Karim Kadar

Wake Forest University School of Medicine, Winston-Salem, NC

Mayumi Kawabe

Department of Dermatology, University of Pittsburgh, Pittsburgh, PA

Akihiro Kawauchi

Department of Urology, Kyoto Prefectural University of Medicine, Kyoto, Japan

Hyung L. Kim

Department of Urologic Oncology, Roswell Park Cancer Institute, Buffalo, NY

Eugene D. Kwon

Departments of Urology and Immunology, Mayo Clinic, Rochester, MN

John S. Lam

Department of Urology, David Geffen School of Medicine at University
of California, Los Angeles, CA

Jean-Baptiste Lattouf

Department of Surgery, Urology Hospital Center of the University of Montreal,
Montreal, QC, Canada

W. Marston Linehan

Urologic Oncology Branch, National Cancer Institute, Bethesda, MD

James W. Mier

Department of Hematology and Oncology, Beth Israel Deaconess Medical Center,
Boston, MA

Yoichi Mizutani

Department of Urology, Kyoto Prefectural University of Medicine, Kyoto, Japan

Tsuneharu Miki

Department of Urology, Kyoto Prefectural University of Medicine, Kyoto, Japan

Robert J. Motzer

Memorial-Sloan Kettering Cancer Center, New York, NY

Peter F. A. Mulders

Department of Urology, Radboud University Nijmegen Medical Center, Nijmegen, the Netherlands

Mark Nogueira

Department of Medicine, Roswell Park Cancer Institute, Buffalo, NY

Jeannette C. Oosterwijk-Wakka

Department of Urology, Radboud University Nijmegen Medical Center, Nijmegen, the Netherlands

Egbert Oosterwijk

Department of Urology, Radboud University Nijmegen Medical Center, Nijmegen, the Netherlands

Marina Parton

Addenbrooke's Hospital, Cambridge, UK

Benedetta Peruzzi

Tumor Institute of Tuscany, Department of Clinical and Preclinical Pharmacology, University of Florence, Florence, Italy

Vanitha Ramakrishnan

Genentech, Inc., San Francisco, CA

W. Kimryn Rathmell

Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, NC

Karen L. Reckamp

City of Hope National Medical Center, Duarte, CA

Brian I. Rini

Cleveland Clinic Taussig Cancer Center, Cleveland Clinic Foundation, Cleveland, OH

Gaurisankar Sa

The Department of Animal Physiology, Bose Institute, Kolkata, India

Phillip E. Shaheen

Cleveland Clinic Taussig Cancer Center, Cleveland, OH

Brian Shuch

Department of Urology, David Geffen School of Medicine at University of California, Los Angeles, CA

Walter J. Storkus

Department of Dermatology, University of Pittsburgh, Pittsburgh, PA

Robert M. Strieter

Department of Internal Medicine, University of Virginia School of Medicine, Charlottesville, VA

Charles Tannenbaum

Department of Immunology, Lerner Research Institute, Cleveland Clinic Foundation, Cleveland, OH

Snehal Thakkar MD

Taussig Cancer Center, The Cleveland Clinic Foundation, Cleveland OH 44195

Susan A. J. Vaziri

Cleveland Clinic Taussig Cancer Center, Cleveland Clinic Foundation, Cleveland, OH

Cathy Vocke

Urologic Oncology Branch, National Cancer Institute, Bethesda, MD

Cheryl Walker

MD Anderson Cancer Center, Science Park Research Division, University of Texas, Smithville, TX

Keith Wilson

PDL Biopharma, Inc., Redwood City, CA

Christopher Wood

MD Anderson Cancer Center, University of Texas, Houston, TX

James C. Yang

National Cancer Institute, National Institutes of Health, Bethesda, MD

Ming Zhou

Tissue Microarray Core, Departments of Anatomic Pathology and Cancer Biology, Glickman Urological Institute, Cleveland, OH

Targeted Therapy for Metastatic Renal Cell Carcinoma: Overview

Ronald M. Bukowski, Robert A. Figlin, and Robert J. Motzer

Abstract The treatment of advanced renal cell carcinoma has evolved over the last decade, and currently a new treatment paradigm utilizing a variety of targeted approaches is in place. Novel agents including sunitinib, sorafenib, temsirolimus, and bevacizumab are utilized for patients with advanced and metastatic clear cell carcinoma. Inhibition of a variety of targets including kinase receptors and their ligands such as vascular endothelial cell growth factor (VEGF), or the intracellular kinase mTOR (mammalian target of rapamycin) are in part responsible for the effects of these agents. The clinical trials responsible for these advances as well as the evolving treatment paradigm are reviewed in this introduction.

Keywords Renal cell carcinoma • VHL gene • VHL protein • VEGF • PDGF • RCC therapies

The purpose of the current textbook is to provide an overview of targeted therapeutics for renal cell carcinoma and review the validated and potential molecular targets in this tumor. The recent shift in the treatment paradigm and availability of multiple targeted agents with significant clinical activity in patients with advanced clear cell carcinoma prompted this undertaking. The novel agents that have altered the treatment landscape for advanced renal cell carcinoma are summarized in [Table 1](#). The majority of these directly or indirectly inhibit the vascular endothelial cell growth factor (VEGF) pathway. Clear cell carcinoma of the kidney appears to be a tumor uniquely sensitive to strategies that target and inhibit tumor-associated angiogenesis.

1 Background

Renal cancer accounts for 3% of all malignant tumors and is the sixth leading cause of death in the United States. There were an estimated 51,000 new cases and 13,000 deaths in 2006 (1). The age at diagnosis ranges from 40 to 70 years,

R.M. Bukowski (✉)

Cleveland Clinic Taussig Cancer Center, 9500 Euclid Ave, Cleveland, OH 44195
e-mail: Bukow464@sbcglobal.net

Table 1 Targeted agents: metastatic RCC

Multikinase inhibitors (VEGFR, PDGFR):
Sunitinib
Sorafenib
Antibodies: (VEGF inhibitor)
Bevacizumab
mTOR inhibitor:
Temozolomide

with a male:female predominance of 1.6:1.0 (1). Renal cell carcinomas generally arise from the renal epithelium and account for ~85% of all renal malignancies (2). One third of patients present with locally advanced or metastatic disease (3), and ~20–40% of those who undergo surgical resection of the primary tumor will develop metastatic disease.

Histologically, these tumors represent a group of subtypes with unique morphologic and genetic characteristics. Clear-cell renal carcinoma is the most common type, accounts for ~70% of renal epithelial malignancies, and arises from the proximal convoluted tubule. These tumor cells are characterized by clear cytoplasm with occasional eosinophilic cytoplasm and ~60% of sporadic clear-cell renal tumors are associated with defects in the VHL gene (4, 5). This contrasts with renal tumors in patients with the VHL syndrome in which the first mutation is inherited. Papillary renal cancer is the second most common type, comprising 10–15% of renal tumors. Understanding histologic subtypes and associated gene alterations has provided the opportunity to develop specific therapeutic agents (Fig. 1).

2 VHL Gene: Role in Renal Cell Carcinoma

Clear cell carcinoma is a very vascular neoplasm, and recent studies have uncovered the molecular basis of this phenotype. This is discussed fully in chapter “Molecular Genetics of Inherited Renal Cell Carcinoma: Identification of Targets in the Hereditary Syndromes” by Linehan, Dhanani, Vocke, and Bratslavsky and in chapter “VHL and HIF in Clear Cell Carcinoma: Molecular Abnormalities and Potential Clinical Applications” by William Kaelin and Daniel J. George. In brief, in sporadic renal cancer, both the maternal and paternal von Hippel–Lindau (VHL) alleles are inactivated by acquired mutations. The VHL protein, the product of the VHL gene, functions as a tumor suppressor gene and is responsible for ubiquitination and proteasome degradation (2, 5) of hypoxia-inducible factor (HIF)¹. HIF- α is a key regulator of hypoxic response and is the primary target of the VHL protein. In conditions of hypoxia or abnormal VHL function, the VHL protein does not bind to HIF- α , resulting in its accumulation. This activates the transcription of hypoxia-inducible genes, including VEGF, platelet-derived growth factor (PDGF), transforming growth factor- α (TGF- α),

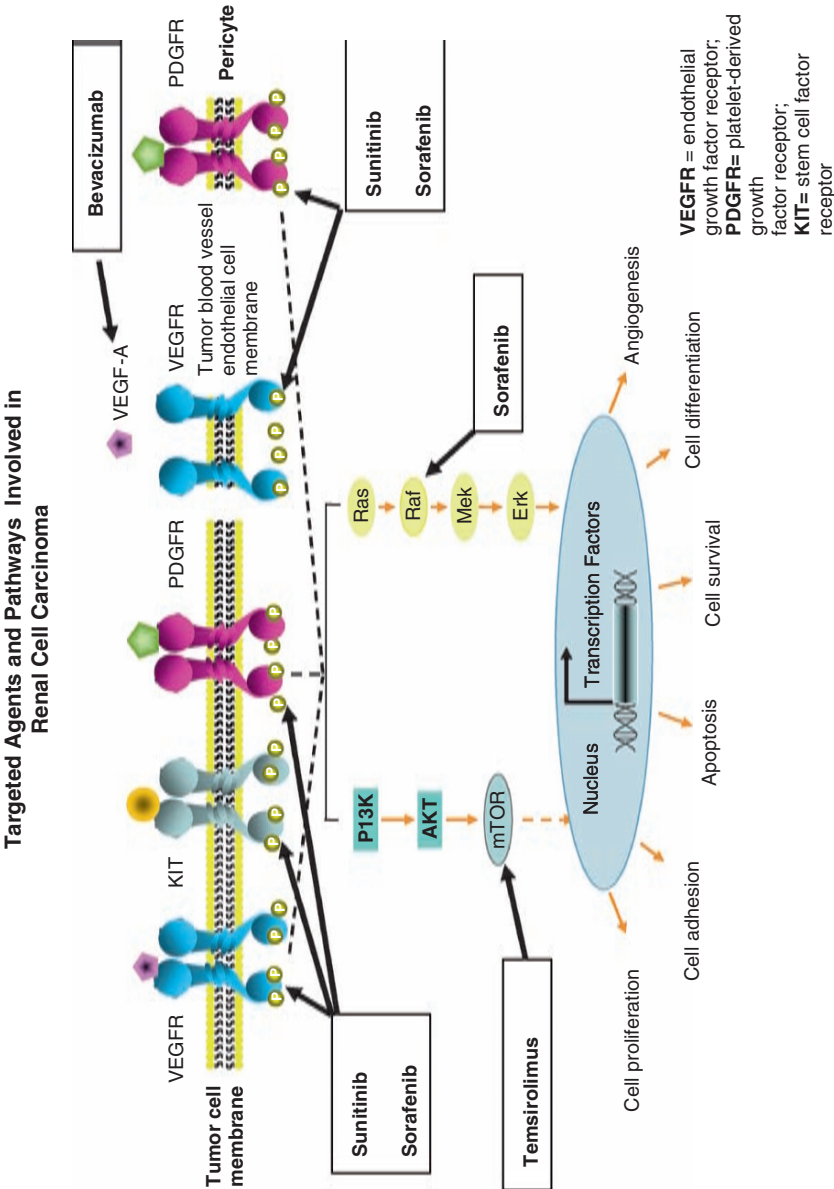


Fig. 1 Targeted agents and pathways involved in renal cell carcinoma

and erythropoietin (EPO). Renal cancers are vascular tumors, and recruit blood vessels. The VHL gene controls this process by suppressing angiogenesis, but loss of the VHL gene or its function allows increased secretion of VEGF, PDGF, TGF- α , and EPO.

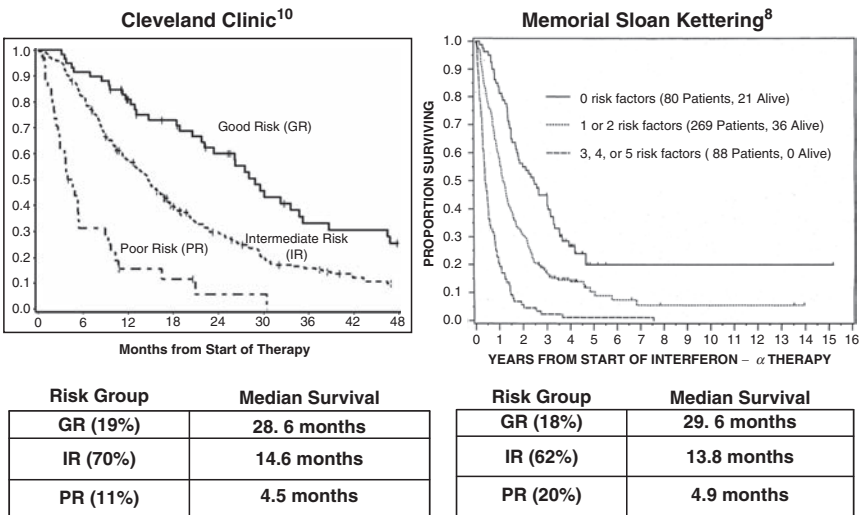
3 Management of Advanced Renal Cell Carcinoma: Historical

The management of patients with metastatic renal cell carcinoma (RCC) has recently changed, and a new treatment paradigm is in place. Blockade of the VEGF pathway and the function of HIF are now utilized as primary therapeutic strategies. In the past, immunotherapy with either interleukin (IL)-2 and/or IFN- α has been the standard approach for systemic treatment. A previous clinical trial has shown that responses were best with high-dose intravenous IL-2 (21%) rather than with low-dose intravenous IL-2 (11%) and subcutaneous IL-2 (10%), although no progression free survival (PFS) or overall survival (OS) advantages were observed (6). Similar response rates were reported comparing high-dose IL-2 (23.2%) with subcutaneous IL-2 plus IFN- α (9.9%) and again, no PFS or survival advantage was found (7). IFN- α monotherapy has been the standard of care for advanced RCC. A median OS of 13.1 months and median TTP of 4.7 months for patients receiving IFN have been noted in retrospective reviews (8). In view of these data and the modest survival advantage for IFN found in recent randomized trials, this cytokine was chosen as the comparator arm in a series of recent Phase III trials of new agents in patients with metastatic disease.

4 Prognostic Factors in Renal Cell Carcinoma

Retrospective analysis of untreated and previously treated patients with metastatic RCC has identified clinical characteristics that can be used to categorize patients into groups with differences in prognosis. For previously untreated patients, an initial prognostic model was developed at Memorial Sloan Kettering Cancer Center, and later validated and expanded by the Cleveland Clinic investigators to include five clinical characteristics (Fig. 2), plus two additional independent prognostic factors (8, 9, 10). These criteria have been utilized in recent Phase III clinical trials of sorafenib, sunitinib, bevacizumab, and temsirolimus. In the future, this scheme will likely be modified so it reflects risk factors for patients receiving targeted therapy, and more than likely include one or more molecular markers.

Prognostic Factor Models : Survival
Advanced Renal Cell Carcinoma



Risk Factors : PS < 80%, LDH $\geq 1.5\times$ ULN, $\uparrow$ corrected Ca⁺⁺, Hgb < LLN, DFI < 1 yr (replaced nephrectomy),

Fig. 2 Prognostic factor models: survival advanced renal cell carcinoma

5 Bevacizumab

Bevacizumab (Avastin, Genentech) is a fully humanized monoclonal antibody that binds all the isoforms of VEGF. The antitumor activity of bevacizumab monotherapy in patients with advanced clear-cell carcinoma was demonstrated in a sequence of Phase II randomized trials (11, 12). In the first of these (11), 116 patients with cytokine refractory disease received either low dose (3 mg/kg) or high dose (10 mg/kg) bevacizumab or a placebo every 2 weeks. Objective responses were confined to the high-dose arm (10% partial response) and were accompanied by a significantly longer TTP as compared to patients receiving a placebo (4.8 vs. 2.5 months, $p < 0.001$). This trial suggested that an agent inhibiting VEGF could change the natural history and biologic behavior of RCC. A survival advantage was not found, possibly related to the small size, and/or the crossover design.

A second randomized trial in untreated patients with advanced clear cell carcinoma compared the combinations of bevacizumab with a placebo or with erlotinib (Tarceva, Genentech, OSI; EGFR tyrosine kinase inhibitor (12). One hundred and four previously untreated patients were enrolled, the overall response rates in both arms were similar, and no differences in PFS were identified. The median PFS for all patients was 8.6 months suggesting an effect of bevacizumab monotherapy. Thus, this agent appeared to have definite therapeutic effects in advanced disease,

but additional evidence was required. To confirm this effect, a series of Phase III trials utilizing bevacizumab in combination with IFN- α have been conducted (13, 14). The preliminary results of the first study conducted by Roche Laboratories (Basel, Switzerland) in Europe have been reported (13). The study was a randomized, blinded placebo-controlled trial comparing bevacizumab plus IFN- α 2a with placebo plus IFN- α 2a in 643 patients. The findings have demonstrated the superiority of the bevacizumab containing arm. The overall response rate was 31% for the combination compared to 13% with IFN- α alone ($p < 0.0001$). The median PFS for the combination was 10.2 months, compared to 5.4 months for the IFN- α alone group (HR = 0.63, $p < 0.0001$). The various prognostic groups were also examined, and improvement of PFS was noted in both the favorable and intermediate subgroups, but not in the poor risk group. The toxicity of bevacizumab and IFN- α appeared to be slightly increased compared to IFN- α 2a monotherapy, but no enhancement of previously noted bevacizumab adverse events such as hypertension, vascular events, or proteinuria were reported. Survival data remain preliminary. A second Phase III trial utilizing bevacizumab and IFN- α was conducted by the Cancer and Leukemia Group B (CALGB). This was an open label randomized trial in which patients received IFN- α with/without bevacizumab (14). The study has accrued over 700 patients with clear cell carcinoma, and a recent letter to participating investigators noted the results were similar to those reported in the Avoren Trial. This study appears to provide independent confirmation of the clinical benefit produced by treatment with bevacizumab and IFN- α .

The benefit of adding bevacizumab to IFN- α appears significant; however, a monotherapy arm utilizing bevacizumab alone was not included in either Phase III trial, and therefore, it remains unclear whether IFN- α is truly necessary. Preclinical studies with this combination to further delineate the mechanisms responsible for the therapeutic effects are needed.

6 Sorafenib

Sorafenib (Nexavar, Bayer) is an orally bioavailable inhibitor of Raf-1, a member of the RAF/MEK/ERK signaling pathway, as well as multiple growth factor receptors including VEGFR-1, VEGFR-2, VEGFR-3, PDGFR, Flt-3, and c-KIT (15). In preclinical and animal models, this multitargeted TKI blocked the RAF/MEK/ERK signaling pathway and inhibited tumor angiogenesis. A randomized Phase III placebo-controlled study (Treatment Approaches in Renal Cell Cancer Global Evaluation Trial) (16, 17) was conducted. A final analysis of survival utilizing a cut-off date of 9/06 was recently reported (18). After 561 deaths, the median OS for the placebo and sorafenib groups were not significantly different (15.9 vs. 17.8 months, HR = 0.88, $p = 0.146$). In the placebo group, 216/452 patients had crossed over to sorafenib therapy after the study was amended, thereby potentially diluting the effect of sorafenib, and potentially decreasing the differences between the two arms. In order to investigate this possibility, a secondary preplanned analy-

sis of survival was conducted. Patients crossing over to sorafenib were censored at time of progression. The median survival for this group is 14.3 months compared to 17.8 months for the sorafenib cohort ($HR = 0.78$, $p = 0.0287$). This difference was statistically significant (O'Brien-Fleming threshold $\alpha = 0.037$). This analysis suggests that sorafenib therapy is associated with an improvement in OS.

The TARGETs trial was conducted in cytokine refractory patients, in order to investigate the effects of sorafenib in the treatment-naïve setting, a Phase II randomized trial comparing monotherapy with sorafenib or IFN- α was conducted (18). One hundred and eighty-nine patients with advanced clear cell carcinoma were randomized, and a recent analysis demonstrated no differences in PFS for the sorafenib patients or IFN- α -treated individuals (5.7 vs. 5.6 months, respectively, $HR = 0.88$, $p = 0.504$). This finding may be related to the type of study conducted (Phase II), the small sample size, or the lack of effects in this patient population.

7 Sunitinib

Sunitinib (Sutent, Pfizer) is a multitargeted oral TKI of VEGFR-2, PDGFR with less potent activity against fibroblast growth factor receptor-1 tyrosine kinase activity. In preclinical studies, this agent demonstrated direct antitumor activity in cells dependent on signaling through PDGFR, KIT, and FLT3 for proliferation and survival, in addition to antiangiogenic effects (19). A sequence of Phase II clinical trials have demonstrated the activity of this agent in patients with cytokine-refractory advanced and metastatic RCC (20, 21). Both trials accrued 168 patients, and the overall response rate (ORR) was 40% (investigator assessment) and 25.5% (independent review). The majority of responses were partial. Stable disease at 3 months or more was seen in 27 and 23% of patients, in these trials. Median time to progression was 8.7 months (95% confidence interval [CI]: 5.5–10.7) and 8.1 months (95% CI: 7.6–10.4) in trials 1 and 2, respectively, and the median survival in trial 1 was 16.4 months. On the basis of these results, and the ability of this agent to induce objective and meaningful responses, the FDA approved sunitinib for the treatment of advanced RCC in January 2006.

A large randomized trial in untreated patients with metastatic clear-cell carcinoma was then conducted comparing sunitinib to IFN- α 2a (22).

A total of 750 patients were randomized to receive either sunitinib or IFN- α in 6-week cycles. Patients were required to have clear-cell histology, and were stratified based upon lactate dehydrogenase, ECOG PS, and the absence of prior nephrectomy. The primary endpoint of the trial was PFS. Secondary endpoints were ORR, OS, safety, and patient-related outcomes. Patient distribution was as follows: 35% had good-risk disease, 58% had intermediate-risk disease, and 7% had poor-risk disease. Sunitinib therapy significantly improved PFS (median 11 months vs. 5 months, $p < 0.000001$) and ORR (39% vs. 8%, $p < 0.000001$). There was only one complete response (CR), which occurred in the sunitinib arm. Median OS had not been reached at the time of analysis, but there was a trend

toward improved survival in the sunitinib group. The marked improvement in PFS and ORR has provided strong evidence for the use of sunitinib as first-line therapy in metastatic RCC.

The Phase III trial comparing sunitinib to IFN- α as first line therapy for metastatic RCC was also analyzed for prognostic factors. The median PFS for patients with good-risk disease was 14 months, for those with intermediate risk 9 months, and for patients with poor risk 4 months (23). Although most responses to sunitinib are in the form of PR or SD, some complete responses (CRs) are also seen. In a retrospective review of metastatic RCC patients who received sunitinib therapy, 2/74 patients (2.7%) achieved a RECIST-defined CR lasting >15 months (24). These two patients were treated in the first line setting, had nonbulky pulmonary metastases and favorable or intermediate MSKCC risk status.

8 mTOR Inhibitors: Temsirolimus

The mammalian target of rapamycin (mTOR), a large polypeptide kinase, is also a therapeutic target in RCC. mTOR is a downstream component in the phosphoinositide 3-kinase (PI 3-kinase)/Akt pathway, and acts by regulating translation, protein degradation, and protein signaling. VEGF-mediated endothelial cell proliferation requires the activity of PI 3-kinase (25). mTOR has also been identified as an upstream activator of HIF, stabilizing the molecule force, preventing degradation, and thereby increasing HIF activity (26).

In a randomized Phase II trial, 111 patients with advanced refractory RCC were treated with three dose levels (25.0, 75.0, 250 mg/week) of temsirolimus (27). Seven percent of patients achieved a response. The median TTP was 5.8 months, with median survival for the entire population 15.0 months. When the various prognostic groups were examined, those with poor-risk status appeared to have benefited the most leading to a Phase III trial in this patient cohort. Temsirolimus has also been combined with IFN- α in a Phase I/II clinical trial in 71 patients with advanced RCC (28). Partial responses were observed in 11% of all patients, with median TTP of 9.1 months.

On the basis of these initial studies in refractory patients, a Phase III trial with temsirolimus was designed. This study compared temsirolimus, IFN- α monotherapy, or the combination as first-line treatment in patients with unfavorable prognostic features (29). Patients treated with temsirolimus had significant improvement of their median survival (10.9 months temsirolimus vs. 7.3 months interferon) compared to IFN- α -treated individuals. In patients receiving the combination, survival improvement was not seen, perhaps related to the lower dose intensity for temsirolimus and increased toxicity of the combination. On the basis of these data, temsirolimus was approved by the FDA on May 30, 2007, for the treatment of patients with advanced renal cell carcinoma.

A total of 626 poor-risk patients (i.e., with ≥ 3 prognostic factors) were entered. The majority of patients (80%) had predominantly clear cell carcinoma, but patients with nonclear cell histologies were eligible.

An updated analysis of this Phase III trial (30) investigated the relationship between tumor histology, prognostic factors, and survival. Median OS and PFS were increased in patients treated with temsirolimus, regardless of tumor histology, and was most pronounced in the subset of patients with nonclear cell histology. Twenty-five to thirty percent of patients randomized to these two treatment arms had an intermediate prognosis as defined by Motzer et al. (8) When the intermediate- and poor-risk patients were examined separately, only the poor-risk subset receiving temsirolimus demonstrated improvement in median OS.

9 Summary

Sorafenib, sunitinib, and bevacizumab were first studied and shown to have activity in cytokine-refractory patients as second-line therapies. The FDA label for the two TKIs describes efficacy for the treatment of “advanced kidney cancer.” In treatment-naïve patients, a series of Phase II and III randomized trials with these three agents have compared their efficacy to first-line cytokine therapy. A randomized Phase III trial of sunitinib compared with IFN accrued 750 patients, and demonstrated improvement in response rates and PFS (primary end point). The Phase III trials comparing bevacizumab and IFN- α to IFN- α alone also suggest efficacy in the untreated RCC patient. The efficacy and safety of sorafenib in treatment-naïve patients were assessed in a randomized Phase II trial and do not demonstrate differences between therapy with either IFN- α and sorafenib. Finally, the Phase III trial comparing temsirolimus alone and with IFN to IFN alone in poor-risk patients demonstrated a survival advantage for patients receiving monotherapy.

In the absence of randomized studies directly comparing these agents in similar patient populations, treatment recommendations are problematic; however, based on the efficacy (ORR, PFS, and OS) observed in the various randomized trials, sunitinib is currently the accepted standard for therapy and can be offered to patients as initial therapy. An acceptable alternative would appear to be bevacizumab plus IFN- α . The reports of these trials are still preliminary, but two independent randomized trials appear to support this conclusion. Studies with temsirolimus were performed in poor-risk patients, and the subset analysis of intermediate-risk individuals appears negative. The efficacy of this agent in nonclear cell histologies is of interest. Sorafenib would appear to be indicated in the treatment-refractory patient based on the findings in the TARGETs trial. These recommendations are summarized in [Table 2](#).

Another question is the role of high-dose IL-2 as first-line treatment. Although less than 10% of patients treated with high-dose IL-2 achieve a durable complete response, the lack of survival benefit, the significant toxicity and morbidity, and the highly specific nature of patients treated, support its use as a secondary therapy at best.

The availability of the targeted agents has significantly changed the treatment paradigm for patients with advanced disease. Many unanswered questions

Table 2 Renal cell carcinoma treatment algorithm: 2007^a

Patient type	Setting	Therapy	Options
Treatment-naïve patient	MSK risk: Good or intermediate	Sunitinib Bevacizumab + IFN- α	HD IL-2
Treatment-refractory patient (≥ 2 nd line)	MSK risk: Poor	Temsirolimus	Sunitinib
	Cytokine refractory	Sorafenib	Sunitinib
	Refractory to VEGF/VEGFR or mTOR inhibitors	Investigational	?Sequential TKIs or VEGF inhibitor

^aAdapted from M. Atkins, ASCO 2006 and R. Bukowski, ASCO 2007

remain however. The mechanisms of response and development of resistance to the targeted agents need to be explored. The issues of stable disease and the value of percent maximal tumor regression in defining clinical benefit are also of interest, and may be relevant surrogate end points predicting clinical benefit.

The effects of sequential targeted therapy are of interest, and recent data suggest patients receiving previous therapy with various targeted agents may respond to a second TKI (31). Phase II trials of AG013736 in patients with progressive RCC following sorafenib, sunitinib in patients progressing after bevacizumab, and sorafenib in patients progressing after bevacizumab or sunitinib are in progress. These preliminary observations suggest sequential TKI therapy may be possible, and cross-resistance may not develop.

Another question is the optimal timing of discontinuing targeted therapy in patients with slowly progressive disease. Data in patients treated with imatinib for gastrointestinal stromal tumors show benefit for continued therapy despite radiographic progression. Alternatively, these patients may experience toxicity without benefit, and lose the opportunity to participate in clinical trials of other agents. The question of whether patients benefit from continued therapy in the setting of progressive disease remains unanswered.

Finally, to date, no adjuvant therapy has proven to be useful in preventing relapse following nephrectomy for completely resected, localized RCC. Several trials are now in progress to assess the role of targeted therapy in the adjuvant setting. One randomized Phase III adjuvant trial compares sorafenib (for either 1 or 3 years) to a placebo, and a second National Cancer Institute-sponsored Phase III trial compares sorafenib and sunitinib to a placebo. Data from both trials will not be available for between 8 and 10 years.

The chapters in this book will explore in detail the clinical and biologic features of renal cancer, the molecular targets identified in the various histologic subtypes, and the rationale for the use of the agents discussed. Finally, the clinical applications of these agents, as well as novel targeted strategies are reviewed. The advances in this field are significant both at the basic and clinical level, and clearly demonstrate that renal cancer now represents a model for application of targeted therapeutic approaches.

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Molecular Genetics in Inherited Renal Cell Carcinoma: Identification of Targets in the Hereditary Syndromes

Nadeem Dhanani, Cathy Vocke, Gennady Bratslavsky,
and W. Marston Linehan

Abstract Kidney cancer affects 51,000 in the United States each year and is responsible for nearly 13,000 deaths annually. Kidney cancer is not a single disease; it is made up of a number of different types of cancer that occur in the kidney. These distinct forms of kidney cancer each have a different histologic type, a different clinical course, respond differently to therapy, and are caused by different genes. The *VHL* gene is the gene for the inherited form of clear cell kidney cancer associated with von Hippel–Lindau as well as for the common form of sporadic, noninherited clear cell kidney cancer. The product of the *VHL* gene forms a complex with other proteins and this complex targets the hypoxia-inducible factors (HIF) for ubiquitin-mediated degradation. A number of novel agents which target the *VHL*-HIF pathway have recently been approved by the FDA for treatment of patients with advanced kidney cancer. The *MET* gene is the gene for the inherited form of papillary kidney cancer associated with hereditary papillary renal carcinoma (HPRC) and has been found mutated in a subset of tumors from patients with sporadic, type I papillary kidney cancer. Clinical trials are currently underway evaluating the role of agents which target the *MET* pathway in patients affected with HPRC as well as sporadic papillary kidney cancer. The *BHD* gene is the gene for the inherited form of chromophobe kidney cancer associated with Birt–Hogg–Dubé (BHD). Biochemical studies have revealed that the BHD pathway interacts with the *MTOR* pathway and agents which block this pathway are currently being evaluated in preclinical models as a potential approach for the treatment of BHD-associated as well as sporadic chromophobe kidney cancer. The Krebs cycle enzyme, fumarate hydratase, is the gene for the inherited form of type II papillary kidney cancer associated with hereditary leiomyomatosis renal cell carcinoma (HLRCC). *In vitro* and *in vivo* studies are currently underway evaluating novel approaches for targeting of this kidney cancer pathway. It is hoped that understanding the genes that cause cancer of the kidney will provide the foundation for the development of effective forms of therapy for patients with this malignancy.

W.M. Linehan (✉)

Urologic Oncology Branch, National Cancer Institute,
10 Center Drive MSC 1107, Bldg 10 CRC Room1-5940, Bethesda, MD 20892
e-mail: WML@nih.gov

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1 Introduction

An estimated 36,000 people will be diagnosed with kidney cancer this year, and close to 30% of these patients will die of their disease (1). On the basis of the data from the National Cancer Institute's SEER Cancer Statistics Review, 1 out of every 82 men and women will be diagnosed with cancer of the kidney over the course of their lifetime, and the incidence continues to rise. Men are affected almost twice as often as women, and the incidence among blacks is slightly higher than that of whites. With the increased use of axial abdominal imaging, kidney tumors are being diagnosed at earlier stages, often times incidentally while the patient is still asymptomatic. Nonetheless, at the time of presentation, 40% of these tumors will no longer be confined to the kidney, either through local extension or distant metastatic spread (2). While extirpative surgery is most often curative in tumors restricted to the kidney, treatment of metastatic disease has proven to be a formidable challenge with the traditional therapies currently available. As our understanding of the genetic basis of kidney cancer increases, exciting advances in molecular therapeutics offer novel approaches to treatment for these patients.

2 Identification of the *VHL* Gene

As recently as 30 years ago, very little was known about the contribution of genetic mutations to the development of renal tumors. Like cancer of the colon, breast, and prostate, kidney cancer was known to occur in both sporadic and familial forms. On the basis of the earlier work by Knudson and Strong (3, 4), the concept of tumor suppressor gene inactivation was recognized as an etiological factor in Wilms' tumor and retinoblastoma. In keeping with this hypothesis, when compared to the sporadic form, familial kidney cancers were more often multifocal, bilateral, and had earlier onset. Still, there was no gene identified that could be implicated in renal cancer.

In 1979, Cohen et al. (5) noted a chromosomal translocation between the short arm of chromosome 3 and the long arm of chromosome 8 in eight of ten affected members of a family known to have heritable kidney cancer. This was followed by several additional reports characterizing chromosomal abnormalities in different families affected with renal cell carcinoma (RCC). In each of these lineages, chromosome 3 was involved, particularly the 3p13-3p14 region. Important insight into the link between the hereditary and sporadic forms of RCC was provided through work by Zbar and colleagues (6) when they reported loss of alleles at loci

on the short arm of chromosome 3 in 11 of 11 evaluable patients with sporadic renal cancers. Shortly thereafter, it was postulated that the genetic mutation responsible for von Hippel–Lindau (VHL) was located in a region on chromosome 3p, distinct from the human homologue of RAF1, but apparently linked to it (7). All evidence was pointing to the existence of a tumor suppressor gene encoded on the short arm of chromosome 3, a mutation of which resulted in renal cell cancer. Unfortunately, gene localization was still not feasible because the region of interest was too large for the cloning techniques available at the time. Research efforts were thus shifted to the heritable form of renal cancer, with VHL being the model for investigation.

2.1 von Hippel–Lindau

VHL is transmitted in an autosomal-dominant pattern with an estimated incidence of 1 in 36,000 live births (8, 9). With a penetrance of over 95% by the age of 65 (10), affected individuals develop neoplastic tumors in multiple organ systems. Central nervous system lesions include retinal hemangioblastomas, endolymphatic sac tumors of the inner ear, and craniospinal hemangioblastomas in the cerebellum, brainstem, spinal cord, lumbosacral nerve roots, and supratentorial lesions. The pancreas may also be affected in these patients, developing cysts, cystadenomas, and neuroendocrine tumors. Benign epididymal papillary cystadenomas occur with increased frequency than the general population and can be bilateral. Rarely, women can have analogous lesions with papillary cystadenomas in the broad ligament. Tumors found in the kidney are solid renal cell cancers, simple cysts, and combinations thereof. In the setting of VHL, it has been estimated that a kidney may contain 600 microscopic tumors and over 1,000 cysts before the age of 40 years (11). Although simple cysts in these patients rarely transform to solid masses (12), complex cysts are known to contain malignant elements and may progress if left untreated. Adrenal lesions found in VHL are pheochromocytomas. Like the kidney tumors, these are frequently multiple and bilateral. Extra-adrenal paragangliomas are also known to occur in these patients, arising in periaortic tissues, the carotid body, and the glomus jugulare (9).

In searching for the gene responsible for VHL, researchers explored the applicability of Knudson's two-hit hypothesis to tumor behavior in VHL patients with kidney cancer. Tory et al. (13) evaluated tissue from patients with multiple kidney tumors, and for each patient compared chromosome 3 from one tumor to another. They found that each patient had loss of the same allele of chromosome 3p in all of their tumors. Further analysis of haplotypes revealed that the lost allele was always from the wild-type chromosome, the contribution of the nonaffected parent. This provided strong support for the notion that alteration of a tumor suppressor gene was the causative factor in VHL, and in accordance with Knudson's theory, an

individual with a germline mutation was at risk for VHL if they incurred a second hit at the same locus thus inactivating the wild-type allele.

Expanding upon earlier work by Seizinger et al. (14), Lerman's group (15) isolated and mapped 2,000 single copy DNA fragments of chromosome 3 from humans, thus generating vital tools which would be used for the future cloning of the *VHL* gene. With these reagents newly available, Hosoe and colleagues (16) performed further multipoint linkage analysis to localize the *VHL* gene to an interval between *RAF1* and a polymorphic DNA marker, D3S18. Finally, in 1993, researchers at the National Cancer Institute reported identification of the *VHL* gene through cloning studies and described its role in RCC (17). This small gene, with 854 coding nucleotides on three exons, was found to be located on the short arm of chromosome 3 and responsible for encoding the VHL protein. The gene is evolutionarily conserved and its product shares homology with only a small region of a surface membrane protein of *Trypanosoma brucei*.

Once the causative gene for VHL had been identified, clinicians were eager to find screening methods to identify patients with genetic mutations. Early laboratory studies generated germline mutation detection rates of 39–75% (18, 19). Mutation analyses showed that the type (e.g., insertion, deletion, missense, or nonsense) and location (e.g., codon position) of mutation correlated well with phenotype, thus allowing health care providers to predict the extent of involvement of the various organ systems for any given VHL family. In a study of 469 VHL families from North America, Europe, and Japan, researchers compared the effects of identical *VHL* germline mutations on different families. On the basis of their findings, VHL was broken down into three distinct phenotypes: pheochromocytoma along with RCC, pheochromocytoma alone, and RCC alone (20). Later studies correlated the relationship between length and location of germline mutations and the incidence of RCCs in VHL patients. A retrospective review of 123 patients from 55 families revealed that individuals harboring a partial deletion suffered a significantly higher rate of RCC when compared to those with complete gene deletions. Moreover, deletion mapping demonstrated the presence of a 30-kb gene on the short arm of chromosome 3, directly adjacent to the *VHL* gene which, when preserved, may promote the development of RCC (21). Further advances were made when Stolle's group (22) developed a new technique which improved germline mutation detection, accurately identifying a mutation in 93 out of 93 (100%) VHL families tested. The method involved a combination of tests that each demonstrated high sensitivity for the various types of mutations implicated in VHL. Qualitative Southern blotting to detect gene rearrangements and quantitative Southern blotting for the detection of entire gene deletions were the newly added components responsible for the dramatic increase in sensitivity. In addition, fluorescence in situ hybridization (FISH) and full gene sequencing completed the battery of tests. The 100% sensitivity of the new technique lent support to the notion that VHL is genetically homogeneous, and clinically allowed providers to counsel patients with reasonable certainty that a family member found to lack the gene mutation with the new test combination was unlikely to have VHL.

2.1.1 Sporadic RCC

Discovery of the *VHL* gene in the setting of familial RCC allowed scientists to then investigate its role in sporadic tumors. Gnarr et al. (23) used PCR amplification of the three exons of the *VHL* genes of 108 patients with sporadic RCC in order to analyze the entire coding region in each gene. They identified somatic mutations in the *VHL* gene in 57% of these patients, and nearly all (98%) were found to have loss of heterozygosity. It was clear that the *VHL* gene played a role in the development of sporadic RCC in a majority of patients; however, questions arose as to why gene mutations were not demonstrable in all renal cell cancers. One explanation is offered by an important mechanism for *VHL* gene inactivation as described by Herman and colleagues (24). They discovered hypermethylation of a CpG island in the 5' region of the *VHL* gene, a region which is normally unmethylated, in nearly 20% of *VHL* patients with RCC. No other mutation of the *VHL* gene could be demonstrated in 80% of these patients, and *VHL* gene expression was absent in all. Furthermore, when treated with 5-aza-2'-deoxycytidine, a hypomethylating agent, the *VHL* gene was once again expressed. Additionally, one has to consider the limitations of current investigative techniques. There are still regions of the *VHL* gene which have not yet been thoroughly examined and this may hinder our ability to fully detect genetic variation. Furthermore, there is always the possibility of normal tissue interspersed with cancerous cells within a given tumor, thus confounding laboratory findings (25).

2.1.2 Cystic Lesions in VHL

In addition to solid RCCs, patients with VHL are also frequently found to have cystic lesions within their kidneys (Fig. 1). These lesions range from simple benign cysts, as characterized by radiographic imaging, to complex cystic masses suspicious for malignancy. In this patient population which can be expected to develop numerous multifocal and bilateral lesions requiring surgical extirpation, maximal nephron preservation relies upon the clinician's ability to predict the malignant potential of a cyst or mass, and the likelihood that treatment of that lesion will improve survival. In order to better characterize the relationship between cysts and solid renal masses, Lubensky et al. (26) analyzed 26 renal lesions from two VHL patients for loss of heterozygosity at the *VHL* region. They found loss of a *VHL* allele in 25 out of the 26 lesions, thereby demonstrating both benign and malignant lesions to share similar genetic aberration. In both sets of lesions, the mutated gene remained while the normal copy was the one that was lost, thus keeping with Knudson's two-hit hypothesis. Further evidence to support the theory that renal cysts potentially represent precursors to malignant RCC in VHL was provided by the work of Lee et al. (27) when they showed the consistent coexpression of erythropoietin and erythropoietin receptor in RCC as well as many renal cysts.

Knowing that simple cysts harbored the same genetic abnormality as solid malignant lesions, clinicians were then faced with the dilemma of when to act on cysts found in the kidneys of VHL patients. If left untreated, simple cysts

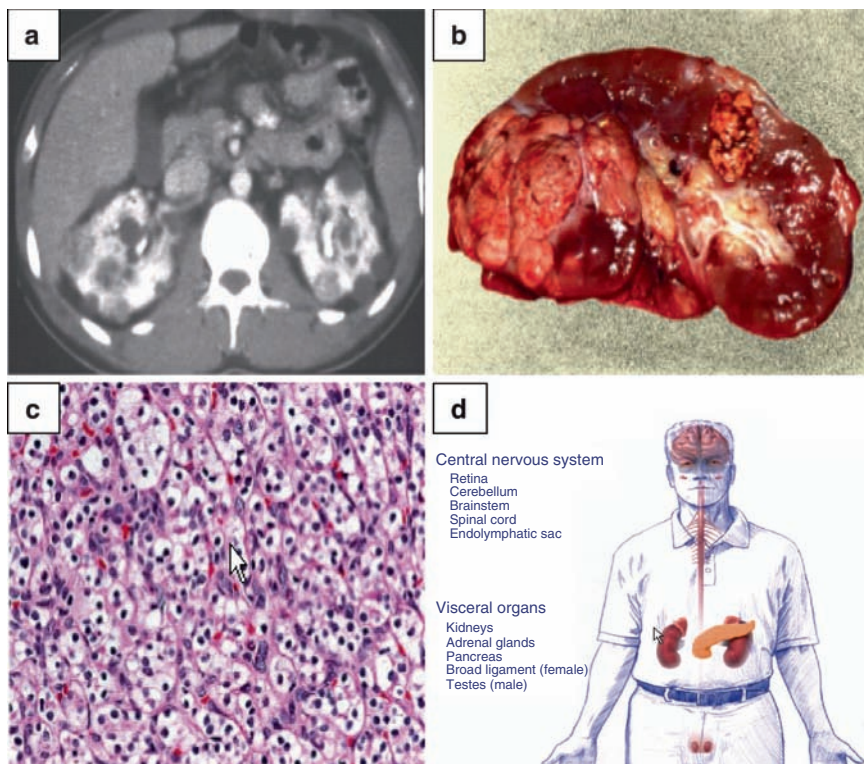


Fig. 1 Phenotypic manifestations of VHL. Renal masses are common in VHL patients. **a** CT scan of a VHL patient demonstrating characteristic bilateral multifocal renal lesions consisting of simple and complex cysts as well as enhancing solid masses. **b** Gross specimen removed from a VHL patient showing classic multiple golden-yellow tumors. **c** H&E stain of a classic clear cell renal carcinoma found in patients with VHL. **d** In addition to renal manifestations, VHL affects organs systems throughout the body. From Linehan et al. (76) (*See Color Plates*)

may develop malignancy over time, and a plan of observation may prove fatal if progression to metastatic disease ensued. On the other hand, unnecessarily operating on benign lesions could lead to a dramatic increase in morbidity for VHL patients, including the perioperative risks of surgery as well as the subsequent renal insufficiency from loss of parenchyma. Thus, investigators focused on determining the natural history of cystic lesions in the VHL population (12). Two hundred and twenty-eight renal lesions from 28 patients were observed for a mean of 2.4 years with serial computed tomography scans. Overall, 74% of the cysts remained stable with respect to size, with an additional 9% actually decreasing in size. Only 2 patients were found to have malignant transformation of their simple cysts based on radiographic criteria. These results supported the practice of conservative management of simple cysts in the VHL population.

2.2 *Function of the VHL Gene*

Once the putative gene for RCC was identified, there was an effort to better define the function of the VHL protein, with the hope that this would eventually uncover potential therapeutic targets. One method of determining the function of a protein is to find out what other proteins it complexes with in order to reveal its role in a cellular pathway. In 1995, Duan and colleagues (28) localized the *VHL* gene product to the cytosol and the nucleus, indicating common translocation of the protein. They were also able to identify two additional proteins of 16 and 9kDa which formed a heterotrimeric complex with VHL. When certain missense mutations of the *VHL* gene were investigated, the complex did not form. Subsequent studies (29) offered a more detailed description of the protein complex. They explained the function of a transcription elongation factor, Elongin (SIII), made up of three distinct protein subunits, Elongins A, B, and C, which serves to prevent transient pauses of RNA polymerase II (Pol II) during transcription. Although VHL protein was shown to displace Elongin A and compete for binding with Elongins B and C *in vitro*, there was no evidence of such function *in vivo*. Iliopoulos et al. (30) demonstrated the effects of VHL protein on certain hypoxia-inducible genes. Under normoxic conditions, intact VHL was shown to downregulate vascular endothelial growth factor (VEGF), platelet-derived growth factor B (PDGF-B), and the glucose transporter GLUT1 by destabilizing their respective mRNAs. Thus, presumably, with a VHL mutation there was unregulated expression of these proteins, a finding which was congruent with the known hypervascular characteristics of VHL-associated RCCs. In a search for proteins that interact with the VHL-B-C complex, Pause and colleagues (31) identified Hs-CUL-2, a newly described gene involved in cell cycle regulation of yeast and *Caenorhabditis elegans*. They observed that in the presence of a *VHL* gene mutation, the VHL-B-C-Hs-CUL-2 interaction was markedly diminished, suggesting a tumor suppressor role for this new protein.

It was known that VEGF, GLUT1, and PDGF are all targets of hypoxia-inducible factor (HIF) and also that the clear cells of RCC express higher levels of these proteins than nonmalignant cells (32). The role of VHL was further elucidated when researchers showed that the previously described protein complex of VHL-B-C-CUL functioned as a ubiquitin ligase that targets HIF1 α and HIF2 α for degradation under normoxic conditions (33). Upon hydroxylation by oxygen-dependent prolyl hydroxylases, HIF1 α binds to VHL and is subsequently degraded (34). If the hydroxylation does not occur, however, VHL binding is inhibited and ubiquitination of HIF1 α fails (35). Transcription of HIF-dependent genes ensues leading to overexpression of VEGF and ultimately increased vascularity. Lending support to this pathway, Maranchie et al. (36) used a competitive inhibitor of the VHL-HIF1 α binding site to assess functional outcomes. In preventing this interaction, they found accumulation of cellular HIF1 α in normoxia and a conversion to the *VHL*-negative phenotype.

3 Hereditary Papillary Renal Carcinoma

While advances were being made in the genetic basis of RCC resulting from *VHL* mutations, in 1994 clinicians were uncovering a distinct familial syndrome which was also manifest by renal tumors. Zbar and colleagues (37) reported on a family in which renal tumors had developed in three generations, and whose tumors were multifocal and bilateral. Pathologically these tumors were papillary variants of RCC, as opposed to the conventional type associated with VHL, and they showed no abnormalities in chromosome 3. This new syndrome, termed hereditary papillary renal carcinoma (HPRC), appeared to have an autosomal dominant mode of inheritance with incomplete penetrance. Further analysis of 10 families with HPRC suggested renal cancers occur in both sexes, with a male:female ratio of 2.2:1, have

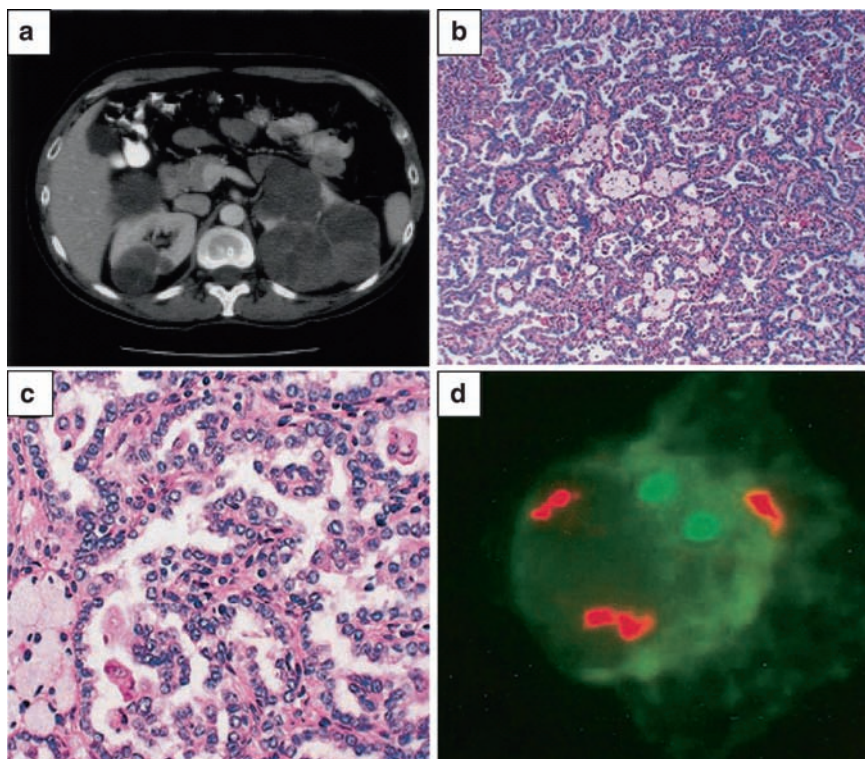


Fig. 2 Manifestations and genetics of HPRC. Patients with HPRC primarily develop bilateral multifocal renal masses. **a** Abdominal CT demonstrates HPRC tumors with characteristic poor enhancement on contrasted study that may frequently be mistaken for simple cysts. The tumors are best seen on late phase images of a contrast CT. **b** Low and **c** high power H&E stain of type I papillary RCC seen in patients with HPRC. **d** Fluorescence in situ hybridization (FISH) using a MET probe demonstrating trisomy of chromosome 7 (red signal) in papillary type I RCC compared with chromosome 11 serving as control (green signal). From Schmidt et al. (42) (See Color Plates)

a late age of onset (50–70 years), are bilateral and multifocal in nature (38). A later study evaluated 88 surgical pathology slides of grossly normal areas of 12 kidneys from patients with HPRC. More than half of these samples were found to contain microscopic papillary renal cancers, thereby predicting the presence of 1,100–3,400 microscopic tumors in a single kidney of a patient with HPRC (39). Histologically these tumors display a distinct phenotype, with a majority of the architecture in a papillary/tubulopapillary pattern and a chromophil basophilic staining, consistent with a type I papillary renal carcinoma phenotype (40). Radiographically in stark contrast to the hypervascular tumors of VHL, tumors of HPRC display poor contrast enhancement and are markedly hypovascular (41, 42) ([Fig. 2](#)).

3.1 Identification of the Gene for HPRC

Three years after describing the disease, researchers reported identification of the gene responsible for HPRC (43). Findings of chromosomal trisomy in malignant papillary renal carcinomas raised suspicions of proto-oncogene gene dysfunction and the defect was mapped to the long arm of chromosome 7. Missense mutations in the tyrosine kinase domain of the *MET* gene ultimately proved responsible for constitutive activation of the MET protein and interference with autoinhibitory mechanisms, resulting in papillary renal cancers. The MET transmembrane protein was found to be a receptor site for hepatocyte growth factor (HGF) also termed Scatter factor (SF) (44). Upon activation by HGF, MET tyrosine phosphorylation induces a host of signaling cascades responsible for embryonic development, cell branching, and invasion (45).

4 Birt–Hogg–Dubé

In 1977, three physicians described a familial syndrome in which affected individuals developed multiple small skin-colored papules on the face, neck, and back (46). Histologically these lesions were found to be fibrofolliculomas, trichodiscomas, and acrochordons, and they were transmitted in an autosomal dominant pattern. Some patients with this constellation of findings, termed Birt–Hogg–Dubé (BHD), were also known to have concurrent visceral tumors, including thyroid carcinoma, colonic polyps, and one case of a renal tumor. In 1999, a group of clinicians noted that a significant number of their renal mass patients had these distinctive skin lesions that had previously been described in dermatologic literature. They therefore set out to evaluate a large cohort of patients with known familial renal tumors and assess the presence of cutaneous findings. As a result, Toro and colleagues (47) found three extended families in whom there appeared to be common segregation of renal tumors and the cutaneous lesions of BHD. They concluded that BHD seemed to be associated with renal tumors, both transmitted in an autosomal dominant manner.

As BHD began to attract more attention and closer scrutiny, numerous additional disease processes were identified in BHD patients. Spontaneous pneumothoraces, parotid oncocytomas, multiple lipomas, angioliomas, parathyroid adenomas, and colonic polyposis were all postulated to have some connection with BHD (48–51). In order to better define the spectrum of disease processes associated with BHD, Zbar and colleagues (52) solicited participation from patients who were under the care of dermatologists from across the United States and Canada for classic BHD skin lesions. The patients were evaluated for concomitant health problems, particularly kidney, lung, and colon manifestations. The group eventually found no correlation between BHD and colon cancer or polyps. There was, however, a strong link

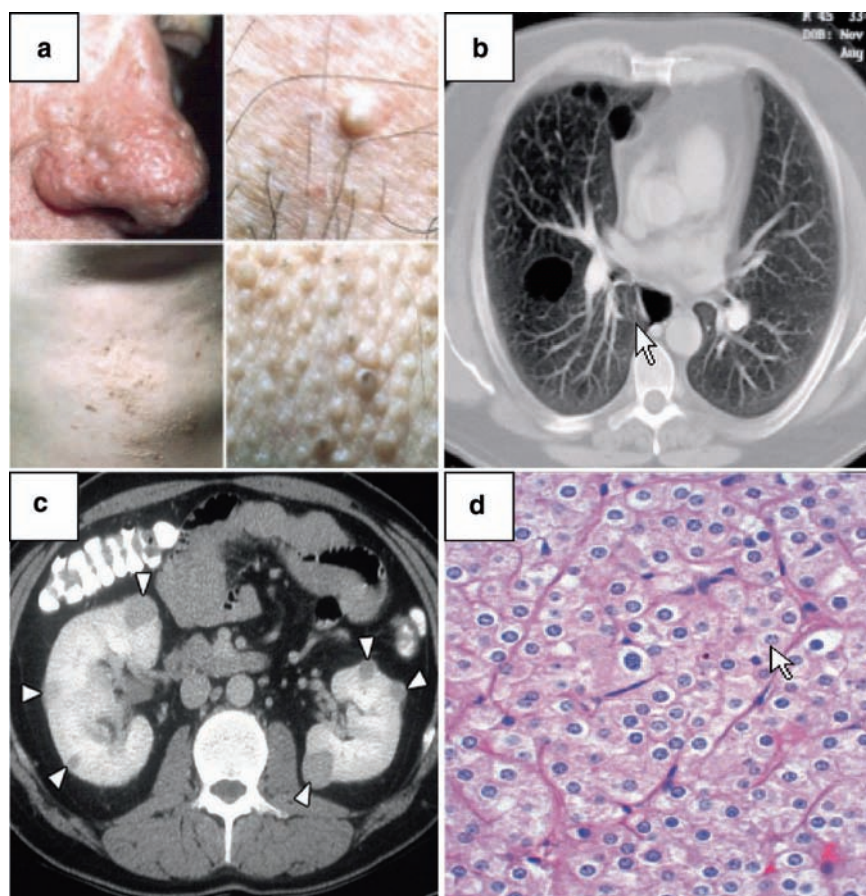


Fig. 3 Phenotypic manifestations of BHD. Classic findings in BHD include (a) characteristic cutaneous fibrofolliculomas, (b) pulmonary cysts that result in a 30-fold increased incidence of spontaneous pneumothoraces, and (c) renal tumors that are usually multifocal and can vary in pathologic subtype, from (d) chromophobe RCC (most common) to oncocytoma, hybrid tumors, or clear cell carcinoma. From Zbar et al. (53) (*See Color Plates*)

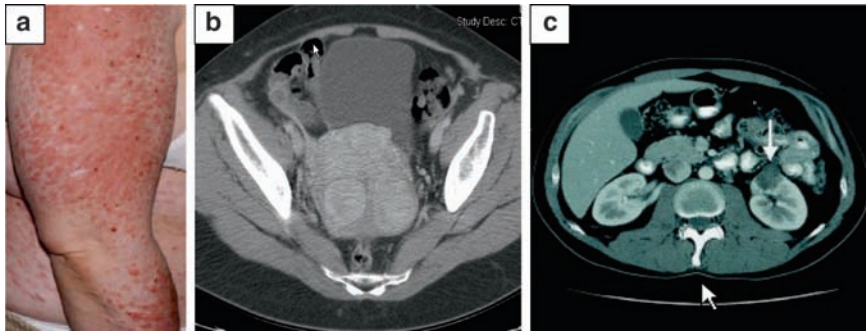


Fig. 4 Phenotypic manifestations of HLRCC. **a** Classic cutaneous leiomyomas presenting as multiple firm and erythematous macules and papules that are frequently painful. **b** Abdominal CT scan showing multiple uterine leiomyomas. This often leads to hysterectomy in HLRCC-affected women in their 20s or 30s. **c** CT abdomen demonstrating anterior upper pole mass in the left kidney. The renal lesions of HLRCC patients may present early and frequently have an aggressive clinical course. From Toro et al. (62) (*See Color Plates*)

between BHD and renal tumors, as previously suspected, as well as spontaneous pneumothoraces. On multivariate analysis, patients with BHD had an odds ratio of ~9.0 for developing renal tumors, and a risk of developing spontaneous pneumothoraces 32 times higher than the general population (Fig. 3).

In order to better characterize the renal neoplasms associated with BHD, researchers examined the pathologic findings of 130 renal tumors from 30 BHD patients from 19 different families (53). Close to 35% of the tumors were pure chromophobe variants of RCC, with an additional 50% being a hybrid of chromophobe RCC and oncocytoma. Less than 10% of the entire cohort had elements of clear cell (conventional) RCC. When present, the clear cell RCC were larger, with a mean diameter of 4.7 cm, versus the chromophobe tumors which averaged 3.0 cm, or the hybrid tumors with a mean diameter of 2.2 cm. Furthermore, analysis of grossly normal appearing surrounding renal parenchyma revealed multifocal oncocytosis throughout a majority of the specimens (Fig. 4).

4.1 Identification of the *BHD* Gene

Knowledge of the genetic basis for BHD came largely in part from work by Schmidt and colleagues (54). Linkage analysis was used to localize the *BHD* gene to a locus on the short arm of chromosome 17 from a screen of the genome of a large BHD kindred. Further work by Nickerson et al. (55) utilized recombination mapping to localize the gene to a region of 17p11.2. A novel gene in this region was determined to exhibit mutations in the germlines of affected patients. The gene product, folliculin, was truncated as a result of insertions, deletions, or nonsense mutations. The frequency with which *BHD* is inactivated as a result of genetic

mutations suggested a tumor suppressor function. Vocke and coworkers (56) found support for this theory when they sequenced the DNA of 77 renal tumors from 12 patients with germline *BHD* mutations. They demonstrated a high frequency of mutations in the wild-type *BHD* allele, thus providing the second “inactivating hit.” The 579 amino acid protein, named for the hallmark dermatologic findings of the syndrome, has no known functional domains, but is highly preserved across species. *BHD* mRNA expression as measured by FISH has been demonstrated in 17 human tissues, including the kidney, lung, skin, and brain (57).

5 Hereditary Leiomyomatosis Renal Cell Carcinoma

A fourth familial syndrome of renal cancer was recently described by Launonen et al. (58). They noted cosegregation of cutaneous leiomyomas and type II papillary renal cell carcinoma in two familial lines (Fig. 4). This syndrome, termed hereditary leiomyomatosis renal cell carcinoma (HLRCC), was mapped to a 14-cM region on the long arm of chromosome 1 (59). Fumarate hydratase, the product of the putative gene for this syndrome, is a catalyst for the conversion of fumarate to malate in the

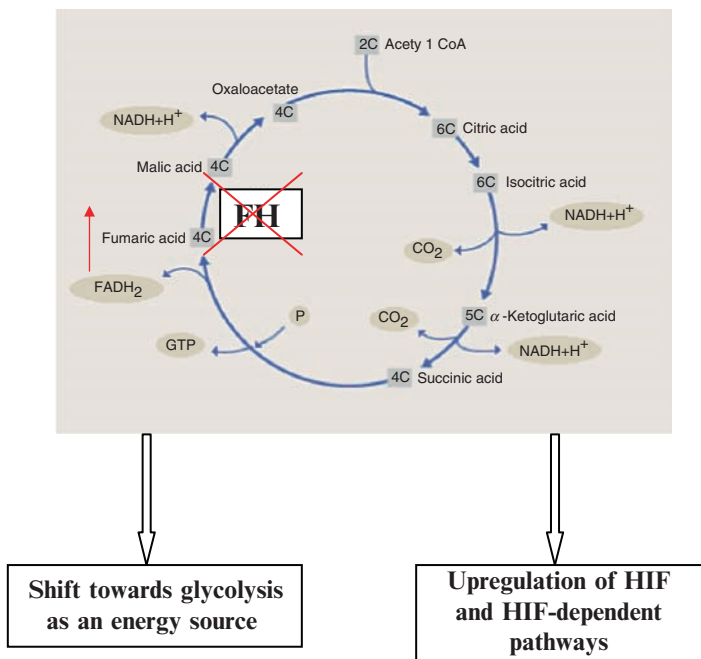


Fig. 5 In HLRCC, mutation of the FH gene leads to dysfunctional fumarate hydratase, one of the key regulatory enzymes in the Krebs cycle, necessary for mitochondrial respiration and oxidative energy production. This in turn leads to accumulation of fumarate but more importantly to preferential energy production from glycolysis, a phenomenon observed in other malignancies as well. It may also lead to upregulation of HIF and HIF-dependent pathways

Krebs cycle, and its activity is diminished in leiomyomatous tumors (60). The loss of FH function and impediment of the Krebs cycle creates reliance upon glycolytic metabolism and upregulation of HIF and HIF-inducible transcripts (61) (Fig. 5). The resultant environment is ideal for tumor cell survival and proliferation. The largest reported series of HLRCC patients revealed a 93% germline *FH* mutation detection rate in families suspected of harboring disease with an autosomal dominant inheritance pattern (62, 63). Details of the molecular mechanisms involved in the downstream pathway of this gene are still under investigation, but the renal cancers associated with it appear to be aggressive and lethal if allowed to progress.

6 Treatment

6.1 Localized Disease

As our understanding of renal malignancies has evolved, so have our treatment strategies. In 1869, Gustav Simon performed the first planned nephrectomy in the treatment of a ureterovaginal fistula. A century later, Robson and colleagues (64) described refined techniques for radical nephrectomy for renal malignancies. Surgical extirpation remains the gold standard for treatment of localized renal cell carcinoma, although the surgical techniques have become more sophisticated. Since the first laparoscopic radical nephrectomy performed by Clayman (65), great strides have been made in minimally invasive approaches to removing kidneys. Laparoscopy offers patients decreased morbidity as compared with the historical open surgical procedures while not appearing to compromise cancer control.

In the setting of localized renal tumors, nephron sparing surgery is becoming more common. In 1890, Czerny performed the first partial nephrectomy for malignancy. Since that time, the scope has increased with surgeons proposing a wide range of acceptable size limits for nephron sparing surgery, with general consensus around 4 cm in diameter (66, 67). Here, too, minimally invasive approaches are being employed and laparoscopic partial nephrectomies are now being performed at specialized centers across the country.

Preservation of renal function and maximal sparing of nephrons during therapy is of paramount importance when treating patients with familial syndromes who are at risk for developing multiple, recurrent, bilateral tumors, and may require numerous therapeutic interventions over their lifetime. Nonetheless, cancer control cannot be compromised. In order to minimize the morbidities associated with renal replacement therapy while maintaining vigilance in the containment of cancer, a threshold of 3 cm has been employed whereby tumors are observed until they reach this size criterion (68). In determining the safety of this guideline, researchers found no patients developed metastatic disease nor did they require dialysis when the 3 cm rule was adhered to.

In addition to surgical extirpation, ablative techniques have also been employed for the treatment of renal tumors. Thermal tissue ablation with radiofrequency

energy can be performed either percutaneously or laparoscopically. With higher wattage generators results for radiofrequency ablation appear promising. Hwang et al. (69) reported favorable outcomes for 23 out of 24 patients treated with RFA at a mean follow-up of 1 year. Nonetheless, this is still considered an experimental technique and further studies will need to be conducted with longer follow-up and validation of post-RFA imaging criteria.

6.2 *Metastatic Disease*

Despite high success rates with treatment of localized renal cancers, the prognosis for patients with metastatic disease is far grimmer. Although immunotherapy has been used, with interleukin-2 being the standard treatment modality, overall response rates are only in the range of 15–22% (70). It is obvious that new strategies are needed for the successful treatment of these patients, and molecular therapeutics seem to hold the key. The success of the tyrosine kinase inhibitor STI-571 in combating gastrointestinal stromal tumors and chronic myelogenous leukemia has fueled enthusiasm for further investigation into the molecular mechanisms of oncogenesis and potential pharmacologic disruption of these pathways (71, 72).

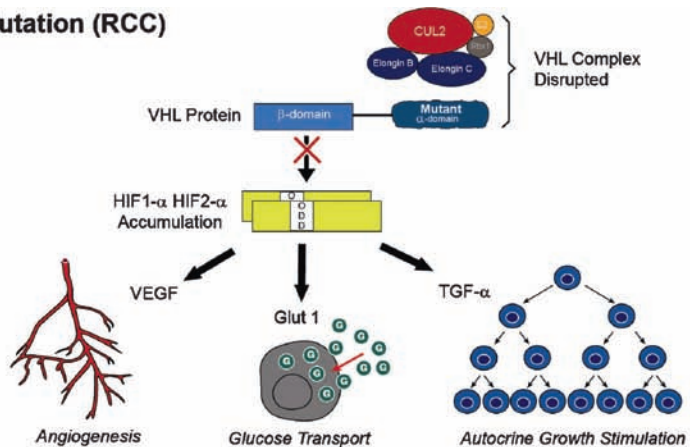
In the paradigm of renal cancers, molecular therapeutics can be thought of in two broad categories: those that seek to interrupt specific pathways of tumorigenesis and the individual proteins involved, and those that affect the cancer cell's adaptability. Given the variability of each distinct type of renal cancer, it should not be surprising that this heterogeneous group of diseases offers a wide range of unique molecular targets. Our understandings of the mechanisms involved in the familial syndromes greatly impact our ability to direct therapies at their sporadic counterparts.

6.3 *Targeting VHL*

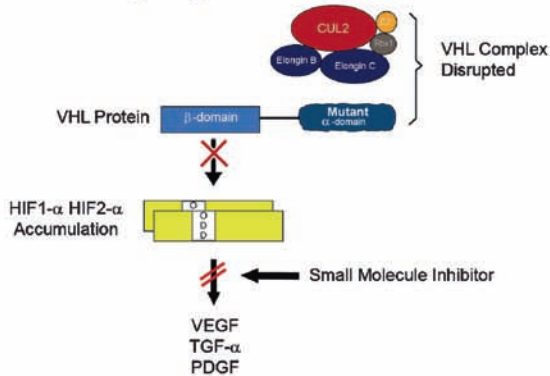
The VHL pathway offers a variety of targets for intervention. In *VHL* negative cells, the protein complex responsible for promoting HIF degradation is nonfunctional, resulting in the overabundance of HIF in a normoxic state. One therapeutic approach was demonstrated by Rapisarda et al. (73) when they used a small molecule inhibitor of the HIF-1 pathway, topotecan, to block the transcriptional activity of HIF-1. Although effective in reducing the accumulation of HIF-1 α in hypoxic environments, the efficacy of topotecan for VHL remains to be determined since in vitro and in vivo studies in human VHL models suggest HIF-2 to be the major factor in oncogenic pathways (74, 75). Efforts are currently under way to better target HIF-2 function (76, 77).

Several components of the downstream pathways in HIF have also been targeted (Fig. 6). Failure to adequately inactivate HIF leads to unregulated expression of

A. VHL Gene Mutation (RCC)



B. VHL/HIF Pathway Molecular Targeting



C. VHL/HIF Downstream Pathway Molecular Targeting

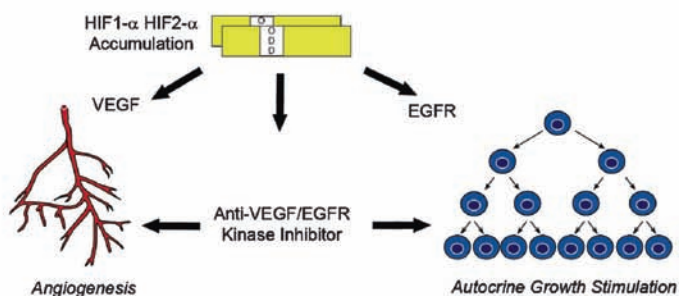


Fig. 6 VHL gene mutation, downstream effects, and molecular targeting of the VHL pathway. **a.** With a VHL gene mutation, the VHL complex is disrupted and allows for accumulation of HIF with subsequent activation of downstream pathways for angiogenesis, glucose transport, and growth. **b.** Inhibition of overaccumulated HIF and prevention of downstream activation with a small molecule is one of the strategies for molecular targeting of the VHL/HIF pathway. **c.** New tyrosine kinase inhibitors as well as direct VEGF and PDGF receptor blockers are examples of downstream targeting. From Linehan et al. (76)

gene products such as VEGF, PDGF, EGF, TGF α , and GLUT1. Pharmacotherapies inhibiting these pathways may offer a systemic modality to combat metastatic disease. Bevacizumab, a monoclonal antibody to VEGF, has been shown to decrease angiogenesis in renal cell carcinoma (78). Another drug, BAY 43-9006, inhibits signal transduction and subsequent cell proliferation by antagonizing the tyrosine kinase receptors for VEGF and PDGF (79). The receptor of EGF can be blocked individually through the function of either ZD1839 or erlotinib, or in combination with the VEGF receptor by ZD6474 (80–82).

6.4 *Altering the c-MET Pathway*

Type I papillary RCC in HPRC has been shown to result from activating mutations in the cell surface tyrosine kinase receptor for HGF, c-MET. Upon activation, the c-MET receptor is autophosphorylated, thus recruiting multiple signaling molecules to its cytoplasmic domain and activating intra- and extracellular cascades which ultimately contribute to cellular proliferation, scattering, and invasion (45). On the basis of this knowledge, several therapeutic strategies have been proposed: inhibition of autophosphorylation by the prevention of ATP binding, inhibition of the interaction between HGF and its receptor, and suppression of the downstream signaling cascade of activated c-MET (76).

6.5 *HSP-90 Inhibition*

An alternative strategy in the molecular targeting of tumorigenesis is to affect the mechanisms used by the cancer cells to adapt and thrive in surrounding environments. One such group of targets is molecular chaperones, termed heat shock proteins (HSPs), which maintain appropriate protein conformation, assist in protein transport, and play a role in antigen presentation. Out of the entire family of molecular chaperones, heat shock protein 90 (HSP-90) has drawn attention for its active role in renal. HSP-90 is part of a complex that stabilizes and promotes the activity of HIF and the receptor tyrosine kinases MET and KIT (76). An inhibitor of HSP-90, 17-allylamino-17-desmethoxygeldanamycin (17-AAG), has been shown to disrupt the function of this complex, thus leading to rapid inactivation and degradation of its client proteins (83). As a result, HIF-dependent transcriptional activity is impaired, thus decreasing the downstream gene products in the HIF pathway.

HSP-90 has also been shown to play a role in chromophobe and papillary RCC through its effects on KIT and MET and their downstream pathways (84, 85). In addition to direct inhibition of KIT, HSP-90 inhibitors also function on AKT and RAF, transcription promoters which are stimulated by KIT but are also themselves client proteins of HSP-90. Finally, with respect to MET, HSP-90 inhibitors may have a potential role as an adjunct to angiogenesis inhibitors. Hypoxia has been shown

to upregulate MET via the HIF pathway, including in vivo after the administration of antiangiogenic agents. Therefore, suppression of MET using HSP-90 inhibitors simultaneously with antagonists of angiogenesis may prove beneficial (76).

7 Conclusion

Great strides have been made in the understanding of the genetic basis for renal malignancy. Through refined surgical techniques, patients afflicted with localized renal cancer have an excellent chance for survival with continued decrease in treatment-associated morbidities. Unfortunately, the current treatment modalities for those with advanced disease are not nearly as effective. Nonetheless, the future looks promising. Unrelenting research and dedication to understanding the cellular mechanisms of oncogenesis have the potential to change the face of renal cancer therapy and may provide these patients with the hope of a cure.

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Molecular Targets in Renal Tumors: Pathologic Assessment

Ming Zhou

Abstract Pathologists play critical roles in the clinical management and research of renal cell carcinoma (RCC). Careful assessment by pathologists of a cancer-harboring kidney specimen not only renders an accurate histological diagnosis and classification, but also provides information important for prognosis and therapeutic decisions. In addition, redundant tumor tissues not required for diagnosis could be procured for clinical trials, experimental therapies, and research. This chapter briefly describes how the RCC specimens are processed in pathology laboratories and how clinicians can facilitate such a process. In addition, important pathological and cytogenetic features of different subtypes of RCC will also be discussed.

Keywords Renal cell carcinoma • Renal neoplasm • Pathology • Cytogenetics • Prognosis

Many different types of benign and malignant tumors have been described in the kidney. Accounting for over 90% of all malignancies in adult kidneys, renal cell carcinoma (RCC) is derived from the renal tubular epithelial cells. It encompasses a group of tumors with heterogeneous clinical, pathologic, and genetic characteristics, as well as diverse prognosis and therapeutic responses.

1 Histological Classification of RCCs

The pathological classification of RCC serves several purposes: (1) to render a diagnosis that is clinically relevant and reflects the underlying pathogenetic mechanisms; (2) to provide prognostic information; and (3) to provide guidance to therapy. The current classification of RCCs, published by the World Health Organization in 2004 ([Table 1](#)) (1), is based primarily on morphology. However,

M. Zhou

Department of Anatomic Pathology, Urology, Cancer Biology and Taussig Cancer Center, Cleveland Clinic, Cleveland, OH

Table 1 2004 WHO classification of renal cell neoplasms^a

Renal cell carcinoma
Clear cell renal cell carcinoma
Multilocular cystic clear cell renal cell carcinoma
Papillary renal cell carcinoma
Chromophobe renal cell carcinoma
Carcinoma of the collecting ducts of Bellini
Renal medullary carcinoma
Xp11 translocation carcinoma
Carcinoma associated with neuroblastoma
Mucinous tubular and spindle cell carcinoma
Renal cell carcinoma, unclassified type
Papillary adenoma/renal cortical adenoma
Oncocytoma

^aFrom Eble et al. (1)

characteristic genetic and molecular features that are associated with different types of RCCs have also been incorporated into this classification. For example, RCC associated with Xp11.2 translocation is a group of RCCs characterized by chromosomal translocations involving the *TFE3* gene on chromosome Xp11.2. Although it morphologically overlaps with and may be mistaken for clear cell or papillary RCC, it is classified as a distinct clinicopathological entity based on this characteristic genetic change (2). The morphological classification will still dominate in a foreseeable near future. However, more molecular and genetic criteria will also be incorporated into the classification scheme. It is hoped that molecular and genetic classification will not only provide more accurate pathological classification, but also offer better prognostic and therapeutic information, and may help design more specific, gene-based therapy.

1.1 Renal Cell Carcinoma, Clear Cell Type (Clear Cell RCC)

Clear cell RCC is the most common histological subtype, accounting for 60–70% of all RCCs. It most commonly affects patients in their sixth and seventh decades of life. Male:female ratio is 2:1. The majority of clear cell RCC arises sporadically, with only <5% of the cases as part of an inherited cancer syndrome (3), including von Hippel–Lindau syndrome, Birt–Hogg–Dube syndrome, and constitutional chromosomal 3 translocation syndrome. As a general rule, familial clear cell RCC presents at a younger age and is more likely to be multifocal and bilateral.

Clear cell RCC presents as a round, circumscribed, and nonencapsulated mass with hemorrhage, necrosis, cystic degeneration, and calcification on gross examination (4). Golden yellow color is characteristic (Fig. 1a). Multicentricity and/or

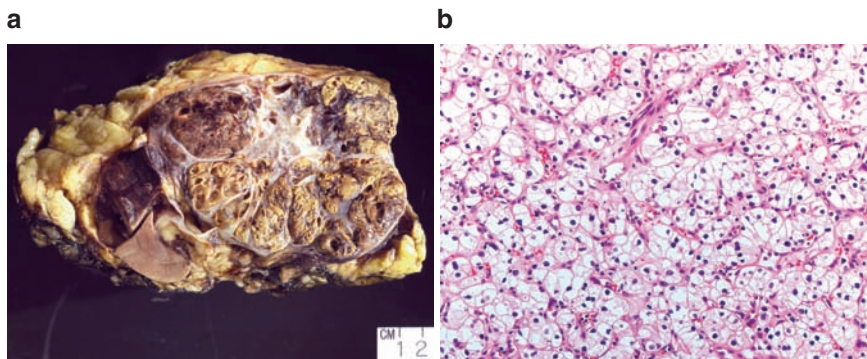


Fig. 1 Clear cell RCC forms a multinodular mass with distinctive yellow coloration. Hemorrhage and necrosis are also seen. A central stellate-shaped scar is also present (a). It is composed of compact nests of tumor cells with clear cytoplasm separated by delicate vasculature (b) (*See Color Plates*)

bilaterality occur in <5% of sporadic clear cell RCC cases, and are more often associated with inherited cancer syndromes.

Microscopically the tumor cells exhibit clear cytoplasm (Fig. 1b) due to loss of cytoplasmic lipid and glycogen contents during the preparation of slides, hence termed “clear cell” subtype. However, poorly differentiated, high-grade clear cell RCC often acquires more eosinophilic and granular cytoplasm. The term “granular cell RCC” was used in the past for RCC with eosinophilic and granular cytoplasm, and high-grade nuclei. Although some RCCs with “granular” cytoplasm are clear cell RCC, many are of other histological subtypes. Therefore, “granular cell RCC” is not a specific subtype. The term is antiquated and its use is discouraged.

Sarcomatoid differentiation is found in ~5% of the cases and is indicative of poor prognosis (5). “Rhabdoid” cells with large eccentric nuclei, macronucleoli, and prominent acidophilic globular cytoplasm are occasionally seen and are associated with poor prognosis (6).

1.2 Multilocular Cystic Renal Cell Carcinoma

This entity is an uncommon subset of clear cell RCC, accounting for 5% of the latter. It forms a sharply circumscribed, entirely cystic mass without solid component (Fig. 2a). Microscopically it comprises of variably sized cysts lined with a single layer or occasionally several layers of attenuated flat or plump clear cells (Fig. 2b). Expansile cellular nodules are not present. The nuclei are almost always small with dense chromatin (Fuhrman grade 1). If these strict diagnostic criteria are applied, multilocular cystic RCC has a favorable prognosis with no local or distant metastasis documented after complete surgical removal (7,8).

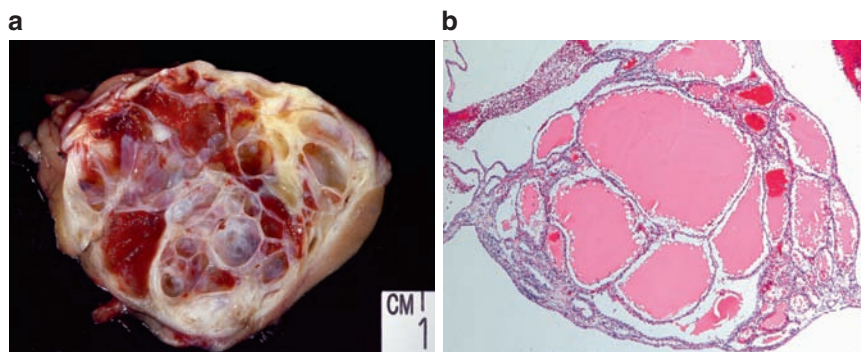


Fig. 2 Multilocular cystic renal cell carcinoma. This tumor is sharply circumscribed, and entirely cystic without solid components (a). Microscopically it contains variably sized cysts lined with a single layer and occasionally several layers of clear cells. The nuclei are small with dense chromatin (Fuhrman grade 1) (b) (See Color Plates)

1.3 Renal Cell Carcinoma, Papillary Type (Papillary RCC)

Papillary RCC accounts for 10–15% of RCCs with the gender and age distribution similar to clear cell RCC. However, papillary RCC has a better prognosis than the latter. The 5-year survival approaches 90%.

Papillary RCC presents as a circumscribed and discrete mass with a thick tumor capsule. Hemorrhage and necrosis are also common, especially in large tumors (Fig. 3a). Bilateral and multifocal tumors are more common in papillary RCC than in other RCC subtypes. Microscopically, papillary RCC comprises variable proportions of papillae and tubules (Fig. 3b). Papillary RCC can be further categorized into types I and II based on the morphological features (9). These two types of papillary RCC differ in genetic characteristics as well as clinical outcomes with a better prognosis observed for the type I papillary RCC.

Most papillary RCCs are sporadic. Infrequently, it occurs in the setting of inherited cancer syndromes, including hereditary papillary renal cell carcinoma syndrome (HPRCC syndrome) and hereditary leiomyomatosis and renal cell cancer (HLRCC syndrome) (3). HPRCC syndrome is caused by mutations in *c-MET* proto-oncogene (10) on chromosome 7q31 and develops papillary RCC of type I histology. HLRCC syndrome is an autosomal dominant disease and contains mutations in *fumarate hydratase* (*FH*) gene on chromosome 17. Patients develop skin and uterine leiomyomas and papillary RCC of type II histology (10).

1.4 Renal Cell Carcinoma, Chromophobe Type (Chromophobe RCC)

Chromophobe RCC accounts for ~5% of RCCs. The prognosis is significantly better than clear cell RCC with mortality <10%.

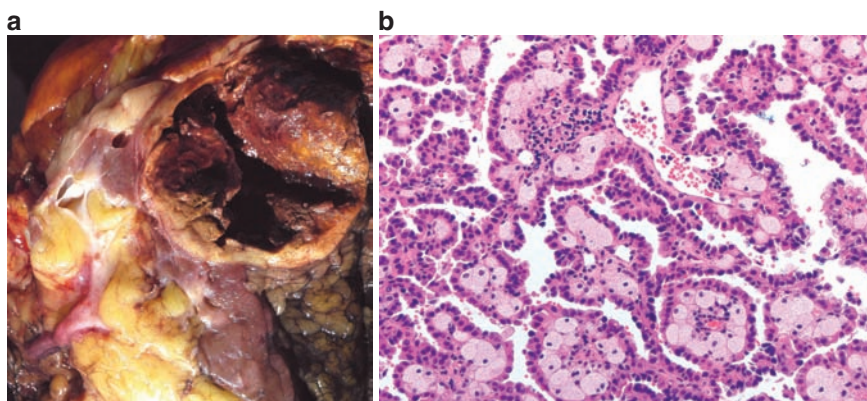


Fig. 3 This papillary renal cell carcinoma is predominantly exophytic and has a thick tumor capsule and extensive hemorrhage and necrosis (**a**). It is composed of papillary structures with foamy histiocytes within the fibrovascular stalks (**b**) (*See Color Plates*)

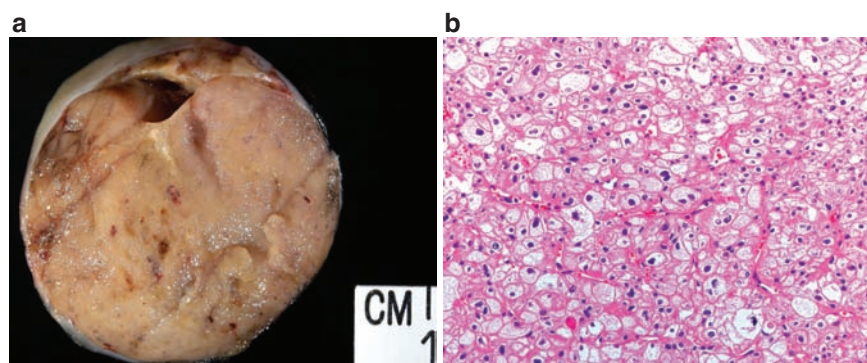


Fig. 4 Chromophobe RCC forms a circumscribed mass with a homogenous light brown cut surface (**a**). The large and polygonal tumor cells have pale cytoplasm, prominent cell border, and irregular nuclei with perinuclear clear halo (**b**) (*See Color Plates*)

Chromophobe RCC often presents as a solitary, circumscribed, and nonencapsulated mass with a homogenous light brown cut surface (**Fig. 4a**). The tumor cells are large and polygonal and have finely reticulated cytoplasm due to numerous cytoplasmic microvesicles, prominent cell border resembling plant cells, irregular, often wrinkled nuclei that are described as “raisin-like,” and clear halos around the nuclei (**Fig. 4b**). The cytoplasm is pale as if it were resistant to hematoxylin and eosin staining, hence termed “chromophobe.”

Occasionally, chromophobe RCC can occur in Birt–Hogg–Dube syndrome (3), an autosomal dominant disorder characterized by benign skin tumors (fibrofolliculomas, trichodiscomas of hair follicles, and skin tag), renal epithelial neoplasms, and spontaneous pneumothorax. Renal tumors are often multifocal and bilateral, and present in the forms of chromophobe RCC, oncocytomas, clear cell RCC, and

occasionally papillary RCC. Hybrid oncocytic tumors with features of chromophobe RCC and oncocytoma have also been described (11,12).

1.5 Carcinoma of the Collecting Duct of Bellini (Collecting Duct Carcinoma)

Collecting duct carcinoma is postulated to derive from the collecting ducts of Bellini. It is rare and accounts for <1% of RCCs. It has a poor prognosis with metastasis at presentation in many patients and two-thirds of them succumb to the disease within 2 years of the diagnosis (13).

Collecting duct carcinoma arises in the central region of the kidney, and appears firm and gray-white with infiltrative borders. Secondary extension into the cortex may occur, especially in large tumors. Tumor cells form complex and angulated tubules or cystic structures embedded in a marked desmoplastic stroma (Fig. 5a). Cytologically they are highly atypical with markedly pleomorphic nuclei, vesicular chromatin, prominent nucleoli, and frequent mitosis.

1.6 Renal Medullary Carcinoma

Medullary carcinoma of the kidney is an extremely rare and highly aggressive renal tumor that almost exclusively affects patients with sickle cell hemoglobinopathies (almost always sickle cell trait) (14,15). The tumor is believed to originate from the collecting ducts but is distinct from collecting duct carcinoma.

Grossly, renal medullary carcinoma appears as a poorly circumscribed, centrally located, gray-white mass with hemorrhage and necrosis. Microscopically it resembles collecting duct carcinoma with high-grade tumor cells arranged in solid sheets or more commonly of microcystic pattern (Fig. 5b). The tumor cells are embedded in a desmoplastic stroma and are infiltrated by neutrophils and lymphocytes. Sickle red blood cells are characteristic.

1.7 RCCs Associated with Xp11.2 Translocation/TFE3 Gene Fusion

This entity is defined by the chromosomal translocation involving the *TFE3* gene on chromosome Xp11.2 that results in overexpression of the TFE3 protein (2,16,17). The translocation partner genes include *PRCC* on 1q21, *ASPL* on 17q26, *PSL* on 1p34, and *NonO* on Xq12. These carcinomas typically affect children and young adults. Although RCC accounts for <5% of pediatric renal tumors, Xp11.2-associated RCCs make up a significant proportion of these cases. RCC involving *ASPL-TFE3* translocation characteristically presents at an advanced stage and also with lymph

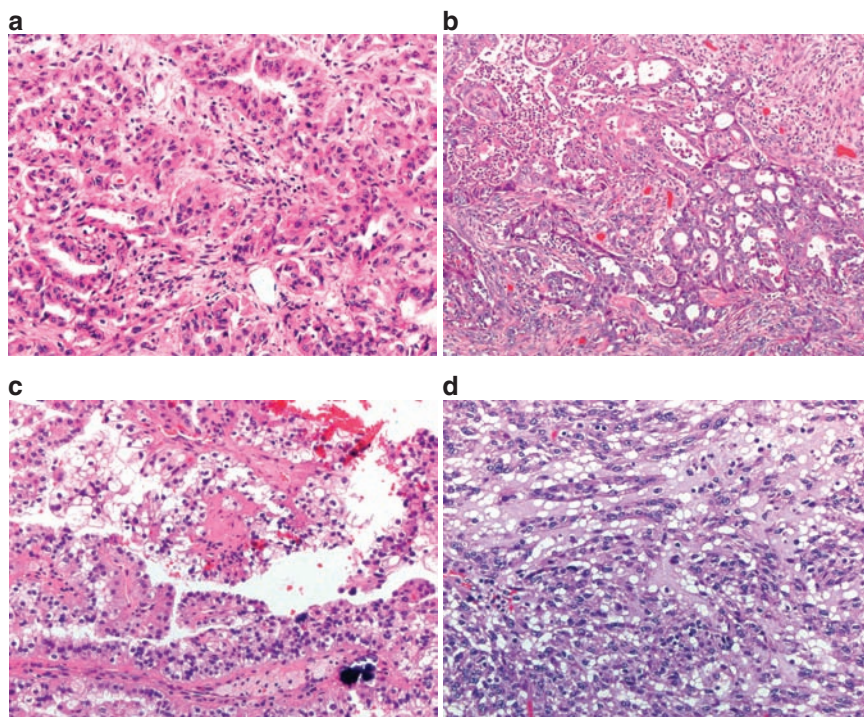


Fig. 5 Collecting duct renal cell carcinoma consists of high-grade tumor cells forming angulated tubules embedded in markedly inflamed and desmoplastic stroma (a). In medullary carcinoma, tumor cells from microcystic structures that are embedded in a desmoplastic stroma and infiltrated by neutrophils and lymphocytes (b). Renal cell carcinoma associated with Xp11.2/TFE3 translocation consists of nested to pseudopapillary structures lined tumor cells with abundant clear, sometimes eosinophilic, cytoplasm (c). Mucinous tubular and spindle cell carcinoma is composed of elongated cords and collapsed tubules with slit-like spaces embedded in lightly basophilic myxoid stroma (d) (See Color Plates)

node metastasis (18). Tumors affecting adult patients may pursue aggressive clinical course (19).

The morphology varies with different chromosomal translocations but the most distinctive histological feature is papillary structures lined with clear cells (Fig. 5c). The diagnosis can be confirmed by positive nuclear immunostain for TFE3 protein.

1.8 Mucinous Tubular and Spindle Cell Carcinoma

This entity is a low-grade tumor of possible distal nephron or loop of Henle origin (19,20). It has a wide age range of 17–82 (mean 53) years and a female

predominance (female-to-male ratio 4:1) in contrast to the male predominance observed in other types of RCCs. As its name implies, the tumor is composed of elongated cords and collapsed tubules with slit-like spaces embedded in a lightly basophilic and myxoid background (Fig. 5d). The tumor cells have low-grade nuclei. The prognosis seems favorable with the majority of the patients free of disease after surgical resection.

1.9 RCC Associated with Neuroblastoma

RCC can affect the long-term survivors of neuroblastoma (21). All affected children were diagnosed with neuroblastoma at age of 2 years or younger and the majority had advanced stage neuroblastoma. RCC was diagnosed at ages ranging from 5 to 14 years and occurred after a period of 3–11.5 years (average 9) following the neuroblastoma diagnosis. Morphologically, many of these tumors are typical clear cell RCC. However, some tumors have solid and papillary architecture with oncocytoid cells. Some cases are RCCs associated with Xp11.2 translocation.

1.10 Renal Cell Carcinoma, Unclassified Type

Two to five percent of RCCs do not fit into any of the subtypes in the 2004 WHO classification; hence are termed renal cell carcinoma, unclassified type. Understandably they represent a group of heterogeneous tumors with little in common in terms of clinical, morphological, or genetic features. It is important to bear in mind that rather than being a true biological entity, RCC of unclassified type is a diagnostic category. Hopefully as our understanding of RCC advances, this category will diminish and perhaps will eventually disappear.

1.11 Papillary Adenoma

Papillary adenoma is benign and the most common renal cell neoplasm, frequently as incidental findings at autopsies. Its incidence increases with age and also in patients on long-term dialysis with acquired cystic disease, or in scarred kidneys in patients with chronic pyelonephritis or renal vascular disease.

Papillary adenomas appear as a well-circumscribed, yellow or white small nodules in the renal cortex. They have a papillary and tubular architecture, similar to papillary RCC, but <5 mm according to the WHO classification (1). The cells lining these structures have uniform small nuclei and inconspicuous nucleoli similar to Fuhrman grade 1 or 2 nuclei.

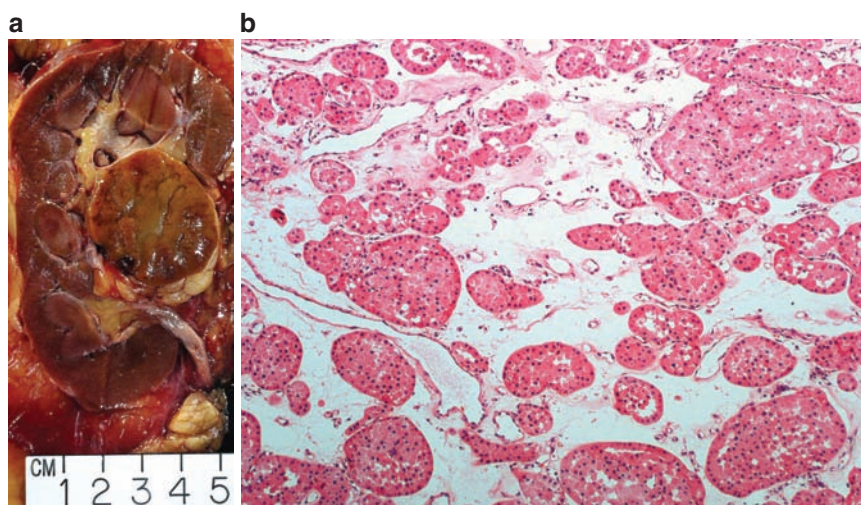


Fig. 6 Renal oncocytoma forms solitary and circumscribed mass with homogeneous dark brown cut surface and a central scar (a). It consists of uniform tumor cells with granular eosinophilic cytoplasm and regular round nuclei nested in a loose hypocellular and hyalinized stroma (b) (See Color Plates)

1.12 Renal Oncocytoma

A benign neoplasm, renal oncocytomas account for 5% of renal cell neoplasms. They occur in a wide age range with a peak incidence in the seventh decade of life. Most cases are sporadic, although familial cases have been reported in association with Birt–Hogg–Dube syndrome and familial renal oncocytoma syndrome (3).

Oncocytomas are typically solitary, well-circumscribed, nonencapsulated tumors (Fig. 6a) with homogeneous cut surface and characteristic mahogany-brown color (4). A central stellate scar is seen in one third of the cases, more commonly in larger tumors. Microscopically it is characterized by bright eosinophilic cells, termed oncocytes, arranged in nested, acinar or microcystic pattern associated with a loose hypocellular and hyalinized stroma (Fig. 6b). Extension of oncocytoma into the perinephric fat, or rarely into vascular space, can sometimes be found and does not seem to adversely affect the benign nature of the lesion.

2 Histological Prognostic Factors for Renal Cell Carcinoma

Many clinical, pathological, and molecular factors impact the prognosis of RCCs. The pathological parameters include tumor size, anatomic extent of the tumor, vascular invasion, involvement of the adrenal gland and adjacent organs, histological subtypes, grade, sarcomatoid differentiation, tumor necrosis, and status of surgical margins.

Those parameters that have been incorporated into the American Joint Committee on Cancer (AJCC)/TNM staging for RCC will not be discussed here.

2.1 Histological Subtypes

Many studies have reported different survival rates among patients with different subtypes of RCC (22,23). Histological subtypes often correlate with TNM stages and other pathological parameters. For example, clear cell RCC usually has worse pathological features than papillary and chromophobe RCC, including larger tumor size, higher Fuhrman grade, pathological tumor stage (pT), and the likelihood to develop distant metastasis. By univariate analysis, most studies found that clear cell RCC fares worse than papillary and chromophobe RCC. In a study by Cheville et al., the 5- and 10-year cancer-specific survival rates were 68.9 and 60.3% for clear cell RCC, 90.1 and 81.9% for papillary RCC, and 88.1 and 83.3% for chromophobe RCC, respectively (22). By multivariate analysis, however, the histological subtypes were not found to be independent predictors of the clinical outcomes. Nevertheless, histological classification of RCC is still clinically important, since different subtypes may exhibit diverse therapeutic response. For example, Upton et al. reported that 20% of advanced-stage clear cell RCC responded to interleukin 2-based therapy in contrast to 6% response rate in nonclear cell RCC (24).

2.2 Fuhrman Grading System

Fuhrman grading is the most common grading system for RCC. It is based on the nuclear size, irregularity of the nuclear membrane, and nucleolar prominence, and categorizes RCCs into grades 1–4. Fuhrman grade can be grouped into low grade (grade 1 and 2) and high grade (grade 3 and 4). Such grade compression not only preserves the prognostic significance of the Fuhrman grading but also improves the interobserver agreement (25).

Most studies have confirmed that Fuhrman grading is an independent prognostic predictor for clear cell RCC and RCC, not otherwise specified (25). However, its prognostic significance for papillary and chromophobe RCC remains controversial. A recent study suggested Fuhrman grading is not prognostically useful for chromophobe RCC (26)

2.3 Sarcomatoid Renal Cell Carcinoma

Sarcomatoid differentiation is present in 1–6.5% of RCCs and can be found in any RCC subtypes (5). It is thought to represent transformation to a more aggressive

and higher-grade RCC. The classification of such RCC should be based on the identifiable nonsarcomatous element. Histologically the sarcomatoid component ranges from malignant spindle cells to those resembling leiomyosarcoma, fibrosarcoma, angiosarcoma, rhabdomyosarcoma, and other sarcomas. Sarcomatoid differentiation is considered as an adverse pathological parameter and is graded as Fuhrman grade 4 (5). RCC with sarcomatoid differentiation often presents as high-stage and high-grade disease with frequent distant metastasis. Even if present as a small component, sarcomatoid differentiation worsens the prognosis significantly.

2.4 Tumor Necrosis

Histologically confirmed tumor necrosis is strongly associated with adverse clinical outcomes on univariate and multivariate analyses (22,27,28). Lam et al. also reported that the presence and extent of histological necrosis in RCC were independent predictors of survival for localized but not metastatic cases (29). However, tumor necrosis is not prognostically useful for papillary RCC since it is commonly observed in this tumor.

2.5 Invasion of Urinary Collecting System

Involvement of the collecting system, including renal calices, pelvis, and ureter, occurs most frequently in high-stage and high-grade RCC. Therefore, Uzzo et al. found that collecting system invasion did not portend a worse prognosis in high-stage (T3 or higher) tumor, whereas it was an adverse pathological feature in low-stage RCC (30). Another study revealed that collecting system invasion provides independent prognostic significance associated with a 1.4-fold greater risk of death compared to that without collecting system invasion (31). However, the study by Terrone et al. did not confirm the independent prognostic value of the involvement of the collecting system, even although the collecting system invasion was associated with a worse prognosis in univariate analysis (32).

2.6 Microvascular Invasion

Microvascular invasion, defined as vascular invasion identified only on microscopic examination, is detected in 13.6–44.6% of RCCs, and is more common in larger RCCs of higher stage and Fuhrman grade. Its presence was associated with worse prognosis and higher likelihood of disease recurrence in univariate analyses, although such findings have not been confirmed in multivariate analysis (33,34).

2.7 Completeness of Tumor Resection

Positive renal parenchymal margin in nephron sparing surgery indicates incomplete resection of RCC and is associated with poor prognosis (35,36). However, the width of the margins does not seem to correlate with the disease recurrence as long as the surgical margins are free of tumor (36).

3 Handling of RCC Specimens

When a RCC specimen is received in the pathology laboratory, the size and weight of the specimen, the size, location, and the physical properties of the tumor will be recorded. Pathologists will also investigate whether the tumor involves the surgical margins, perinephric and renal sinus fat, adrenal gland, renal vasculature, and adjacent organs. Renal hilar lymph nodes will also be searched. Representative sections will then be submitted for microscopic examination.

It is important to realize that not only pathologists, but urological surgeons, oncologists, and other clinical staff also play an important role in the optimal handling of RCC specimens. Clinical information is critical and should accompany the surgical specimens. Previous medical history, including prior renal tumors and family history, is extremely useful for identifying familial RCC cases. Awareness of such history will prompt pathologists to perform additional molecular and genetic studies to work up the case. Urologists should try to preserve the anatomy of the nephrectomy specimen by keeping the specimen intact without sectioning into it. If the perinephric fat has to be removed from the kidney for any reason, the zone of tumor contact should be indicated. Lymphadenectomy specimen should be submitted separately. If the lymphadenectomy is not separated from the perihilar tissue, pathologists should be made aware of this information so that they could look for the lymph nodes in the nephrectomy specimens. The specimen should be sent to pathology fresh without fixative in a sterile fashion immediately after its removal from the patient. Fix the specimen in formalin only if the delivery of the specimen to the pathology laboratory is expected to be delayed. The importance of the availability of fresh tumor tissue for molecular and cytogenetic studies could not be overemphasized. Approximately 2–5% of renal tumors remain unclassified using the current WHO classification. Molecular and genetic approaches offer the only hope that these “unclassified” RCC would be classified based on the genetic and molecular features.

4 Summary

Renal cell carcinoma represents a group of heterogeneous tumors with distinct clinical, pathological, and genetic characteristics, and diverse prognosis and therapeutic response. The 2004 World Health Organization classification is the most

updated classification system based on morphology and genetics. Pathological examination of the RCC specimens not only renders diagnosis, but also provides information important for prognosis and therapeutic decisions. In addition, tumor tissues could be procured for experimental studies and research. Pathologists, urologists, and oncologists play equally important role in the optimal handling and processing of RCC specimens.

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Interferons and Interleukin-2: Molecular Basis of Activity and Therapeutic Results

Thomas E. Hutson, Snehal Thakkar, Peter Cohen, and Ernest C. Borden

Abstract Metastatic renal cell carcinoma (RCC) is a tumor refractory to standard cytotoxic chemotherapy regimens. The rationale for the use of cytokines in this cancer is based upon compelling evidence that RCC is sensitive to immunological manipulation.

Cytokine-based therapy with either interleukin-2 (IL-2) or interferon- α (IFN- α) can result in objective tumor responses in up to 15% of patients, and in selected patients, these responses may be durable. Subsequently these cytokines have impacted positively on both quality and quantity of life for thousands of RCC patients – and it seems probable that their full clinical potential has yet to be reached. The development of “targeted” therapies for clear cell RCC with the potential for greater antitumor activity and less toxicity has brought into question the role of cytokines in this patient population. However, no noncytokine therapy to date has proven curative in patients with metastatic RCC. A series of recently completed and ongoing clinical trials will partially define the future role of IFNs and IL-2 in sequence and in combination with newer “targeted” agents. As the knowledge of the role that IFN and IL-2 contribute to both innate and acquired immunity continues to expand, it seems probable that the structure and/or application of these cytokines will be further modulated for therapeutic advantage in RCC.

Keywords nterferon • Interleukin • IFN- α 2, IL-2 • Angiogenesis inhibition
• Clinical effects of IL-2 and IFN

1 Introduction

Interferon- α 2 (IFN- α 2) and interleukin-2 (IL-2) were the first therapeutics to result in regressions of metastatic renal cell carcinoma (RCC) in multi-institutional studies. The role of biological response modification, as an effective approach to treatment of human malignancies, was to a significant extent established by these studies. Since initial approval for clinical marketing of IL-2 by the Food and Drug

Administration (FDA) was for metastatic RCC, more recently expanded to metastatic melanoma, this from statement is particularly true for RCC. Subsequently these cytokines have impacted positively on both quality and quantity of life for thousands of RCC patients – and it seems probable that their full clinical potential has yet to be reached. FDA approval of any biological response modifier was less than 25 years ago; clinical application of biochemical modulation and second-generation fluoropyrimidines, which began almost 50 years ago, continues to this day with third generation products of 5-fluorouracil having recently reached clinical approval. Second generation IFNs with improved pharmacodynamic and clinical properties were introduced just over 5 years ago and, because of improved convenience and effectiveness, have subsequently been approved for chronic hepatitis. As the knowledge of the role of the IFN system and IL-2 in innate and acquired immunity continues to expand, it seems probable that the structure and/or application of these cytokines will be further modulated for therapeutic advantage in RCC.

Reaching the full potential of IFNs and IL-2 as therapeutics for RCC will also result from additional understanding of the mechanisms of immune augmenting and antitumor actions. Suppression of the IFN system and acquired T-cell-mediated immunity in and by malignant cells has emerged as an important contributor to the development of the clinical disease. Gene expression profiling and cytogenetic analyses have identified decreases in IFN-stimulated genes (ISGs) in malignancies including RCC (1–18). Epigenetic and genetic silencing of ISG expression in RCC may influence tumor development (19–24). Such observations are probably the partial basis for effectiveness of IFNs and/or inducers for chemoprevention from carcinogen-induced tumors in preclinical models and decreases in hepatocellular carcinoma (25–30). Suppression of T-cell infiltration and function occurs in RCC and other malignancies (31–37). Activation of IL-2-mediated events involving T-cell immunity or the IFN system, as the latter may augment immunomodulatory effects such as dendritic cell (DC) function, continue to have promise and to be explored for future clinical applications (38–40).

This chapter will aim to identify some of the bases for the foregoing predictions through summaries of potential mechanisms of action and clinical results of IFN- γ production by T cells, whether stimulated by IL-2 or other activators, that certainly plays an important role in the immune response, immunosurveillance, and clinical immune augmentation for some pathogens (41–45). However, despite substantial efforts at clinical translation, these preclinical studies of IFN- γ have yet to have to impact oncological disease outcomes (46–52). The focus, thus, in what follows will be on IFN- α 2 and IL-2 and RCC. However, as many of the general principles related to cellular effects of these cytokines have not always been derived from studies of RCC, included will be studies from other histologies for broader understanding of concepts and development of future studies in RCC. Even further insights for understanding how IFNs and IL-2 will further reduce morbidity and mortality from RCC can be obtained from the more than 3,000 articles on RCC and “IFN” or “IL-2” entered in the National Library of Medicine data base (www.ncbi.nlm.nih.gov/entrez). However, recent reviews relevant to biology and application of these cytokines may be found useful as a guide to

and a larger bibliography of pertinent articles underlying biology contributing to clinical activity (53–55).

2 IFNs: Mechanisms of Actions

2.1 *Signaling*

IFNs interact with transmembrane glycoprotein receptors of two types (type I and type II) (56). These receptors are found on both normal and malignant tissue (57). They have been identified on RCC cells (58, 59). Binding of various subtypes of IFNs- α to the receptors have been relatively equivalent although IFN- β likely has greater affinity (58, 59). When assessed against antiviral units, PEGylated IFN- α 2b had essentially the same effects as IFN- α 2 (60). The receptors modulate their activity via cytoplasmic tyrosine kinases of the Janus kinase (JAK) family including JAK1, JAK2, and JAK3 as well as Tyk2 (61, 62). These kinases activate cytoplasmic signal transducers, designated signal transducers and activators of transcription (STATs), that subsequently migrate to the nucleus and induce transcription of ISGs (5).

There are between 100 and 2,000 IFN receptors on any given cell. These transmembrane proteins bind exclusively to IFNs; the type I receptor is specific for IFNs- α , - β , and - ω and the type II receptor binds IFN- γ (1). The type I receptor has two subunits, IFNAR-1 and IFNAR-2 (6–8). These subunits act independently and are associated with the Tyk2 and JAK1 kinases, respectively (8, 9). On the other hand, the type II receptor associated with IFN- γ has two subunits, IFNGR-1 and IFNGR-2, which couple together upon activation (10–12). This intracytoplasmic cascade due to the coupling of IFNGR-1 and 2 is facilitated by activation of JAK1 and JAK2 kinases (13).

The integral role of the JAK family kinases in facilitating activation of the intracellular cascade after IFN stimulation has been demonstrated in vitro in cell lines lacking the JAK family kinases. In these cell lines, the response to IFNs was attenuated (14, 15). The downstream phosphorylation of intracellular tyrosine residues after stimulation of the JAK family kinases facilitates DNA transcription. One of these tyrosine residues phosphorylated is contained within the STAT () protein.

STATs are a family of proteins that convey signals from cell surface activation to DNA transcription by binding to DNA regulatory elements (3). STAT protein recruitment to the activated JAK complexes occurs when phosphorylation of the JAK kinases unveils binding sites for the SH2 domain of the STAT protein. Phosphorylation of the tyrosine residues on the STAT protein facilitates STAT dimerization and subsequent translocation to the nucleus. There, binding to the promoter elements of the DNA yields control of gene transcription. These IFN activated STATs and hence control the transcription of ISG (3).

At least seven STAT proteins have been identified and cloned to date (16). These include STATs 1–4, 5a and 5b, and 6. In vitro experiments delineating mechanism

of intracellular DNA transcription activation have yielded results demonstrating the necessity of particularly STAT1 and STAT2. In fact, STAT1 is essential for the normal functioning of IFN- α , IFN- β , and IFN- γ , whereas STAT2 is only essential for IFN- α and IFN- β (17, 18). Signaling is downregulated by both suppressors of cytokine signaling and SHP-1 and SHP-2 proteins, both of which, if inhibited, could provide therapeutics advantage (18A–18C).

2.2 Angiogenesis Inhibition

Angiogenesis is the physiological process involving the growth of new blood vessels from preexisting vessels. This is an integral component in the natural evolution of a tumor (19). IFNs act on different tumor subtypes to diminish new blood vessel formation by downregulating growth factors or by upregulating inhibitory factors, thereby keeping the net result of angiogenesis in the “off” position. Inhibition precedes the antiproliferative effects of IFN and can be objectively characterized as early as 24 h after IFN exposure (20).

Basic fibroblast growth factor (bFGF) is an angiogenic growth factor (21). Its activity has been linked to several tumor subtypes including bladder, colon, and prostate (21, 22). By way of STAT1 manipulation, the cell surface activation by IFN- α and IFN- β downregulates bFGF. This was demonstrated in vitro where the effects of IFN on bFGF correlated with the presence of STAT1 (23). This downregulation has been correlated in vivo with diminished tumor growth and tumor vascularity (21).

Vascular endothelial growth factor is also a major regulator of tumor-associated angiogenesis (24). Recently developed antitumor agents, such as sunitinib, have activity against VEGF and have been FDA approved for the treatment of VEGF-associated tumors including RCC and gastrointestinal stromal tumor (25). Effect of IFN- α 2 on VEGF gene expression has been investigated as a possible mechanism of action for the observed clinical response of tumor regression with IFNs. In vitro experiments have demonstrated that there are lower VEGF plasma levels and reduced VEGF mRNA levels after exposure to IFN- α (26). This effect is due in part to Sp1/Sp3-mediated inhibition of VEGF gene transcription (26). Not only these angiogenic factors but also chemokines have been implicated in antiangiogenic effects of IFNs in RCC (26A–26B).

2.3 Immune Modulation

In vivo effects of IFNs in tumor surveillance is not entirely understood. Much of the influences of IFNs on tumor surveillance, prevention, and treatment stems from effects on immune modulation. As has been demonstrated in mouse models, the addition of anti-IFN antibodies potentiates the growth of several murine tumors

regardless if the model is athymic or immunocompetent (27). The role of IFNs in immunosurveillance stems from its ability to influence various cell types.

Numerous immune effector cell types including cytotoxic and helper T cells, natural killer (NK) cells, as well as DCs are influenced by IFNs in RCC and other malignancies (28–30). Cell-mediated cytotoxicity is also influenced by the induction of Apo2L/TRAIL expression on immune effector cells as well as the upregulation of major histocompatibility complexes (MHCs) (31, 32). These modulations sensitize tumor cells to cellular-mediated cytotoxicity by T cells and NK cells.

The role of IFNs in immunosurveillance is in part due to its ability to induce the expression of tumor-specific antigens (Ags) on the cell surface of tumor cells. These include carcinoma embryonic Ag and Tag-72 (33, 34). Immune surveillance is also influenced by IFN upregulation of Fc receptors on tumor cell surfaces so as to make them more susceptible to destruction, potentially by way of antibody cross-linking (35).

IFNs also induce several growth factors and cytokines, including ISG-15, some of which can influence proliferation and cellular-dependent cytotoxicity of NK cells and lymphokine-activated killer (LAK) cells (36). In addition, IFNs also have an effect on chemokines that can function as chemoattractants. Several chemokines, including IP-10 and I-TAC, are chemoattractive to lymphocytes and monocytes and influence tumor surveillance (37, 38). Many immune effector genes have been identified as induced by IFNs in RCC cells by expression profiling.

2.4 Apoptosis

The effect of IFNs on direct cellular apoptosis is not completely understood. One possible mechanism deals with nuclear bodies (NBs). These NBs are multiprotein complexes located in the nucleus and are associated with different diseases including leukemia and viral infections (39). The NBs have associated organizer proteins, most of which represent ISGs. One of these organizer proteins is promyelocytic protein. As promyelocytic protein is believed to have a role in cell death, this may represent a mechanism of IFN-associated apoptosis over a broad spectrum (40).

For the most part, the influence of IFNs on cellular apoptosis is a delayed effect, occurring approximately 48 h after IFNs. This delay is a manifestation of gene transcription that is a requirement for IFN-induced apoptosis. These apoptosis-facilitating ISGs have been identified in several disease states including CML and multiple myeloma, however remain elusive in several malignancies (41–43).

In RCC, induction of apoptosis by IFNs occurs through mechanisms involving ISGs. Apo2L/TRAIL (TNF-related apoptosis inducing ligand) has been implicated in other cell types, and although its definitive role has not been established, it is likely an important mediator (41). In RCC cells sensitive to IFN- α 2, apoptosis-associated ISGs were identified but were suppressed in resistant RCC cell lines (38A). Recently, RASS1FA, a tumor suppressor gene, has shed some new insight²². In vitro RCC resistant to IFNs was shown to have an epigenetically silenced tumor

suppressor gene labeled RASS1FA. The addition of a demethylator (5-AZA-dC) or the depletion of DNA methyltransferase 1 (DNMT1) resulted in the activation RASS1FA and hence, an increased sensitization to the effects of TRAIL-induced apoptosis (22)

2.5 Summary

The antitumor effects of IFNs for RCC can be divided into the three broad categories: antiangiogenic, immunomodulatory, and antiproliferative/apoptosis. The first two involve alteration of host response to tumor whereas the latter reflects a direct antitumor cell effect. Which of these – or all – are most important for the antitumor effects in RCC or other malignancies has not been resolved. Regardless, underlying the antitumor effects are the several hundred genes, some of which are discussed above, that are modulated in expression at the transcriptional level. These genes influence cellular and clinical activity of IFNs through effects on cell morphology, cell viability, cell-cycle progression, differentiation and survival, and intercellular interactions.

3 IL-2: Mechanism of Action

3.1 Preclinical Studies of IL-2: In Vitro Studies

Extensive study of the physiology of IL-2 preceded its evaluation as a therapeutic agent. Initially named T-cell growth factor, IL-2 was the first cytokine identified which could numerically expand T cells when exogenously added to cultures (63, 64). IL-2 was observed to be produced by T cells after they were experimentally exposed to plant-derived lectins, proteins which cross-link sugars to provide structural support to carbohydrates (63, 64). By cross-linking then unspecified glycoproteins on the T-cell surface, lectins such as concanavalin A and phytohemagglutinin A simulated antigenic stimulation and consequent IL-2 production. Supernatants from such cultures could be provided to numerically expand CD8⁺ T cells which killed tumor or allogeneic cell targets, but which were not by themselves either IL-2 producers or proliferative in culture (63, 64). This led to early hopes that tumor-specific killer T cells might be raised to therapeutic numbers either in vitro or in vivo by the pharmacological use of IL-2, mimicking its presumed physiological role (65).

Because of the observed inability of classically prepared killer T cells to produce IL-2, it was initially believed that IL-2 production constituted solely a helper response of Ag-activated CD4⁺ T cells, leading to both autocrine (CD4⁺ helper) and bystander (CD8⁺ killer) proliferation (66). Subsequent studies have indicated

that this helper paradigm is only one of many facets of IL-2 physiology, and that additional, supraphysiological impacts are possible when it is administered systemically.

3.2 *Expression and Function of IL-2 Receptor Subunits*

Many different cell types besides T cells can express IL-2 receptor (IL-2R), including subsets of B cells, other lymphocytes, oligodendroglia, and myeloid cells such as macrophages and DCs (67–69). The complete IL-2R complex consists of three chains which may be expressed singly or in combination (70). Whereas the γ -chain (p64, CD132) is also a component of many other cytokine receptors (e.g., IL-4, IL-7, IL-9, IL-13, and IL-15) (71), the α -chain (p55, CD25) uniquely binds IL-2, and the β -chain (p75, CD122) binds only IL-2 and IL-15 (72, 73). In the absence of the α -chain, IL-2 binding to the β -chain or $\beta\gamma$ complex is of relatively low affinity. The α -chain is also of low affinity, but when it complexes to β or $\beta\gamma$, it confers vastly higher binding affinity to the aggregate, probably by inducing a favorable conformational change within the β -chain (74).

From such binding kinetics, it will be apparent that several categories of IL-2 responsiveness can exist in cells (73, 75–77). Cells which constitutively express β or $\beta\gamma$ but not α -chain are, at that juncture, unlikely to be responsive to lower concentrations of rIL-2 (e.g., 24 IU/ml); nonetheless, high IL-2 concentrations (e.g., 6,000 IU/ml) can achieve stimulation through these low affinity forms of the receptor. In contrast, cells that express the high-affinity $\alpha\beta\gamma$ aggregate in a steady state are equipped to respond in real time to lower concentrations of IL-2. Finally, cells which are inducible for α -chain may have divergent responses depending on their α -chain status and the concentration of IL-2 to which they are exposed. These three circumstances are respectively illustrated by large granular lymphocytes (LGLs), CD25^{high} regulatory T cells, and naive effector T cells. Circulating LGLs typically express only the low-affinity $\beta\gamma$ complex, which can be activated by *high-dose* (HD) IL-2 exposure either in vitro (e.g., 6,000 IU/ml) or in vivo to induce Ag-unrestricted lytic activity against a wide variety of tumor targets (75). Such IL-2-activated LGLs were designated “LAK” cells (78). In contrast to LGL, CD25^{high} CD4⁺ regulatory T cells were originally identified by their high steady state expression of the α -chain; the sustained survival of this subpopulation depends upon continued IL-2 stimulation, which is favored by its steady-state coexpression of the β - and γ -chains (79). In contrast, resting effector T cells typically require antigenic T-cell receptor stimulation (Signal 1) and costimulation through surface CD28 (Signal 2) for induction of α -chain and coregulation of $\beta\gamma$ complex expression (76). Therefore, proliferation of resting effector T cells to *low-dose* (LD) IL-2 does not occur in the absence of antecedent signaling. However, at least some resting T cells which preexpress low levels of β -chain can develop LAK activity in response to HD IL-2.

3.3 Helper Role of IL-2 in T1-Type Cell-Mediated Immunity and the Reversal of Anergy

CD4⁺ T “helper” cells naturally differentiate into discrete states, currently designated as Th1, Th2, and Th3, and additional differentiation states such as Th17 have recently been identified (80, 81). The Th1 state is associated with those cell-mediated and complement-mediated immune responses needed to eradicate established infections; the Th2 state is associated with the capacity to neutralize certain infections before they are established, and the Th3 state is a negative regulatory state. In each of these differentiated states, the Ag-activated CD4⁺ T cell is stimulated to produce a different set of cytokines, and only the Th1-type subset constitutes IL-2 and IFN- γ producers. Such helper IL-2 production can stimulate proliferation of Ag-activated CD8⁺ cells, CD4⁺ T cells themselves, and the subset of B cells which secrete complement-fixing classes of antibody, since such B cells express IL-2R (80, 82, 83). In contrast, Th2-type CD4⁺ T cells produce IL-4 and IL-5, proliferogenic for IL-4R or IL-5R expressing B cells which produce neutralizing, secreted or mast cell-binding classes of antibody (certain IgG isotypes, IgA and IgE) (80, 82, 83).

Although animal studies often focus on predominant Th1 versus Th2 responses to particular pathogens, Th1 and Th2 responses can and must coexist under normal circumstances for effective control of infections. For example, a Th1 response to virus may lead to eradication of infected host cells, whereas a coordinated Th2 neutralizing response can prevent continually shed virus from infecting additional cells. Nonetheless, it has long been recognized that IL-2 production by Th1-type CD4⁺ T cells is under much tighter regulation than Th2-type production of IL-4 and IL-5 (80). This is unsurprising, since IL-2 production arms Th1-type CD4⁺ T cells with a nearly autocrine capacity to augment bystander T-cell populations. Whereas Th2-induced responses typically resolve when neutralizing antibody eliminates the extracellular source of stimulatory Ag, even small amounts of persistent intracellular Ag, presented in an MHC-restricted context by infected host cells, may be sufficient to trigger perpetuation of a Th1-type response, well illustrated by the host response to mycobacteria (84).

Before sanctioning the bystander toxicity of an amplified Th1-type response, it is essential that the host discriminate, to the best of its ability, whether persistent Ag presentation in an MHC-restricted context reflects a resolving or persistent infection. Therefore, an intricate and incompletely understood series of antigenic and costimulatory signals are employed by the host to determine whether perpetuated Th1-type responses are worth the risk. For example, exposure of Th1-type CD4⁺ T cells to relevant Ag (Signal 1) in the absence of sufficient costimulation (Signal 2) often results in the Th1-type T-cell's anergy rather than full activation (84). Such costimulatory Signal 2 is normally provided by host Ag-presenting cells (APCs) at the same time that they process and present relevant Ag to T cells. Robust costimulation is more likely when, in addition to antigenic proteins, host APCs are exposed to confirmatory nonantigenic elements of infection, referred to as pathogen-associated molecular patterns (PAMPs). PAMPs include a variety of

molecules normally absent in eukaryotic cells, including lipopolysaccharides (endotoxins), CpG-rich oligodeoxynucleotides, double-stranded RNA, and flagellum-associated protein (85). It may be noted that such PAMPs are seldom present when tumor Ag is subjected to natural processing by ambient host APC (86).

When exposed to Ag in the absence of robust costimulation, or to a nonoptimal concentration of Ag in the presence of costimulation, Th1-type CD4⁺ T cells become tolerized and fail to make the IL-2 needed to achieve further autocrine proliferation (87). Such T cells survive in a nonproliferative state in which they are partially activated, but await additional signals which they cannot provide for themselves. Such tolerance can often be reversed *in vitro* or *in vivo* by exposing the partially activated T cell to a cycle of exogenous IL-2, which may be provided by bystander, nontolerized Th1-type CD4⁺ cells (87, 88). Following that rescue cycle, the previously tolerized CD4⁺ T cell again achieves a resting state in which adequate Signal 1 and 2 stimuli will trigger IL-2 production.

The recurrent risk for suboptimal stimulation and tolerance represents a host-protective physiological checkpoint for Th1-type CD4⁺ T cells. Recent studies also suggest that many CD8⁺ cells cycle through a parallel cycle, but in the case of CD8⁺ T cells, a form of recurrent anergy may occur as an inevitable event (89). Freshly activated CD8⁺ T cells often display a transient, "helper-independent" capacity to produce IL-2, and as a consequence, a transient capacity for autocrine-induced proliferation. Following such abbreviated proliferation, CD8⁺ T cells actively retain signature elements of effector function, including Ag-restricted lytic activity and IFN- γ secretion, but normally become dependent upon an exogenous source of IL-2 for further proliferation, deemed "split tolerance" (90). Exogenous exposure to IL-2, normally provided by activated Th1-type CD4⁺ T cells, overcomes split tolerance and enables CD8⁺ T cells to reattain their own abbreviated capacity for IL-2 secretion upon subsequent antigenic restimulation (90–92). Therefore, CD8⁺ T cells may routinely cycle between helper-dependence and helper-independence.

Since exogenous IL-2 has the capacity to reverse anergy or tolerance in both CD4⁺ and CD8⁺ T cells, it is unsurprising that many CD4⁺ or CD8⁺ T-cell cultures can be perpetuated indefinitely simply by continuing to add exogenous IL-2 during culture expansion (93). It has become increasingly apparent, however, that this convenient strategy for expanding T-cell numbers may contribute to a progressive loss of T-cell therapeutic potency, probably due to deconditioning from the absence of cyclic physiological signaling. Furthermore, recent studies have indicated that, besides Signals 1 and 2, T cells are often dependent *in vivo* upon additional physiological signal(s), presently referred to as Signal 3 (94), not only to prevent the onset of anergy but also to prevent apoptosis (activation-induced cell death) during T-cell proliferation. In the absence of adequate third signaling, proliferation proceeds, but T-cell numbers fail to increase, due to a steady-state apoptosis that offsets any net increase in surviving T cells (94, 95). Many different factors, often expressed by activated host DCs, can provide efficient third signaling, including IL-12, OX40 ligand, 4-1BB ligand, and IFN- α (94–99). The mechanisms of T-cell protection by these agents appear to employ a variety of nonconvergent molecular pathways, each of which leads to resistance against

apoptosis. In addition, it has been reported that IL-2 itself can provide effective third signaling (99). In contrast to the other Signal 3 agents, however, which are typically effective in a wide range of dosing and schedules, IL-2's efficacy as a Signal 3 agent is limited to particular dosing and timing schedules, and varies considerably for each model under study (99–101).

3.4 Characterization of IL-2 Monotherapy in Mouse Tumor Models

In prototypic studies employing IL-2 monotherapy against established mouse tumors, significant antitumor effects of IL-2 were found to require near lethal dosing (600,000–900,000 IU per injection) (102). Vascular leak and hypotensive effects of such HD IL-2 were not yet identified, nor that supportive hydration would have reduced this toxicity. Equivalent doses can be administered with relative safety to patients who receive appropriate hydration and blood pressure support (103).

In subsequent murine studies, the impact of HD IL-2 monotherapy against established, weakly immunogenic methylcholanthrene (MCA)-induced sarcomas was linked by monoclonal antibody (mAb) depletion studies to host CD8⁺ T cells, although not to CD4⁺ T cells (104). In addition, therapeutic responsiveness in these models required the participation of host NK cells, identified by their surface expression of the ASGM-1 glycosphingolipid (104). In the case of even less immunogenic tumors, which stimulated no detectable T-cell responses, the effectiveness of HD IL-2 monotherapy was fully attributable to host NK cells. This pointed to the importance of a non-T-cell mechanism in HD IL-2 antitumor therapeutic effect, but it was also observed, paradoxically, that all of the MCA sarcomas under investigation were fundamentally resistant to lysis by NK cells (104).

3.5 Characterization of LAK Cells

This seeming paradox was quickly resolved by the observation that *in vitro* IL-2 treatment of NK cells (or their human LGL equivalent) armed these cells with a generic capacity to kill most tumor cells. This capacity, deemed LAK cell activity, paralleled a phenomenon previously reported in which monocytes or macrophages treated with IFN- γ were rendered tumoricidal (105). Although their killing mechanisms are different [e.g., LAK cells kill tumor targets within hours whereas tumoricidal macrophages (TM ϕ) often require several days], LAK cells or TM ϕ prepared from a particular mouse or human are capable of recognizing and killing tumor cells not only from that individual but also tumor cells from unrelated donors of the same species (allogeneic) and even tumor cells from other species (xenogeneic) (105–108). Thus, this pattern of tumor killing is totally non-MHC-restricted and does not involve any identifiable tumor Ag.

While nonactivated NK cells themselves have an ability to recognize and kill tumor targets with downregulated MHC expression, they acquire the expanded ability to recognize and kill a vastly wider panoply of tumor cell targets after they experience LAK differentiation (106–108). In addition to targeting malignantly transformed cells, both LAK and TM ϕ also have the capacity to distinguish infected host cells from normal host cells and kill the former (109–111). Recognizable infected targets include not only virally transformed cells but also host cells infected by microbial parasites such as *Toxoplasma gondii* and *Listeria monocytogenes*.

Data point to a greater importance for LAK cells than T cells when HD IL-2 is the primary modality used to treat 3–10 days established mouse tumors: (a) LAK cell activity is strongly promoted in such mice (104); (b) administration of *lower dose* IL-2 can strongly potentiate the therapeutic effect of adoptively transferred anti-tumor T cells in the same models, but has no detectable antitumor effect by itself (112); and (c) even when HD IL-2 results in tumor regression of established animal tumors, long-term antitumor T-cell memory is not generated, and subsequent tumor challenges are not rejected (102, 104). This is in striking contrast to IL-6 or IL-12 monotherapy, each of which promotes long-term antitumor CD8⁺ and CD4⁺T-cell memory as a component of tumor rejection (113–115).

Such observations encouraged in-depth investigations of the therapeutic potential of LAK cells. LAK cells were generated from normal mouse or patient lymphocyte sources by several days of in vitro exposure to HD IL-2, then adoptively transferred into tumor-bearing syngeneic mice or the patients themselves in tandem with exogenous IL-2 (116, 117). Clinical trials demonstrated that there was no therapeutic benefit from cultured LAK cells in excess of that achievable with IL-2 treatment alone (117). However, animal studies also demonstrated that such in vitro-generated LAK cells were extremely limited in their capacity to migrate out of the bloodstream into the tumor bed (116). Therefore, the role of natural LAK cell activation in HD IL-2's therapeutic benefit cannot be surmised from the disappointing therapeutic impact of adoptively transferred, cultured LAK cells.

3.6 IL-2 In Vivo Impact on Defined T Cell Populations

The above animal studies suggest that de novo sensitization of host T cells is not a prominent feature of HD IL-2 monotherapy, even though T-cell sensitization is routinely observed in the same tumor models during successful IL-6 and IL-12 monotherapy. Nonetheless, IL-2 can impact the function of previously sensitized T cells. Recent mouse studies examined the impacts of both vaccination and HD IL-2 upon presensitized, adoptively transferred CD8⁺ T cells specific for the melanoma-associated Ag gp100 (118). Adding HD IL-2 to gp100 vaccination only modestly increased the frequency of anti-gp100 T cells detected within the tumor bed, but the activation status of such T cells was markedly enhanced, as evidenced by their increased spontaneous secretion of IFN- γ upon extracorporealization (118). This IL-2 attributed effect correlated to an enhanced therapeutic outcome, but it was not

determined whether this constituted a reversal of tolerance or merely a potentiation of already licensed effector activity. In the case of CD8⁺ T cells, however, there may be a limited distinction between these mechanisms [see above (90–92)]. Such investigations raise the possibility that exogenous HD IL-2 administration may reverse tolerance and clonally expand T cells in a subset of cancer patients with presensitized, tumor-specific T cells.

Formative culture techniques for propagating antitumor T cells often employed only exogenous IL-2 as a stimulus, giving rise to T cells which were highly lytic for antigenically relevant tumors *in vitro*, but which displayed limited therapeutic efficacy *in vivo*, both in mice and in melanoma patients (119–125). Specifically, adoptive transfer of such T cells was typically ineffective against extrapulmonary tumors, and was also highly dependent upon administration of exogenous IL-2 for its therapeutic effect against pulmonary tumors. When, in contrast, anti-CD3 was added as an ancillary polyclonal stimulus to IL-2-driven cultures, it became possible to propagate both CD4⁺ and CD8⁺ T cells from tumor-bearing animals with remarkably improved therapeutic efficacy. Such anti-CD3 plus IL-2-driven T cells could provide stand-alone adoptive therapy to cure aggressive, established tumors at every tested anatomic location, including the brain (126, 127). This did not require coadministration of exogenous IL-2 or any other factors when provided in adequate doses. Efforts to improve outcomes in melanoma patients have also benefited from the implementation of such second generation culture techniques (128–130).

The biology of such second generation-cultured effector T cells is reviewed in detail elsewhere (119, 131), but is relevant to the present discussion in two regards: (a) adjunct agents can be tested with subtherapeutic doses of stand-alone T-cell preparations to identify which agents are immunopotentiating (100) and (b) the same adjuncts can also be tested with therapeutic (stand-alone) T-cell doses to identify any agents capable of subverting T-cell function (100). Since the newer T-cell preparations can provide effective stand-alone therapy against tumors at any tested location, anatomic disparities for individual adjuncts can also be identified.

Many newer agents are now available for study which deliver third signals to T cells, including IL-12, OX40 ligating mAb, and 4-1BB ligating mAb (94–99). Recent studies compared the impacts of adjunct IL-2 and OX-40 ligating mAb upon T-cell adoptive therapy of both established pulmonary metastases and established intracranial tumors. Coadministration of OX-40 ligating mAb rendered subtherapeutic doses of adoptively transferred T cells highly effective against both advanced pulmonary and advanced intracranial tumors (Fig. 1). In contrast, coadministration of IL-2 rendered subtherapeutic T-cell doses effective against pulmonary tumors, but did not render subtherapeutic T-cell doses effective against intracranial tumors. Coadministration of IL-2 rendered therapeutic doses of T cells ineffective against established intracranial tumors (Fig. 1). Trafficking studies with fluorescently labeled T cells demonstrated that coadministration of IL-2, but not OX40 ligating mAb, subverted accumulation of the T cells within intracranial tumors (100). Because IL-2 has detectable chemotactic properties for T cells, it is possible that pharmacological IL-2 administration disrupts normal patterns of effector T-cell recirculation through at least certain peripheral tissues.

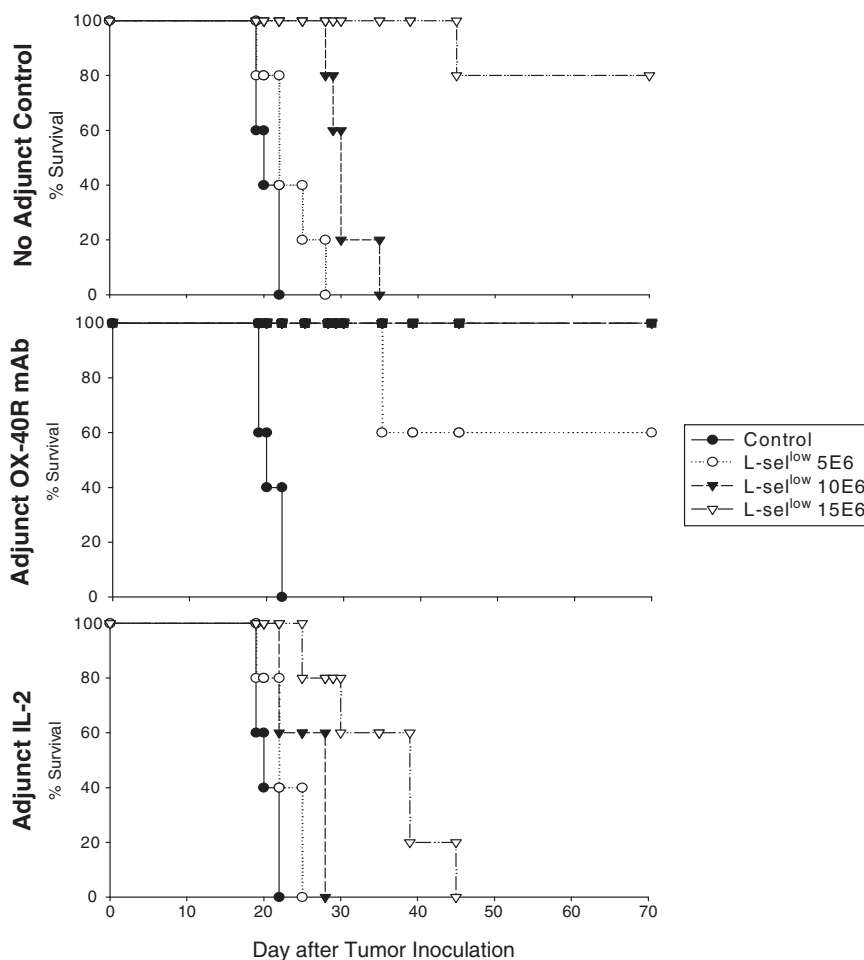


Fig. 1 Effects of conjunctival OX-40R mAb or rIL-2 treatment on adoptive immunotherapy advanced (10-day) intracranial MCA 205 mouse tumors with culture-activated T cells. OX-40R mAb (150 µg) was given i.p. twice on days 0 and 3 after cell transfer. rIL-2 (10,000 U) was given i.p. twice daily for four consecutive days commencing on the day of cell transfer. Each group consisted of five mice. A dose-dependent therapeutic effect of T cells is apparent. Sufficient dosing can cure without adjunct treatments. Adjunct OX-40R mAb enabled lower doses of T cells to be therapeutically effective, whereas adjunct rIL-2 impaired the therapeutic impact of otherwise curative doses of T cells [modified from reference (110)]

3.7 Summary: Preclinical Studies of IL-2

In composite, the above-described studies support several modes by which pharmacological administration of IL-2 could favorably stimulate the immune system for therapeutic purposes in RCC. Exogenous IL-2 may hypothetically serve a

Th1-like function of enabling preactivated CD4⁺ and CD8⁺ memory T cells to proliferate. It may furthermore serve to reverse anergy or tolerance in those effector T cells which have been suboptimally reactivated. Such suboptimal reactivation is suspected to be a frequent natural event in the tumor-bearing host, where tumor Ag is usually presented in the absence of inflammatory danger signals or PAMPs which, in contrast, facilitate T-cell sensitization to infectious agents. Furthermore, IL-2 has the potential to provide third signaling and an associated antiapoptotic effect for proliferating T cells. Finally, higher dose IL-2 can activate tumoricidal (LAK) activity in accessory lymphocytes and monocytes, as described for the LGL subpopulation above.

Recent clinical studies suggest that IL-2 can be employed in a nondetrimental manner to potentiate second generation T-cell adoptive therapy in advanced melanoma patients when also combined with nonmyeloablative chemotherapy. An ongoing trial at the National Cancer Institute has observed a high response rate, with likely prolonged survival in many patients, when anti-CD3 restimulated tumor-infiltrating lymphocytes are adoptively transferred in tandem with cyclophosphamide, fludarabine, and HD IL-2 (128–130). It remains to be determined whether newer Signal 3-type agents will prove more effective and less toxic than IL-2, as suggested by preclinical studies, and whether higher dosed, further refined T-cell preparations will preempt any clinical need for exogenous cytokine administration.

4 IFNs: Clinical Effects

The first cytokine studied in patients with advanced RCC were IFNs. In 1983, documented responses in up to 26% of RCC patients treated with buffy coat impure leukocyte IFN- α on a schedule of 3 MU intramuscularly daily (137, 138). These studies demonstrated for the first time that cytokine therapy could effect tumor regression, and heralded the modern era of immunotherapy in RCC. Since then, a variety of both natural and recombinant IFNs have been evaluated as treatment for metastatic RCC.

The most commonly used IFNs in clinical practice are recombinant IFN- α -2a and recombinant IFN- α -2b, which differ only by a single nonessential amino acid. Both have been studied extensively in patients with metastatic RCC in doses ranging from 3 to 50 MU per day. Response rates range from 0 to 30% (137, 139–153) and the overall response rate is 14.5% (13 complete and 81 partial response, 95% CI 12–17%) in 648 patients (154). Optimal results appear to be associated with doses from 5 to 10 MU/m² (155). Responses occur most frequently in patients with pulmonary metastases and good performance status (139, 155). Median response duration is generally between 6 and 10 months but occasionally durable complete regressions over 2 years occur (139).

Several randomized trials have been conducted in which IFN- α 2 has been compared with other therapeutic approaches. These include comparisons to either

a noncytokine regimen or other cytokines. A trial in which 160 patients received either the combination of IFN- α and vinblastine or vinblastine alone resulted in improvement in both response rates and median survivals in IFN- α -treated patients were noted. The response rates were 16.5% and 2.5%, respectively, and median survivals were 15.8 and 8.8 months. These findings suggest that IFN as a single agent may enhance survival because the clinical activity of vinblastine is minimal (156). A second trial compared IFN- α [10MU subcutaneous (SC) three times weekly] with medroxyprogesterone (300 mg daily) in 335 patients (157). Results with IFN- α were significantly better than medroxyprogesterone. In the group with measurable disease, IFN- α produced responses in 13% of patients compared with 7% in patients treated with medroxyprogesterone. Although only modestly effective, these results suggest that IFN- α can enhance survival in patients with metastatic RCC (8.5 vs 6.0 months) (20).

In a recent meta-analysis (158), results from six randomized trials using SC IFN α involving 963 patients with metastatic RCC suggest that IFN- α is superior to controls (odds ratio for death at one year = 0.67, 95% CI 0.50–0.89). The pooled hazard ratio for survival from this analysis was 0.78 (0.67–0.90) and the weighted average median improvement in survival was 2.6 months. No evidence of a dose–response effect with IFN- α was identified nor was there any correlation between objective response rate and overall survival. Additionally, there was no statistically significant difference when IFN- α was compared with SC IL-2 and there was no difference between IFN- α 2a and IFN- α 2b (159).

Over the last several years, research has focused on identifying prognostic factors predictive of survival and response to cytokine-based treatments. The most widely used prognostic model is that derived from a study performed at the Memorial Sloan-Kettering Cancer Center (MSKCC), with 670 patients treated during clinical trials from 1973 to 1996 (160). Multivariate analysis identified five pretreatment patient-related factors associated with a shorter survival: Karnofsky performance status < 80%, high lactate dehydrogenase (LDH) level (>1.5 times the upper limit of normal), low hemoglobin level (below the lower limit of normal), high corrected calcium level (>10 mg/dl), and absence of nephrectomy. On the basis of these five risk factors, patients were assigned into three groups: those with zero risk factors (favorable risk), those with one or two risk factors (intermediate risk), and those with three to five risk factors (poor risk). After categorization of patients by risk, median survival was found to be 22 months for patients with zero risk factors, 11.9 months for patients with one of the prognostic factors, and 5.4 months for patients with two or three prognostic factors. In 2005, these five risk factors were independently confirmed to predict survival in patients with metastatic RCC (161).

In an attempt to further define the role of cytokines in the treatment of metastatic RCC of intermediate prognosis (>1 metastatic site; >1 year from renal tumor to metastasis), the French Immunotherapy Intergroup conducted a Phase III trial (162). A total of 456 patients were randomized to receive medroxyprogesterone acetate, IFN- α , LD SC IL-2, or the combination of IFN- α plus IL-2. The response rate at 3 months in the four treatment groups were 2.5% for MPA, 4.4% for IFN- α , 4.1% for IL-2, and 10.9% for IFN- α plus IL-2. Unexpectedly, the overall response

rate did not differ between groups (median, 15 months; $p > 0.05$). Although these results would suggest that cytokines should not be used in metastatic RCC of intermediate prognosis, concerns regarding the applicability of this data have been raised (163).

Two randomized trials have demonstrated a statistically significant survival advantage in RCC patients with synchronous metastatic disease who received cytoreductive nephrectomy followed by IFN- α (164, 165). The first trial reported by the Southwest Oncology Group (164) randomized 246 patients to nephrectomy followed by IFN- α compared with single agent IFN- α without surgery. In the 120 eligible patients undergoing adjunctive nephrectomy followed by IFN- α , improvement in survival was noted (11.1 vs 8.1 months; $p = 0.05$). In the second trial, 85 patients were randomized to nephrectomy followed by IFN- α or single-agent IFN- α without surgery. Both time to progression (5 vs 3 months, hazard ratio 0.60, 95% CI 0.36–0.97) and median duration of survival were significantly better in the patients receiving nephrectomy and IFN- α (17 vs 7 months, hazard ratio 0.54, 95% CI 0.31–0.94) (166). These trials suggest that patients with primary tumors in place and metastatic disease should be considered for nephrectomy (if medically possible) before IFN- α . Retrospective reviews of data also suggest this approach can be considered in patients receiving other cytokines such as IL-2 (163).

Both pegylated IFN- $\alpha 2$ and IFN- $\alpha 1$ have yielded objective responses in RCC (167–170). Although only a single Phase II trial has been conducted (170), the added convenience of weekly administration and essentially equivalent efficacy argue for its greater use. In a Phase I trial, IFN- $\alpha 1$ yielded objective responses in RCC with tolerance which suggested an advantage over IFN- $\alpha 2$ (171). Thus, future clinical studies should consider incorporation of these IFNs into trials.

5 IL-2 Clinical Effects

Results of 255 assessable patients enrolled into seven separate Phase II clinical trials involving HD IL-2 administered at a dose of 600,000 or 720,000 IU/kg by 15-min IV infusion every 8 h over 5 days as tolerated have been summarized (172). Patients were scheduled to receive a second identical treatment cycle following 5–9 days of rest, and treatment courses were to be repeated each 6–12 months for stable or responding patients. An overall objective combined response rate of 14%, with 5% complete responses and 9% partial responses. On the basis of these encouraging results, the FDA approved HD IL-2 in 1992 for the treatment of metastatic RCC, and it became the first agent (and biological therapeutic) to be approved for this disease. The only predictor of response and tolerability to HD treatment from these initial studies was performance status, with good performance status patients receiving benefit. The rate of drug-related deaths was 4%, and more than half of patients required administration of vasopressors.

Since then, clinical trials evaluating HD IL-2 therapy in RCC have reported variable objective tumor responses (172–175). Complete responses to this therapy

range from 0% to 13% of treated patients, while partial responses range from 0% to 30% of patients. The early studies reported objective tumor responses after therapy with IL-2 plus LAK cells in cohorts of patients with various cancers (176, 177). However, adoptive transfer of autologous IL-2-activated LAK cells along with bolus infusion of IL-2 (33 $\mu\text{g/kg}$) yielded an OR of only 16% (178) comparable to studies using bolus IL-2 without LAK cells. These results coupled with the difficulty in their growth *in vitro* have resulted in the abandonment of LAK cell therapy for RCC.

Because the objective complete response rate for IL-2 in RCC patients is only 9%, and is only 7% for melanoma patients, statistical powering to confirm these response rates as well as any associated survival advantage motivated a clinical experience in excess of 1,050 patients (103, 132, 133). This constituted an historic enterprise at the National Cancer Institute, driven both by the goal of FDA approval, the novelty of cytokine responsiveness, and the absence of effective alternative treatments for these types of cancer. Follow-up composite survival in the NCI studies has been monitored in excess of 20 years, illuminating a definite and sustained survival advantage for a subset of RCC and melanoma patients who initially had objective responses to IL-2, even without further IL-2 or additional therapy. Such a large-scale clinical experience is unlikely to be repeated for other modestly effective cytokines, particularly since more broadly effective first line treatments such as sorafenib have now become available.

As the consequence of this uniquely extended clinical observation, much is known about the distinctive therapeutic impacts and toxicities of IL-2 treatment, but little is still certain about the mechanism(s) by which IL-2 achieves its therapeutic effect in a fraction of RCC and melanoma patients. Even though the physiological role of IL-2 in T-cell proliferation motivated its therapeutic assessment, there is still no evidence which directly links its therapeutic impact to clonal expansion of antitumor T cells *in vivo*. Further complicating any mechanistic insights is the observation that the pattern of clinical responses is different for melanoma and RCC patients. In the case of RCC, a survival advantage was appreciated not only in patients who attained CRs but also in a group of RCC patients who originally attained stable PRs (103, 132, 133), and has recently been reaffirmed (134, 135). Many of these responsive RCC patients continue to perform symptomatically and radiographically as if their cancer is permanently controlled, whereas, in contrast, IL-2 responding melanoma patients with PR typically recur rapidly. Paradoxically, whereas it is relatively easy to demonstrate antitumor T-cell responses in melanoma patients (136), only in recent years it has become possible to do so sporadically in RCC patients, and no particular T-cell modulation by IL-2 has yet been correlated to therapeutic effectiveness. Finally, the predicted responsiveness in RCC patients to HD versus LD IL-2 varies with the location of disease burden (103, 132–135). Such data indicate that IL-2 may have a number of therapeutic mechanisms, each of varying relevance to individual types of cancer.

Due to the significant toxicity from HD IL-2, alternative LD regimens have been developed and studied in patients with metastatic RCC. The efficacy of LD IV bolus IL-2 (72,000 IU/kg/dose every 8 h for 5 days each cycle, two cycles per

course of treatment, two or more courses of therapy as tolerated) was compared with HD infusion (720,000 IU/kg/dose) in patients with RCC (179). Although patients in the LD IL-2 therapy group did not receive more than 3.6 million cumulative IU of IL-2 (median of 27 doses per course) compared with accumulated doses up to 21.6 million IU in the HD group (median of 13 doses per course of therapy), the two regimens yielded comparable objective response rates (15% and 17%, respectively) with an actuarial 1 year survival rates of 74% and 78%, respectively. Complete responses were durable (≥ 7 months). HD IL-2 induced significantly greater thrombocytopenia, hypotension, and malaise than LD IL-2, but the latter group experienced a greater number of bacterial infections.

The toxicity of bolus intravenous IL-2 depends on the dose used. A flu-like syndrome that includes fever, chills, and myalgias is experienced by most patients. Cardiovascular, pulmonary, and central nervous system toxicities are associated with HD IL-2 (179). Cardiovascular toxicity includes hypotension requiring vasopressors, cardiac arrhythmias, myocardial infarction, and myocarditis. Pulmonary toxicity secondary to capillary leak syndrome may develop and require mechanical ventilation. Grade 3 renal toxicity with oliguria can occur in more than 20% of treated patients, with creatinine levels over 8 mg/dl reported in 2%. Confusion and neuropsychiatric complaints are also common. Careful patient selection is required to minimize the morbidity and mortality associated with administration of HD IL-2. Recent guidelines for the management of HD IL-2 toxicity have been developed (180).

The short half-life of intravenous bolus IL-2, as well as the potential to deliver equivalent biological doses without the need for intensive care monitoring, resulted in the use of continuous intravenous (CIV) infusion of this cytokine. Various administration schedules and doses have been used. As approved for use in Europe, most of the early trials were conducted with IL-2 at a dose of 18 MIU/m²/day for 5 days, which was repeated after a 1 week rest. Reported response rates vary, but in a group of 922 patients recently reviewed, the overall response rate was 13.3% (171, 181). In a randomized trial using CIV IL-2, IFN α , or the combination, the response rate in 138 patients receiving IL-2 was 6.5% (similar to SC IFN α) with 94 of 138 patients developing hypotension resistant to vasopressor agents (182). To date, no dose-response relationship has been observed with CIV IL-2.

SC IL-2 has also been used in metastatic RCC patients, but published experience is limited. Most reports involve fewer than 25 patients, and in a group of 290 patients recently reviewed (183), a response rate of 16.8% was found. In several series (184, 185), SC IL-2 is initially administered at a higher dose level for 2–5 days followed by maintenance IL-2. Butler et al. (186) noted two complete responses lasting for 29.0 to >35.0 months, suggesting some of these responses may be durable. The SC route is associated with less toxicity; however, it does produce significant fatigue, fever, and malaise. Development of SC nodules at injection sites has also been noted. Hypotension and the capillary leak syndrome are uncommon with this administration route.

In an attempt to help clarify the role of HD and LD IL-2 regimens for the treatment of patients with metastatic RCC, two randomized trials have been conducted. Yang et al. (187) recently reported the results of a randomized trial to determine the

effectiveness of SC IL-2 (94 patients) compared with LD (150 patients) and HD bolus IL-2 (156 patients) in a total of 400 patients with metastatic RCC. There was a higher response rate with HD IL-2 (21%) versus LD IL-2 (13%; $p = 0.048$) but no overall survival difference. The response rate of SC IL-2 (10%, partial and complete response) was similar to that of LD IL-2. Response durability and survival in completely responding patients was superior with HD IL-2 compared with LD IL-2 therapy ($p = 0.04$). As expected, toxicities were significantly less frequent with both LD IL-2 and SC IL-2, especially hypotension. The second trial, conducted by the Cytokine Working Group and recently by Bui et al. (188), randomized 192 patients with metastatic RCC to receive either outpatient SC IL-2 and SC IFN- α combination therapy (96 patients) or HD IL-2 (96 patients) therapy. The response rate was 23.2% (22 of 95 evaluable patients) for HD IL-2 versus 9.9% (9 of 91 evaluable patients) for the combination of SC IL-2 and SC IFN α . Ten patients receiving HD IL-2 were progression free at 3 years compared with three patients receiving combination therapy, and the median survival favored HD IL-2 although was not statistically significant. In this study, patients with bone or liver metastasis and primary tumor in place had a superior survival with HD IL-2 ($p = 0.040$). Neither study has demonstrated a clear survival advantage to HD IL-2, although objective tumor responses and the durability of response in complete responders appear to be improved.

Recently, RCC response to IL-2 and patient survival has been correlated to histology (clear cell and alveolar features) (189), as well as carbonic anhydrase IX (G250 Ag) expression (190). Retrospective analysis of paraffin-embedded tissue sections of RCC from 66 patients enrolled in a previously reported Cytokine Working Group trial (190) demonstrated high carbonic anhydrase IX (CAIX) expression in 78% (21 of 27) patients who responded to HD IL-2 therapy compared with only 51% (20 of 39) patients who did not respond to therapy. In this study, the percentage of CAIX positive tumor cells was utilized to separate high (>85%) versus low ($\leq 85\%$) expressers. When combining good and intermediate pathology (190) with high expression of CAIX, the resultant group contained 96% of responders to HD IL-2 therapy compared with only 46% of nonresponders. Prospective study of CAIX expression as a surrogate to predict response to HD IL-2 is ongoing.

6 Perspective

IFN- $\alpha 2$ and IL-2 were the first therapeutics effective for treatment of RCC. As the first human clinical product for cancer from recombinant DNA technology, IFNs were a prototype for the clinical development of IL-2. The groundwork for future advances has been laid by demonstration of clinically useful activity of IFNs and IL-2 in increasing survival from RCC through biological modulatory effects on gene expression, apoptosis, angiogenesis, and immunological function. Potent modulation of gene expression, which is reviewed above, must underlie the clinical activities of IFNs and IL-2. A significant regulatory pathway for gene induction, the

JAK-STAT pathway, was originally elucidated by the study of IFNs but has proven to be critical for signaling by IL-2 in addition to other cytokines.

A series of recently completed and ongoing clinical trials will partially define the future role of IFNs and IL-2 in sequence and in combination with newer “targeted” agents. Improved selection of patients for IL-2 based on molecular markers such as CAIX could allow exclusion of patients unlikely to show a response to HD IL-2. The role of IFNs as primary therapy for patients with metastatic RCC will depend upon the results of Phase III trials in combination with newer targeted agents (sorafenib, sunitinib, CCI-779, and bevacizumab). IFN- α 1 could offer equivalent or improved responses with fewer side effects. Much potential for additional application of IFNs, IL-2, or their inducers as anticancer protein therapeutics thus remains, particularly when one considers that their cellular and molecular effects, have yet to be fully elucidated. Additional innovative ideas, when translated into therapeutic trials, should substantially broaden the application IFNs and IL-2 for RCC over the next decade.

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The Molecular Biology of Kidney Cancer and Its Clinical Translation into Treatment Strategies

William G. Kaelin Jr. and Daniel J. George

Abstract The most common histologic type of kidney cancer is clear cell renal carcinoma. Most clear cell renal carcinomas are linked to somatic inactivation of the von Hippel–Lindau tumor suppressor gene (*VHL*), either as a result of mutations or, less commonly, hypermethylation. The *VHL* gene product, pVHL, has multiple functions. The best understood function, and the one most tightly linked to renal carcinogenesis, is to serve as the substrate recognition component of an ubiquitin ligase complex that targets the HIF α transcription factor for destruction when tissue oxygenation is adequate. In hypoxic tumor cells, or in tumor cells lacking functional pVHL, HIF α becomes stabilized, binds to its partner protein HIF β (also called ARNT), and transcriptionally activates ~100–200 genes that promote adaptation to hypoxia including the genes encoding vascular endothelial growth factor (VEGF) and platelet-derived growth factor B (PDGF B). This probably explains the neoangiogenesis that is typical of clear cell renal carcinomas and their sensitivity to drugs, such as sorafenib, sunitinib, and bevacizumab, that inhibit VEGF or its receptor KDR. In addition to pVHL, HIF α levels are also influenced by activity of the mTOR kinase. pVHL-defective tumor cells are sensitive to mTOR inhibitors in preclinical models and mTOR inhibitors have recently demonstrated activity in the clinic for the treatment of kidney cancer.

Keywords von Hippel–Lindau • Hypoxia • HIF • VEGF • KDR • mTOR • Angiogenesis

1 Introduction

There are ~30,000 new cases of kidney cancer, and 12,000 kidney cancer-related deaths, each year in the United States. The most common form of kidney cancer is clear cell renal carcinoma. Forty to eighty percent of clear cell renal carcinomas are linked to biallelic inactivation of the von Hippel–Lindau tumor suppressor

W.G. Kaelin Jr. (✉)

Dana Farber Cancer Institute, 44 Binney Street, Boston, MA 02115

e-mail: william_kaelin@dfci.harvard.edu

gene (*VHL*), either as a result of somatic mutation or hypermethylation. The importance of this gene with respect to renal carcinoma first came to light as a result of studies of von Hippel–Lindau disease, which is caused by germline *VHL* mutations and characterized by an increased risk of several tumors, including clear cell renal carcinoma. Laboratory studies have begun to elucidate the biochemical functions of the *VHL* gene product, pVHL. This knowledge has provided new mechanistic insights into the pathogenesis of clear cell renal carcinoma and has already motivated successful clinical trials of drugs that inhibit vascular endothelial growth factor (VEGF), which is an angiogenic polypeptide that is overproduced when pVHL function is compromised.

2 von Hippel–Lindau Disease

von Hippel–Lindau disease is a hereditary cancer syndrome that was first described about 100 years ago and affects approximately 1 in 35,000 people (1, 2). The disease is transmitted in an autosomal dominant manner although, like most hereditary cancer syndromes, it is actually caused by recessive mutations (see below). The cardinal features of this disease are visceral cysts in organs such as the kidney and the pancreas and neoplasms including clear cell renal carcinomas, central nervous system and retinal hemangioblastomas, pancreatic islet cell tumors, endolymphatic sac tumors, and pheochromocytomas. Renal cell carcinomas (RCCs) and hemangioblastomas are the two leading causes of death in this disease.

Essentially all individuals with a clinical diagnosis of VHL disease have inherited a defective *VHL* allele, which resides on chromosome 3p25, from one of their parents (3). Pathological changes ensue when the remaining wild-type *VHL* allele is inactivated or lost in a susceptible cell, thereby depriving the cell of the normal *VHL* gene product, pVHL. The organ-specific risk of tumor development in VHL disease is influenced by the nature of the *VHL* mutation (genotype–phenotype correlations). Some *VHL* mutations cause Type 1 VHL disease (low risk of pheochromocytoma) and others Type 2 VHL disease (high risk of pheochromocytoma). Type 2 VHL disease has been subdivided into 2A (low risk of RCC), 2B (high risk of RCC), and 2C (pheochromocytoma only without other usual disease manifestations) (4). The *VHL* gene is expressed widely throughout the body and it remains a mystery as to why certain organs are susceptible to tumor development when *VHL* is mutated and most others not.

3 VHL mutations in Sporadic Cancers

In keeping with the Knudson 2-Hit Model, inactivation of both the maternal and paternal *VHL* alleles as a result of somatic mutations (or, less frequently, hypermethylation) is common in nonhereditary hemangioblastomas and clear cell renal

carcinomas (5). The frequency of loss of heterozygosity (LOH) at the 3p25 locus in sporadic kidney cancer (>90%) is even higher than the frequency of *VHL* mutations (~50%) (6). This suggests that 3p harbors additional tumor suppressor genes relevant to kidney cancer and/or that haploinsufficiency at the *VHL* locus contributes to kidney cancer pathogenesis in some settings.

In contrast to hemangioblastoma and clear cell renal carcinoma, somatic *VHL* mutations are rare in sporadic pheochromocytomas unless there is also a previously unrecognized germline *VHL* mutation. A similar conundrum exists for other genes linked to familial pheochromocytoma and the related tumor paraganglioma (*NFI*, *c-Ret*, *SDH B*, *SDH C*, *SDH D*) (7). A recent study suggested that the genes linked to familial pheochromocytoma/paraganglioma, including *VHL*, regulate the survival of sympathoadrenal precursor cells during a specific developmental window characterized by extensive apoptosis (8). Conceivably these genes are no longer relevant to pheochromocytoma/paraganglioma pathogenesis once this window has passed.

Somatic *VHL* mutations are rare in tumors other than clear cell renal carcinoma and hemangioblastomas. *VHL* mutations are, however, occasionally observed in colorectal carcinoma (9, 10). A recent paper suggested that there is significant crosstalk between pVHL and the Wnt pathway in colorectal carcinoma and that genetic alterations that directly (*VHL* mutations) or *indirectly* downregulate pVHL contribute to colorectal carcinogenesis (11).

4 The VHL Protein

The *VHL* gene encodes a protein containing 213 amino acid residues (12). A shorter form of the protein is also generated as a result of translational initiation from an alternative, in-frame, start codon (13–15). In many cells this shorter pVHL isoform is actually the major *VHL* gene product. Both isoforms behave very similarly in most of the biochemical and functional assays performed to date. For simplicity, both isoforms will therefore be referred to generically as “pVHL” in this review.

pVHL resides in both the cytoplasm and nucleus and dynamically shuttles between these two cellular compartments (12, 15–20). In addition, some pVHL associates with mitochondria and with the endoplasmic reticulum (21, 22). A recent study showed that pVHL subcellular localization is influenced by changes in extracellular pH, with low pH promoting its sequestration in nucleoli (23, 24). In addition, pVHL abundance seems to be influenced by cell density (25).

pVHL forms a complex with elongin B, elongin C, Rbx1, and Cul2 (2). This complex possesses ubiquitin ligase activity and targets the alpha subunits of the heterodimeric transcription factor called HIF (hypoxia-inducible factor) for proteasomal degradation when oxygen is available (2). Interestingly, pVHL contains two mutational hotspot regions called the alpha domain and the beta domain. The alpha domain binds directly to elongin C and nucleates the formation of the complex (26, 27). The beta domain is a substrate docking site and binds directly to HIF α family members (of which there are three, called HIF1 α , HIF2 α , and HIF3 α) (26, 28).

The interaction of pVHL with HIF α is oxygen dependent because HIF α must be prolyl hydroxylated, which is an oxygen-dependent posttranslational modification, in order to be recognized by pVHL (29, 30). This modification, which can occur on either of two prolyl residues within HIF α , is catalyzed by members of the EglN family (also called PHD family or HPH family). EglN1 appears to be the primary HIF prolyl hydroxylase under normal conditions and is highly sensitive to changes in oxygen availability as well as to changes in cellular redox status (31–33). Under low oxygen conditions, or when pVHL function is compromised, HIF α subunits accumulate and heterodimerize with a HIF β (also called ARNT) family member. These heterodimers bind to specific DNA sequences (hypoxia-response elements) and transcriptionally activate a suite of genes involved in adaptation to hypoxia including genes linked to erythropoiesis (such as erythropoietin), angiogenesis [such as VEGF and platelet-derived growth factor B (PDGF B)], glucose uptake and metabolism (such as GLUT1), pH control (such as carbonic anhydrase), and extracellular matrix control (such as MMP2 and MMP9). Gene expression profiling suggests that perhaps 100–200 genes are regulated by HIF (34–38).

pVHL can interact with other cellular proteins, some of which might also be ubiquitination targets (39, 40). These pVHL-binding partners include fibronectin, atypical PKC family members, Rpb1, Rpb7, and VDU-1. Biochemical and genetic data suggest that pVHL regulates the formation of a stable extracellular matrix via both HIF-dependent and HIF-independent pathways (41–49). pVHL has also been implicated in the regulation of hypoxia-inducible mRNA stability (in addition to its effect on hypoxia-inducible mRNA transcription) (50, 51), possibly through a physical interaction with the mRNA-binding protein HuR (52, 53), and in the regulation of microtubule stability through a direct physical interaction with tubulin (54).

The genotype–phenotype correlations described above presumably reflect the degree to which various pVHL functions are altered by different *VHL* mutations. In this regard, the products of Type 1, Type 2A, and Type 2B alleles are defective with respect to HIF regulation. A recent study, however, suggested that Type 2A pVHL mutants retain greater residual HIF-binding capability than do Type 2B pVHL mutants (55), which might account for their lower risk of RCC (see also below). Type 2A and Type 2B pVHL mutants also differ with respect to microtubule stabilization (54). Type 2C pVHL mutants are seemingly normal with respect to HIF regulation but are compromised with respect to fibronectin matrix assembly and regulation of aPKC (8, 43, 56). Dysregulation of aPKC appears to be responsible for the enhanced survival of pVHL-defective sympathoadrenal precursors that has been postulated to result in pheochromocytoma development (8).

5 VHL and the Biology of Clear Cell Renal Carcinoma

VHL patients with Type 1 or Type 2B *VHL* mutations develop numerous preneoplastic renal cysts. Immunohistochemical and laser capture microdissection studies indicate that the cells lining these cysts have lost the remaining wild-type *VHL* allele and overproduce various HIF-responsive gene products (57–60). It is presumed that

mutations at other genetic loci, occurring stochastically, are required to convert these cysts into RCCs.

Restoration of pVHL function in *VHL*^{-/-} clear cell renal carcinoma lines using gene transfer techniques does not affect their proliferation under standard cell culture conditions but suppresses their ability to form tumors in nude mice xenograft assays (12, 50). pVHL does inhibit cell proliferation and promote differentiation of *VHL*^{-/-} renal carcinoma in vitro under special cell culture conditions, such as under low serum conditions or in three-dimensional spheroid assays (47, 61, 62). *VHL*^{-/-} renal carcinoma cells have lost certain epithelial cell characteristics, perhaps reflecting an epithelial–mesenchymal transition, and produce a disorganized extracellular matrix (42, 47, 49, 63). These abnormalities, which are corrected after reintroduction of wild-type pVHL, appear to reflect the loss of both HIF-dependent and HIF-independent pVHL functions (42).

A number of earlier observations regarding clear cell renal carcinoma can now be rationalized based on the knowledge that pVHL regulates HIF, and consequently HIF target genes. For example, it has been appreciated for some time that these tumors are highly angiogenic and produce very high levels of VEGF. In addition, these tumors sometimes elaborate erythropoietin, leading to paraneoplastic erythrocytosis. Carbonic anhydrase IX, which is a HIF-responsive gene product, is frequently overproduced by clear cell renal carcinomas and an antibody against this protein is being developed as a potential diagnostic and therapeutic agent for this disease (64). A recent study also suggested that HIF dysregulation contributes in some fashion to increased NFκB activity in pVHL-defective cells (65), which might play a role in the chemoresistance and radioresistance that is typical of clear cell renal carcinoma.

pVHL-defective renal carcinomas appear to have a better prognosis, however, than pVHL-proficient renal carcinomas (66). Although the molecular basis for this observation is not clear it will need to be considered when evaluating the impact of *VHL* status on response and survival after treatment with investigational drugs, such as those described below.

6 Validation of HIF as a Therapeutic Target in Renal Carcinoma

There are three HIFα family members in the human genome (HIF1α, HIF2α, and HIF3α). HIF2α appears to be especially important with respect to renal carcinogenesis based on the following observations. First, HIF1α appears to be upregulated in the earliest recognizable *VHL*^{-/-} preneoplastic renal lesions (57). The subsequent appearance of HIF2α in such lesions is associated with increased dysplasia (57). Second, *VHL*^{-/-} renal carcinomas either express both HIF1α and HIF2α or exclusively HIF2α (67). Third, tumor suppression by pVHL can be overridden by a HIF2α variant that cannot be prolyl hydroxylated, but not the corresponding HIF1α variant (68–70). Finally, elimination of HIF2α in *VHL*^{-/-} renal carcinoma cells, like restoration of pVHL function, inhibits their growth in vivo in nude mice xenograft assays (68, 71).

Collectively, these results validate HIF2 α as a potential therapeutic target in *VHL*^{-/-} renal carcinomas. Unfortunately, DNA-binding transcription factors have not proven to be highly tractable as drug targets with the exception of the steroid hormone receptors. Alternative strategies would include the use of drugs that *indirectly* downregulate HIF α and the use of agents that target HIF-responsive proteins implicated in tumorigenesis.

6.1 *Targets that Affect HIF α Accumulation*

A number of agents have been identified that downregulate HIF α protein levels. For example, HIF α , perhaps because of its high metabolic turnover rate, is very sensitive to agents that inhibit protein synthesis (such as mTOR inhibitors) (72–75) or inhibit protein folding (such as HSP90 inhibitors) (76–79). A recent report showed that pVHL-defective renal carcinoma cells display increased sensitivity to mTOR inhibitors relative to *VHL*^{+/+} renal carcinoma cells and that this sensitivity is due to downregulation of HIF α (80). HDAC inhibitors also downregulate HIF α levels (81, 82), apparently by affecting HSP90 acetylation (83, 84). In addition to downregulating HIF α , HSP90 inhibitors can, inhibit a number of proteins that act downstream of HIF, such as EGFR and Cyclin D1 (see below) (85, 86). There are also a number of agents that downregulate HIF α where the mechanistic link between their ostensible target and HIF accumulation remains obscure. These include drugs that inhibit thioredoxin (87, 88), topoisomerase I (89, 90), and certain microtubule agents (91). A caveat with all these agents, especially those with unclear mechanisms of action, relates to specificity. For the reason cited above, HIF α is one of the first proteins to disappear when protein translation rates fall. Drugs that interfere with cellular homeostasis and secondarily inhibit protein translation might appear to inhibit HIF when, in fact, their effects are quite broad.

6.2 *HIF-Responsive Targets*

As mentioned above, there are perhaps 100–200 genes that are HIF responsive and consequently deregulated when pVHL function is compromised. A number of these genes encode proteins that can be inhibited (directly or indirectly) with drugs and for which there is a credible link to renal carcinogenesis. A prototypical example is VEGF, which promotes endothelial cell proliferation and is implicated in tumor angiogenesis. Upregulation of VEGF probably minimizes the selection pressure on pVHL-defective cells to develop additional means to promote angiogenesis (such as the elaboration of alternative angiogenic peptides) during tumor progression. This might explain why clear cell renal carcinoma is the only solid tumor where inhibitors of VEGF, or its receptor KDR, have single agent activity (see below).

The HIF responsive growth factor PDGF stimulates pericyte coverage of newly sprouting blood vessels. In preclinical models, dual inhibition of both VEGF (and hence endothelial cells) and PDGF (and hence pericytes) is required to regress established blood vessels, in contrast to immature vessels (92, 93). Fortuitously, KDR and the PDGF receptor are structurally related and some small molecule KDR inhibitors also inhibit PDGF signaling. An organized extracellular matrix can serve to sequester angiogenic factors such as VEGF and inhibition of the HIF-responsive matrix metalloproteinase MMP2 has been shown to inhibit VEGF-dependent angiogenesis (94).

The growth factors listed above are believed to act in paracrine fashion. Another HIF-responsive growth factor, TGF α , is believed to establish an autocrine loop by binding to the EGFR and promoting renal carcinoma cell proliferation (95, 96). In preclinical models, interruption of this loop is sufficient to inhibit pVHL-defective tumor growth (96). The growth factor SDF, and its receptor CXCR4, are both HIF-responsive and are also suspected of establishing an autocrine loop that might play a role in tumor cell invasion and metastasis (97, 98).

In renal epithelium, but not other epithelia, pVHL loss leads to HIF-dependent upregulation of Cyclin D1 (25, 38, 99, 100). This might help to explain why pVHL inactivation is tightly linked to kidney cancer and not other epithelial cancers. Cyclin D1, when bound to its catalytic partners cdk4 or cdk6, promote the phosphorylation, and hence inactivation, of the RB tumor suppressor protein (101). This, in turn, accelerates cell-cycle progression and proliferation. These observations suggest that drugs that inhibit cdk4/6 activity, or drugs that indirectly downregulate Cyclin D1 (such as HSP90 inhibitors or, theoretically, Pin1 prolyl isomerase inhibitors (102)), might eventually play a role in the treatment of clear cell renal carcinoma.

7 VEGF-Targeted Therapy in RCC

Inhibition of VEGF signaling has proven to be clinically effective as a molecularly targeted strategy in the treatment of patients with metastatic kidney cancer, and clear cell carcinoma in particular, and is based on the strong biologic rationale outlined above. Composed of a family of at least five different ligands (VEGF A–E) and three different receptors (VEGFR1–3), VEGF signaling has pleiotrophic effects, but is most prominently associated with promoting endothelial proliferation, survival, and angiogenic physiology (103). Therapeutic strategies have included sequestration of VEGF (most notably VEGF-A growth factor) using a neutralizing antibody and inhibition of VEGF tyrosine kinase receptor activation by small molecules, typically with a multitargeted agent affecting additional, structurally related, tyrosine kinase receptors. Inhibition of other VHL-regulated growth factor targets has not yet demonstrated substantial clinical efficacy as monotherapy; however, mTOR inhibition alone has demonstrated clinical benefit. The combined effects of VEGF inhibition with mTOR inhibitors and other potential modulators of HIF is actively being pursued in ongoing Phase I and II clinical trials in patients with advanced RCC.

8 VEGF Neutralizing Antibody Therapy in RCC

Bevacizumab (Avastin®, Genentech, San Francisco, CA) is a murine monoclonal IgG antibody, 93% humanized, with specificity for VEGF-A. This agent, which has a terminal half-life of 21 days, demonstrated antitumor activity in preclinical mouse models and was the first antiangiogenic agent to demonstrate clinical benefit in cancer patients in clinical trials (104).

In the mid 1990s, multiple Phase I and II trials were initiated using bevacizumab in patients with a variety of solid tumors. Of these, one of the more encouraging initial results came from a Phase II randomized, placebo-controlled trial of 116 patients with metastatic RCC, performed by Yang et al. (105). All patients were required to have measurable disease to enter the study and to have received prior cytokine therapy (108 patients) or to have been ineligible for prior treatment with cytokines (8 patients). Patients were randomized to receive either placebo, low-dose bevacizumab (4.5 mg/kg Day 1 followed by 3 mg/kg Day 7 and alternating weeks thereafter), or high-dose bevacizumab (15 mg/kg Day 1 followed by 10 mg/kg Day 7 and alternating weeks thereafter) in a blinded fashion.

The primary end point of the study was the time to disease progression (TTP). The study was stopped early at the second planned interim analysis due to a significant improvement in the TTP identified for patients in the bevacizumab arms compared to placebo. In those patients receiving high doses of bevacizumab (10 mg/kg), the median TTP was 4.8 months versus 2.5 months for the placebo group ($p < 0.001$) (see Table 1). The median TTP in the low-dose group was 3 months, which was also a statistically significant improvement over placebo ($p = 0.041$). Bevacizumab was well tolerated although the occurrence of side effects expected for a VEGF inhibitor, such as hypertension (36% of patients, 20% of which met criteria for Grade 3 hypertension) and proteinuria, was significantly greater in the high-dose

Table 1 VEGF-targeted, mTOR-targeted, and multitargeted therapy in RCC

Cytokine-refractory RCC (Phase II or III)	Partial or complete response (%)	Stable disease 3 months or longer (%)	Median time to progression (months)	Median overall survival (months)
Bevacizumab 10 mg/kg versus placebo (105)	10 versus 0	NA	4.8 versus 2.4 ($p < 0.001$)	NA
Bevacizumab 3 mg/kg versus placebo (105)	0	NA	3.0 versus 2.4 ($p = 0.04$)	NA
Sunitinib ^a (112)	34	29	8.2	16.4
Sorafenib versus placebo ^a (120, 128)	2 versus 0	78 versus 55	6.0 versus 3.0 ($p < 0.000001$)	19.4 versus 15.9 ($p = 0.15$)
Temsirolimus (126)				
25 mg	5.6	52.8 ^b	6.3	13.8
75 mg	7.4	55.3 ^b	6.7	11.0
250 mg	8.1	43.2 ^b	5.2	17.5

^aIndependent assessment

^bCR, PR, or SD for 6 months

arm compared with the placebo group. Malaise (fatigue), fever, and epistaxis were also more frequent in the treated group.

On the basis of these encouraging Phase II results, two randomized Phase III trials were initiated to investigate the efficacy and safety of bevacizumab in combination with interferon alpha versus interferon alpha alone as first-line therapy for patients with metastatic RCC. The first is a Phase III intergroup study (CALGB 90206), which has recently completed accrual (732 patients) but has not yet been reported (106). The second study, a European study supported by Roche pharmaceuticals (690 patients), is placebo controlled. Neither study includes a crossover and both are powered to detect differences in survival. Results are expected in 2007.

Meanwhile, bevacizumab has been tested in combination with additional targeted agents in RCC, most notably with the epidermal growth factor receptor (EGFR) inhibitor, erlotinib (Tarceva, OSI Pharmaceuticals, Melville, NY). In single arm, Phase II, testing bevacizumab was combined with erlotinib in patients with metastatic RCC (107). Prior therapy was not an eligibility criteria and patients continued on daily oral erlotinib (150 mg) and biweekly bevacizumab (10 mg/kg) until overall disease progression or intolerable adverse events. In 59 evaluable patients, most of whom were good or intermediate risk by Motzer first-line criteria, 25% (95% CI 16–37%) demonstrated an objective response, with a median progression-free survival (PFS) of 11 months. While encouraging, these results needed a randomized trial to definitively demonstrate additive benefit to the combination.

Toward this end, Bukowski et al. conducted a randomized Phase II trial of the combination of high dose bevacizumab with or without erlotinib at standard doses in untreated metastatic RCC patients with clear cell histology and predominantly low to intermediate risk based on Motzer criteria (108). Preliminary results of this study were reported at ASCO 2006. In 104 patients, PFS was not significantly different between the two arms, in patients treated with bevacizumab alone demonstrating a median PFS = 8.5 months and in those treated with the combination demonstrating a median PFS = 9.9 months (HR 0.86, 95% CI 0.50–1.49, $p = 0.58$). Overall response rates were similar for the two arms (14% vs. 13%) but toxicities, including diarrhea, proteinuria, hypertension, and rash, were more common and higher grade in the combination arm. While the results do not support a synergistic effect of combined VEGF and EGFR inhibition, the single arm results of bevacizumab alone in this front-line setting compare favorably against historical controls for interferon and further support its evaluation in this setting.

9 Multitargeted Tyrosine Kinase Inhibition in RCC

Since imatinib mesylate (Gleevec®, Novartis Pharmaceuticals, East Hanover, NJ) demonstrated robust clinical activity in patients with chronic myelogenous leukemia and subsequently in patients with metastatic gastrointestinal stromal tumor, enormous interest has grown around the development of orally available tyrosine kinase inhibitors (109, 110). Similar agents have been developed since with

selectivity to specific tyrosine kinase receptors, including most notably the VEGF receptor family. These agents typically act as competitive inhibitors of the ATP-binding domains of their receptor tyrosine kinase targets. Variant amino acids that flank kinase ATP-binding domains can affect small molecule binding and therefore form the basis for selectivity. Nonetheless, none of the agents developed to date are completely selective. The number and affinity of additional targets inhibited by these agents is likely to be one of the more important features separating their activity and tolerance from others in their target class.

VEGF has demonstrated clinical importance in a number of solid tumors. However, RCC appears to be particularly susceptible to VEGF blockade. Over the past 5 years, several multitargeted agents with VEGFR as a target have reported activity in Phase II RCC clinical trials (111–115). At the same time, several other multitargeted tyrosine kinase inhibitors that do not inhibit VEGFR have not demonstrated clinical activity in this population (116–118), supporting the importance of VEGFR in the multitargeted profile. In particular, two multitargeted agents, sunitinib malate (Sutent®, Pfizer Pharmaceuticals, New York, NY) and sorafenib (Nexavar®, Bayer/Onyx Pharmaceuticals, West Chester, PA), have demonstrated clinical benefit in Phase III studies and have now been FDA approved for the treatment of advanced RCC (119, 120). Further review of these clinical studies involving these agents provides proof of concept that the VEGF receptor tyrosine kinase is a therapeutic target in RCC.

9.1 Sunitinib

Sunitinib is a multitargeted, orally available tyrosine kinase inhibitor with activity against PDGF (IC_{50} 8 nM) and VEGF (IC_{50} 9 nM) receptors, as well as KIT and FLT3 pathways (see Table 1) (121). Sunitinib has demonstrated growth-inhibitory activity against a variety of tumors in preclinical models including human colon xenografts and small cell lung cancers (122).

Phase I testing of sunitinib monotherapy revealed a number of safe regimens with scheduled interruptions, including 2 weeks on and 1 week off (2 + 1), 1 week on and 1 week off (1 + 1), and 4 weeks on and 2 weeks off (4 + 2), with doses given up to 75 mg daily (123, 124). Using the 4 + 2 regimen at 50 mg daily, a number of responses were seen in Phase I, including 4 PRs in patients with RCC (125). Based upon these early results, this regimen was tested in the Phase II–III setting in patients with metastatic RCC and patients with imatinib-refractory gastrointestinal stromal tumor (GIST).

Two multicenter, single arm Phase II trials of sunitinib in patients with metastatic RCC who had failed one prior cytokine chemotherapy were conducted (112) (see Table 1). Patients with measurable disease received 50 mg/day for 4 weeks followed by a 2-week rest. Response was evaluated by RECIST criteria. In the first trial of 63 patients, 40% achieved PR with median duration of 10+ months and 33% had stable disease (investigator assessed). The median overall survival (OS)

was 16.4 months. The second trial was similar in design except that it required clear cell histology, prior nephrectomy, and evidence of progressive disease prior to treatment. The results in this study, which included 106 patients, were similar with a 44% overall response rate (investigator assessed). In both trials, Grade 1 and 2 adverse events included fatigue, diarrhea, nausea, stomatitis, left ventricular ejection fraction (LVEF) decline, hypertension, bone marrow suppression, and elevated pancreatic enzymes. Based upon these results, accelerated approval was granted by the FDA for treatment of all forms of advanced RCC.

A Phase III trial of sunitinib versus interferon alpha in patients with untreated metastatic RCC was reported at ASCO 2006 (119). Eligibility for this study required documented clear cell histology with radiographic evidence of measurable disease, good performance status (ECOG = 0–1), and adequate blood counts. Patients were stratified by performance status, LDH levels, and presence or absence of prior nephrectomy. Seven hundred and fifty patients were randomized to either interferon or sunitinib. A preplanned interim analysis revealed a statistically significantly improved PFS in patients treated with sunitinib versus interferon (11 months vs. 5 months, $p < 0.000001$) by independent central review. Superior ORR (31% vs. 6%) was also seen in sunitinib versus interferon-treated patients by central review. No statistically significant difference was seen in OS by the time of this interim analysis. Safety analysis of sunitinib-treated patients revealed rates of adverse events similar to those previously reported (111, 112) with the exception for fatigue, which was less common in this previously untreated population (51% overall and 7% Grade 3). The robust effects seen in this trial unequivocally demonstrate the effectiveness of VEGFR inhibition and support the potential additive effect of PDGFR inhibition in combination.

9.2 Sorafenib

Sorafenib is another multitargeted, orally available, kinase inhibitor. It has activity against VEGFR-2 and -3, as well as RAF kinase, FLT3, PDGFR, and c-kit. Initially selected for its RAF kinase inhibitory properties, sorafenib demonstrated significant antitumor activity in RCC patients as part of a randomized discontinuation study in patients with advanced cancers (113). The randomized discontinuation design was performed to determine whether sorafenib inhibited tumor growth in patients with metastatic solid tumors who achieved stable disease status (bidimensional tumor measurements remaining within 25% of baseline) during a 12-week run-in period of treatment with sorafenib 400 mg bid. After the 12 weeks of initial therapy such patients were randomized, in a double-blind fashion, to sorafenib 400 mg bid or placebo, and the progression-free rate (percentage of patients with SD or response) at 24 weeks (12 weeks after randomization) was compared.

Secondary endpoints included overall PFS, PFS after randomization, tumor response rate, and safety. After initial enrollment revealed objective responses in

patients with metastatic RCC, these patients were prioritized and RCC ended up being the predominant tumor type assessed in this study (202/502).

Results of the run-in phase demonstrated that 71% of patients (144/202) exhibited tumor shrinkage or disease stabilization at 12 weeks and 4% of patients had independently confirmed PRs. In patients with stable disease who were subsequently randomized to sorafenib or placebo, Sorafenib 400 mg p.o. bid was also shown to be associated with a significantly prolonged median PFS in metastatic RCC compared to placebo (24 weeks vs. 6 weeks from the time of randomization, $p = 0.0087$). Grade 3/4 adverse events included hypertension (24%), fatigue (4%), rash (2%), hand-foot skin reaction (13%), and diarrhea (3%). Drug-related adverse events led to discontinuation in 2% of patients, and no deaths.

Based upon these encouraging Phase II results, a large, multinational, randomized double-blind, placebo-controlled, Phase III study of sorafenib in patients with advanced RCC was initiated to assess OS. Low or intermediate risk patients who had received one prior systemic therapy for advanced RCC were randomized to continuous oral sorafenib 400 mg bid versus placebo and best supportive care (BSC). Study endpoints included OS, PFS, and response rate determined by independent, blinded, radiologic review (120).

At the time of the PFS analysis, 769 of the planned 884 patients were randomized and 342 PFS events were reported. The study demonstrated a median PFS of 24 weeks for sorafenib versus 12 weeks for placebo as assessed by independent review (hazard ratio 0.44; $p < 0.00001$) (see [Table 1](#)). The 12-week progression-free rate was 79% for sorafenib versus 50% for placebo. More of the patients in the sorafenib arm had a decrease in the size of the tumor (74% vs. 20%); however, only 2% of patients had documented partial response by RECIST criteria assessed by independent review. Based upon these results, the study was halted and placebo-controlled patients were allowed to crossover to sorafenib open label.

Drug-related adverse events in patients treated with sorafenib included reversible skin rash (31%), diarrhea (30%), hypertension (30%), hand-foot skin reaction (26%), fatigue (18%), and sensory neuropathic changes (9%). The incidence of cardiac ischemia/infarction events was higher in the sorafenib group (2.9%) compared with the placebo group (0.4%). Overall it was concluded that sorafenib significantly prolonged PFS compared with placebo in patients with previously treated advanced RCC and displayed a favorable safety profile (120).

10 mTOR Inhibition in RCC

As described above, mTOR represents a possible therapeutic target in RCC for several reasons, including its effects on HIF and actions downstream of VEGFR signaling. Rapamycin is a natural inhibitor of mTOR. In fact, rapamycin was pivotal in the original identification of this protein. Temsirolimus (Torisel®, or CCI-779, Wyeth Pharmaceuticals, Philadelphia, PA) is an ester of rapamycin and has been studied in several trials with RCC patients. In an initial Phase II study, 111 patients with advanced refractory RCC were randomly assigned to receive

temsirolimus 25, 75, or 250 mg by weekly intravenous infusion (126). The objective response rate was only 7%, regardless of dose. However, 51% of the patients had either complete response, partial response, minor response, or stable disease >24 weeks (see Table 1). The overall median TTP was 5.8 months and overall median survival was 15 months. The most common adverse events were maculopapular rash, mucositis, asthenia, nausea, acne, pruritis, diarrhea, vomiting, anemia, hypertriglyceridemia, thrombocytopenia, and hyperglycemia. Of the 111 patients, 21 discontinued therapy because of adverse events. While not conclusive, these results supported a possible clinical benefit and warranted a randomized trial of mTOR inhibition versus standard of care.

Based upon a subset analysis that suggested the greatest benefit might be seen in poor risk patients, a randomized Phase III trial of temsirolimus versus interferon alpha versus the combination of temsirolimus and interferon alpha was initiated in poor risk, previously untreated, patients with advanced RCC (127). Key eligibility criteria included histologically confirmed RCC, measurable disease, Karnofsky performance status (KPS) ≥ 60 , adequate bone marrow function and at least 3 of 6 poor risk factors (KPS < 80, elevated LDH, calcium, decreased hemoglobin, 3 or more sites of disease, or metastasis < 1 year from diagnosis). Six hundred and twenty-six patients were randomized to 1 of 3 arms. At the second planned interim analysis, results revealed a statistically significant OS advantage for the temsirolimus arm alone over the interferon alone arm (median survival of 10.9 months vs. 7.3 months, $p = 0.0069$). Asthenia (54% all grades, 12% Grade 3) was the most common side effect in the temsirolimus alone arm, while nausea, rash, dyspnea, diarrhea, peripheral edema, vomiting, or stomatitis were seen in >20% of patients. However, these toxicities were rarely (<10%) Grade III or higher. Overall, temsirolimus was well tolerated even in this poor risk patient population and demonstrated a survival advantage versus standard therapy.

11 Summary

Loss of VHL protein function is a frequent and early event in the pathogenesis of RCC. Understanding of the biochemical consequences of this altered function has led to the identification of important therapeutic targets in this disease. Inhibition of VEGF, PDGF, and mTOR pathways has been linked with clinical benefit including robust and durable tumor regressions, significant delays in tumor progression, or increased OS. Sequential and concomitant use of agents to inhibit these and other targets related to the VHL and HIF pathways might improve clinical outcomes in the future.

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VEGF: Biologic Aspects and Clinical Approaches

W. Kimryn Rathmell and Brian I. Rini

Abstract Vascular endothelial growth factor (VEGF) is a key mediator in the tumorigenesis of renal cell carcinoma (RCC). VEGF is upregulated almost universally in clear cell type RCC as a result of loss of the *VHL* tumor suppressor and subsequent unabated activation of the hypoxia response pathway. Within the tumor, VEGF expression and local secretion drives the migration and proliferation of endothelial cells to support the extensive angiogenesis for which RCC is well known. VEGF is a complex set of isoforms with highly regulated patterns of expression, secretion, and receptor activation activities. Thus, the systemic impact of derangements of VEGF levels is not completely understood. As a factor so closely tied to the genetic events involved in the development of RCC, and a key mediator of tumor angiogenesis, many strategies have been developed to rein in the expression of VEGF. Two approaches to bind and neutralize VEGF have been investigated in RCC with promising results. This chapter will provide an overview of the molecular biology of VEGF regulation, the development of the inhibitory antibody for use in RCC, and an innovative new technology, VEGF-Trap, developed as an alternative VEGF inhibitory strategy for use in the treatment of RCC.

Keywords RCC • VEGF • VHL • Bevacizumab

1 Introduction

A growing understanding of the underlying molecular biology of renal cell carcinoma (RCC) has identified a number of pathways pertinent to the pathophysiology of clear cell RCC. Activation of the hypoxia response pathway by mutations of the von Hippel–Lindau (*VHL*) tumor suppressor gene results in the transcriptional activation

W.K. Rathmell (✉)

University of North Carolina, Lineberger Comprehensive Cancer Center, CB 7295,
Rm 21-237, Chapel Hill, NC 27599
e-mail: rathmell@med.unc.edu

of a variety of genes important in tumor progression, including vascular endothelial growth factor (VEGF). VEGF is the most potent promoter of tumor-associated angiogenesis, and as such has been a much sought-after target of drug development. Inhibitors targeting the VEGF protein have recently undergone clinical testing in metastatic RCC. This chapter will provide an overview of the genetic and molecular biology of VEGF activity and current strategies of targeting VEGF for clinical use.

2 Biologic Consequences of *VHL* Gene Inactivation in RCC

A detailed description of *VHL* activity is provided elsewhere in this text. Briefly, the *VHL* gene encodes a 213 amino acid protein (pVHL) which plays an integral role in regulating the normal cellular response to oxygen deprivation. In conditions of physiologic oxygen availability and normal *VHL* gene function, pVHL is the substrate recognition component of an ubiquitin ligase complex that targets a family of protein transcription factors, the hypoxia-inducible factors (HIF1 α and HIF2 α) for proteolysis (1–3). In conditions of cellular hypoxia, the pVHL–HIF interaction is disrupted as a result of loss of oxygen-dependent hydroxylation on HIF, thus leading to brisk stabilization of the HIF transcription factors (4–6). With defective *VHL* gene and protein function, the interaction between pVHL and HIF is disrupted even in the presence of adequate oxygen supplies. In this situation, HIF is not subject to proteolysis and is thus constitutively activated. Activated HIF translocates into the nucleus and leads to transcription of a large repertoire hypoxia-

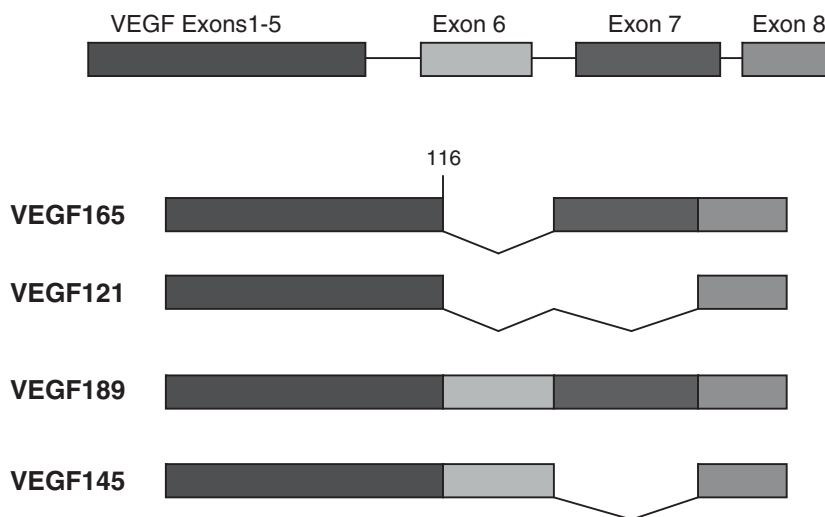


Fig. 1 Structures of related VEGF-A family members. VEGF-A family members are derived from alternate splicing events from a single genetic allele

inducible genes (7). Several of the hypoxia-inducible genes induced by this process have been identified as critical mediators of the tumorigenesis process, perhaps most notably VEGF (7).

In sporadic (noninherited) RCC, *VHL* gene allele deletion (loss of heterozygosity) has been demonstrated in 84–98% of sporadic renal tumors, and examination of RCC tumors for mutation in the remaining *VHL* allele has been observed in 34–57% of clear cell RCC tumors (8–12). *VHL* gene inactivation in RCC may also occur through gene silencing by methylation (10, 12–14). Taken together, the above data suggest that biallelic *VHL* gene inactivation occurs in the majority of clear cell RCC tumors. There is no evidence that nonclear cell RCC tumors harbor mutations in the *VHL* gene (15).

The above data provide compelling evidence for *VHL* gene inactivation in the majority of clear cell RCC tumors leading to overexpression of VEGF and other factors as a driving force in renal tumor angiogenesis. In fact, RCC almost universally develops highly vascular features in both the primary and metastatic sites of disease, an observation which led to the initial suggestion that liberation of an angiogenic factor may be uniquely correlated with this disease (16). Thus, with the development of effective agents targeting the angiogenesis signaling pathway, inhibition of VEGF has been aggressively pursued as a therapeutic target in RCC.

3 The VEGF Pathway in RCC

3.1 VEGF – Basic Principles

In 1948, Dr. I.C. Michaelson reported on the finding of a soluble “angiogenic factor X” which could promote the growth of vessels in the developing retina (17). In his seminal observation, regional tissues liberated a substance which promoted the expansion of existing blood vessel networks (angiogenesis), supported the formation of new vessels (neovascularization), and directed this process along a substrate gradient to reach a region in need of vascular supply. These concepts continued to form the framework for the next 50 years of research into the complex process of angiogenesis and continue to compel the current investigations to understand the factors which function to mediate it. The angiogenic factor Dr. Michaelson identified promoting this process is the VEGF.

VEGF is also referred to as VPF (vascular permeability factor) and functions as an important regulator of endothelial cell physiology. VEGF was identified in 1989 as a secreted mitogen of endothelial growth (18, 19). This secreted ligand has attracted major attention as the dominant factor regulating angiogenesis in both normal development and tumor growth. This factor exerts its mitogenic growth promoting influence not on the tumor cells themselves (except in rare circumstances), but instead upon the vascular endothelial cells, promoting both proliferation and new vessel formation. This mitogenic ligand-mediated response occurs in

many physiological processes in which expansion of blood and nutrient delivery is necessary, including development, wound healing, maternal–fetal placenta formation, and uterine decidua formation, as well as pathologic processes such as diabetic retinopathy, tissue recovery from ischemic insult or injury, and cancer. In the development of cancer, because oxygen diffusion is limited to little more than 1 mm of tissue penetration, collections of cancer cells any larger than this must recruit and acquire the means of delivering oxygen and nutrients to deeper layers. The process of tumor angiogenesis, like other processes which integrate endothelial cell vascular network expansion, is dependent on secreted VEGF to promote existing vessel ingrowth into the tumor as well as potentially neovascularization. Like enhanced cellular proliferation, evasion of cell death, independence from growth factor requirement, insensitivity to antigrowth signals, and tissue invasion, the ability of a tumor to promote its own angiogenesis is one of the critical hallmarks of cancer (20). Because of the importance of VEGF in this critical role, it has become a central player in the arena of targeted drug development for many tumors, including RCC. For years, the influence of angiogenesis and the impact on oxygen and nutrient delivery to rapidly growing tumors has been a basic factor of tumor biology as it impacts such key clinically relevant factors such as drug delivery, surgical resection strategies, and sensitivity to radiation therapy. This chapter will provide an overview of the biology of VEGF and relevant proangiogenic co-factors, as well as the current status of therapeutics specifically targeting this key molecule in the treatment of RCC.

3.1.1 Protein Structure and Isoforms

Originally called VPF, VEGF actually refers to a family of related peptides, each with restricted tissue expression and receptor specificity. VEGFA is structurally related to the platelet-derived growth factor family, sharing homology with both PDGF-A and PDGF-B. The original VEGFA was identified as a secreted peptide from bovine pituitary follicular cells by Ferrara and Henzel as a 45-kDa protein, although the apparent molecular mass is ~20 kDa under reducing conditions (21). The active portion of the protein is a 26-amino acid signal sequence at the N terminus of the multiple isoforms of the protein which were eventually identified.

The VEGFA gene is located at 6p21.3 (22). A pro-angiogenic gene transcript was cloned by Leung et al., identifying the first of a family of isoforms encoding the primary protein of 165 amino acids (VEGF165) (18). Human VEGFA has at least nine subtypes due to the alternative splicing of a single gene (23). In addition to VEGF165, these include two common 121 amino acid (VEGF121) and 189 amino acid (VEGF189) forms; these latter two isoforms resulting from a deletion of 44 amino acids and insertion of 24 amino acids at residue 116, respectively. A 145 amino acid isoform (VEGF145) was identified which lacked exon 7 was identified by Poltorak et al. in carcinoma cell lines (24). The various isoforms all arise from one 8-exon gene identified by Tischer et al., and arise through alternative splicing (25). VEGF189 represents the complete 8-exon transcript. VEGF165 lacks exon 6,

VEGF145 lacks exon 7 as mentioned above, and VEGF121 lacks both exons 6 and 7 (Fig. 1). VEGF165 is the predominant isoform and has basic charge and moderate affinity for heparin, owing to 15 basic amino acids within the 44 residues of exon 7 (21). By contrast VEGF121 has little affinity for heparin and is liberated freely from the cell, but VEGF189, as a result of additional basic residues contributed from exon 6 binds heparin strongly and sequestered in the extracellular matrix (26). This sequestered pool of VEGF can be released upon exposure to heparin or heparinases (27).

The activity of the various isoforms continues to be an area of active investigation. Certain of the isoforms act in a dominant negative fashion, for example, VEGF165b. This isoform binds to VEGF-R2 with the same affinity as VEGF165, but fails to activate the downstream signaling pathways (26). Additionally, support from a variety of murine models suggests that the various isoforms play distinct roles in vascular patterning and arterial development. Mice engineered to express exclusively the VEGF121 isoform die within the first 2 weeks of postnatal life due to ischemic cardiomyopathy presumed to be as a result of failure of myocardial angiogenesis (27, 28). The lack of the VEGF165 or VEGF188 isoforms, modeled in similar murine systems, also produce severe defects in directed extension of developing blood vessels, suggesting that the extracellular matrix-bound versions of the protein are essential in directing spatial orientation of vascular branching. In contrast, mice which exclusively express the VEGF165 isoform develop normally.

Related family members, VEGFB, VEGFC, and VEGFD interact with a separate group of related receptors involved in lymphatic vessel development (29, 30). Although, investigations continue to examine potential activities of these proteins in tumor biology, a definitive role for these related proteins in tumor development or maintenance has not been conclusively demonstrated. Therefore, although targeting these pathways may eventually be developed in cancer therapy, there is no current role in RCC, and the focus of this chapter will be exclusively on VEGFA.

3.2 Regulation of VEGF: Management at Many Levels

3.2.1 Cellular Sources of VEGF

VEGF is primarily produced and secreted by fibroblast cells. Primary evidence for this was provided by an elegant experiment performed by Fukumura et al. (1998), in which a line of transgenic mice was established expressing the green fluorescent protein (GFP) under the control of the VEGF promoter (31). The mice demonstrated GFP expression around healing wound margins and in the granulation tissue of superficial wounds. Solid tumors implanted in the transgenic mice led to accumulation of GFP expression around the tumor resulting from induction of host VEGF promoter activity, and with time the fluorescent cells could be seen throughout the tumor mass. In both implanted tumor and spontaneous tumor models in this system, GFP expression remained primarily limited to stromal cells or fibroblasts.

In this investigation limited to transcriptionally affected VEGF regulation, for the first time the cells which initiate and direct vessel formation were identified in a very visual way.

Much of what we understand about VEGF regulation does come from investigations of the expression in tumors or tumor-related tissues. Major advances have been made in the quantitative assessment of parameters or factors directly associated with tumor angiogenesis. Histological quantitation of intratumoral microvessel density (MVD) and the measurement of angiogenic factors in the serum of cancer patients have consistently supported a major role for sustaining this process in tumors from a spectrum of tissues. Most of the attention has been directed to VEGF and its cognate receptors in tumor angiogenesis.

3.2.2 VEGF: Transcriptional Regulation

Gene expression of VEGF is regulated by a variety of factors including growth factors, p53 mutation, estrogen receptor activation, thyroid stimulating hormone, nitric oxide, and hypoxia. Inappropriate activation of the hypoxia response pathway, as discussed above, and elsewhere in this text, is a major mechanism of VEGF transcriptional regulation in RCC (32, 33). The HIF α subunits activated as a result of either *VHL* inactivation or conditions of hypoxia heterodimerize with a constitutively available partner, HIF1 β (aryl hydrocarbon receptor nuclear transferase, ARNT). This HIF complex binds to a conserved hypoxia response element proximal to a CREB element (cAMP response element binding protein) where the hypoxia-modified C-terminal transactivation domain of HIF α interacts with the p300/CBP (CREB binding protein) co-activator to induce transcription at the VEGF locus (34–36). Further, inhibition of binding at the consensus hypoxia response element promoter sequence suppresses transcriptional activation of VEGF (37). On the basis of nuclear run-off transcriptional assays, the transcriptional rate for VEGF is increased two- to threefold by hypoxia (38).

3.2.3 VEGF: Regulation of mRNA Stability

VEGF is also regulated in response to hypoxia at the level of mRNA stability. Both the 5' and 3' untranslated regions of VEGF confer increased mRNA stability. In particular, the increase in VEGF level in response to hypoxia is at least partly contributed by posttranscriptional mechanisms (39). Under normoxic conditions VEGF mRNA is extremely labile, with half life of 15–45 min (40, 41), and experiments have demonstrated an increase in half life by as much as eightfold under hypoxic conditions. The normally short half life of VEGF mRNA is mediated through nonameric segments in the 3' untranslated region (3'UTR) with the sequence UUAUUUAUU. Mutational analysis has demonstrated increased mRNA stability of the VEGF transcript when either of these sequences is disrupted. Correspondingly, deletion analysis identified an additional region of the 3' untranslated region distinct but proximal to the nonameric element (42). A protein complex

which binds to this region was identified by affinity purification. One of these proteins, HuR, was identified as a binding partner in this complex with specificity to the region of the VEGF 3' UTR that is critical for stabilization (43). Mapping of purified protein binding to the VEGF mRNA revealed a 45 nucleotide binding site that maps only four nucleotides 5' to one of the consensus nonameric instability sequences.

3.2.4 VEGF: Translational Regulation

In comparison with hypoxia-regulated transcriptional control, the effect of hypoxia on translation has been understudied and much less is known about the mechanisms and significance of these translational controls. There are reports that hypoxia plays opposite roles on cap-dependent translation and translation initiated by an internal ribosomal entry (IRE) mechanism. Global protein translation that involves the traditional 5'-m7-GTP capping processing is inhibited during hypoxia, whereas translation by IRE is spared from this downregulation. During hypoxia, translation of a relatively small number of factors critical for the physiological or pathological response to O₂ deprivation, such as VEGF, basic fibroblast growth factor (bFGF), and HIF-1 α , is maintained. These genes generally have long and GC rich nucleotide sequences in their 5'-UTRs and significant secondary structures expected to impede ribosomal scanning for the initiation codon and, as such, they are predicted to be subject to translational regulation (44).

IRE accounts for the translation of such mRNAs during hypoxia. Translation of genes with IRES does not require eIF4E and the 5'-m7-GTP cap, therefore it evades the inhibition of eIF4F caused by hypophosphorylation of 4E-BP1. Verification of IRES-mediated translation for HIF-1 α and VEGF during hypoxia was demonstrated using polysomal profiling and/or bicistronic reporter assays. While actin mRNA, whose translation is not induced during hypoxia, shifts to less actively translated monosomal fractions, HIF-1 α mRNA remains in fractions of actively translated polysomes. In the bicistronic reporter assay, 5'-UTRs for HIF-1 α or VEGF were inserted between two luciferase reporters and the ratio of expression driven by cap-dependent mechanisms and IRES-mediated translation was examined. In these studies, 5'-UTRs for HIF-1 α and VEGF promoted the expression of the downstream reporter and the IRES activities were not affected by hypoxic conditions that caused a reduction in cap-dependent translation. Thus, the presence of IRES in HIF-1 α and VEGF selectively maintains their translation under conditions of low O₂ availability.

4 VEGF Function

The secreted VEGF protein is a potent mitogen of capillary and vascular endothelial cells (18, 21). It is perhaps the only chemokine with direct activity to stimulate proliferation of endothelial cells, promoted through binding and dimerization of cell

surface transmembrane receptors. The receptors FLT1 (VEGFR1) and KDR/FLK1 (VEGFR2) comprise the major classifications of receptors which bind VEGFA. These receptors are only present on endothelial cells, and evidence suggests that the isoforms of VEGF compete for binding on the receptor. Specifically, VEGF145, the major tumor-associated isoform, will inhibit the binding of VEGF165 to the KDR/FLK1 receptor. Both VEGF145 and VEGF189 will bind efficiently to the extracellular matrix, and thus spatially fixing them as chemokines to direct vessel formation. As an elegant demonstration of the pro-angiogenic activity of VEGF145, Poltorak and colleagues injected purified VEGF145 into mouse skin demonstrating that this protein acting alone can induce angiogenesis (24). When nonproliferating endothelial cells in culture are deprived of oxygen and nutrients, VEGF acts independently of its mitogenic activity to stimulate elongation, network formation, and branching morphogenesis (45). In solid tumors that are exposed to chronic or intermittent hypoxic conditions, Helmlinger proposed that autocrine endothelial VEGF production contributes to the formation of tumor blood vessels and promotes tumor growth. Investigations of tissue and cell culture oxygen gradients have demonstrated the induction of a VEGF gradient, followed by establishment of new vascular networks. The addition of an anti-VEGF neutralizing antibody abolished vascular network formation, thus establishing the role of this important protein in network formation (45).

In addition to its role as a mitogen and a chemokine, the VEGF family regulates the permeability of both mature and developing vessels (46–51). VEGF increases the permeability of endothelial cells in a dose-dependent manner. These activities are additionally mediated through interactions with the FLT1 and KDR/FLK1 receptor family. Receptor activation and signal transduction via the phosphoinositol-3-kinase signaling cascade was required for the permeabilization of these cells as inhibitors of PI-3 kinase and MAP kinase both were inhibitory of this process in vitro (52). Microvessel hyperpermeability is the critical step for the abnormal transport of molecules and cells across the blood vessel wall and, therefore, the crucial step for many diseases including tumor growth and metastasis (53). Understanding the mechanisms of microvessel hyperpermeability from various approaches is important in combating these malignant diseases.

Additionally, VEGF has been implicated in maintaining the blood–brain barrier, and facilitating glucose transport across this critical barrier during periods of physiological crisis. Dantz et al. demonstrated a brisk increase in serum VEGF levels in response to insulin-induced hypoglycemia (but not to hyperinsulinemic euglycemia) (54). During a period of hypoglycemia, VEGF levels correlated with preserved neurocognitive function, in contrast to all other counterregulatory hormone measured.

VEGF performs much of its pro-angiogenic effect through cooperative mechanisms involving other molecules. Specifically, the angiopoietins collaborate with VEGF during angiogenesis, with angiopoietin-1 performing an antiapoptotic role in vessel development (55). In most solid tumor models, VEGF is largely expressed in the hypoxic periphery. Angiopoietin-2, however, antagonizes the role of angiopoietin-1, and its expression in the few remaining internal vessels and in the angiogenic

vessels at the tumor margin suggests a critical role of this destabilizing activity in facilitating the formation of fresh vessel growth promoted by VEGF at the tumor rim. In a model of implanted mammary tumor cells into rat brains, Holash observed that the tumor cells rapidly associated with and migrated along existing cerebral blood vessels with minimal upregulation of VEGF. However, there is widespread regression of the initially co-opted vessels, and a brisk conversion to VEGF promoted angiogenesis.

5 VEGF and Cancer

Folkman noted in 1995 the importance of VEGF and its receptor system in tumor vascularization and suggested to potential role of therapeutic mechanisms specifically targeting this aspect of tumor biology (56). Since then a revolution in targeted therapy has evolved many strategies to achieve the inhibition of this key aspect of tumor biology. Because of the unique molecular characteristics of HIF activation and VEGF expression in RCC, this cancer is an ideal system in which to examine these emerging therapeutics and provide important proof of concept work. A detailed overview of the currently utilized and emerging strategies of VEGF targeted therapy will be overviewed in the latter part of this chapter.

As eluded above, the vascularization of tumors provides a provocative target for therapy for several reasons. First, although new vessel formation is an important factor in development and wound healing, in the mature animal, the formation of new vessels is an event essentially relegated to pathologic conditions. None demonstrate the efficiency of this process more elegantly, however, than the growth of solid tumors and solid tumor metastases. The cancers cells within tumors require access to blood vessels for continued growth as well as for the establishment of further metastatic sites, and inhibiting vessel formation offers an opportunity to reduce the morbidity and mortality of cancer. To obtain nutrients for their growth, cancer cells co-opt host vessels, sprout new vessels from existing ones, and/or recruit endothelial cells from the bone marrow (57). Second, the resulting vasculature is structurally and functionally abnormal, typically dilated, and highly permeable with global disorganization. Therefore, this unique vasculature may provide a unique target for inhibitory therapy (32).

A large amount of data has supported the importance of VEGF and its cognate receptors in tumor angiogenesis. Historically, other angiogenic factors have also been implicated in the tumor angiogenic process, including members of the FGF family, and other cytokines with a positive role in regulation of angiogenesis in the experimental setting, including transforming growth factor- β , tumor necrosis factor- α , and hepatocyte growth factor (58, 59). High levels of these factors have been observed in the serum of cancer patients – in particular bFGF, which is elevated in the serum of patients with renal, breast, and prostate cancer (60–64), and in the urine of patients with a wide variety of tumors including kidney, bladder, breast, and lung cancer, as well as hematologic malignancies (60, 61, 65).

Examination of the cerebrospinal fluid of children with brain tumors has detected high levels of bFGF in this body fluid as well (66). As more recent attention has turned to the more potent activator of angiogenesis, VEGF, measurable increases in this molecule have been observed in association with cancer as well. VEGF is elevated in the serum of patients with non-small-cell lung cancer, colorectal, breast, ovarian, uterine, and RCC (66–71). As detailed above, a variety of mechanisms account for the increase in VEGF, with activation of the hypoxic response pathway via the transcription factors HIF1 and HIF2 as the most classic mechanism of induction. Despite the consistency with which VEGF is elevated in the tumor phenotype, it remains an unpredictable tumor marker (72). Many other pathologic or inflammatory states can cause serum elevations of VEGF and levels themselves vary somewhat widely in inpatient measurement. One potential problem with serum measurement of VEGF is the high concentration of VEGF in platelets, which release their contents during the collection of serum samples. Attempts to integrate the plasma and urinary pattern of angiogenic factor expression into a profile of angiogenesis have been somewhat more successful. First, the use of citrate in sample collection of plasma effectively prevents platelet degranulation. Second, the analysis of multiple factors reduces the statistical error probability. However, with only two factors, VEGF and HGF, it was possible to distinguish cancer patients from healthy individuals (72), such that a threshold exists to separate these two populations based on plasma measurement. The profiles were even more distinctly separate for patients with advanced disease.

RCC presents a unique clinical setting in which a tumor type nearly universally usurps a pro-angiogenic cellular homeostatic mechanism. Through mutations in *VHL* or other genetic events which result in the dysregulated expression of the hypoxia-inducible transcription factors HIF1 α and/or HIF2 α (described in another chapter), a large cohort of hypoxia responsive genes is induced, including VEGF as one of the classic transcriptional targets (33). Cell culture model systems of RCC have demonstrated a direct link between *VHL* mutation and upregulation of VEGF. RCC cells in which *VHL* is mutant express abundant levels of VEGF mRNA and protein, and reconstitution of these cells with a wild-type *VHL* cDNA restores predicted patterns of VEGF hypoxia responsive regulation (7). Thus, increased expression of VEGF and the consequences of that increased expression are expected and predictable events in the development of most RCC.

6 Clinical Approach to VEGF Inhibition in RCC

6.1 *Anti-VEGF Antibody (Bevacizumab)*

A recombinant human monoclonal antibody against VEGF (rhuMAb VEGF, bevacizumab, Avastin®; Genentech, South San Francisco, CA) binds and neutralizes all biologically active isoforms of VEGF (73). In vitro studies have demonstrated

that bevacizumab causes decreased survival of human vascular endothelial cells (HUVEC) and decreases VEGF-induced HUVEC permeability (74). This humanized antibody was demonstrated to inhibit bovine capillary endothelial cell proliferation in response to VEGF and has showed antitumor effects in sarcoma and breast cancer cell lines (73). In addition, preclinical studies have shown that bevacizumab has activity against metastases (reviewed by Gerber and Ferrara (75)). For example, Rowe and colleagues showed that the antibody (A4.6.1) prevented lung metastases from Wilm's tumors implanted into kidneys of nude mice (76).

The clinical utility of bevacizumab in metastatic RCC was initially investigated in a randomized phase II trial in which 116 patients with treatment-refractory, metastatic clear cell RCC were randomized to receive placebo, low dose (3 mg/kg) bevacizumab or high dose (10 mg/kg) bevacizumab given intravenously every 2 weeks (77). There were four partial responses, all in the high dose bevacizumab arm (4/39; 10% objective response rate). A substantial proportion of patients had tumor shrinkage not meeting objective response criteria. Figure 2 demonstrates tumor burden changes over time for each of the three patient cohorts, demonstrating a gradual positive effect on tumor burden with increasing doses of bevacizumab. An intent-to-treat analysis demonstrated a significant prolongation of time to progression in the high dose bevacizumab arm compared to placebo (4.8 months vs. 2.5 months; $p < 0.001$ by log rank test). In the high-dose bevacizumab arm, hypertension of any grade occurred in 36% of patients, and grade 3 hypertension, defined as hypertension not controlled by one standard medication, was observed in 21% of patients. Asymptomatic proteinuria without renal insufficiency was observed in

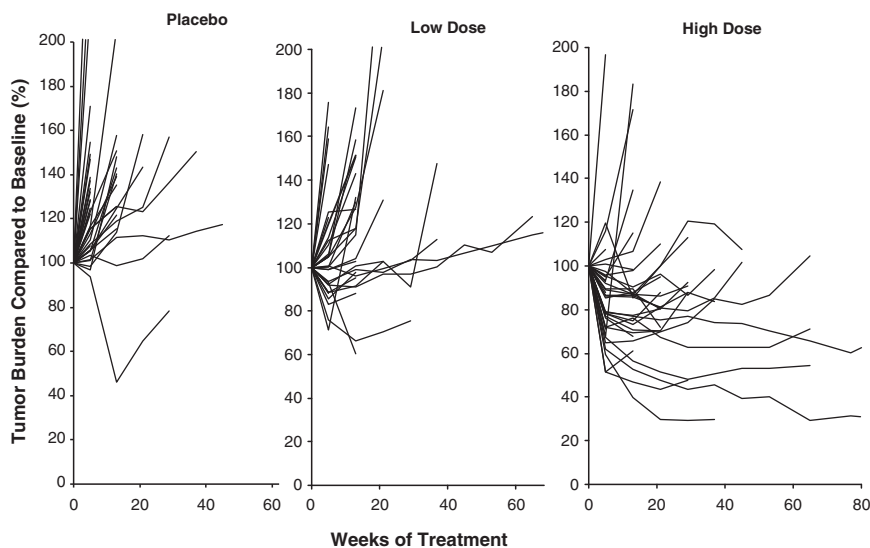


Fig. 2 Change in tumor burden over time for metastatic RCC patients treated with placebo or bevacizumab. Reprinted with permission (79).

64% of patients in the high-dose bevacizumab arm. All toxicities were reversible with cessation of therapy.

Preclinical studies show that VEGF maintains tumor vessel morphology even in established vascularized tumors, prompting a rationale for patients to remain on anti-VEGF therapy (78). It is, however, important to determine whether prolonged therapy is feasible in the clinic. Follow-up from the Yang et al. trial has demonstrated no further toxic side effects, including four patients who have continued on therapy without progression for 3–5 years (79). Toxicity in these patients was limited to proteinuria with normal renal function.

Bevacizumab has been further investigated in RCC in combination with an anti-epidermal growth factor receptor (EGFR) strategy. Preclinical investigation in human RCC xenograft models of bevacizumab and erlotinib, a small molecule EGFR inhibitor, have demonstrated potential benefit of combination therapy on tumor growth inhibition (80). A clinical trial in metastatic RCC with bevacizumab 10 mg/kg IV every 2 weeks in combination with erlotinib 150 mg daily reported a 25% partial response rate (81). Although they were generally mild to moderate, the most common adverse events were skin rash and diarrhea. However, recent findings from a randomized, multicenter, double-blind phase II trial further evaluating bevacizumab with or without erlotinib as first-line therapy for metastatic RCC showed that patients in both arms had similar progression-free survival and response rates. These data suggest one or more of the following: that erlotinib does not add to the efficacy of bevacizumab, that the single-agent activity of bevacizumab has been underestimated, or that the randomized phase II trial design was underpowered to detect a small benefit in the combination arm. A complete analysis of the data is expected in 2006.

On the basis of these data, an Intergroup phase III trial investigating the addition of bevacizumab to initial systemic therapy in RCC recently completed accrual (82). Patients with metastatic clear cell RCC without prior systemic therapy were randomized to either low-dose interferon-alpha 2b (Intron A, Schering-Plough, Kenilworth, NJ), 9 MU subcutaneously three times weekly, or the same dose and schedule of interferon-alpha 2b in combination with bevacizumab, 10 mg/kg IV every 2 weeks. The primary endpoint of the trial is overall survival, designed to detect an improvement in median survival from 13 months for interferon-alpha alone to 17 months for the combination. A similarly designed phase III trial is underway in Europe using interferon-alpha 2a (Roferon; Hoffmann-LaRoche, Grenzach-Wyhlen, Germany) instead of interferon-alpha 2b. Both trials are nearing planned analysis and results are eagerly awaited on the effect of adding bevacizumab to front-line therapy in RCC.

Clinical trials of another approach to initial therapy in metastatic RCC, high-dose interleukin-2 (IL-2), in combination with bevacizumab are also underway. The rationale for this combination includes the suggestion that bevacizumab may prevent much of the tumor-induced immunosuppression attributed to VEGF and thereby enrich the immune-enhancing effects of IL-2 (83, 84). In addition, IL-2 toxicity may be reduced by the vascular effects of bevacizumab. Bevacizumab may decrease the significant vascular leak syndrome associated with IL-2 and allow

more IL-2 doses to be administered with less toxicity. Bevacizumab 10 mg/kg IV every 2 weeks will be integrated with standard high-dose IL-2 regimens with both progression-free and overall survival as primary endpoints. Bevacizumab in combination with low-dose IL-2 is also planned for analysis in a separate trial.

Combination studies are also evaluating the feasibility of combining bevacizumab with an inhibitor of the tyrosine kinase portion of the VEGF receptor (sorafenib, Nexavar® Bayer Pharmaceuticals, West Haven, CT and Onyx Pharmaceuticals, Richmond, CA). To date, 12 patients (including 2 patients with metastatic RCC) have been enrolled in a phase I study combining these two agents (85). No grade 4 toxicities have been observed. Dose-limiting toxicities with bevacizumab 10 mg/kg every 2 weeks and sorafenib 200 mg bid have been observed and include hypertension, hand-foot syndrome, fatigue, diarrhea, elevated lipase, proteinuria, and thrombocytopenia. Other grade 1/2 toxicities included neuropathy, rhinorrhea, weight loss, and anorexia. Partial responses were seen in 2 of 7 heavily pretreated patients. Nine remaining patients had stable disease or minor response. Preliminary data show that all 12 patients have experienced clinical benefit, but the regimen is associated with greater toxicity than monotherapy administered at the same dose. A phase I trial of bevacizumab in combination with another VEGFR inhibitor, sunitinib malate (Sutent®, Pfizer Inc. New York, NY), is also planned.

6.2 VEGF-Trap

VEGF-Trap (Regeneron Pharmaceuticals, Tarrytown, NY and Sanofi-Aventis, Bridgewater, NJ) is a fusion protein composed of the human VEGF receptor VEGFR-1 (Flt-1) extracellular immunoglobulin (Ig) domain 2 and the VEGFR-2 (KDR) extracellular Ig domain 3 fused to human IgG1 Fc molecule (Fig. 3) (86). VEGF-Trap thus acts as a soluble decoy receptor to bind VEGF and disrupt subsequent VEGF signaling. VEGF-Trap binds to VEGF with great affinity ($K_d \approx 1$ pM) and also binds another angiogenic protein, placental growth factor. In cultured endothelial cell assays, VEGF-Trap demonstrated inhibition of VEGF-induced VEGFR-2 phosphorylation and endothelial cell proliferation. In xenograft models, VEGF-Trap-treated mice exhibited significant inhibition of tumor growth and tumor-associated angiogenesis in implanted rat C6 glioma, human A673 rhabdomyosarcoma, and mouse B16 melanoma tumors compared to vehicle-treated controls (86, 87). A murine xenograft model employing SK-NEP-1 cells (human Wilms tumor cell line derived from embryonic renal cells) demonstrated involution of existing mature vasculature and apoptosis of endothelial cells coincident with regression of established tumors after VEGF-Trap treatment (88, 89). VEGF-Trap treated animals demonstrated a significant regression of established tumors (mean tumor weight decreased by 79.3% by day 36; $p < 0.0002$).

Two phase I studies with VEGF-Trap have been reported in patients with refractory solid tumors. In the first trial (90), 38 patients, including 9 patients with metastatic RCC, received 1 (or 2) subcutaneous dose(s) of VEGF-Trap followed

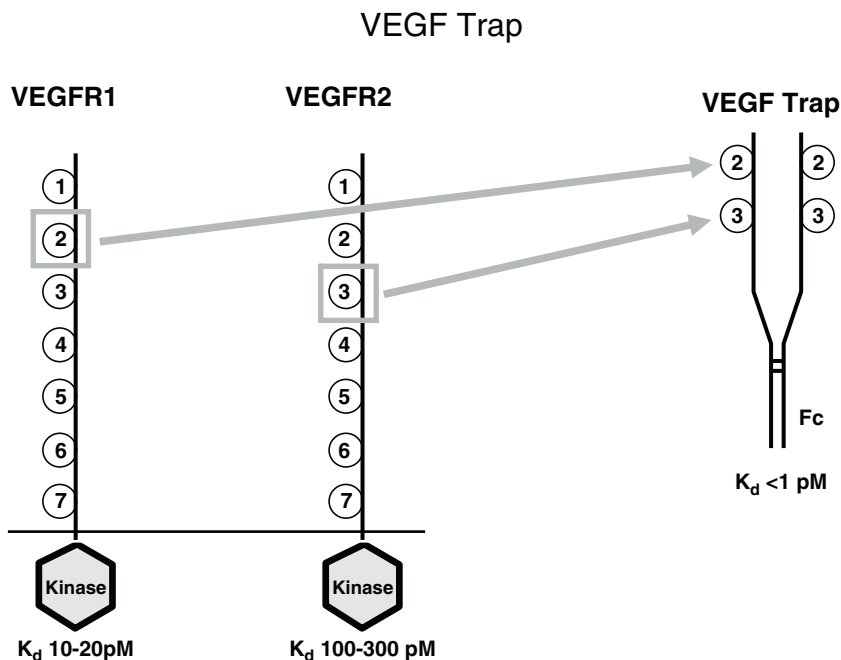


Fig. 3 VEGF trap structure. VEGF-Trap is a fusion protein composed of the human VEGF receptor VEGFR-1 (Flt-1) extracellular immunoglobulin (Ig) domain 2 and the VEGFR-2 (KDR) extracellular Ig domain 3 fused to human IgG1 Fc molecule. Reprinted with permission (93).

4 weeks later by six weekly injections (escalating dose levels of 0.025, 0.05, 0.1, 0.2, 0.4, and 0.8 mg/kg) or six twice weekly (0.8 mg/kg) injections. Drug-related grade 3 adverse events included hypertension and proteinuria without a maximum tolerated dose determined. No anti-VEGF-Trap antibodies were detected. No objective responses have been observed in this trial. Fourteen of 24 assessable patients, including five of six patients at the highest dose level, have maintained stable disease for 10 weeks.

In the second trial (91, 92), 30 patients have been treated to date with intravenous VEGF-Trap every 2 weeks at one of five dose levels (0.3, 1.0, 2.0, 3.0, and 4.0 mg/kg). Drug-related grade 3 adverse events included arthralgia and fatigue. One patient with metastatic RCC has maintained stable disease for over 11 months (at the 1.0 mg/kg dose level). Objective antitumor activity included a partial response in an advanced ovarian cancer patient and minor responses in metastatic bladder cancer and uterine leiomyosarcoma. Vascular imaging with dynamic contrast-enhanced MRI performed at baseline and after 24 h indicated effective inhibition of tumor perfusion at higher dose levels (≥ 2.0 mg/kg). Complete binding of circulating VEGF was documented at higher dose levels (≥ 2.0 mg/kg) with the observation of free in excess of bound VEGF-Trap in plasma. Further investigation is ongoing to define the clinical utility of VEGF-Trap in RCC.

7 Conclusions

VHL gene inactivation is a frequent event in clear cell RCC leading indirectly to overexpression of VEGF. Strategies to bind VEGF protein have undergone investigation in metastatic RCC. Bevacizumab has demonstrated prolongation of TTP versus placebo and is being investigated in multiple RCC settings. VEGF-Trap is a distinct VEGF binding molecule with evidence of effects on vasculature leading to tumor regression. Results from ongoing and future trials will more precisely define the role of VEGF binding strategies in RCC.

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VEGF and PDGF Receptors: Biologic Relevance and Clinical Approaches to Inhibition

John S. Lam, Robert A. Figlin, and Arie S. Beldegrun

Abstract The dependence of tumor growth on the development of a neovasculature is well established. One of the best studied angiogenic factor is vascular endothelial growth factor (VEGF). VEGF belongs to a family of homodimeric glycoproteins that bind to three different VEGF receptor (VEGFR) tyrosine kinases in an overlapping pattern. VEGFRs share regulatory mechanisms with other well-characterized receptor tyrosine kinases, such as the platelet-derived growth factor receptor (PDGFR). These mechanisms include receptor dimerization and activation of tyrosine kinase, as well as creation of docking sites for signal transducers to direct cellular function including cell migration, survival, and proliferation. Tumor biology studies, drug development, and clinical studies have established VEGFR and PDGFR as validated drug targets. Well-interconnected preclinical and clinical efforts are necessary for the continued development of this exciting process. This chapter will review the biological role of VEGFR and PDGFR signal transduction and the interplay between the different receptors as it relates to kidney cancer.

Keywords Platelet-derived growth factor • Receptors • Signal transduction • Tyrosine kinase • Vascular endothelial growth factor

1 Introduction

The dependence of tumor growth on the development of a neovasculature is a well-established aspect of cancer biology (1). Angiogenesis is important for supply of oxygen, nutrients, growth factors and hormones, proteolytic enzymes, influences on hemostatic factors that control the coagulation and fibrinolytic system, and

J.S. Lam (✉)

Department of Urology, David Geffen School of Medicine at University of California-Los Angeles, 10833 Le Conte Avenue, 66-124 CHS, Box 951738, Los Angeles, CA 90095
e-mail: drjohnlam@gmail.com

dissemination of tumor cells to distant sites (2, 3). Angiogenesis occurs in a series of complex and interrelated steps (4). Stressed, diseased, or injured cells release a number of angiogenic factors that either interact directly with endothelial cells and stimulate their proliferation and migration or act indirectly through the activation of other cells involved in angiogenesis. A variety of proangiogenic and antiangiogenic molecules emanate from cancer cells, endothelial cells, stromal cells, blood cells, and the extravascular matrix (5). The best studied angiogenic factors are vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF or FGF-2) (6, 7). Platelet-derived growth factor (PDGF) also participates in vessel generation and maturation (8). Antiangiogenic therapy for malignancy has been extensively researched during the last three decades, and novel agents representing different approaches to blocking tumor neovascularization are now available as well as several that are in clinical development (5, 9, 10).

VEGF denotes a family of homodimeric glycoproteins that consists of five mammalian members [VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placenta growth factor (PlGF)], a parapoxvirus-encoded member (VEGF-E), and one in the venom of snakes (VEGF-F). These ligands bind to three different VEGF receptor (VEGFR) tyrosine kinases in an overlapping pattern (Fig. 1), as well as to co-receptors, such as heparin sulfate proteoglycan (HSPG), and neuropilin (NRP). VEGFRs share

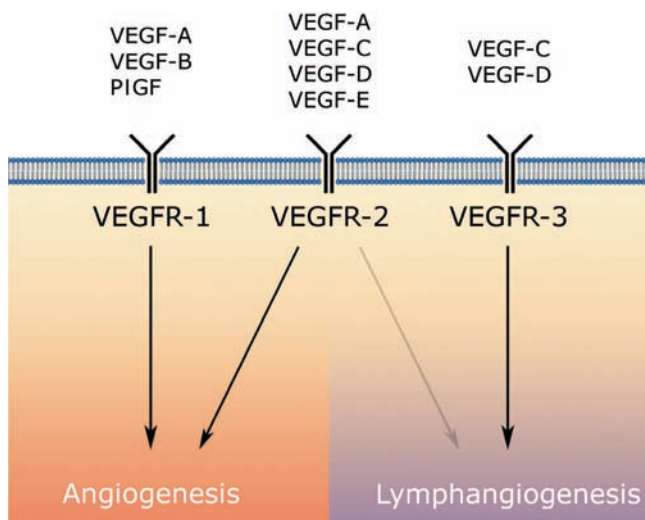


Fig. 1 Vascular endothelial growth factor receptor (VEGFR) binding properties. The vascular endothelial growth factor (VEGF) family consists of five mammalian members [VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placenta growth factor (PlGF)] as well as a parapoxvirus-encoded member (VEGF-E), and one in snake venom (VEGF-F), which bind to three different VEGF receptor (VEGFR) tyrosine kinases in an overlapping pattern. Binding results in receptor dimerization and activation of tyrosine kinase, as well as creation of docking sites for signal transducers to direct a variety of cellular functions that include angiogenesis and/or lymphangiogenesis

regulatory mechanisms with other well-characterized receptor tyrosine kinases, such as the PDGF receptor (PDGFR) and the epidermal growth factor receptor (EGFR). These mechanisms include receptor dimerization and activation of tyrosine kinase, as well as creation of docking sites for signal transducers to direct cellular function including cell migration, survival, and proliferation (6).

2 VEGF Ligands

In 1983, VEGF was partially purified as a tumor product known as vascular permeability factor (VPF). VPF increased the permeability of tumor vessels, allowing large molecules, such as fibrin, to escape and form a protective and nurturing network around and within the growing tumor (11). VPF was later found to be identical to VEGF, a highly specific mitogen for vascular endothelial cells (12). The recognition that VEGF belongs to a family of proteins led to the parallel designation of VEGF as VEGF-A. These glycoproteins belong to a structural superfamily of growth factors that also include PDGF and transforming growth factor- β . VEGF-A exists in five different isoforms (comprising 121, 145, 165, 189, and 206 amino acids in humans) with different biological activities, which are generated by alternative splicing of a single pre-mRNA species. The activities of the VEGF-A isoforms are dictated by their different abilities to interact with VEGFR co-receptors HSPG and NRP. In addition, the bioactivity of VEGF family members is regulated by proteolytic processing, which may enable specific interactions with different types of receptors (13).

3 Expression, Signal Transduction, and Function of VEGFRs

Key signals regulating embryonic cell growth and differentiation, as well as the remodeling and regulation of adult tissues by members of the VEGF family, are mediated by VEGFR tyrosine kinases and by non-tyrosine kinase receptors, NRP-1 and NRP-2 (14–16). The VEGFR family includes VEGFR-1 (also known as Flt-1), VEGFR-2 (also known as Flk-1 in mouse or KDR in human), and VEGFR-3 (also known as Flt-4), which belong to a superfamily of receptor tyrosine kinases characterized by extracellular (Ig) immunoglobulin homology domains and a split tyrosine kinase intracellular domain (17, 18). VEGFRs are equipped with a ~750-amino acid residue extracellular domain, which is organized into seven Ig-like folds. The fifth Ig domain is replaced by a disulfide bridge in VEGFR-3. The extracellular domain is followed by a single transmembrane region, a juxtamembrane domain, a split tyrosine-kinase domain that is interrupted by a 70-amino acid kinase insert, and a C-terminal tail. The Ig domain-2 of VEGFR-1 has been shown to constitute the ligand-binding site on the receptor (19). Biochemical analyses have also shown that the Ig domain-3 in VEGFR-2 is important for the

determination of ligand-binding specificity (20). Alternative splicing or proteolytic processing of VEGFRs gives rise to secreted variants of VEGFR-1 (21) and VEGFR-2 (22), and to a C-terminal truncated VEGFR-3 (23). VEGFRs have partly overlapping, but independent roles in vascular development and maintenance, as well as regulating vascular permeability.

The activity of receptor tyrosine kinases is regulated by the availability of ligands. A feature of VEGF-A is the upregulation of its expression under hypoxic conditions. Hypoxia allows for the stabilization of hypoxia-inducible factor (HIF) and binding to specific promoter elements that are present in the promoter region of VEGF-A (24). Recent work in understanding the von Hippel–Lindau (VHL) gene has demonstrated a role for angiogenesis in the pathophysiology of renal cell carcinoma (RCC) (10, 25). Loss of VHL gene function or hypoxia correlates with constitutive activation of HIF-1 α and a variety of genes important in tumor progression that include VEGF and PDGF. Hypoxia-driven deregulated expression of these genes enhance neovascularization of proliferating renal tumors (26–28). Several studies have demonstrated that VEGF expression results from inactivation of the VHL tumor suppressor gene observed in the majority of RCC (29–31) thereby identifying VEGF as a critical component of RCC angiogenesis and a relevant therapeutic target in RCC. Initial attempts to inhibit VEGF in RCC employed agents with multiple postulated antitumor and antiangiogenic mechanisms. Subsequently, therapies directed specifically against VEGF have been developed. Most recently, small molecules with inhibitory effects on the VEGFR and downstream protein signaling have been investigated. These agents have been characterized with regard to effects on VEGF, VEGFR, and endothelial cells with the presumption that the resulting action on tumor vasculature leads to an antitumor effect. Importantly, VEGFR expression has been identified on the surface of RCC (32–35), suggesting that VEGF may augment RCC tumor growth through an autocrine loop.

Expression of VEGFR-1 is also directly regulated by HIF (36). VEGFR-2 is also upregulated during hypoxia, but the role of different HIFs in this regulation remains uncertain. VEGFR-3 expression is upregulated in differentiating embryonic stem cells that are cultured in a hypoxic atmosphere (37), but the contribution of hypoxia in the regulation of *in vivo* VEGFR-3 expression remains unclear. Guided by the binding properties of the ligands, VEGFRs are able to form both homodimers and heterodimers (38). The signal-transduction properties of the VEGFR heterodimers compared with homodimers remains to be elucidated. Dimerization of receptors is accompanied by activation of the receptor-kinase activity that leads to the autophosphorylation of the receptors (6). Phosphorylated receptors recruit interacting proteins and induce the activation of signaling pathways that involve an array of second messengers.

Positive regulation of VEGFR-2 activity has been shown to be achieved through interaction with the trimeric G proteins G α q/G α 11 (39). Negative regulation of receptor tyrosine kinases is important for limiting the response of the target cells and induction of kinase activity is known to be counteracted by rapid dephosphorylation of the receptors by tyrosine-specific phosphatases. It has been shown that the activation of VEGFR-2 is negatively regulated by the phosphotyrosine

phosphatases Src-homology phosphatase (SHP)-1 and SHP-2 (40, 41). Receptor tyrosine kinase activity is also downregulated by rapid degradation through the proteasome pathway, as well as through internalization of the receptor and degradation in lysosomes. Rapid and controlled endocytosis and inactivation of VEGFR-2 is also likely important for accurate and localized responses to VEGF gradients (42). Internalization and degradation of VEGFR-2 requires protein kinase C (PKC)-dependent phosphorylation of the receptor C-terminal tail (43).

3.1 Vascular Endothelial Growth Factor Receptor 1

VEGFR-1 is a 180-kDa high-affinity receptor for VEGF-A, VEGF-B, and PlGF. It is expressed in vascular endothelial cells and a range of nonendothelial cells. VEGFR-1-deficient mouse embryos die at day 8.5–9 due to obstruction of vessels by an overgrowth of endothelial cells and do not assemble into tubules and functional vessels (44). The increase in the number of endothelial progenitors in the absence of VEGFR-1 (45) implies a negative regulatory role for the receptor during vascular development that appears to be exerted by the soluble form of VEGFR-1 (sVEGFR-1), which lacks the transmembrane and intracellular part of the receptor. During pregnancy, sVEGFR-1 is expressed at considerable levels in the placenta (46) and has been associated with the pathogenesis of preeclampsia by decreasing levels of VEGF-A and PlGF, resulting in endothelial dysfunction by preventing binding to the functional VEGFRs (47).

Evidence suggests that VEGFR-1 may modulate blood vessel formation in both embryos and adults (48–50). The deletion of the intracellular tyrosine kinase domain of VEGFR-1 remains compatible with normal vascular development (51), which suggests that the physiological role of VEGFR-1 in development may be to sequester excess VEGF. Hematopoietic stem cells (52, 53) as well as circulating CD97⁺ monocytes or macrophages (54) express VEGFR-1, which appears to be essential for migration of these cells (55, 56). Hematopoietic cells accumulate in tumor tissue and have been shown to promote tumor vascularization (57). Furthermore, growth of tumors expressing PlGF is impaired in mice with deletion of the intracellular domain of VEGFR-1 (58), which suggests that the intracellular domain of VEGFR-1 may regulate tumor angiogenesis. Inhibition of VEGFR-1 function by neutralizing antibodies has also been shown to reduce tumor growth and decrease the number of perivascular hematopoietic cells in the tumor (59, 60).

VEGFR-1 binds VEGF-A with high affinity ($K_d = 10$ pM), but ligand binding results only in a maximal two-fold increase in kinase activity (61–64). The phosphorylation sites on VEGFR-1 have been mapped in cellular models engineered to overexpress the receptor (65, 66). VEGFR-1 does not appear to be phosphorylated on consensus positive regulatory tyrosine residues, which could explain its low level of kinase activity. Analyses of chimeric VEGFR-1/VEGFR-2 have implicated the juxtamembrane domain of VEGFR-1 in repression of its kinase activity (67). Repression may be exerted through folding of the intracellular domain of VEGFR-1

preventing exposure of regulatory sequences in the kinase domain (66). Another interesting but unclear feature of VEGFR-1 is that its different ligands (VEGF-A, VEGF-B, and PlGF) transduce distinct biological responses. In an attempt to understand the potential mechanisms that underlie these different responses, VEGF and PlGF have been shown to possibly induce different phosphorylation-site patterns in VEGFR-1 (68).

The biological outcome of stimulating or inhibiting the activity of VEGFR-1 may be influenced through “crosstalk” with VEGFR-2. Several groups have reported that VEGFR-1 negatively regulates VEGFR-2 signals. VEGFR-1 has been shown to negatively influence VEGFR-2 function in cells expressing chimeric VEGFR (49, 50). Antagonizing signals from VEGFR-1 that override mitogenic signals from VEGFR-2 have been shown to depend on phosphoinositide 3-kinase (PI3K) (50). Notably, receptor heterodimerization is not a prerequisite for the negative influence of VEGFR-1. The signaling capacity of heterodimers of VEGFR-1 and -2 is similar to that of VEGFR-2 homodimers, at least with regard to signal transducers such as phospholipase C- γ 1 (PLC- γ 1) (69). Moreover, embryonic stem cells that lack VEGFR-1 show increased levels of VEGFR-2 phosphorylation (70). However, other reports have indicated that VEGFR-1 may instead promote VEGFR-2 activity during pathological conditions (71). Activation of VEGFR-1 by PlGF results in increased phosphorylation of VEGFR-2 (72), possibly through the displacement of VEGF-A that is bound to VEGFR-1, thereby making it available for VEGFR-2. Alternatively, signals that are induced by VEGFR-1 may positively modulate the output of VEGFR-2 through attenuation of intracellular negative regulatory pathways that possibly involve phosphotyrosine phosphatases.

3.2 Vascular Endothelial Growth Factor Receptor 2

VEGFR-2 (also known as KDR or Flk-1) is the first endothelial receptor activated in the early embryo and a 200–230-kDa high-affinity receptor for VEGF-A, the processed forms of VEGF-C and -D, and VEGF-E. It is expressed in both vascular and lymphatic endothelial cells, as well as several other nonendothelial cell types (34, 35, 73–75). VEGFR-2-deficient mouse embryos die by embryonic day 8.5–9.5 exhibiting defects in the development of endothelial and hematopoietic precursors indicating that VEGFR-2 is crucial for vascular development (76). VEGF-A binds to the second and third extracellular IgG-loop of VEGFR-2 with a K_d of 75–125 pM, which is ten-fold lower than the affinity for VEGFR-1 (64).

The possibility that VEGFR-2 blocking therapies could be tailored to attenuate only specific signal-transduction pathways to reduce side effects of the treatment has promoted an interest in dissecting VEGFR-2 signaling. In contrast to VEGFR-1, autophosphorylation of VEGFR-2 after stimulation with VEGF-A is readily detected in intact cells, and the positions of several phosphorylated tyrosine residues in the receptor have been mapped. To date, Tyr951 and Tyr996 in the kinase-insert domain, Tyr1054 and Tyr1059 in the kinase domain, and Tyr1175 and Tyr1214 in

the C-terminal tail, have all been identified as autophosphorylation sites. Of these, phosphorylation of Tyr1054 and Tyr1059 is required for maximal kinase activity (77). In addition, several proteins have been found to associate, through their Src homology 2 (SH2) domain, with specific autophosphorylated tyrosine residues. Tyr951 creates a binding site for the VEGFR-associated protein (VRAP) (78) and Tyr1175 creates a binding site for Sck (79) and PLC- γ 1 (80).

VEGFR-2 is considered to be the major mediator of several physiological and pathological effects of VEGF-A on vascular endothelial cells. Only a few SH2-domain-containing molecules have been shown to interact directly with VEGFR-2. PLC- γ 1 binds to phosphorylated Tyr1175 and mediates the activation of the mitogen-activated protein kinase (MAPK)/extracellular-signal-regulated kinase (ERK)-1/2 cascade and proliferation of endothelial cells (80). PLC- γ 1 activates PKC by the generation of diacylglycerol and increased concentrations of intracellular calcium. VEGFR-2 also activates PI3K, which results in increased lipid phosphatidylinositol-3,4,5-triphosphate (PIP3) and activation of several important intracellular molecules such as AKT (protein kinase B or AKT/PKB) and the small GTP-binding protein Rac. The AKT/PKB pathway regulates cellular survival by inhibiting proapoptotic pathways such as B-cell lymphoma 2 (Bcl-2)-associated death promoter homologue (BAD) and Caspase 9 (81). The AKT/PKB pathway regulates nitric oxide (NO) production by direct phosphorylation and activation of endothelial NO synthase (eNOS) (82, 83), which is implicated in the increase in vascular permeability and cellular migration observed with VEGF-A.

The significance of the Ras-Raf-MEK-MAPK pathway downstream of VEGFR-2 is unclear. In the case of VEGFR-2, it was originally reported that Raf was directly activated via PKC in a Ras-independent manner (84). However, data has shown that VEGF-A can stimulate Ras (85) via a pathway that requires PKC and sphingosine kinase (86). Conflicting results exist in the literature with respect to the interaction of VEGFR-2 with ShcA or Grb2, which recruit the Ras-activating nucleotide-exchange factor son of sevenless (SOS) to the receptor (79, 87). The small GTP-binding protein Rac has also been implicated in regulating vascular permeability (88) and cellular migration.

Another important phosphorylation site on VEGFR-2 is Tyr951, which is a binding site for the signaling adaptor TSA γ 1 (T-cell-specific adaptor; also known as VEGFR-associated protein (VRAP)) (89). The phosphorylated Tyr951-TSA γ 1 pathway has been shown to regulate endothelial-cell migration (89, 90). TSA γ 1-deficient mice had reduced vascularization and tumor growth, which demonstrate that this is an important pathway for endothelial cells engaged in active angiogenesis (89). VEGF-A induces the formation of a complex between TSA γ 1 and Src (89), which suggests that TSA γ 1 may regulate Src activation and vascular permeability downstream of VEGFR-2.

Phosphorylation of Tyr1214 has been implicated in VEGF-induced actin remodeling through the sequential activation of CDC42 and p38 MAPK (91). Inhibition of the p38 MAPK augments VEGF-induced angiogenesis in chicken chorioallantoic membrane (CAM) (92, 93), without an accompanying increase in vascular permeability (92). Moreover, p38 MAPK-induced phosphorylation of heat-shock protein-27 (HSP27), a molecular chaperone that positively regulates VEGF-induced actin reorganization and migration (94, 95). Other signaling molecules that have

been implicated in VEGF-induced migration through VEGFR-2 include the focal-adhesion kinase (FAK) and its substrate paxillin (96–98), which are involved in focal-adhesion turnover during cellular migration. Endothelial-cell motility seems to be also regulated through IQ motif containing GTPase activating protein 1 (IQGAP1), a newly identified binding partner of phosphorylated VEGFR-2, which binds to and activates Rac1 by inhibiting its intrinsic GTPase activity (99). IQGAP1 co-localizes with phosphorylated VEGFR-2 at the leading edge of migrating endothelial cells and IQGAP1 knockdown by siRNA prevented VEGF-induced migration (100). Several other important intracellular signaling molecules are activated by VEGFR-2, notably Src. Although it is not yet clear exactly how the VEGFR-2 interacts with Src or its downstream role, mice lacking the Src family members Src and Yes show impaired VEGF-A-induced vascular permeability (101).

VEGFR-2 function is modulated through co-receptors such as HSPG, which interact with certain VEGF isoforms and VEGFR-2 (102). The functional VEGF–VEGFR-2 complex includes NRPs (15). NRP-1 is essential for vascular development (103), whereas NRP-2 may be required for lymphatic development (104). There is currently no evidence that NRPs exert direct signaling function in endothelial cells, but NRPs may act by stabilizing the VEGF–VEGFR-2 complex. The signaling capacity of VEGFR-2 is influenced by the adhesion state of endothelial cells. Vascular endothelial (VE)-cadherin is an endothelial cell-specific adherence junction component that is involved in complex formation with VEGFR-2. The crucial function of this complex is indicated by the fact that elimination of VE-cadherin expression blocks transduction of VEGF-induced signals for cell survival (105). The VE-cadherin complex includes β -catenin, which is phosphorylated and translocated to the nucleus in VEGF-A-stimulated cells (106, 107). Nuclear β -catenin binds to the T-cell factor or the closely related lymphoid-enhancer factor (TCF/LEF) (108), thereby regulating transcriptional activity of genes that are implicated in the angiogenic response.

3.3 Vascular Endothelial Growth Factor Receptor 3

VEGFR-3 is a 195-kDa high-affinity receptor for VEGF-C and VEGF-D. Distinct features of VEGFR-3 include cleavage during synthesis within the fifth extracellular Ig loop, the two regulating polypeptides are kept together by a disulfide bridge (109). There are two VEGFR-3 splice variants in humans, one short and one long; the latter has a C-terminal extension of 65 amino acids (110, 111) that is created by a retroviral insertion (112). VEGFR-3-deficient mouse embryos die at embryonal day 9.5 due to deficient vessel remodeling and cardiovascular failure at mid-gestation suggesting a significant embryonic role of VEGFR-3 in cardiovascular development (113). These effects may be due to a direct loss of VEGFR-3 function or an indirect effect caused by an increased availability of VEGF-C and -D for activation of VEGFR-2 (114). In embryos, VEGFR-3 is initially expressed in all vessels, but during development its expression in blood vessels decreases and

becomes restricted to developing lymphatic vessels (115). In the adult, VEGFR-3 expression is detected primarily in lymphatic endothelial cells, and its expression is induced in proliferating endothelial cells, such as the angiogenic vessels of various malignancies (116, 117). These cells are physiologically distinct from blood vascular endothelial cells.

VEGFR-3 is the only VEGFR for which naturally occurring mutations have been described. Therefore, dysfunction of lymphatic vessels may be caused by congenital inactivating mutations in VEGFR-3 (118). The homeodomain transcription factor Prox-1 has been shown to regulate the lymphatic endothelial cell phenotype (119). Inactivating point mutations in the VEGFR-3 leads to chronic lymphedema (120). Blocking VEGFR-3 function by overexpression of a soluble recombinant VEGFR-3 in the skin of transgenic mice leads to regression of lymphatic vessels and features of lymphedema with no apparent effect on the blood vasculature (121). The soluble VEGFR-3 also suppresses tumor lymphangiogenesis, with decreased metastasis to regional lymph nodes (122). Enhanced VEGFR-3 function by tumor-specific overexpression of VEGF-C or VEGF-D in various tumor models increases lymphangiogenesis and promotes the spread of metastasis (123–125).

Activation of VEGFR-3 induces proliferation, migration, and survival in lymphatic endothelial cells (119). The kinase activity of VEGFR-3 is likely regulated by phosphorylation of conserved residues in the kinase domain (Tyr1063 and Tyr1068). Phosphorylation of the Tyr1337 is required for association of the Shc–Grb2 adaptor protein complex to VEGFR-3 (109, 126), whereas the role of other phosphorylation sites (Tyr1230, Tyr1231, Tyr1265, Tyr1337, and Tyr1363) in signal transduction downstream of VEGFR-3 remains to be identified. VEGFR-3 forms homodimers or heterodimers with VEGFR-2 in response to processed VEGF-C (38). These heterodimeric receptors may form *in vivo* both in lymphatic endothelial cells and in certain endothelial cells, such as in fenestrated capillaries, which express both receptor types (127). The dimerization partner directs the use of potential phosphorylation sites, which is a reflection of the different substrate specificities of kinases. Therefore, in the heterodimer, VEGFR-3 is not phosphorylated at the two C-terminal tyrosine residues Tyr1337, which is the Shc-binding site, and Tyr1363 (38).

VEGFR-3 has been reported to mediate activation of the ERK1/2 in a PKC-dependent manner of p42/44 MAPK, as well as activation of the PI3K–AKT/PKB pathway (119), which could be important for lymphatic and blood endothelial cell survival. These pathways may be important during embryonic development, when VEGF-C guides migration and sprouting of lymphendothelial precursor cells from restricted regions of the cardinal vein (128). VEGFR-3 binds both the full length and proteolytically processed forms of VEGF-C and VEGF-D, whereas VEGFR-2 binds the proteolytically processed forms only. Thus, the relative availability of the ligands may indirectly modulate the signal-transduction capacities of the two receptors (129). The signal-transduction capacity of VEGFR-3 is directly influenced by heterodimeric complex formation with VEGFR-2 (38). Certain VEGFR-3 phosphorylation sites such as Tyr1337 are not phosphorylated by VEGFR-2, which affects the ability of VEGFR-3 to interact with substrates such

as Shc. Other signal transducers that are potentially used by VEGFR-3 include PLC- γ 1, SHP-2 (130) and the transcription factors STAT3 (signal transducer and activator of transcription-3) and STAT5 (131). Signal transduction by VEGFR-3 is also modulated by co-receptors such as NRP-2. The crucial role of this interaction has been shown by the phenotype of NRP-2-deficient mice, which fail to form normal lymphatic vessels and capillaries (104).

3.4 *Neuropilins*

NRP-1 and NRP-2 are a class of high-affinity non-tyrosine kinase receptors for VEGFs on endothelial and neuronal cell surfaces (15, 132). NRPs appear to be required for axonal guidance and for regulation of angiogenesis (15, 133). Both receptors bind certain isoforms of VEGF-A, VEGF-B, PlGF, and VEGF-E (15, 134, 135). NRP-1 was identified initially as a 130- to 140-kDa cell-surface glycoprotein that served as a receptor for several types of semaphorin/collapsins, a large family of secreted and transmembrane proteins that serve as repulsive guidance signals in axonal and neuronal development (136–138). During embryonic development, NRP-1 is expressed in the nervous, cardiovascular, and skeletal systems (136, 139, 140), whereas in adults it is also expressed in endothelial cells, tumor cells, lung, heart, liver, kidney, pancreas, placenta, osteoblasts, and bone marrow stromal cells (15, 141). NRP-2 has a similar expression pattern.

The role of NRP-1 in vascular development was established by transgenic mouse studies. Mouse embryos lacking functional NRP-1 died due to defects in VEGF-mediated angiogenesis and cardiac failure (103). Overexpression of NRP-1 leads to an excess of dilated blood vessels and hemorrhage, as well as neuronal abnormalities due to overactivity of VEGF-A (140). NRP-2 has been shown to bind VEGF-C and is expressed together with VEGFR-3 in endothelial cells of lymphatic vessels (16, 104). Mice lacking NRP-2 were viable, but shown to have lymphatic defects (104). Similar to VEGFR-1, a naturally occurring soluble isoform of NRP-1 (sNRP-1) acts as a natural antagonist to inhibit VEGF-A₁₆₅ binding to endothelial and tumor cells as well as VEGF-A₁₆₅-induced tyrosine phosphorylation of VEGFR-2. Rat prostate carcinoma cells that expressed recombinant sNRP-1 were characterized by extensive hemorrhage, damaged vessels, and apoptotic tumor cells (142).

NRP-1 differs greatly from VEGFRs in that it does not have an intracellular signaling domain and its activity is likely mediated as a co-receptor for VEGFR-1 and VEGFR-2 by enhancing the binding affinity of ligands to the receptors (15, 143). The binding of VEGF-A isoforms and other VEGF family members to NRP-1 is highly specific in that NRP-1 binds to VEGF-A₁₆₅ and PlGF-2, but not VEGF-A₁₂₁ (15, 132). VEGF-A₁₆₅ binds to NRP-1 by way of the VEGF exon 7-encoded domain, whereas VEGFR-1 and VEGFR-2 bind by way of the exon 3- and 4-encoded domains, respectively (144). NRP-2 binds to VEGF-A₁₆₅, as well as a rarer isoform, VEGF-A₁₄₅. Similar to NRP-1, however, it does not bind VEGF-A₁₂₁ (133). Evidence

also suggests that VEGF may signal directly through NRP without VEGFR-1 or VEGFR-2 serving as a co-receptor.

Although the specific functions of NRP in vessel development and angiogenesis are not fully known, *in vitro* inhibition of VEGF-A₁₆₅ binding to NRP-1 in endothelial cells decreased binding to VEGFR-2 and subsequent mitogenic activity (145), whereas co-transfection of NRP-1 into VEGFR-2-expressing endothelial cells enhanced the binding of VEGF-A₁₆₅ to VEGFR-2 and subsequent mitogenesis and chemotaxis (15, 137). Endothelial cells that expressed NRP-1 alone did not respond to either VEGF-A isoform, which suggests that NRP-1 may not act as a signaling receptor for chemotaxis, but rather as a co-receptor for VEGFR-2 (15, 146). Although far less is known about the signaling of VEGFs via NRP receptors, the evidence to date suggests that modulation of VEGF activity through NRP receptors may be a therapeutic target for proangiogenic and antiangiogenic strategies.

3.5 *Co-receptors for VEGFRs*

VEGF signaling is complicated by the fact that the ligands and their receptors interact with additional cellular proteins such as NRP, HSPG, integrin, and cadherin. These interactions allow coordination of signal strength, timing, and specificity with extracellular cues arising from soluble ligands, cell–cell, and cell–substratum interactions. VEGF-A₁₂₁, encoded by exons 2–5 and 8, consists of a receptor-binding domain specific for VEGFR-1 and -2 and is a poor mitogen. The longer VEGF-A₁₆₅ isoform, that also contains sequences encoded by exon 7, binds the receptors with similar affinity as VEGF-A₁₂₁, but displays increased signaling potential (144, 146, 147). All isoforms carrying exon 7, or 6 plus 7, such as VEGF-A₁₆₅, VEGF-A₁₈₃, VEGF-A₁₈₉, and VEGF-A₂₀₃, interact with NRPs and HSPG (15, 132, 133, 137, 146–151).

Integrins play an important role in cell signaling linking intracellular signaling pathways activated by soluble factors to output elicited by cellular interactions with the extracellular matrix and neighboring cells. Specific integrins bind to the extracellular domain of VEGFR-2 and augment receptor signaling (152). Integrins of the $\beta 3$ subfamily specifically bind to the extracellular domain of VEGFR-2 resulting in increased receptor activation upon VEGF stimulation (153–155). Direct interaction between $\beta 3$ integrin and VEGFR-2 is restricted to integrin $\alpha v\beta 3$ and was shown to be either ligand independent (154) or dependent (155). VEGFR-2 signaling in the context of $\alpha 1\beta 1$ and $\alpha 2\beta 1$ integrins has been shown to regulate lymphangiogenesis during tissue repair, further demonstrating how output from VEGFRs is modulated by cellular interactions with the extracellular matrix (156). VEGFR-2-mediated angiogenesis is also directly regulated by integrins as proposed by work performed in knockout mice lacking $\beta 3$ or $\beta 5$ integrin (157, 158). Animals that did not express these integrins showed increased VEGFR-2 activity and tumor vascularization. These studies suggest that integrins prevent aberrant stimulation of resting endothelial cells under nonpathological conditions or facilitate angiogenesis during vessel repair in disease (159).

Cadherins are involved in the formation of adherens junctions in endothelial and epithelial cells and play an essential role in VEGF signaling (105, 160–163). Blood flow and shear stress can influence vascular development and remodeling in part through the creation of mechanosensory complexes that involve VEGFR-2, platelet–endothelial cell adhesion molecule-1 (PECAM-1) and VE-cadherin, which functions upstream of integrin activation (164). Interaction of VEGFR-2 with VE-cadherin is regulated by β -catenin. At high cell density, the phosphatase PTP1/Dep1/CD148 associates with VE-cadherin and attenuates tyrosine phosphorylation of VEGFR-2 thereby suppressing signaling through PI3K, MAPK, and PLC- γ 1 (165). At low cell density, such as at the tip of developing blood vessels, VEGFR-2 associates with integrin α v β 3 instead of cadherin and signal output is directed toward stimulation of cell migration and mitogenesis (159). Reduced turnover of VEGFR-2 at high cell density has also been demonstrated to depend on cadherin and enhance VEGF-mediated activation of MAPKs (166).

3.6 Prognostic Role of VEGF–VEGFR Expression in RCC

Understanding the expression of the VEGF–VEGFR family in RCC will individualize the selection of target-specific therapies based on the tumor biology and optimize the benefit of agents that target these pathways. Different patterns of VEGF and VEGFR mRNA expression levels between different RCC histological subtypes have been demonstrated and were shown to be associated with tumor stage and survival (167). In patients with clear cell RCC, VEGF mRNA levels below the median had a significantly shorter survival time compared to those with higher levels. By contrast, in patients with papillary RCC, VEGF, VEGFR-1, and VEGFR-2 mRNA levels above the median were related to adverse survival.

Tissue microarrays constructed from paraffin-embedded clear cell ($n = 340$) and papillary ($n = 42$) RCC nephrectomy specimens evaluated the role of the VEGF–VEGFR family in RCC (35). Immunohistochemistry was performed with antibodies directed against VEGF-A, VEGF-C, VEGF-D, VEGFR-1, VEGFR-2, and VEGFR-3. Protein expression was analyzed within the tumor epithelium, as well as in tumor-associated endothelium. Expression of VEGFRs was also scored in tumor-associated endothelium. In the VEGF angiogenesis pathway, papillary RCC demonstrated a higher mean expression of VEGF-A (57% vs. 37%, $p < 0.0001$) and VEGFR-2 (49% vs. 37%, $p < 0.0001$) than clear cell RCC, whereas there was no difference observed in the expression of VEGFR-1 ($p = 0.53$) (35). Within the lymphangiogenesis pathway, clear cell RCC demonstrated a higher mean expression of VEGF-D (51% vs. 41%, $p = 0.033$), whereas papillary RCC demonstrated a higher mean expression of VEGFR-3 (13% vs. 3%, $p = 0.0002$) within the tumor epithelium and no difference was seen in the mean expression of VEGF-C ($p = 0.33$) (35). In addition, VEGFRs were identified on the surface of RCC cells, suggesting that VEGF may augment RCC tumor growth through an autocrine loop. These data suggest that other signaling pathways aside from VHL may be promoting VEGF

production and patients with papillary RCC should also be considered for therapies that target the VEGF ligands and/or their receptor tyrosine kinases, which have thus far been limited to patients with clear cell RCC.

Survival and metastatic pattern in clear cell RCC were also evaluated. Logistic regression analysis was used to correlate marker expression with hematogenous metastasis (M stage = 1) and lymphatic metastasis (N stage > 0). Each marker was first analyzed as a continuous variable with the generated hazard ratios corresponding to a 20% increase in marker expression. For each marker found to be significant in this model, recursive partitioning survival tree analysis was performed to identify the cutoff value with the greatest ability to dichotomize patients according to marker expression. When analyzed as continuous variables, univariate predictors of hematogenous spread to distant metastases included VEGFR-1 ($p = 0.007$) and VEGFR-2 ($p = 0.03$) expression on the tumor epithelium. Using the mean percent expression cutoff identified by recursive partitioning, VEGF-A (cutoff > 45%, $p = 0.014$), VEGFR-1 (cutoff > 60%, $p = 0.006$) and VEGFR-2 (cutoff > 5%, $p < 0.001$) expression on the tumor epithelium as well as VEGFR-1 (cutoff > 2%, $p < 0.001$) and VEGFR-2 (cutoff > 5%, $p < 0.004$) expression by the tumor-associated endothelium predicted metastasis. Multivariate analysis established VEGFR-1 ($p < 0.038$) and VEGFR-2 ($p < 0.036$) expression by the tumor epithelium as independent predictors of distant metastasis. VEGF-A ($p = 0.009$) and VEGFR-1 ($p = 0.006$) expression by the tumor epithelium and low tumor-associated endothelial expression of VEGFR-3 ($p < 0.0001$) were univariate predictors of lymph node involvement when analyzed as continuous variables (34). In the dichotomized univariate model, VEGF-A (cutoff > 65%, $p = 0.005$), VEGFR-1 (cutoff > 80%, $p = 0.001$) and VEGFR-2 (cutoff > 5%, $p = 0.04$) expression by the primary tumor, and endothelial expression of VEGFR-3 (cutoff > 25%, $p = 0.0001$) proved statistically significant. Low VEGFR-3 expression was retained as an independent predictor of lymph node involvement in multivariate analysis ($p = 0.0017$) with a fourfold increase in risk of lymphatic metastasis (34). When evaluating disease-specific survival, only low endothelial expression of VEGFR-3 was an independent predictor of survival in multivariate analysis ($p = 0.02$) (34).

4 PDGF Ligands

PDGFs constitute a family of growth factors with each member containing one of four different polypeptide chains: PDGF-A, PDGF-B, PDGF-C, and PDGF-D (168–170). Each chain is encoded by an individual gene located on chromosomes 7, 22, 4, and 11, respectively (171, 172). These polypeptide chains are linked with an amino acid disulfide bond forming homodimers or heterodimers, of which five have been described so far: PDGF-AA, PDGF-AB, PDGF-BB, PDGF-CC, and PDGF-DD. These factors exert their cellular effects through two tyrosine kinase receptors: PDGFR- α and PDGFR- β . PDGFR- α can be activated by PDGF-AA, PDGF-AB, PDGF-BB, and PDGF-CC, while PDGF-BB and PDGF-DD bind and

activate PDGFR- β . A heterodimeric PDGFR- α/β complex has also been identified which can be activated by PDGF-AB, PDGF-BB, and PDGF-CC. Ligand binding to receptors induces receptor dimerization, activation, and autophosphorylation of the tyrosine kinase domain, which leads to the subsequent recruitment of SH-2 domain containing signal-transduction proteins and activation of signaling enzymes including Src, PI3K, and PLC- γ 1 (173). Tissue culture and in vivo murine models have suggested that PDGFR- α and PDGFR- β activate distinct signaling pathways, which ultimately promote cell migration, proliferation, and survival (174). These findings, together with the observations that PDGFRs participate in different tumor-associated processes, have prompted the development of different kinds of PDGF antagonists.

5 Role of PDGF Ligands and Receptors in Tumor Development

The role of PDGF in angiogenesis and embryonic development of kidney, brain, and cardiovascular and respiratory systems have been demonstrated in gene knockout studies. Expression of PDGFR is found on erythroid and myeloid precursors in bone marrow, as well as in monocytes, megakaryocytes, fibroblasts, endothelial cells, osteoblasts, and glial cells. Aberrant or overexpression of PDGFR is associated with a variety of disorders including atherosclerosis (175), fibrotic disease (176), and malignancy (177). Expression of PDGF has been shown in a number of cancers (178–180). PDGF–PDGFR play physiological roles in a number of processes that lead to tumor development including autocrine stimulation of cancer cells, development of angiogenesis, recruitment of tumor fibroblasts, and control of tumor interstitial pressure.

5.1 Autocrine PDGFR Signaling

The first indications of autocrine PDGFR signaling as a transforming mechanism arrived with the discovery of the homology between the simian sarcoma virus oncogene (v-sis) and the PDGF B-chain (181, 182), and subsequent demonstrations of the transforming ability of human PDGF-B cDNA (183–185). Similar studies have shown that expression of PDGF-AA, -CC, or -DD in NIH 3T3 fibroblasts leads to a transformed phenotype (186–188). These findings have been followed-up in a series of studies demonstrating co-expression of PDGF and PDGFR in malignancies derived from PDGF-responding cells (178, 189–194). Evidence has also been provided for autocrine PDGFR signaling in ovarian (195, 196) and prostate cancer (180, 197, 198), as well as in neuroblastomas (199). Furthermore, it has recently been shown that autocrine PDGFR signaling plays an essential role in

the development of metastasis (200). Tissue culture and xenograft tumor models exhibit reduced growth when treated with PDGFR inhibitors (201–207). Different malignancies have also been shown to be associated with mutational activation of PDGF or PDGFR (208–214).

5.2 Role of PDGF Ligands and Receptors in Angiogenesis

There is increasing evidence that implicate PDGFR signaling in tumor angiogenesis. PDGF has been found to stimulate angiogenesis in vivo (215–217), and a role for PDGF in the recruitment of tumor pericytes has been demonstrated in knockout mice models (218). A recent study with a selective PDGFR tyrosine kinase inhibitor (CP-673,451) confirms that selective blockade of PDGF can inhibit angiogenesis in a sponge angiogenesis model (219). Furthermore, tumor growth inhibition has been seen in a number of tumor models regardless of PDGFR status suggesting that the antitumor effect is due to antiangiogenic properties rather than direct inhibition of autocrine stimulation.

The extent and characteristics of pericyte coverage of tumor vessels remains to be fully described. Confocal microscopy combined with markers for pericytes and endothelial cells have shown mural cells to be associated with tumor capillaries, but abnormalities in the pericyte coverage of tumor vessels have also been noted (220, 221). Pericytes surrounding normal capillaries were well organized and closely attached with endothelial cells, whereas tumor pericytes showed a lower density, looser connection with endothelial cells, and displayed cytoplasmic processes extending away from the vessel wall. Differences in marker protein expression, including expression of α -smooth muscle actin on tumor pericytes, were also noted. Immunohistochemical analysis of a tumor tissue array revealed that more than 90% of samples displayed PDGFR- β staining surrounding capillaries, most likely representing tumor pericytes (222).

Pericyte recruitment is part of the development of normal functional capillaries. The analyses of PDGF-B and PDGFR- β knock-out mice have documented the importance in this process of PDGF-BB-mediated activation of PDGFR- β on pericytes (218). The functional roles of PDGFR- β signaling in tumor pericyte recruitment have been analyzed by comparing tumor pericyte recruitment in tumor models in which the extent of PDGFR activation has been manipulated (223–225). Pericytes are thought to be required for normal microvessel stability. Mouse models with pericyte deficiency demonstrate a range of abnormalities within the microvasculature (226, 227). In tumors transplanted into PDGF-B retention motif deficient mice, fewer pericytes were seen and conversely, transgenic expression of PDGF-B into tumor cells was able to increase pericyte density (223). By comparing the pericyte recruitment in vessels of subcutaneous tumors in wild-type mice and in mice expressing PDGFR- β deficient in activation of PI3K, it was concluded that tumor pericyte recruitment was dependent on this pathway (225). Furthermore, a B16 mouse melanoma model showed that

production of PDGF-BB or PDGF-DD by tumor cells was associated with an increase in pericyte concentration within the tumor and increased tumor growth rate occurred in the absence of increase in vessel density, suggesting that the increased pericyte coverage affected tumor vasculature in a functional, rather than quantitative manner (225). These studies demonstrate that PDGF is a critical regulator of pericyte recruitment to tumor vessels.

Antiangiogenic approaches targeting of VEGFRs are attracting attention as novel cancer therapies. However, studies suggest that VEGFR-targeted therapies are more effective in immature tumor vessels that lack pericyte coverage and that pericyte recruitment and/or survival may confer resistance to VEGFR antagonists (4). Interestingly, inhibition of VEGFR-2 increases pericyte formation and vessel normalization (228). On the other hand, inhibition of PDGFR is associated with pericyte detachment from tumor vessels. Thus, the combination of VEGFR inhibitors and PDGFR antagonists is an attractive concept that is being evaluated (229–232).

Whereas PDGFR- β expression on tumor pericytes is very common, the expression of PDGFR- β on tumor endothelial cells appears to be more restricted. More recently, PDGFR- β expression has been reported in mouse models of prostate cancer bone metastasis, ovarian and pancreatic cancer (205, 222, 233). Interestingly, in the prostate cancer study, upregulation of endothelial cell PDGFR- β expression was seen in the bone metastases, but not in tumors growing in adjacent muscle, suggesting tissue-specific components in the regulation of PDGFR expression. In both mouse models, treatment with a combination of a PDGFR inhibitor together with chemotherapy enhanced the effects of chemotherapy alone. Combination effects occurred in association with increased tumor endothelial cell apoptosis suggesting a sensitization of endothelial cells to chemotherapy, by PDGFR antagonists (205). Further studies are warranted to further investigate the occurrence of PDGFR on endothelial cells in other tumor models and in clinical settings.

5.3 Recruitment of Tumor Fibroblasts

A fibroblast-rich stroma constitutes a large part of most solid tumors and most likely contributes functionally to tumor growth and maintenance of tumors. Prominent PDGFR- β expression in the stroma of a variety of malignancies have been demonstrated in series of immunohistochemical studies (234–241). Analyses of tumor cells lacking PDGFR, where PDGF production has been manipulated, provide support for a functional role of PDGFR signaling in tumor stroma recruitment (242–244). Overproduction of PDGF-BB by WM9 melanoma cells led to a reduced latency time of in vivo tumor formation (242). PDGF-BB transfected cells also formed tumors characterized by less necrosis and a more abundant tumor stroma. Similarly, transfection of nonmalignant HaCaT epithelial cells induced the ability to form benign cysts with high blood vessel density and proliferating fibroblasts (243).

A causal role for PDGFRs in the desmoplastic response associated with breast cancer was provided through the downregulation of PDGF production in a desmoplasia-inducing breast cancer cell line (244). Tumor cells with reduced PDGF production formed nondesmoplastic tumors with reduced fraction of myofibroblasts and a lower collagen content.

5.4 Role of PDGF Ligands and Receptors in Control of Tissue Interstitial Fluid Pressure

The widespread expression of PDGFR- β in tumor stroma implies that large therapeutic benefits could be obtained if critical tumor properties could be assigned to these receptors. Studies have shown that a function of these receptors is the control of tumor transvascular transport. One of the parameters determining transvascular transport, and also transport of standard chemotherapy drugs, is interstitial fluid pressure (IFP) which controls the convection rate across capillary walls. Activation of PDGFR plays a key role in the elevation of IFP in tumors. The IFP of normal tissues is under the control of a number of mechanisms. Connective tissue cells can modify IFP via $\beta 1$ integrins (245). In a rat model, administration of PDGF-BB normalizes dermal IFP that has been artificially lowered, an effect which appears to be specific to PDGF-BB (246). The mean IFP in solid tumors ranges between 14 and 30 mmHg compared to a normal tissue IFP of -1 mmHg. Although the precise cause for such high IFP is not well understood, the high IFP can cause poor uptake of anticancer drugs and hence reduced cell kill (247).

On the basis of previous evidence that a function of PDGFR is control of IFP in normal loose connective tissue and that most solid tumors show PDGFR- β expression in tumor stroma, a set of studies have investigated the effects of PDGFR antagonists on tumor IFP and drug uptake (248–250). PDGF inhibition represents a promising therapeutic adjunct by normalizing tumor IFP and thereby increasing drug delivery to the tumor. Systemic treatment with imatinib or an oligonucleotide aptamer antagonist of PDGF-B was shown to reduce tumor IFP in an experimental rat PROb colon carcinoma model (248). Accordingly, imatinib increased the tumor capillary-to-interstitium transport of ^{51}Cr -EDTA in the same tumor model. The effects of PDGFR antagonists on chemotherapy response have been investigated in two subcutaneous tumor models that demonstrated an increase in tumor transvascular transport through monitoring the distribution of tracer compounds or cytotoxic drugs (249, 250). The increased uptake occurred in the absence of effects on tumor vessel density or pericyte coverage, and was associated with an $\sim 30\%$ reduction in tumor IFP. Furthermore, correlative evidence was provided through a set of experiments using varied dosing schedules of PDGFR antagonists in which enhanced drug uptake was paralleled by reduced IFP and vice versa (250).

A series of studies were performed in two animal tumor models to investigate if PDGFR antagonist-induced drug uptake was associated with enhanced antitumor effects of chemotherapy drugs. In SCID mice, the uptake and antitumor effects of paclitaxel on subcutaneous KAT-4 human anaplastic thyroid carcinomas were enhanced by inhibitory PDGF aptamers or imatinib treatment (249). The antitumor effects of 5-fluorouracil were also enhanced by imatinib in a PROb colon carcinoma model in syngenic BDIX rats (249). In a further study, the antitumor effect of epothilone B was potentiated when co-administered with imatinib in SCID mice with subcutaneous KAT-4 tumors (250). The improved efficacy correlated with reduced tumor IFP and a three-fold increase in the tumor concentrations of epothilone B. Furthermore, combination effects were not observed when combined drug regimens were tested on cultured tumor cells supporting the notion that the beneficial effects of combining PDGFR antagonists with chemotherapy drugs occurred as a result of increased tumor uptake of the cytotoxic drugs. Of potential clinical importance, there was no significant increase of epothilone B levels seen in normal organs and the tolerability of epothilone B was not reduced.

The correlations observed between the effects of imatinib on IFP and tumor drug uptake are in agreement with the hypothesis that reduction in the tumor IFP can enhance drug delivery to the tumor. However, the mechanism whereby PDGFR blockade causes a reduction in IFP remains to be clearly established. Nevertheless, these data suggest that inhibition of PDGF is associated with a reduction in tumor IFP that improves the intratumoral delivery of concurrently administered cytotoxic drugs. Such a combination could improve the efficacy without increased toxicity in comparison with conventional treatments. Clinical trials are warranted to investigate if these experimental findings can be validated in patients. It should also be cautioned that the intrinsic insensitivity of tumor cells to cytotoxic drugs, such as a reduced susceptibility to apoptotic stimuli, may limit the beneficial effects of an increased tumor drug uptake.

6 Perspectives and Future Directions

Effective forms of therapy are limited for the majority of patients with advanced RCC, but it is hoped that continuing research will help provide the rationale for new forms of therapy for kidney cancer based on understanding the molecular pathways involved in tumorigenesis. The success of the program that led to the development of imatinib to target the c-Kit protooncogene in chronic myeloid leukemia and gastrointestinal stromal tumors is viewed as “proof of principle” for the molecular targeting approach to treatment of solid tumors, but have also highlighted the importance of proper patient selection (251). There has been an intense focus in the past few years on the development of therapies based on modulation of VEGFR and PDGFR function. It is likely that inhibition of tumor angiogenesis will be a useful part of combination therapy in tumor treatment in the future, but may be insufficient alone. For long-term treatments involving VEGFR and PDGFR

antagonists, it is important to learn more about expression patterns of these receptors, as well as the nonendothelial cell functions that these receptors may exert. Clearly, many downstream signaling pathways from the receptors have now been identified. However, many of these appear to be pathways used by several different growth factors in a range of cell types and important in functions such as migration and proliferation. Equipped with high quality reagents and using tumor models that closely mimic the in vivo situation, it will be possible to pinpoint the role of known signal transducers in this response. Furthermore, using new explorative techniques based on proteomics and microarray will allow for the identification of novel proteins and genes regulating tumorigenesis. Such molecules will constitute new and, potentially, highly specific drug targets for the development of future antitumor therapies.

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Sunitinib and Axitinib in Renal Cell Carcinoma

Robert J. Motzer

Abstract Sunitinib and axitinib are two oral, small-molecule agents that emerged from the same drug discovery program. They were rationally designed for selective inhibition of receptor tyrosine kinases (RTK), critically involved in human tumor malignancies. Sunitinib, the more extensively studied of the two, has demonstrated unprecedented antitumor activity in two phase II studies of cytokine-refractory metastatic renal cell carcinoma (mRCC) and statistically significant superiority over interferon-alfa as first-line therapy in patients with mRCC. These data have established sunitinib as a new reference standard of care in the first-line mRCC setting. Axitinib has also demonstrated significant antitumor activity in the cytokine-refractory mRCC setting, as well as shown encouraging results as second-line therapy in mRCC patients, refractory to prior RTK inhibition. In this chapter, we will discuss each compound in turn, briefly reviewing the preclinical and phase I development of each, before summarizing the clinical data available to date for the use of each in mRCC, including biomarker data for both drugs and discussion of data for sunitinib in different patient populations, in combination with other targeted agents, and at different dosing schedules.

Keywords Sunitinib • SU11248 • SUTENT • Axitinib • AG-013736, • Renal cell carcinoma

1 Introduction

Sunitinib malate (SU11248, SUTENT®) and axitinib (AG-013736) both emerged from a drug discovery program to identify rationally designed, small molecules with suitable pharmacologic properties that would inhibit the activity of selected

R.J. Motzer

Memorial Sloan-Kettering Cancer Center, 1275 York Ave, New York, NY 10021

e-mail: motzerr@mskcc.org

receptor tyrosine kinases (RTKs), known to have critical involvement in human tumor malignancies. Sunitinib was approved in 2006 by the US FDA for the treatment of advanced renal cell carcinoma (RCC) and by the European Medicines Agency (EMA) for use in advanced and/or metastatic RCC (mRCC). At the same time, sunitinib was also approved by both authorities for the treatment of gastrointestinal stromal tumors (GIST) after disease progression on or intolerance to imatinib mesylate therapy. Clinical development of sunitinib continues in these and other malignancies in order to optimize treatment regimens and identify all of the potential anticancer benefits that sunitinib may offer. Axitinib is currently in phase II development for mRCC, as well as for other solid malignancies (e.g., thyroid, metastatic breast, and non-small cell lung cancers).

In this chapter, we will discuss each compound in turn, briefly reviewing the preclinical and phase I development of each, before summarizing the clinical data available to date for the treatment of mRCC.

2 Sunitinib (SU11248)

Sunitinib is a small molecule with the molecular formula $C_{22}H_{27}FN_4O_2$. The free base has a molecular weight of 398.48 and the L-malate salt, the form used in clinical trials (Fig. 1), has a molecular weight of 532.56. The chemical name of the L-malate salt is (Z)-N-[2-(diethylamino)ethyl]-5-[(5-fluoro-2-oxo-1,2-dihydro-3H-indol-3-ylidene)methyl]-2,4-dimethyl-1H-pyrrole-3-carboxamide (S)-2-hydroxysuccinate.

2.1 Preclinical Activity of Sunitinib

Sunitinib was evaluated against more than 80 kinases and was shown to be a potent inhibitor of several members of the class III/V split kinase domain family of RTKs, including vascular endothelial growth factor receptors (VEGFRs) -1, -2, and -3, platelet-derived growth factor receptors (PDGFRs) α and β , stem cell factor receptor (KIT), colony-stimulating factor receptor-1 (CSF-1R), Fms-like tyrosine kinase-3 receptor (FLT3), and the glial cell-line derived neurotrophic factor receptor (rearranged during transfection; RET); inhibition of function has been demonstrated in cell proliferation assays (1–4). Pharmacokinetic studies showed that sunitinib is metabolized by cytochrome P450 (CYP) 3A4 to an active primary metabolite (SU12662), with similar potency to sunitinib and similar binding to human plasma protein *in vitro* (90% and 95%, respectively) (5).

Sunitinib has shown antitumor activity against a broad range of tumor models *in vivo*, causing tumor regression in murine models of renal (786-O), colon (Colo205 and HT-29), breast (MDA-MB-435), lung (NCI-H226 and H460), and prostate (PC3-3M-luc) cancers, while suppressing tumor growth in others, such as human glioma xenografts (SF763T) and melanoma lung metastases (B16) (6). In addition,

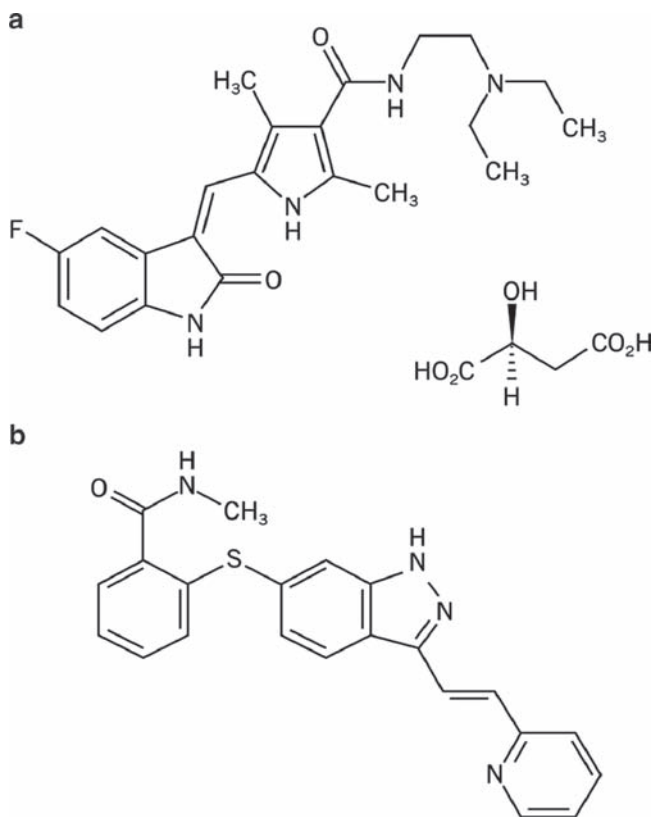


Fig. 1 Chemical structures of (a) sunitinib and (b) axitinib [reproduced from the SUTENT prescribing information (5) and the United States Pharmacopeia (USP) Dictionary (23) with permission of Pfizer, Inc. and USP, respectively]

a 30% reduction in tumor microvessel density confirmed inhibition of tumor-related angiogenesis in an experimental model of colon carcinoma (7). In mouse xenograft models, sunitinib inhibited the phosphorylation of PDGFR- β , VEGFR-2 and KIT at plasma concentrations ≥ 50 ng/mL in a time- and dose-dependent manner, and daily administration was sufficient to inhibit receptor phosphorylation for 12 h.

2.2 Safety and Clinical Activity of Sunitinib: Phase I Studies

The pharmacokinetics and safety of sunitinib were investigated in phase I dose-escalation studies (8–10). Sunitinib was administered once daily at doses ranging from 25 to 150 mg in three different dosing regimens: 2 weeks on treatment followed by 1 week off (2/1 schedule); 2 weeks on treatment followed by 2 weeks

off (2/2 schedule); or 4 weeks on treatment followed by 2 weeks off (4/2 schedule). The off-treatment period was incorporated into the regimens based on preliminary preclinical toxicology and pharmacokinetic data. Adverse events (AEs) were monitored throughout the phase I studies and were graded according to the National Cancer Institute (NCI) Common Toxicity Criteria (CTC), Version 2.0.

The most common AEs experienced by patients included fatigue, nausea, vomiting, diarrhea, neutropenia, thrombocytopenia, and hair/skin discoloration. The severity of AEs was generally dose dependent, and fatigue/asthenia tended to increase and decrease in association with on-treatment and off-treatment periods, respectively. The maximum tolerated dose was defined as 50 mg/day. Patients receiving this dose achieved trough plasma concentrations >50 ng/mL (sunitinib plus SU12662); in preclinical studies, this concentration was found to be sufficient to inhibit receptor phosphorylation and thus induce tumor regression. Based on preliminary tumor response data in phase I studies (10), the recommended dosing schedule for phase II and III studies was the 4/2 schedule, which provided optimal plasma concentration levels and acceptable toxicity. Additional schedules are being investigated, for example, continuous dosing at 37.5 mg/day.

Pharmacokinetic data obtained from phase I and subsequent clinical trials show that sunitinib absorption is unaffected by food, with peak plasma concentrations of sunitinib and SU12662 reached between 6 and 12 h after dosing. The terminal half-life of sunitinib is 40–60 h and of SU12662 is 80–110 h. Sunitinib and SU12662 pharmacokinetics are not significantly altered by repeated daily dosing or repeated cycles, and steady-state plasma concentration levels are achieved after 10–14 days (5).

The phase I population comprised patients with various advanced solid tumors, including RCC, GIST, neuroendocrine tumors, colorectal cancer, and prostate cancer. Twenty-five (21%) of 117 patients enrolled achieved either a partial response (PR; $n = 17$) or a stable disease (SD; $n = 8$) based on the Response Evaluation Criteria in Solid Tumors (RECIST) (11). Four patients with mRCC achieved a PR, lending further support to the therapeutic strategy of development in RCC (9, 10).

2.3 Efficacy and Safety of Sunitinib in Cytokine-Refractory mRCC: Phase II Studies

Two consecutively conducted, independent, open-label, multicenter phase II studies evaluated the efficacy and safety of single-agent sunitinib in patients with cytokine-refractory mRCC (12, 13). The similar design of the trials permitted a subsequent independent analysis of the pooled data. Patients in both trials were required to have measurable disease and failure of one prior cytokine therapy. The first study permitted patients with any RCC histology, while clear-cell histology was a specific requirement in trial 2. Other eligibility criteria specific to trial 2 included radiographic documentation of disease progression and prior nephrectomy. Treatment consisted of oral sunitinib 50 mg once daily in repeated 6-week cycles of the 4/2 schedule and continued until disease progression, unacceptable toxicity or withdrawal of consent.

Dose reduction to 37.5 mg/day and then 25 mg/day was permitted depending on individual patient tolerability. The primary endpoint of each trial was objective response rate (ORR) by RECIST (assessed every 1–2 cycles), defined as the proportion of patients with a confirmed complete response (CR) or PR. Secondary endpoints included time to tumor progression (TTP) in the first study, progression-free survival (PFS) in the second study, overall survival (OS), and safety.

2.3.1 Efficacy

In trial 1 (12), 63 patients with mRCC (87% with clear-cell histology) received a median of 9 months of therapy (range <1 to 24+ months). The ORR was 40% (25 patients with PR), and a further 17 patients (27%) had SD ≥3 months (Table 1). Median TTP was 8.7 months (95% CI: 5.5–10.7) and median OS was 16.4 months (95% CI: 10.8–not available). The majority of patients in trial 1 had a reduction in measurable disease following sunitinib treatment.

In trial 2 (ongoing) (13), 106 patients received a median of five cycles of therapy (range 0–11 cycles), equivalent to 7 months. Among 105 patients evaluable for efficacy (one patient with a diagnosis of clear-cell RCC was withdrawn after a repeat biopsy showed a diagnosis of a different cancer type), one patient (1%) achieved a CR, and 45 patients (43%) a PR, giving an ORR of 44%. A further 23 patients (22%) had SD ≥3 months (Table 1). Median PFS based on third-party independent assessment was 8.3 months (95% CI: 7.8–14.5). At the time of analysis, median OS had not been reached; the 6-month survival was 79% (95% CI: 70–86%).

In a pooled analysis (13), which combined the demographics and efficacy data from the two phase II trials (N = 168), the ORR was 42% (Table 1) and the median PFS was 8.2 months (95% CI: 7.8–10.4) (Fig. 2). Patients who responded to treatment had a longer period without disease progression; median PFS for responders was 14.8 months compared with 7.9 months for patients with SD ≥3 months

Table 1 Investigator-assessed best tumor response with sunitinib in phase II studies of patients with cytokine-refractory metastatic renal cell carcinoma (mRCC)^a

Response, n (%)	Trial 1 (N = 63)	Trial 2 (N = 105) ^b	Pooled analysis (N = 168)
Overall response	25 (40)	46 (44)	71 (42)
Complete response	0	1 (1)	1 (<1)
Partial response	25 (40)	45 (43)	70 (42)
Stable disease ≥3 months	17 (27)	23 (22)	40 (24)
Progressive disease, stable disease <3 months, or not evaluable	21 (33)	36 (34)	57 (34)

^aAdapted and reproduced from Motzer et al. (13) with permission of the American Medical Association

^bOne patient enrolled with a diagnosis of clear-cell renal cell carcinoma was withdrawn after a repeat biopsy showed a diagnosis of a different type of cancer

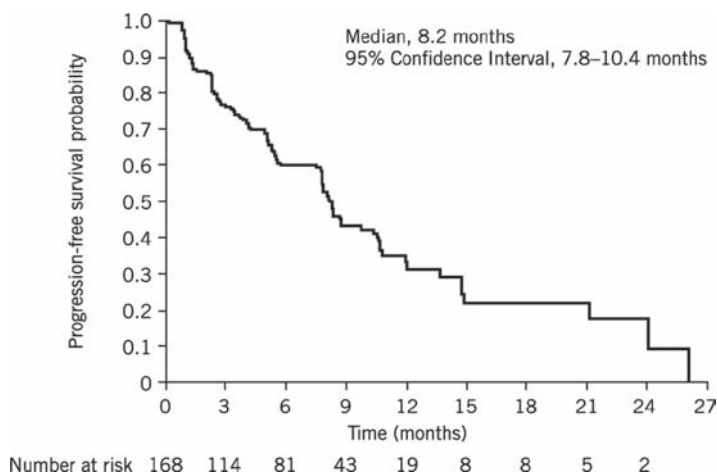


Fig. 2 Kaplan-Meier estimate of investigator-assessed progression-free survival (PFS) in a pooled analysis of two phase II metastatic renal cell carcinoma (mRCC) studies ($N = 168$) [reproduced from Motzer et al. (13), with permission of the American Medical Association]

and 2.1 months for patients with SD <3 months or progressive disease (PD). The pooled analysis also evaluated various baseline clinical features as potential indicators of response. Normal baseline hemoglobin levels rather than levels below the lower limit of normal were present in 78% of responders compared with 43% of nonresponders ($P < 0.001$, 2-tailed Fisher exact test), and 66% of responders compared with 45% of nonresponders ($P < 0.008$, Fisher exact test) had an Eastern Cooperative Oncology Group performance status (ECOG PS) of 0 rather than 1. A univariate analysis also found that these clinical characteristics (i.e., normal serum hemoglobin and favorable ECOG PS) were associated with longer PFS. Poor performance in addition to anemia has been previously reported to be associated with reduced survival in second-line therapy for mRCC (14).

The objective response rates and durability of response achieved by sunitinib treatment in these phase II trials, compared with historical observations of conventional second-line therapies, led to accelerated US FDA and EMEA approval for the use of sunitinib in advanced RCC.

2.3.2 Safety

The tolerability profile of sunitinib was similar, and favorable, in trials 1 and 2; the majority of AEs reported were manageable with temporary delays in dosing, dose reduction, and/or standard medical interventions. In trial 1, the most commonly reported NCI CTC grade 3, non-hematological treatment-related AEs were fatigue (11%), diarrhea (3%), nausea (3%), and vomiting (3%); there were no non-hematological grade 4 AEs. The most commonly reported grade 3/4 laboratory abnormalities were lymphopenia (32%), elevated lipase (21%; not associated with

symptoms of pancreatitis), neutropenia (13%), and anemia (10%). The dose of sunitinib was reduced to 37.5 mg/day in 22 patients, and subsequently to 25 mg/day in two of these patients. The reasons for dose reductions were asymptomatic elevated lipase or amylase, and fatigue. The dose of sunitinib was increased to 62.5 mg/day in five patients and to 75 mg/day in one patient; however, no evidence of improved response was observed at these doses.

In trial 2, the most commonly reported grade 3 (NCI Common Terminology Criteria for Adverse Events Version 3.0 [CTCAE]), non-hematological treatment-related AEs were fatigue (11%), hand-foot syndrome (7%), hypertension (6%), stomatitis (5%), and diarrhea (3%); there were no grade 4 non-hematological AEs. The most commonly reported grade 3/4 laboratory abnormalities were elevated lipase (17%; not associated with symptoms of pancreatitis), neutropenia (16%; not associated with fever or sepsis), thrombocytopenia (6%), and anemia (6%). Sunitinib was discontinued following AEs in 12 patients (11%).

2.3.3 Biomarkers of Response to Sunitinib

A study of molecular tumor markers to explore their potential to be used as surrogate markers for response was undertaken. In trial 1, plasma levels of VEGF and soluble VEGFR-2 (sVEGFR-2) were measured in serially collected samples and were analyzed for correlation with drug exposure and objective responses (15). At the end of the first cycle, VEGF levels had increased more than threefold relative to baseline in 24 of 54 cases (44%), and levels of sVEGFR-2 had decreased by $\geq 30\%$ in 50 of 55 cases (91%) and by $\geq 20\%$ in all cases. In addition, significantly larger changes in both VEGF and sVEGFR-2 were observed in 25 patients achieving a PR than in 32 patients who had SD or PD (only patients with a baseline and one post-treatment time point were included).

2.3.4 Patient Reported Outcomes

In trial 1, patient reported fatigue was assessed weekly using the Functional Assessment of Chronic Illness Therapy-Fatigue (FACIT-Fatigue) scale during the first four cycles of treatment (24 weeks). The FACIT-Fatigue scale is a 13-item, validated questionnaire that assesses the impact of patient reported fatigue on daily activities and function. The possible score range is 0–52, with 0 representing the most fatigue and 52 the least. As a reference, the US general population has a mean score of 43.6, non-anemic cancer patient 40.0, and anemic cancer patients 23.9. For the FACIT-Fatigue scale, the established minimally important difference is a 3–4 point change (16). The mean baseline FACIT-Fatigue score in trial 1 was 40.4, representing more fatigue than the US general population. Baseline FACIT-Fatigue scores correlated with baseline ECOG PS ($P < 0.001$); patients with a better ECOG PS reported less fatigue (17). During the first four cycles of therapy, a mean change from baseline ($\pm$ standard deviation) of -2.2 ± 7.8 was reported, which is less than the established minimally important difference (16). In general, within treatment cycles, mean scores

decreased (more fatigue) and increased (less fatigue) during on- and off- treatment periods, respectively, but not to a level that was considered significant.

2.4 Efficacy and Safety of Sunitinib as First-Line Therapy in mRCC: Pivotal Phase III Randomized Trial

As a result of the exceptional activity of sunitinib in second-line treatment of mRCC, which exceeded the response rates reported for cytokines as first-line treatment, sunitinib has been compared with interferon- α (IFN- α) as a first-line treatment in an international, randomized, phase III trial (18). Patients with clear-cell mRCC received oral sunitinib at a dose of 50mg/day on the 4/2 schedule or subcutaneous injection of IFN- α at a dose of 9MU three times per week (3MU in the first week, 6MU in the second week, and 9MU in all subsequent weeks). The primary endpoint was PFS, and secondary endpoints included ORR (by RECIST), OS, and safety.

A total of 750 patients were randomized to treatment, 375 to sunitinib and 375 to IFN- α , and baseline characteristics were well balanced between the two groups; the median age was 60 years and 90% of patients had had prior nephrectomy. Sunitinib treatment resulted in a statistically significant improvement in both PFS and ORR. Median PFS by independent central review was 11 months for sunitinib compared with 5 months for IFN- α (hazard ratio [HR] 0.42; 95% CI: 0.32–0.54; $P < 0.001$). The ORR by independent central review was 31% for sunitinib compared with 6% for IFN- α ($P < 0.001$), and by investigator assessment was 37% for sunitinib versus 9% for IFN- α ($P < 0.001$). Eight percent of patients in the sunitinib group withdrew from the study because of an AE compared with 13% in the IFN- α group ($P = 0.05$).

At follow up, median OS was greater with sunitinib vs. IFN- α , 26.4 months vs. 21.8 months, respectively (HR = 0.821; 95% CI, 0.673 to 1.001; $P = 0.0510$) per the primary analysis of the unstratified log-rank test ($P = 0.0128$ per unstratified Wilcoxon test) (19).

These data complement the findings of the second-line phase II trials and establish sunitinib as a new reference standard for first-line treatment of clear-cell mRCC.

2.5 Other Sunitinib Studies in mRCC

Treatment tailored to the individual patient may result in further improvements in mRCC therapy. To this end, a number of additional phase II clinical trials with sunitinib have been initiated, including sunitinib administered on a different dosing schedule, in different patient populations, and in combination with other targeted agents.

2.5.1 Sunitinib Administered in a Continuous Dosing Regimen

In a continuous dosing study (20), 107 patients with measurable mRCC, an ECOG PS of 0 or 1, and failure of one prior cytokine-based regimen were randomized to

receive sunitinib either in the morning ($n = 54$) or in the evening ($n = 53$), given once daily at a starting dose of 37.5 mg. Dose reduction to 25 mg/day was permitted based on individual patient tolerability. The primary endpoint was RECIST-defined ORR.

Seventy-three patients (68%) discontinued treatment, 53 because of lack of efficacy and 12 because of AEs (one patient withdrew consent and seven patients died while on treatment). The dose was reduced to 25 mg/day in 48 patients (45%) because of grade 2 or 3 AEs. Overall, the most commonly reported grade 3 treatment-related AEs were asthenia/fatigue (16%), diarrhea (10%), hypertension (10%), and hand-foot syndrome (9%). There were no differences in tolerability or health-related quality of life between patients receiving sunitinib in the morning or in the evening.

At the time of analysis, 21 patients (20%) had a confirmed PR and 43 patients (40%) had maintained SD for >3 months. The median investigator-assessed PFS was 36 weeks (95% CI: 28–44), which is comparable to that for sunitinib 50 mg/day on the 4/2 schedule.

2.5.2 Sunitinib in Patients with Bevacizumab-Refractory mRCC

In a phase II study (21), 61 patients with measurable, clear-cell mRCC and disease progression (as defined by RECIST) within 3 months of bevacizumab-based therapy received sunitinib 50 mg/day on the 4/2 schedule. Fourteen patients (23%) had a PR and 36 patients (59%) had SD, of whom 32 patients (52%) experienced some degree of tumor shrinkage. The median PFS was 30.4 weeks (95% CI: 18.3, 35.6). These preliminary efficacy data suggest that sunitinib may inhibit signaling pathways involved in bevacizumab resistance, although additional studies are needed to determine the precise mechanism of response to sunitinib.

2.5.3 Combination Study of Sunitinib Plus Gefitinib

While the above studies have investigated sunitinib as monotherapy, a phase I/II study has also explored sunitinib plus gefitinib (an inhibitor of epidermal growth factor receptor [EGFR]) as combination therapy in cytokine-refractory mRCC (22). The primary objective of phase I was to determine the maximum tolerated dose and overall safety and tolerability of sunitinib plus gefitinib; the objective of phase II was to assess the antitumor activity of this combination. During phase I, 11 patients with measurable, clear-cell mRCC were given sunitinib on the 4/2 schedule at one of two doses: 37.5 mg/day ($n = 4$) or 50 mg/day ($n = 7$). Gefitinib was co-administered at a daily dose of 250 mg, starting on day 10 of the first sunitinib cycle. The maximum tolerated dose of sunitinib, when given in combination with gefitinib, was established as 37.5 mg/day, and 31 patients were subsequently enrolled in phase II at this dose combination. The most common treatment-related AEs were diarrhea (48%) and generalized rash (26%); and laboratory abnormalities included neutropenia and hyperlipasemia (each of which occurred in 36% of patients). Overall, 15 of 42 patients (36%) had achieved a PR and 20 patients (48%) had SD. The median

PFS was 11.2 months (95% CI: 5.7, 16.0). The preliminary data support preclinical observations that the combination of VEGFR and EGFR inhibition may provide a broader spectrum of tyrosine kinase inhibition and act synergistically to enhance antitumor activity.

2.5.4 Sunitinib Expanded-Access Study

The aim of this expanded-access study, which was ongoing at the time of writing, was to make sunitinib available to patients with mRCC who were ineligible for participation in any ongoing phase I, II, or III clinical trials, and to allow patients to be treated in countries where regulatory approval had yet to be granted (23). In addition, this study further assessed the safety and efficacy (endpoints: OS, TTP, PFS, and ORR) of sunitinib according to each study site's standard of care. Sunitinib 50 mg/day was administered on the 4/2 schedule with dose reduction permitted for toxicity.

At the time of writing, preliminary safety data were available for 2,341 patients, following four cycles of treatment (median; range, 1–17). A total of 1,537 patients (66%) had discontinued treatment, including 904 because of lack of efficacy and 100 because of AEs (94 patients had withdrawn consent and 375 had died). The most common treatment-related AEs were diarrhea (39%), fatigue (36%), and nausea (34%), and the most frequent laboratory abnormality was thrombocytopenia (16%). Of note, the toxicity appeared to be no worse in patients of concern who had not been previously studied (i.e., patients with a poor performance status, brain metastases, and/or who were >65 years of age). The preliminary median PFS for patients with prior cytokine therapy ($n = 1,840$) was 8.9 months (95% CI: 8.3, 9.9).

3 Axitinib (AG-013736)

Axitinib is a small molecule with the molecular formula $C_{22}H_{18}N_4OS$, chemical name *N*-methyl-2-[[3-[(1*E*)-2-(pyridine-2-yl)ethenyl]-1*H*-indazol-6-yl]sulfanyl]benzamide (Fig. 1b); it has a molecular weight of 386.5 (24).

3.1 Preclinical Activity of Axitinib

Axitinib is a potent small molecule tyrosine kinase inhibitor of all known VEGFRs (VEGFR-1, -2, and -3), at picomolar concentrations, and of PDGFR- β and c-Kit, at low nanomolar concentrations (25). *In vitro*, axitinib selectively blocked VEGF-stimulated receptor autophosphorylation in endothelial cells, and inhibited their proliferation and survival (25). *In vivo* experiments have shown that axitinib has both antitumor and antiangiogenic activities; in mice, axitinib delayed the growth

of subcutaneous human xenograft tumors of the breast, colon, skin, ovaries, and glioblastoma and of a syngeneic model of murine lung cancer (26–28).

In a transgenic mouse model of spontaneous islet cell tumors, treatment with axitinib eliminated endothelial fenestrations, and suppressed vascular sprouting within 24 h (29). Blood flow and tumor vessel patency were reduced. After 7 days, vascular density had decreased by more than 70%, and significant tumor shrinkage was seen at 21 days. VEGFR-2 and VEGFR-3 expression was reduced in surviving endothelial cells. These results suggest that axitinib not only stops angiogenesis but also has a potent anti-vascular action leading to blood vessel regression.

3.2 Clinical Data: Phase I Safety and Pharmacokinetics

The safety, clinical activity, and pharmacokinetics of axitinib were investigated in a phase I dose-escalation study (30). Thirty-six patients with advanced, solid malignancies received oral axitinib on a continuous dosing schedule at doses ranging from 5 to 30 mg, twice daily.

Axitinib was absorbed rapidly, with peak plasma concentrations observed 2–6 h after dosing in the fed state, but within 1–2 h in the fasted state. The terminal half-life was between 2 and 5 h, and axitinib reached steady state plasma concentrations within 15 days, with no unexpected accumulation. Pharmacokinetics was generally linear. Although food had no appreciable effect on the plasma half-life, absorption was greater in the fasted state, with a median 49% increase in plasma exposures compared with the fed state. The major route of elimination was through systemic metabolism, with less than 1% of the drug appearing unchanged in the urine.

Dose-limited toxicities included hypertension, hemoptysis, and stomatitis, and were observed primarily at the higher dose levels. Overall, the most common AEs reported included hypertension, fatigue, nausea, diarrhea, vomiting, headache, stomatitis, and erythema. Most cases of hypertension were controlled easily with antihypertensive medications. The recommended dose for phase II development was 5 mg twice daily, administered in a fasted state, on a continuous dosing schedule.

Three patients achieved a confirmed PR to axitinib during the phase I study, one with adenoid cystic carcinoma, and two (of six) with RCC. Other signs of clinical activity included cavitation of lung lesions in two patients with non-small cell lung cancer, tumor regression (less than a PR) in three additional patients, and healing of skin lesions in one patient with breast cancer.

3.3 Efficacy and Safety in Cytokine-Refractory mRCC: Phase II Data

Following encouraging preclinical and phase I data, the efficacy and safety of axitinib in mRCC was investigated in a phase II study (31). All patients had meas-

urable mRCC and failure of one prior cytokine-based therapy because of disease progression or unacceptable toxicity. Axitinib was administered orally in a fasted state at a starting dose of 5 mg twice daily on a continuous dosing schedule until disease progression or unacceptable toxicity occurred. The primary endpoint was RECIST-defined ORR; secondary endpoints included TTP, OS, and safety.

At the time of analysis, 52 patients were enrolled of whom 50 had clear-cell histology and 49 had undergone prior nephrectomy. Twenty-three patients (44%) discontinued treatment, including 16 patients because of disease progression and 7 because of AEs (6 due to treatment-related AEs and 1 due to disease-related AEs). The most common treatment-related AEs were diarrhea (46%), hypertension (42%), fatigue (38%), nausea (36%), and proteinuria (36%).

Twenty-four patients (46%) achieved a PR (95% CI: 32, 60) and 21 patients (40%) had SD, of whom 20 patients (38%) experienced some degree of tumor shrinkage. Median TTP had not been reached after 12–18 months of follow-up.

3.3.1 Biomarkers of Response to Axitinib

Tumor perfusion and serum levels of VEGF and sVEGFR-1 and -2 were analyzed for correlation with clinical improvement in a subgroup of 13 patients with mRCC treated in the axitinib phase II study described above (32). Six patients were categorized as responders to axitinib treatment, having achieved CR or PR by RECIST, while the seven remaining patients were categorized as nonresponders with RECIST-defined SD or PD. Decreased tumor perfusion (TP) was observed in all six responders; in addition, decreased TP was strongly correlated with clinical improvement in four of the seven nonresponders. There was no correlation between response and serum levels of VEGF, sVEGFR-1, or sVEGFR-2.

3.4 *Efficacy and Safety in Sorafenib-Refractory mRCC: Phase II Data*

Having demonstrated efficacy in patients with cytokine-refractory mRCC, axitinib has also been investigated as second-line therapy in mRCC patients, refractory to prior RTK inhibition. Sixty-two patients were enrolled in a multicenter phase II study to determine the ORR with axitinib treatment in patients with histologically documented mRCC who had undergone prior nephrectomy and had subsequently failed sorafenib-based therapy (33). Axitinib was administered orally (with food) at a starting dose of 5 mg twice daily on a continuous dosing schedule until the occurrence of tumor progression or unacceptable toxicity.

At the time of analysis, 13 patients (21.0%) had achieved a confirmed PR (95% CI: 11.7, 33.2) and 21 patients (34%) had SD. The median PFS was 7.4 months (95% CI: 5.9, 9.1) and median OS had not been reached. (The median PFS in patients with prior sunitinib therapy was 6.1 months [95% CI: 3.9, 9.1]). The most

common treatment-related AEs were fatigue (68%), diarrhea (56%), hypertension (39%), and anorexia (39%).

4 Discussion and Future Directions

The preclinical and clinical data in mRCC with single-agent sunitinib and axitinib show that their target receptors are integral to the signaling cascades that directly and indirectly regulate tumor growth, survival, and angiogenesis, and that inhibition of these multiple RTKs results in clinical activity. The efficacy results reported here for sunitinib and axitinib are among the most encouraging presented, to date, for second-line treatment of mRCC. Recent phase III first-line data with sunitinib confirm the results of the second-line phase II trials and show clinical and statistically significant (more than twofold) improvement in PFS for sunitinib compared with conventional IFN- α treatment. These data demonstrate that sunitinib is now a new reference standard for first-line therapy of mRCC. Activity in bevacizumab-refractory disease and in combination with gefitinib further confirms that sunitinib has substantial antitumor activity and is well tolerated in patients with mRCC.

The safety profile of sunitinib has been generally consistent across a variety of solid tumor types, with similar AEs reported in a number of phase II trials in mRCC, a phase III trial in mRCC, and a placebo-controlled phase III study of sunitinib in patients with GIST (34). Sunitinib has a favorable tolerability profile; the majority of AEs experienced by patients are mild to moderate in severity and are generally manageable with standard medical intervention, dose interruptions, or dose reductions. The safety profile of sunitinib in a dosing schedule of 37.5 mg/day administered continuously is comparable to that observed with the current recommended intermittent dosing regimen of 50 mg/day on the 4/2 schedule. In addition, administration of sunitinib orally once a day with no regard to meals offers greater convenience for patients.

Although, the safety data currently available for axitinib are limited in comparison with those available for sunitinib, its safety profile is very similar to that now becoming familiar for this type of RTK inhibitor. As with sunitinib, most of the AEs reported in the phase II trial in mRCC were manageable with dose reduction or interruption, and/or with standard medical intervention.

Ongoing and future approaches with sunitinib in RCC include further combination studies (with chemotherapy, biologic therapy, and with other targeted agents), studies in the adjuvant and neo-adjuvant setting, and further biomarker research to identify patients who are most likely to respond to sunitinib therapy. Meanwhile, further evaluation of axitinib in refractory mRCC patients and in other tumor types is ongoing.

The past 5 years have seen rapid advances in our understanding of the molecular mechanisms associated with RCC. The future of anticancer therapeutics may largely focus on drugs such as sunitinib and axitinib, which target the molecules or mechanisms that drive tumor proliferation and survival. Data obtained to date have validated mechanism-directed RCC therapy based on tumor-specific molecular features.

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Sorafenib in Renal Cell Carcinoma

Saby George and Ronald M. Bukowski

Keywords Renal cell carcinoma • Sorafenib • Tyrosine kinase inhibitor • VEGF • Antiangiogenic therapy

1 Introduction

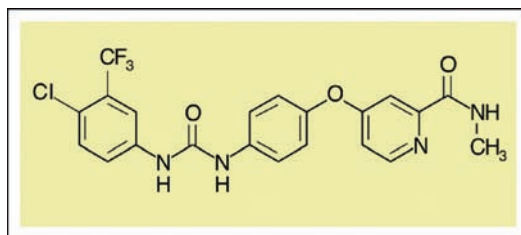
Sorafenib (BAY 43-9006) is a novel tyrosine kinase inhibitor which was recently approved for the treatment of advanced renal cell carcinoma (RCC), after a decade in which cytokine therapy was the standard of care. Sorafenib prolongs the progression-free survival (PFS) in treatment-refractory patients, but RECIST (response evaluation criteria in solid tumors) responses are limited (1, 2). The actions of this drug vary, and are dependent on several factors including its pharmacologic behavior (PK/PD), the receptor tyrosine kinases (RTKs) inhibited, and the extent and duration of target inhibition (3). The significant improvement in PFS is an important milestone in the contemporary therapy of advanced RCC. It is now accepted that PFS is a surrogate of overall survival (OS) in patients with metastatic RCC, as it is in other solid tumors. This chapter reviews the use of sorafenib in RCC and also future development of this agent in this neoplasm.

2 Background

2.1 Molecular Structure

Sorafenib (Nexavar®) is a water-insoluble tosylate salt, with a molecular weight of 464.825 g/mol (Fig. 1). It was developed and marketed by the collaborative efforts of

R.M. Bukowski(✉)
The Cleveland Clinic Tausong Cancer Center, Cleveland, OH 44195
e-mail: bukow464@slcglobal.net

Fig. 1 Sorafenib structure

Onyx and Bayer. The mean relative bioavailability is 38–49% for the tablet form as opposed to the oral solution. The bioavailability decreases to 29% with a fatty meal and 99.5% is protein bound. The half-life of sorafenib is between 25 and 48 h. Drug metabolism involves the P450 cytochrome system. Sorafenib initially undergoes oxidation via cytochrome P450 (CYP3A4) and is subsequently conjugated by UGT1A9. There is evidence of CYP3A4 inhibition when sorafenib was administered with ketoconazole, but the pharmacokinetics of sorafenib were unchanged (4). In this study, the dose level of sorafenib utilized was below the clinically recommended level.

2.2 Preclinical Data

A series of *in vivo* studies utilizing the NCr-nu/nu mice xenograft model have been conducted. Sorafenib was administered orally and tumors allowed to grow to assess antitumor activity, microvessel density, and extracellular signal-regulated kinases (ERKs) phosphorylation (5). Dose-dependent antitumor activity was noted in orthotopic models of human colon, lung, breast, melanoma, ovarian, and pancreatic neoplasms (5). Analysis of microvessel density with anti-CD31 antibodies demonstrated inhibition of neovascularization by sorafenib (5). Immunohistochemical studies demonstrated a close link between inhibition of tumor growth and inhibition of the ERK phosphorylation in two out of three models (5). There was also evidence of cell death and necrosis in response to sorafenib (5). The activity of sorafenib was also investigated in RCC xenograft models (ectopic and orthotopic) (6). Tumor volume and tumor growth delay after 3–5 days of dosing with sorafenib were used as indicators of efficacy. Sorafenib had an inhibitory effect on tumor growth and downregulatory effect on the tumor vasculature of both ectopic and orthotopic RCC tumor models, at doses as low as 15 mg/kg daily. TUNEL assays demonstrated correlation of a decrease in tumor vasculature with apoptosis and central necrosis. Sorafenib did not have any effect on phosphor-ERK, phosphor-histone H3, or Ki67 levels.

Recent reports have demonstrated *in vitro* effects on tumor cell growth and proliferation, with differential effects on von Hippel–Lindau (VHL)-mutant and wild-type RCC tumor cell lines (7). In this study, the clear cell RCC cell lines CAKI-1 (wild-type pVHL), CAKI-2 (mutant pVHL) and the isogenic pair, 786-0-neo

(vector control), and 786-0-VHL (pVHL expressing) cell lines were exposed to sorafenib in various concentrations in normoxic condition and also hypoxic conditions. Proliferation was inhibited by sorafenib in a dose-dependent manner. The CAKI-1 and 786-0-VHL were approximately twofold more resistant to the antiproliferative effects of sorafenib under hypoxic conditions. Interestingly, there was no difference in the effects of sorafenib under normoxia.

The clinical and biologic effects produced by this drug in RCC are the result of its inhibitory activities on a variety of downstream mediators upregulated in response to the molecular abnormalities in sporadic clear cell carcinoma. These include mutations and epigenetic silencing of the VHL gene, with the subsequent overexpression and production of growth factors, such as vascular endothelial growth factor-receptor (VEGF), platelet-derived growth factor (PDGF), and a variety of hypoxia-related genes (TGF α , GLUT-1, CA IX, etc.), all of which may be responsible for tumor-associated neovascularization and growth (3). Sorafenib's mechanisms of action may include reduction of angiogenesis and cellular proliferation; however, the exact mechanisms remain undefined.

2.3 Molecular Targets

Sorafenib is a multikinase inhibitor that targets several serine/threonine and RTKs. Sorafenib targets the intracellular kinases including CRAF, BRAF, and mutant BRAF. It also inhibits the cell surface kinases such as KIT, FLT-3, VEGF receptor -1 (VEGFR-1), VEGFR-2, VEGFR-3, and PDGF receptor beta (PDGFR- β) (8, 9). These kinases appear to play an important role in angiogenesis and cellular proliferation (Table 1). Sorafenib may affect tumor cell proliferation by its effects on the Raf/MEK/ERK pathway, as well as inhibiting angiogenesis (5).

2.4 Pharmacokinetics/Pharmacodynamics

The pharmacokinetic and pharmacodynamic characteristics of this Tyrosine kinase inhibitor (TKI) are unique. Sorafenib demonstrates considerable interpatient variability with respect to its pharmacokinetics (10). Both $C_{\max}$ and area under the curve (AUC) increase with dose escalation. Dosing twice daily (BID) also increased the AUC and $C_{\max}$ (10), and BID dosing also maintained a greater $C_{\min}$ compared to single daily dose (11). The $C_{\max}$ and AUC values were higher on day 7 compared to day 1, and the mean half-life was 20–38.1 h at 400 mg BID (11). The recommended dosing regimen is 400 mg BID, with dose reductions to 400 mg daily and 400 mg every other day dependent on toxicity. The dose levels of 600 mg BID and also 800 mg BID are currently under investigation.

Table 1 compares the in vitro IC50s for various target RTKs, using a variety of assay procedures. Sorafenib was developed as primarily a c-RAF inhibitor but

Table 1 Depicting the various kinases inhibited by sorafenib and the respective IC₅₀s

Target	Pathway significance	Sorafenib IC ₅₀
		Receptor blockade
PDGFR	Angiogenesis/RTKs	57 ± 20 nmol/L
PDGFRα	Angiogenesis/RTKs	NA
FGFR1	Signal transduction at pathway/extracellular at growth factor	580 ± 100 nmol/L
VEGFR-1	Angiogenesis/RTKs	NA
Flk-1KDR or VEGFR-2	Angiogenesis/receptor tyrosine kinases (RTKs)	90 ± 15 nmol/L
VEGFR-3	Angiogenesis/receptor Tyrosine kinases (RTKs)	20 ± 6 nmol/L
c-KIT	Stem cell factor receptor	68 ± 21 nmol/L
Raf-1	Cellular proliferation/signal transduction	6 ± 3 nmol/L
BRAF wild	RAS pathway/intracellular signaling receptors	22 ± 6 nmol/L
BRAF mutant	RAS pathway/intracellular signaling receptors	38 ± 9 nmol/L
FLT-3	Fms-like tyrosine kinase	58 ± 20 nmol/L
c-RET	RET signaling pathways/ Stress response pathway	NA

was subsequently found to inhibit a variety of kinases controlling angiogenesis (5). Sorafenib may affect tumor cell proliferation by its effects on the Raf/MEK/ERK pathway, as well as inhibiting angiogenesis (5). Recent reports have demonstrated in vitro effects on tumor cell growth and proliferation, with differential effects on VHL mutant and wild-type RCC tumor cell lines (7).

3 Sorafenib Approval

Sorafenib tosylate was approved as treatment for patients with advanced RCC in December 2005. Data from a phase III randomized placebo-controlled trial in treatment-refractory patients (*see Fig. 2a*) demonstrated sorafenib increased PFS to 24 weeks compared to 12 weeks in a placebo ($p < 0.000001$)-treated group (*see Fig. 2b*). Sorafenib also produced tumor shrinkage in 4 out of 5 patients (78%), with a 10% (investigator assessed) RECIST response rate (1, 12).

4 Sorafenib Clinical Trials in RCC

The efficacy and safety of this novel TKI in RCC has been demonstrated in various clinical trials. Studies both in the treatment-refractory setting and naive setting have been conducted. Sorafenib has been used as single agent and also in combination with a variety of other drugs in patients with metastatic RCC.

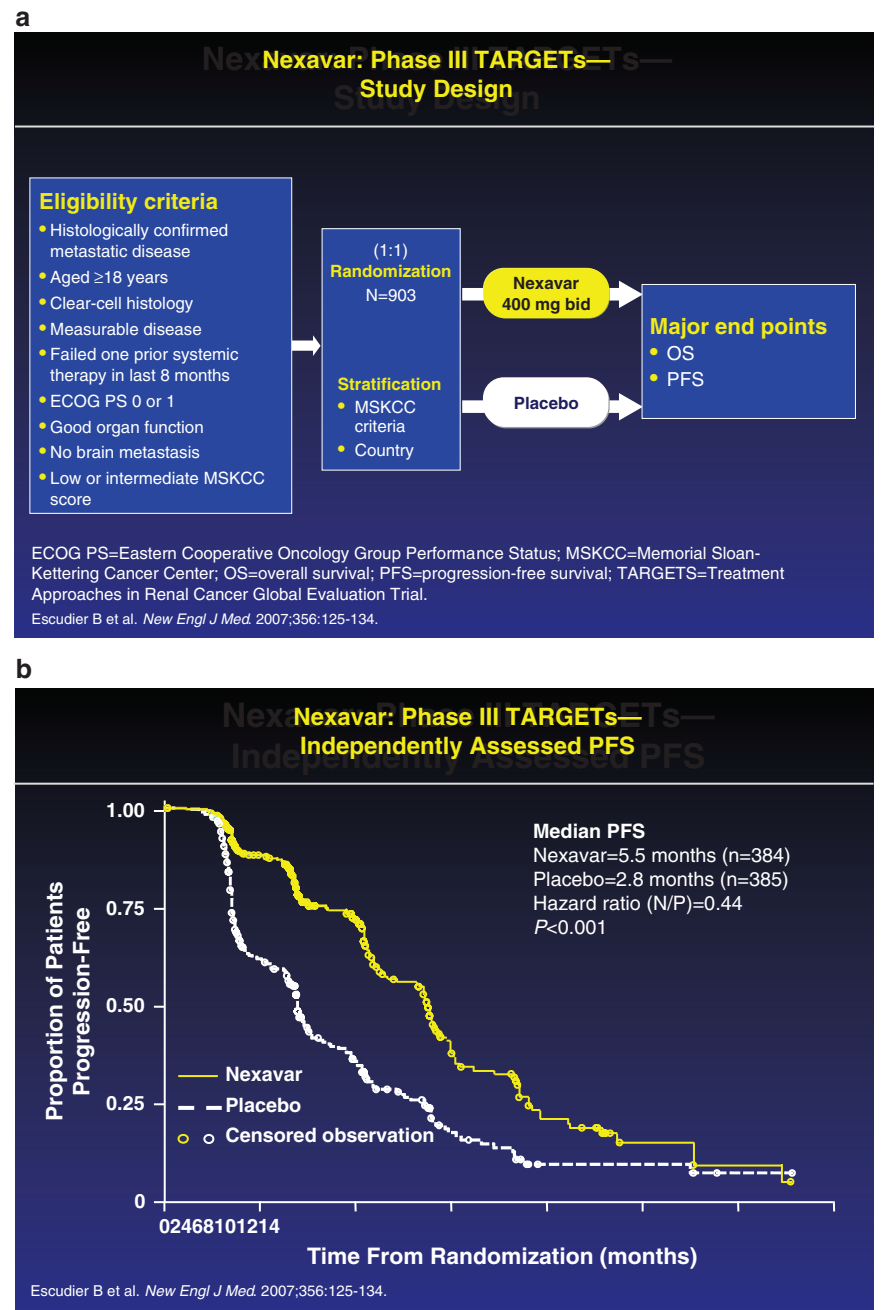


Fig. 2 **a** Schema for the TARGETs trial. **b** PFS on the TARGETs trial. **c** Kaplan-Meyer plot for overall survival (OS) at the time of crossover. **d** OS, K-M plot 6 months after crossover. **e** Tumor shrinkage data of sorafenib in the TARGETs trial

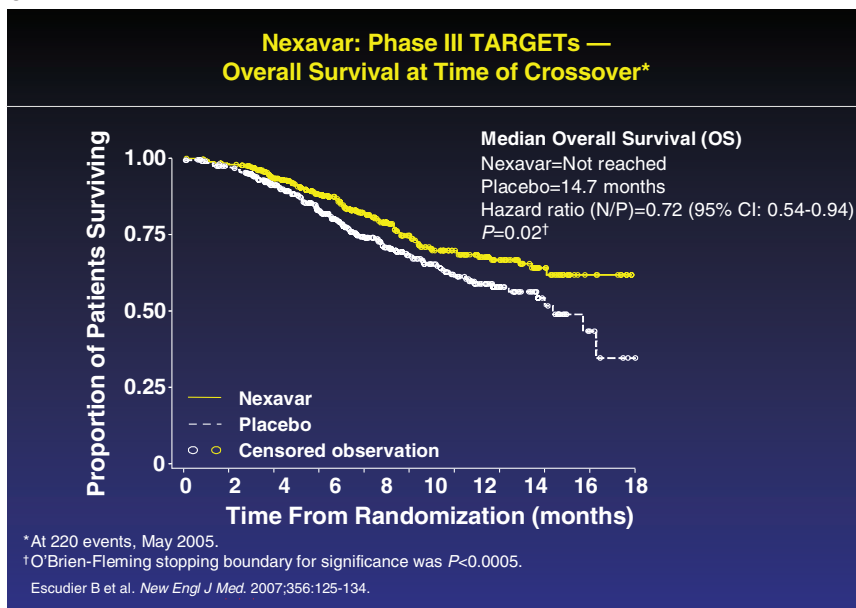
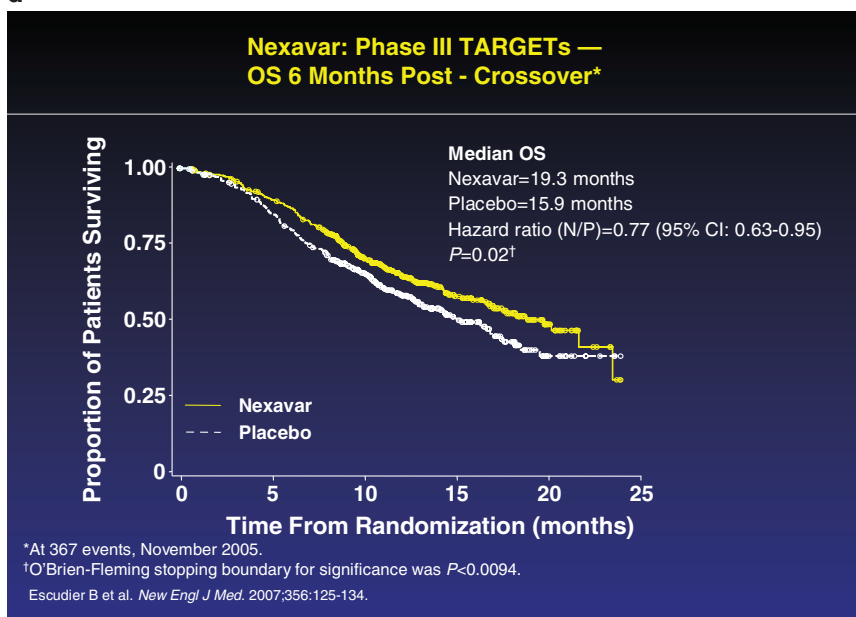
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Fig. 2 (continued)

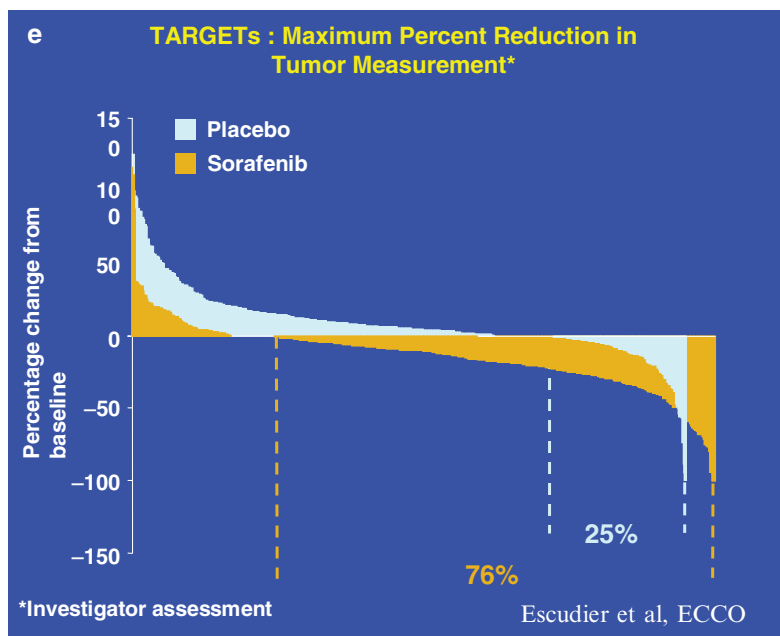


Fig. 2 (continued)

4.1 Phase I

The rationale for clinical trials with sorafenib was its ability to prevent tumor growth in preclinical models (5). The four phase I trials reported with sorafenib (BAY 43-9006) included a total of 10 patients with RCC as well as patients with other malignancies (13–16). The maximum tolerated dose (MTD), safety profile, pharmacokinetic variables, effect on biomarkers, and tumor response were evaluated. The MTD of sorafenib on a BID schedule was found to be 400 mg and dose-limiting toxicities (DLTs) were observed at 600 mg BID and 800 mg BID, respectively. The DLTs observed were hand-foot syndrome, fatigue, diarrhea, hypertension, rash, and pancreatitis. There was one PR (lasting 104 days) and one stable disease (SD) (heavily pretreated patient, who showed disease stabilization for 2 years) among the patients with RCC. The safety profile of sorafenib and the preliminary activity in patients with RCC supported additional studies in patients with RCC.

4.2 Sorafenib Monotherapy

4.2.1 Phase II RDT

The phase I trials were followed by a phase II randomized discontinuation trial (RDT) in patients with solid tumor. This trial included 202 patients with metastatic

RCC of whom, 170 had received prior therapy (17), and 32 patients were treatment naive. In the open label portion of the study, all patients received 400 mg BID of sorafenib. The analysis of 193 evaluable patients demonstrated 36% ($n = 73$) had $\geq 25\%$ decrease in tumor size, 34% SD ($n = 69$), and 25% progressive disease (PD) ($n = 51$), with an overall RECIST response rate of $<5\%$. At week 12, 65 patients with SD were randomized to either continue sorafenib ($n = 32$) or a placebo ($n = 33$). Twelve weeks after randomization, 50% ($n = 16$) of the patients in the sorafenib arm and 18% ($n = 6$) in the placebo remained progression free ($p = 0.0077$). The median PFS was significantly improved in the sorafenib group (24 weeks) compared to the placebo-treated patients (6 weeks, $p = 0.0087$). In the treatment-naïve subset, there were 27 evaluable patients, with 6 PR (18.8%), 18 patients with SD (56.3%), and 3 with PD (9.4%) for a 75% clinical benefit rate (CR + PR + SD) (17, 18). In this group, the median time to progression (TTP) was 40 weeks. The results in this trial suggested sorafenib had a significant effect in controlling disease progression despite a response rate of $\leq 10\%$ (in the treatment-refractory setting), and a randomized phase III study was then conducted.

4.2.2 Phase III

The phase III placebo-controlled trial (TARGETs) evaluating sorafenib in treatment-refractory patients, randomized 903 individuals with clear cell carcinoma to either sorafenib ($n = 451$) or a placebo ($n = 452$) (12, 19). The primary endpoint was OS, and a secondary endpoint was PFS. The first interim analysis demonstrated a significant improvement in PFS for sorafenib-treated patients compared to those receiving a placebo (24 vs 12 weeks, HR = 0.44, $p < 0.000001$) (1). Following this, patients were permitted to crossover from placebo to sorafenib. A prespecified interim analysis of OS using a stratified logrank test was performed 6 months after the crossover was initiated, and 216 of 452 placebo patients had crossed over to the sorafenib. The median OS for sorafenib patients was 19.3 months, and for the placebo group 15.9 months (HR = 0.77, 95% CI: 0.63, 0.95; $p = 0.015$, level of significance required $\alpha = 0.009$). After censoring the crossover patients, the median OS for placebo group was 14.3 months (HR = 0.74, 95% CI: 0.58, 0.93; $p = 0.010$), suggesting a potential effect of crossover (see Table 2). The final analysis of OS (see Fig. 2c and d) demonstrated an improvement of 13.5% for sorafenib versus placebo (median 17.8 versus 15.2 months; HR = 0.88, $p = 0.146$; $\alpha = 0.037$), but not significant (20). In the mean time, secondary analysis censoring the placebo data from June 2005 showed an OS benefit for sorafenib versus placebo (HR = 0.78, 95% CI: 0.62, 0.97; $p = 0.0287$; $\alpha = 0.037$), suggesting that crossover had confounded OS. The PFS benefit seen was independent of prior use of cytokines, age, Memorial Sloan-Kettering Cancer Center (MSKCC) score, presence or absence of lung or liver metastases and time to diagnosis. Subset analysis of the sorafenib-treated patients with/without cytokine therapy demonstrated the PFS was similar in the group received prior cytokine therapy (24 weeks, HR = 0.54, 95% CI: 0.45, 0.64) and that with no prior cytokine treatment (25 weeks, HR = 0.48, 95% CI: 0.32, 0.73) (21). Another subgroup analysis investigating the effects of age was conducted (<65 years vs ≥ 65 years) (22). It was noted that the PFS in patients

<65 years (23.9 vs 12 weeks, HR 0.61, 95% CI: 0.50, 0.74) and ≥65 years (25.9 vs 11.9 weeks, HR 0.37, 95% CI: 0.27, 0.51) for the sorafenib versus placebo arms were similar. Disease control rates (SD + PR + CR) in younger versus older patients in the sorafenib group were 60.7% and 64.4%, respectively, and 38% in placebo group. The findings were consistent with benefit from sorafenib independent of age.

The major adverse events (AEs) were diarrhea, rash, fatigue, and hand-foot reactions [palmo-plantar erythrodysaesthesia (PPE) ([Fig. 3](#))] (*see Table 3*). Treatment discontinuation due to AEs was reported in 10% of patients receiving sorafenib and 8% receiving the placebo. The principal events responsible for discontinuation were constitutional, gastrointestinal, dermatologic, or pulmonary. Dose reduction were required in 13% of sorafenib-treated patients and 3% in the placebo group ($p = 0.001$). Dose interruption was required in 21% of patients in the sorafenib group (mostly

Table 2 Illustrating the survival data from the crossover analysis of the targets trial (19)

Parameter	OS at crossover	OS at 6 months post-crossover	OS at 6 months postcrossover, placebo censored
Placebo OS	14.7 months	15.9 months	14.3 months
Sorafenib OS	Not reached	19.3 months	19.3 months
Hazard ratio	0.72	0.77	0.74
<i>p</i> -value	0.018	0.015	0.01
O'Brien Fleming stopping boundary	0.0005	0.0094	N/A



Fig. 3 Palmo-plantar erythrodysaesthesia (hand-foot syndrome). This is an example of a grade 3 hand-foot syndrome in a patient treated with sorafenib

Table 3 For the adverse events seen in phase II RDT and the targets trial (12)

Adverse events	All grade toxicities % in (grade 3)	
	Sorafenib (<i>N</i> = 451)	Placebo (<i>N</i> = 452)
Fatigue	37 (5)	28 (4)
Diarrhea	43 (2)	13 (1)
Nausea	23 (<1)	19 (1)
Hypertension	17 (4)	2 (<1)
Hand-foot syndrome	30 (6)	7 (0)
Rash/desquamation	40 (1)	16 (<1)
Alopecia	27 (<1)	3 (0)
Pruritus	19 (<1)	6 (0)
Neuropathy	13 (<1)	6 (1)
Anemia	8 (3)	7 (4)

for PPE/rash/diarrhea) and 6% in the placebo group ($p = 0.001$). Cardiac toxicities included hypertension and ischemia. All grade hypertension was observed in 17% of patients with 4% having grade 3 toxicity on sorafenib, while only 2% had all grade hypertension in the placebo arm. Cardiac ischemia was observed in 12 patients on sorafenib (3%) and <1% in placebo. Hypertension was managed by dose interruption per protocol for grade 3 toxicity in addition to the use of antihypertensives which suited the patient best and only <1% required permanent discontinuation of sorafenib.

This study clearly demonstrated the effects sorafenib on disease stabilization, with >75% of patients developing measurable tumor shrinkage. The significant increase in PFS for sorafenib-treated patients is consistent with these findings. An example of a patient with tumor shrinkage in response to sorafenib is illustrated in Fig. 4. The high disease control rate is mostly attributable to the predominance of SD in the sorafenib-treated patients. Stable disease is defined as the range of tumor volume from -30% to +20%. Figure 2 clearly shows that there are at least 51% patients who had some sort of tumor shrinkage and another subset of patients who had SD but no tumor shrinkage or with increase in tumor volume. Often times, the tumors develop central necrosis as evidenced by the CAT scan images and the varying Hounsfield units in different parts of the tumor (Fig. 5). The center of the tumor undergoing necrosis occurs commonly, with or without tumor shrinkage, but after initiation of the treatment. Many patients with SD have this kind of response to the therapy, though they did not qualify as a response per the RECIST. This reiterates the need to improve the response evaluation in RCC patients treated with agents like sorafenib. So the use of RECIST poses a potential for underestimating response to TKI.

4.2.3 Phase II Interferon Versus Sorafenib

A phase II trial was conducted in the treatment-naïve patients with metastatic clear cell carcinoma (23). One hundred and eighty-nine untreated patients were stratified based on the MSKCC prognostic score and randomly assigned to receive sorafenib 400mg

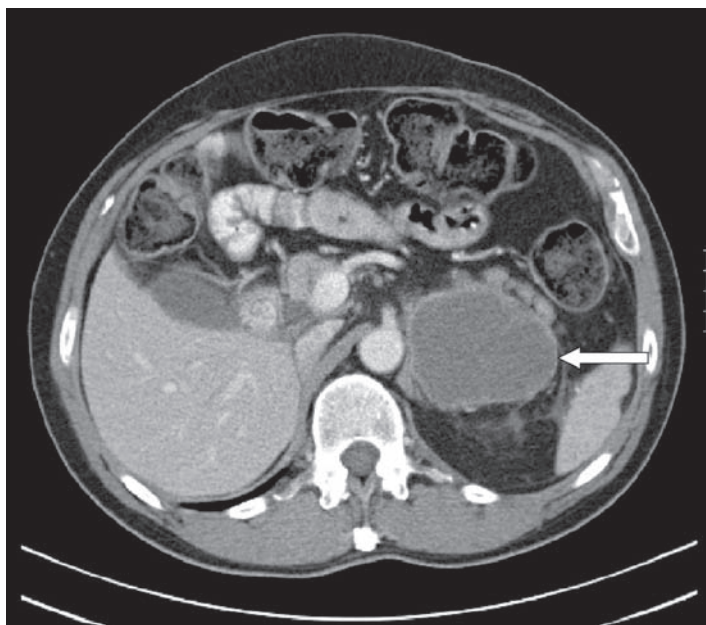


Fig. 4 Example of central necrosis in the tumor in a patient treated with sorafenib

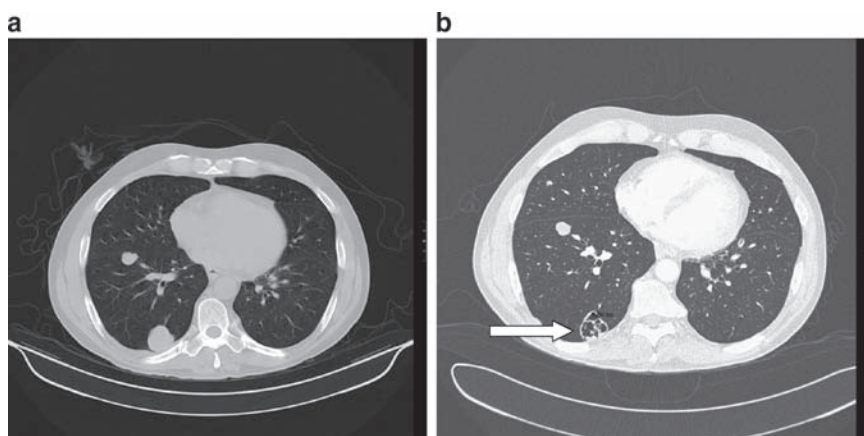


Fig. 5 Tumor reduction of a patient with metastatic RCC to sorafenib at 800mg BID when the low dose (400mg BID) had failed initially after 6 months. This lesion was solid before starting treatment with high dose of sorafenib and then became cystic/cavitary after a two cycles of sorafenib at 800mg BID

BID ($n = 97$) or interferon (IFN) 9 MU TIW ($n = 91$) in part 1. PFS was the primary objective in this study. Dose escalation of sorafenib to 600mg BID upon progression (part 2) was permitted, and crossover to sorafenib for IFN- α patients was also

included. Preliminary toxicity data demonstrate the sorafenib arm had more grade ≥ 3 toxicity, including diarrhea (24.7%), hypertension (13.4%), and hand-foot syndrome (6.2%), and in control the IFN arm had a higher incidence of grade ≥ 3 fatigue (20.9%), fever (18.7%), nausea (13.2%), and flu-like syndrome (6.6%). Drug discontinuation was required in 11% in sorafenib group and 15% in IFN group due to AEs (24). A final analysis of this trial was recently reported, and the PFS in the sorafenib arm was 5.7 months (CI: 5.0–7.4 months), and in the IFN arm 5.6 months (CI: 3.7–7.4 months) (24). The progression-free rates for sorafenib versus IFN were 90% versus 70.4%, 45.9% versus 46.5%, and 11.5% versus 30.4% at 3, 6, and 12 months, respectively.

In part 2 upon crossover to sorafenib from IFN the PFS was 5.3 months ($n = 50$, CI: 3.6–6.1 months). The 600 mg BID was well tolerated and the median PFS in this group was 3.6 months ($n = 44$, CI: 1.9–5.3 months). The primary endpoint was not met by this study. Improvement of PFS was also demonstrated in patients who crossed over from IFN to sorafenib, attesting to its ability to show response in the second-line setting after treating with IFN.

4.2.4 Phase IV

Advanced Renal Cell Carcinoma Sorafenib Study (ARCCS) Trial is a large expanded access program for therapy of patients with advanced RCC (NCT00352859) (25, 26). This is an open label noncomparative phase IV study of sorafenib, and included 1,239 previously untreated patients and 1,249 treatment-refractory patients. The majority had clear cell histology (78%) with 7% having papillary carcinoma. The most common sites of metastases were lung (68.2%), kidney (27.4%), bone (26.8%), and liver (23.1%). Eighty-one percent of patients had prior nephrectomy, 821 (35.1%) had prior radiotherapy and the median time from diagnosis was 1.4 years with 51.6% having received some sort of prior therapy. Toxicity included diarrhea (16.3%), rash/desquamation (15.8%) fatigue (13.2%), hand-foot syndrome (10.5%), and hypertension (9.7%). Out of the 2,488 patients who were evaluable for toxicity, grade 3 AEs were reported in <2% of patients. Response was assessed in 1,850 patients (921 frontline patients). RECIST responses included 1 CR (<1%), 67 PR (4%), 1479 SD (80%), and 303 PD (16%). This translated into 84% clinical benefit rate/disease control rate. Common grade 3 AEs included hand-foot syndrome (7.2%), rash (4%), fatigue (5.3%), hypertension (4.4%), dehydration (2.7%), dyspnea (2.7%), and diarrhea (2.5%). There were 713 (30.5%) drug-related grade 3 or 4 AEs, 672 (28.8%) serious AEs, and 396 (16.9%) AEs leading to drug discontinuation.

4.3 Sorafenib Combination Studies

Sorafenib has been investigated in combination with other antiangiogenic agents as well as cytokines. A few examples of various combination trials are listed below (see Table 4). Preclinical data have suggested it will be possible to combine sorafenib

Table 4 Illustrates use of sorafenib in various settings of RCC

Setting of sorafenib use	Published or ongoing trial
Cytokine refractory setting	TARGETs trial (phase III)
Treatment-naïve RCC	Phase II trial of sorafenib vs IFN
Combination with IFN in frontline setting	SWOG-0412. Phase II trial no longer recruiting.
Combination with IFN in the second-line setting	Gollob et al. Ongoing phase II trial – NCT00098618
Neoadjuvant setting	Ongoing phase II trial – NCT00405366 and NCT00126659
Adjuvant setting	Ongoing ECOG E2805 phase III trial
Sorafenib × 3 years vs sorafenib × 1 year vs observation after surgery	EORTC adjuvant trial
Sorafenib in sunitinib or bevacizumab refractory RCC	Ongoing phase II by Rini et al.
Sorafenib in combination with Ril2	Ongoing phase I/II trial – NCT00389285
Sorafenib in combination with bevacizumab	Ongoing phase I/II trial – NCT00126503
Sorafenib in combination with temsirolimus	Ongoing phase I trial – NCT00255658

with a variety of other agents without dose reductions being required. A series of phase I and II trials have investigated this issue in RCC.

4.3.1 IFN- α and Sorafenib

Preliminary results of three studies combining IFN- α and sorafenib have been reported. A phase I trial combining IFN- α and sorafenib has been reported (27). This study enrolled 13 patients with advanced RCC ($n = 12$) and malignant melanoma which were unresectable and/or metastatic. Treatment regimen included continuous dosing of sorafenib with IFN- α 2a given SC TIW in various cohorts for dose escalation. MTD, one of the primary endpoints has not been reached at the time of this report. The most commonly seen drug-related toxicity was $\leq$ grade 2 fatigue, diarrhea, nausea, alopecia, and PPE. There was 1 PR (patients with RCC) and 8 SD (included patients with melanoma). It was also found that IFN had no effect on the pharmacokinetics of sorafenib and there was no drug–drug interaction.

The Southwest Oncology Group (SWOG) conducted a single arm multicenter trial of sorafenib in combination with IFN- α in 61 untreated patients with RCC (28). The therapy included IFN- α at 10 MIU SC TIW and sorafenib 400 mg PO BID. After three cycles of therapy, only 16 patients remained on treatment. The most frequent grade ≥ 3 toxicities included fatigue, leucopenia, anorexia, diarrhea, and hyponatremia. The overall response rate (ORR) was 19% ($n = 11$, 95% CI: 9%, 32%). Of interest was the noted decrease in severe hand-foot syndrome.

A second phase II study in predominantly second-line and treatment-refractory patients ($n = 24$) also utilized sorafenib in combination with IFN- α 2b at similar dose levels. Patients tolerated the combination reasonably well, and the ORR was 38% with CR rate of 4% (29). In the same trial, there were 46% SD which included 8% patients

who demonstrated a tumor shrinkage of $\geq 20\%$. Dose reduction was required in 58% of the patients for toxicities. Toxicities were mostly grade 1 or 2 and the commoner ones were fatigue (78%), anorexia (74%), rash (70%, 11% grade 3), diarrhea (67%), weight loss (63%), hypophosphatemia (59%, 33% grade 3), nausea (56%), neutropenia (48%, 19% grade 3), alopecia (44%), and oral mucositis (26%). This regimen was overall well tolerated with less number of grade 3 toxicities including PPE.

4.4 Avastin and Sorafenib

Sosman et al. (30) initiated a phase I/II trial combining sorafenib with bevacizumab in patients with advanced RCC. Dose level -1 is 200 mg QD of sorafenib and 3 mg/kg of bevacizumab every 14 days. Dose level 1 is 200 mg BID of sorafenib continuously and 5 mg/kg of bevacizumab every 14 days. The dose level 2 has sorafenib at 400 mg BID and bevacizumab at 10 mg/kg. The phase I part enrolled 24 patients at three dose levels, all patients had significant toxicity including PPE, stomatitis, and anorexia, leading to weight loss of $>15\%$ and reduction in the performance status. Other grade toxicities included hypertension ($n = 4$) and proteinuria (2). There has been 10 PR (41.67%), 11 SD (45.8%), and 3 PD (12.5%). This study is still ongoing in an attempt to define the MTD of both the agents when administered in combination. It appears that dose reductions of either sorafenib or bevacizumab will be required when administered concurrently.

4.5 Temsirolimus and Sorafenib

There is one phase I trial combining sorafenib with CCI-779 (temsirolimus), which is recruiting patients with unresectable or metastatic solid tumors. The primary goal is to study the safety and efficacy of the combination. A randomized phase II trial with multiple arms, which includes bevacizumab, sorafenib, and temsirolimus in various combinations in advanced RCC, is now underway.

5 Clinical Issues

5.1 Treatment-Refractory Patients

The evaluation of clinical response in patients with RCC still utilizes the RECIST (30). In clinical trials with sorafenib, the PFS is improved but significant tumor shrinkage is limited (response rate of around 10%) (1, 2). In the phase II and III studies, the number of patients who achieved disease control (CR + PR + SD) was 70% and 84%, respectively (12, 17, 19), and the PFS was significantly prolonged compared to

placebo (17). Prolongation of PFS may relate to the cytostatic effects of agents such as sorafenib; however, the issue of optimal dosing remains. It is unclear whether at the molecular level suboptimal inhibition of the therapeutic targets was occurring.

The response rates observed in the phase II RDT appeared superior to those seen in the phase III trial; however, the patient populations treated were different. In the phase III study, only treatment-refractory patients were included. The treatment-naïve group in the RDT showed an ORR of 18% while the phase III trial demonstrated an ORR of 10%. The possibility that sorafenib may be more active in the frontline setting than in treatment-refractory patients, does exist, but the phase II randomized trial does not demonstrate this.

Figure 2e shows the tumor shrinkage data from the TARGETs trial of sorafenib versus placebo in the treatment-refractory setting. It is quite conceivable from this waterfall diagram that, even though the number of patient achieving RECIST responses is limited, the percentage of patients having some degree of tumor shrinkage as well as arrest of growth is high (76% in sorafenib opposed to 25% in placebo arm). This may account for the prolonged PFS seen with sorafenib.

5.2 Treatment-Naïve Patients

There are no randomized phase III trials demonstrating significant effects of sorafenib in treatment-naïve patients. The only existing data are from the randomized phase II trial of sorafenib versus interferon in the first-line setting of advanced RCC. This trial randomized 187 patients to sorafenib ($n = 97$) and IFN ($n = 92$). Baseline characteristics were not different in these groups. Fifty-six patients progressed on IFN and then were crossed over to sorafenib. The ORR was 5% versus 9% favoring IFN, but the disease control rates were 79% versus 64% favoring sorafenib. The median PFS was 5.7 months (CI: 5–7.4 months) versus 5.6 months (CI: 3.7–7.4 months) for sorafenib and IFN. The common AEs seen with sorafenib were PPE, rash, and diarrhea while IFN arm was associated with flu-like syndrome. Although this trial failed to reach the primary endpoint, sorafenib appears to have activity in the frontline setting.

The subset analysis of the treatment-naïve patients in the phase II RDT was conducted for assessing its activity in the frontline setting. Out of the 32 patients who were treatment-naïve, there were 6 PR (18.8%), 18 SD (56.3%), and 3 PD (9.4) among the 27 evaluable patients. The clinical benefit rate (CR + PR + SD) was 75% from sorafenib. In this group, the TTP was found to be 40 weeks. Current data do not support use of sorafenib as frontline therapy in metastatic RCC patients.

5.3 Dose Optimization

Current dose for use is 400mg BID as continuous dosing. This is a dose that is tolerated reasonably well. The MTD was 600 BID from the phase I studies with most of the dose-limiting toxicities observed at 800mg BID (13).

Amato and group conducted a phase II trial for dose escalation of sorafenib in patients with advanced RCC (31). The dose was escalated from 400mg BID to 600mg BID after 28 days and further to 800mg BID after day 56 and then continued. Out of the 46 enrolled patients, 22 are still on sorafenib after a median of 6+ months of therapy. They were able to escalate the dose to higher levels in 91% patients and the reported ORR is 52%.

There have been anecdotes of sorafenib being more active at the higher dose than 400mg BID. That is sorafenib at higher doses was able to shrink metastatic RCC when the 400mg BID, which is the standard dose and other antiangiogenic agents failed initially. There are some patients who could tolerate doses of 800 mg BID with multiple grade 1 and grade toxicities. An example of a patient with tumor shrinkage with the higher dose of 800 BID is illustrated in Fig. 4.

In view of the data and experience, the optimal dosing of sorafenib is being reexamined. Studies examining the effects of dose escalation on ORR and PFS are now in progress.

5.4 Biomarkers

Biomarkers of response to therapy in RCC are an area of interest as more and more TKIs and targeted therapies are investigated in this disease. Jaeger et al. had analyzed DNA and RNA from primary tumor blocks before starting treatment with SU11248, AG013736 and/or IFN- α , and bevacizumab (32). Twenty-five (58%) of the 43 evaluable tumors had VHL mutation, of which 15 were frameshift, and 10 were nonframeshift. The median TTP in patients with frameshift mutation was 13.7 months, 7.4 months in nonframeshift mutations, and 5.5 months in patients with no VHL mutation ($p = 0.06$). In the same study, when they compared the expression of single gene with the protein production, it was found that CA IX expression correlated best while hypoxia-inducible factor-2 α (HIF2 α) and VEGFR-2 correlated moderately. This pointed more toward a higher response rate to antiangiogenic therapy in the presence of VHL mutations.

Karashima et al. studied the effects of VEGFR-2 blockade on the development of bone metastasis in a mice model. TSU-68, a TKI against VEGFR-2, demonstrated activity against renal tumor in kidney, but it did not inhibit either growth of bone tumor or bone destruction in the tibia (33). This proved that anti-VEGFR-2 therapy did not inhibit bone metastasis.

Choueiri et al. did an analysis of the VHL status of patients with RCC on various TKIs and correlated with the tumor response (34). Of the 122 evaluable patients for VHL status, 59 were mutated, 12 were methylated, and 51 were wild type. The response rate was highest in the mutated group (46%) followed by wild type (31%) and methylated (17%). The subset analysis of the mutated group ($n = 59$) showed that the majority were frameshift (28) followed by inframe (7), nonsense (6), splice (5), and missense (13). They could not find any association

between VHL status and PFS or OS. There were 28 patients who had received sorafenib in this analysis. The response rate to sorafenib based on VHL status were mutated 2/10 (20%), methylated 0/2 (0%), and wild type 0/16 (0%).

The development of a biomarker test for sorafenib would help in tailoring patient-specific therapy in clear cell RCC. This method when fully developed would be able to predict the best therapy and the therapeutic outcome based on the genetic profiling in RCC.

5.5 Sequential TKI Therapy

Tamaskar et al. reviewed the activity of TKIs in patients with prior antiangiogenic therapy failure (35). This review examined patients with metastatic RCC currently receiving either sunitinib or sorafenib who had previously received “antiangiogenic” therapy, including sunitinib, sorafenib, AG13736, thalidomide, lenalidomide, or M200. Of the 27 evaluable patients, at least 20% ($n = 7$) achieved a PR and 43% ($n = 15$) showed SD. All the SD patients had tumor shrinkage (range 4–28%). Thus, 63% of patients who failed prior antiangiogenic therapy had tumor shrinkage on subsequent sunitinib or sorafenib therapy. A subgroup analysis showed benefit of sunitinib when sorafenib failed and the benefit of sorafenib when sunitinib failed initially. The response of three patients to sunitinib following sorafenib was evaluated and the tumor shrinkage was seen in all patients (4%, 15%, and 17% shrinkage). Similarly, the response to sorafenib following sunitinib ($n = 3$) was evaluated and the tumor shrinkage rates were 12%, 27%, and 27%, respectively, in these patients. This means that there is no cross-resistance between sorafenib and sunitinib.

Most recent data of sequential therapy with sunitinib and sorafenib were recently presented at American Society of Clinical Oncology (ASCO) 2007 (36). Patients with disease progression or unacceptable toxicity after frontline therapy with either sunitinib or sorafenib were then crossed over to the other therapy. Group A had 23 patients who started off with sorafenib and then crossed over to sunitinib. Group B had 14 patients who started with sunitinib and then crossed over to sorafenib. Three patients in group A and 2 in group B were crossed over to the other therapy due to toxicity, while others for progression. For the patients who received sunitinib as second-line therapy, the median duration of stable disease on sunitinib was 32 weeks (range 6–37 weeks, median follow-up 266 days). This group also had four partial responders. The patients who received sorafenib in the second-line, median duration of stable disease after starting sorafenib was 8 weeks (range 4–10 weeks, median follow-up 179 days). There were no responders in this group.

This could mean that sunitinib and sorafenib when given sequentially has moderate activity after failing the other in the past. The activity of sunitinib may be lower when used in the second-line; however, these data are premature and anecdotal at this time.

5.6 Adjuvant and Neoadjuvant Trials

There has been benefit from the use of sorafenib in the cytokine refractory patients, but in the cytokine naive setting no improvement compared to IFN was noted (1, 12, 17–19). Various studies are in progress to evaluate the effects of sorafenib in neoadjuvant and adjuvant settings (see Table 4). Two adjuvant trials in RCC are ongoing. The Eastern Cooperative Oncology Group (ECOG) adjuvant trial in RCC is a randomized phase III study with three arms, sorafenib versus sunitinib versus placebo. The primary aim is to evaluate the adjuvant (postnephrectomy) use of the two TKIs after nephrectomy in preventing recurrence. The European Organisation for Research and Treatment of Cancer (EORTC) adjuvant trial is trying to evaluate the utility of sorafenib given for 3 years versus 1 year versus observation after nephrectomy.

6 Conclusion

Sorafenib is a novel TKI which has significant activity in metastatic RCC in the setting of prior therapy failures. The response rate and PFS are increased in this setting, additionally, improved survival when censoring techniques were used, has been noted for sorafenib-treated patients. The future trials might answer the optimal dose and also the optimal setting for use of sorafenib in RCC, and further investigate its activity in untreated patients. The use of sorafenib in combination with other agents in the antiangiogenic pathways is also being investigated.

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Additional Tyrosine Kinase Inhibitors in Renal Cell Carcinoma

Brian I. Rini

Abstract The biology of clear-cell renal cell carcinoma (RCC) supports that anti-angiogenic agents may have therapeutic effect. Indeed, several agents have been shown to provide robust response rates and progression-free survival advantages over historic therapy. In addition, several newer agents are being tested in metastatic RCC. These agents including pazopanib, AZD2171 and PTK787 have demonstrated preclinical activity and have entered clinical trials. These novel agents are likely to add to the therapeutic armamentarium in RCC.

Keywords VEGF inhibitors • Tyrosine kinase inhibitor • TKI • Pazopanib • PTK787 • AZD2171 • ZK222584

1 Introduction

The biology of renal cell carcinoma (RCC) predicts that agents targeting angiogenesis-related cellular receptors could be of therapeutic benefit. As described in preceding chapters, several agents have undergone extensive clinical evaluation in metastatic RCC, resulting in FDA approval of sunitinib and sorafenib. Several other compounds which inhibit the tyrosine kinase portion of the angiogenesis-related receptors such as vascular endothelial growth factor receptor (VEGFR) and platelet-derived growth factor receptor (PDGFR) have also entered clinical testing in RCC.

2 Pazopanib

Pazopanib (GW786034, GlaxoSmithKline, Research Triangle Park, NC) is a potent, oral multi-target receptor tyrosine kinase inhibitor (TKI) of VEGFR-1,

B.I. Rini

Department of Solid Tumor Oncology, Cleveland Clinic Taussig Cancer Institute, 9500 Euclid Ave., Desk R35, Cleveland, OH 44195

e-mail: rinib2@ccf.org

-2, -3, PDGFR and c-kit. In preclinical experiments, Pazopanib has demonstrated significant inhibitory effect on endothelial cell proliferation and inhibits VEGF-induced VEGFR-2 phosphorylation in mouse lungs and cornea in a dose-dependent manner (1). Pazopanib also showed significant growth inhibition of a variety of human tumour xenografts in mice including Caki-2 renal, and also inhibited basic fibroblast growth factor (bFGF)- and VEGF-induced angiogenesis in two different mouse models of angiogenesis, the Matrigel plug assay and the cornea micropocket model.

In a recent phase I trial (2), 55 patients with refractory solid tumours (including 10 patients with metastatic RCC) were enrolled at escalation doses ranging from 50 mg three times weekly to 2000 mg daily. The most frequent drug-related adverse events included nausea (18%), diarrhea (18%), emesis (15%), fatigue (15%) and hypertension (24%). A single dose-limiting toxicity of grade 3 fatigue was observed at 2000 mg QD dose. Prolonged disease stabilization/minor responses have been observed in 12 of the 52 patients with a variety of advanced cancers. Specifically, of the ten patients with metastatic RCC enrolled, five patients had evidence of tumour shrinkage, qualifying for either stable disease or partial response by response evaluation criteria in solid tumours (RECIST) criteria. Three of these patients with RCC were entered at doses of 800, 1400 and 2000 mg, respectively, and two patients received 300 mg BID. All five patients with RCC with tumour shrinkage achieved trough plasma drug concentrations of $>40\mu\text{M}$, a minimum concentration required for activity in mouse models. On the contrary, five patients with RCC were treated at lower doses (100 mg three times a week, $n = 1$; 50 mg, $n = 2$; and 400 mg, $n = 2$). None of these patients achieved trough concentrations of $>40\mu\text{M}$ and none had anti-tumour activity observed. Further, preliminary assessment of tumour perfusion using dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) showed that patients dosed with 800 mg QD or 300 mg BID had a decrease in tumour perfusion. In addition, biopsy samples demonstrated inhibition of phosphorylated-VEGFR2 in patients dosed with 800 mg QD. Together, these observations suggest that doses $>800\text{ mg QD}$ are needed to reach an exposure of $>40\mu\text{M}$ plasma concentration, which is associated with inhibition of angiogenesis and clinical anti-tumour activity. This is consistent with results from preclinical models that optimal biological activity was observed at Pazopanib plasma concentration $>40\mu\text{M}$. As increasing the dose beyond 800 mg QD does not increase the plasma concentration, 800 mg QD dose was selected for further study in clinical trials.

Based on the above results, a multi-centre, international phase II trial utilizing a randomized discontinuation design was conducted. The trial enrolled 225 patients with the goal to evaluate the safety and efficacy of Pazopanib in locally recurrent or metastatic clear-cell RCC. (ASCO 2008). The objective response rate was 35%, with a duration of response of 68 weeks. Based on these data, a phase III trial of pazopanib versus placebo in RCC patients with 0–1 prior treatments has completed account (results pending). In addition, a phase III trial of pazopanib versus sunitinib, with a primary endpoint of non-inferiority of Pts has begun.

3 AZD2171

AZD2171 (Astra Zeneca...) is an indole-ether quinazoline which inhibits VEGFR-1–3, PDGFR- α and -B as well as FGFR1 *in vitro* in low nanomolar concentrations (3). This compound also inhibits growth factor-induced receptor phosphorylation (VEGFR-2 and PDGF- α and -B) and cellular proliferation in endothelial cells and MG63 human osteosarcoma cells. AZD2171 inhibited tumour cell proliferation directly only at very high drug concentrations, suggesting that the inhibition of tumour growth observed *in vitro* is due to inhibition of VEGF signalling on endothelial cells. Experiments in a number of murine orthotopic xenograft models (colon, lung, prostate, breast and ovary) demonstrated vascular regression, tumour growth inhibition and regression of existing tumours after treatment with AZD2171. A murine orthotopic model employing RENCA (RCC) cells has also been studied (4). In two separate experiments, inoculation of RENCA cells into the left kidney of mice was followed 8 or 10 days later by treatment with AZD2171 or control for a period of 7 or 11 days. AZD2171-treated mice demonstrated a significant reduction in primary and metastatic (lung) tumour volume as well as reduction in tumour vessel density as measured by CD 31 immunostaining.

A phase I clinical trial in patients with refractory solid tumours has been conducted (5). Thirty-six patients received a single oral dose of AZD2171 (0.5–60 mg) followed by a 7-day washout period, then continuing daily oral therapy in 28-day cycles until toxicity or disease progression. The most common toxicity was fatigue, hypertension, nausea, diarrhea and vomiting. Plasma half-life was 12–36 h. Dose-proportionate increases in $C_{\max}$ and AUC support linear pharmacokinetics for single and multiple doses. There was no evidence of time-dependent changes in pharmacokinetics following multiple oral dosing supporting once-daily oral administration. Assessment of tumour blood flow/permeability demonstrated reductions with treatment at doses higher than 20 mg. Disease activity seen in higher dose levels included two partial responses and three minor responses. A randomized cohort expansion at the 20 mg, 30 mg and 45 mg dose levels in 47 total patients is being conducted. Investigation of AZD2171 is also ongoing in a wide variety of malignancies including prostate cancer, non-small cell lung cancer, breast cancer and hepatocellular carcinoma. Further investigation in RCC is being considered.

4 PTK787/ZK222584

PTK787/ZK222584 (PTK787; Novartis Pharmaceuticals, Hanover NJ) is an oral, selective inhibitor of VEGFR-1, VEGFR-2. It also inhibits PDGFR- β tyrosine kinases, c-Kit and c-Fms but at higher concentrations.(6) In a murine RCC model, oral daily therapy with PTK787 at 50 mg/kg resulted in a 67% decrease in primary kidney tumours after 21 days of exposure. The occurrence of lymph nodes and lung metastases was also significantly inhibited. Vessel density in tumour tissues,

detected by immunohistochemistry with an anti-CD31 antibody and blood flow in the tumour feeding renal artery using doppler ultrasound imaging, were significantly decreased by PTK787.(7)

PTK787 was the first TKI to undergo significant clinical investigation in metastatic RCC. A phase I/II trial of PTK787 enrolled 45 patients with metastatic RCC (8). The most common adverse events observed (all grades 1 or 2) were nausea (67%), fatigue (61%) and vomiting (50%). The maximum-tolerated dose was not reached at 1,500 mg/d. The sum of bidimensional measurement of tumours was used to document responses in this study. Partial responses were documented in 5% of patients, minor responses (25–50% tumour shrinkage) in 14% and stable disease in 60%. Correlative perfusion imaging undertaken with contrast-enhanced magnetic resonance imaging provided anecdotal evidence of a change in vascular permeability and/or decrease in tumour blood flow preceding decrease in tumour size. Further development of this agent in RCC is unclear at present given lack of survival benefit in a randomized trial in metastatic colorectal cancer (9).

5 Conclusion

The biology of renal carcinoma supports inhibition of the VEGF receptor as therapeutically relevant. Indeed, robust clinical activity has been observed with agents with this mechanism. Several additional agents are also now in development. Differences in the spectrum of and potency against targets, toxicity profile and mechanisms of resistance will allow for development of these agents in RCC.

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Integrin $\alpha 5\beta 1$ as a Novel Therapeutic Target in Renal Cancer

Vanitha Ramakrishnan, Vinay Bhaskar, Melvin Fox, Keith Wilson, John C. Cheville, and Barbara A. Finck

Abstract Anti-angiogenics have shown efficacy in the treatment of renal cancer. Most current therapies target the VEGF pathway. In this chapter, we discuss the role of integrins, specifically $\alpha 5\beta 1$, in angiogenesis. The integrin $\alpha 5\beta 1$ is up-regulated on proliferating endothelial cells. The monoclonal antibody volociximab is a potent, high-affinity inhibitor of $\alpha 5\beta 1$. Inhibition of $\alpha 5\beta 1$ function by volociximab results in the apoptosis of proliferating, but not resting, endothelial cells in vitro. Inhibitory activity of volociximab appears to be independent of the growth factor milieu, suggesting a potential role for this agent under pathological conditions where multiple growth factors are present. Additionally, in pre-clinical models of angiogenesis such as the cynomolgus choroidal neovascularization model, volociximab is effective at inhibiting lesion development. An assessment of the expression of $\alpha 5\beta 1$ by immunohistochemistry in annotated samples of renal cancer suggest a relationship between $\alpha 5\beta 1$ expression and clinical grade of the tumour. The analysis shows that the expression of this integrin was very high in the tumour vasculature in all the clinical grades of renal cancer tested. However, we found an increase in target expression on the tumour cells that correlated with worsening clinical grade. These results suggest an additional mechanism by which volociximab may provide clinical benefit. Volociximab may not only exert an anti-angiogenic effect but may also have a direct inhibitory effect on the tumour cells. A phase 1 clinical trial with volociximab has been completed in patients having a variety of tumour types. The results show that the antibody is well tolerated at doses up to 15 mg/kg weekly. No drug-related adverse events were noted, and the best clinical response seen so far is stable disease. Volociximab is currently undergoing further clinical testing, and is being assessed in pilot open label trials, in indications including renal cancer.

Keywords Integrins in angiogenesis • Anti-angiogenic therapy • Renal cancer $\alpha 5\beta 1$ • Volociximab

V. Ramakrishnan (✉)

Formerly at Department of Research, PDL BioPharma, Inc., Fremont, CA 94555
e-mail: vanitha.ramakrishnan@gene.com

1 Introduction

Angiogenesis is a process driven by multiple growth factors (1). This mechanism has been well clarified, and targeted therapies against growth factors such as vascular endothelial growth factor (VEGF) and its receptors (bevacizumab, sunitinib) have shown efficacy in the treatment of renal cancer (2–6). While growth factors play a key role in the initiation of angiogenesis, a critical step for the survival of the proliferating endothelial cell is the up-regulation of the integrin $\alpha 5\beta 1$ (7–9). Integrins are cell surface adhesion molecules that mediate the binding of cells to the extracellular matrix. They are heterodimeric, consisting an alpha and a beta chain. The alpha chain mediates the binding specificity for a particular ligand(s). Integrins play a key role in cell functions such as adhesion and migration, platelet aggregation (mediated by $\alpha \text{IIb}\beta 3$) and lymphocyte homing (10, 11).

Endothelial cells express a number of different integrins, including the collagen receptor ($\alpha 2\beta 1$), the vitronectin receptor ($\alpha \nu\beta 3$) and the fibronectin receptor ($\alpha 5\beta 1$). The role of these different integrins in the process of angiogenesis has not yet been fully clarified (12). Much has been elucidated by gene deletion experiments in mice (13). It has been shown that mice deficient for the $\alpha \nu$ integrin are viable, with half the pups surviving to birth, although they die postnatally. Death has been shown to occur due to intracranial bleeds; however, the animals do develop a normal vasculature, hence vasculogenesis and angiogenesis appear to proceed relatively normally (14). Mice with a targeted deletion of the $\beta 3$ gene, on the contrary, show the classic profile of Glanzmann's thrombasthenia, with impaired platelet function and bleeding (15). Interestingly, tumour growth appears accelerated in these animals, and it is hypothesized that this occurs due to leaky vasculature enhancing the supply of nutrients to the tumour (10, 16). Targeted deletion of the $\alpha 2$ gene results in the ablation of platelet responses to collagen, probably due to loss of one of two platelet $\alpha 2\beta 1$, but the mice are fertile and otherwise normal (17). In contrast to the viable offspring resulting from an $\alpha \nu$, $\alpha 2$ or $\beta 3$ gene deletion, targeted deletion of the promiscuous $\beta 1$ gene (18) that comprises the $\alpha 2\beta 1$ and several other integrins leads to early embryonic death, indicating the importance of the $\beta 1$ integrins in development. Conditional knockouts are not yet available.

However, targeted deletion of $\alpha 5$ gene in mice showed some interesting features that were suggestive of a critical role in developing vasculature (19). The phenotype is embryonic lethal, but the embryo survives to E9-10. The development of the vascular tree is impaired, suggesting that $\alpha 5\beta 1$, but not $\alpha \nu\beta 3$, plays a key role in the survival of proliferating endothelial cells (20). Further, work from Dr. Varner's lab has shown that blockade of $\alpha 5\beta 1$ via antibodies results in apoptosis of proliferating endothelial cells as measured by DNA fragmentation and protein kinase A activation (8, 9, 21, 22). On the basis of these data, we have sought to inhibit neoangiogenesis by targeting $\alpha 5\beta 1$ using a chimaeric human IgG4, high-affinity, function-blocking antibody known as volociximab derived from the parent mouse antibody IIA1 (23). In this chapter, we show that inhibiting the function of $\alpha 5\beta 1$ in vitro and in vivo results in inhibition of angiogenesis. An additional role is proposed based on expression of the target on the tumour cells of renal cancer samples where the antibody may exert a direct anti-tumour effect.

2 Biology of $\alpha 5 \beta 1$ in Renal Cancer

2.1 Gene Expression Profile of $\alpha 5 \beta 1$

We have modelled angiogenesis using a well-established in vitro assay known as the tube formation assay (24). In this assay, human umbilical vein endothelial cells (HUVEC) are seeded into a three-dimensional matrix supplemented with growth factors such as VEGF and basic fibroblast growth factor (bFGF). Under these (and other) conditions, the endothelial cells divide and migrate into tubes that can be visualized and quantified. We determined the gene expression profile of a number of integrins in a time course of tube formation using this assay. The isolated mRNA was used for transcript profiling using a custom-designed oligonucleotide micro-array using Affymetrix GeneChip technology (EosHu03) (25).

2.2 Up-Regulation of $\alpha 5 \beta 1$ Gene Expression in Proliferating HUVEC

The data showed that resting endothelial cells expressed both $\alpha 5$ and $\beta 1$ mRNA, but that a significant up-regulation of the $\alpha 5$ and $\beta 1$ gene expression (Fig. 1a) occurred during the time course of tube formation under the influence of growth factors. The expression pattern determined by this novel method further supported a key role for $\alpha 5 \beta 1$ in angiogenesis. The gene expression profiles of all integrins were also evaluated (Fig. 1b). The expression of the $\alpha 2$, $\beta 4$, $\beta 5$ integrin appeared slightly elevated. The $\alpha 5$ and $\beta 1$ genes, however, show significant up-regulation in gene expression under these conditions compared to baseline. An interesting observation was that under the conditions tested, no significant changes were detected in the gene expression pattern of αv . Additionally, $\alpha 5 \beta 1$ protein expression was measured in HUVEC by flow cytometry. While resting cells did express $\alpha 5 \beta 1$, up-regulation of protein expression occurred when cells were induced to proliferate actively (Fig. 1c). These data support the published role of $\alpha 5 \beta 1$ in the proliferation of endothelial cells.

2.3 Effect of Volociximab on HUVEC Proliferation and Tube Formation

Volociximab was tested for direct effects on proliferating HUVEC in vitro. Two different assays were employed. The first was a direct proliferation assay in the presence of growth factors VEGF and bFGF, singly or in combination, and proliferation was evaluated using an (3-(4,5-dimethylethiayol-2-yl)-2,5-diphenyl-tetrazolium bromide) MTT read-out. The activity of volociximab was compared to that of an anti-VEGF antibody HUMV833. Representative data of multiple experiments are shown here (Fig. 2). Under all conditions, volociximab (and the parent mouse

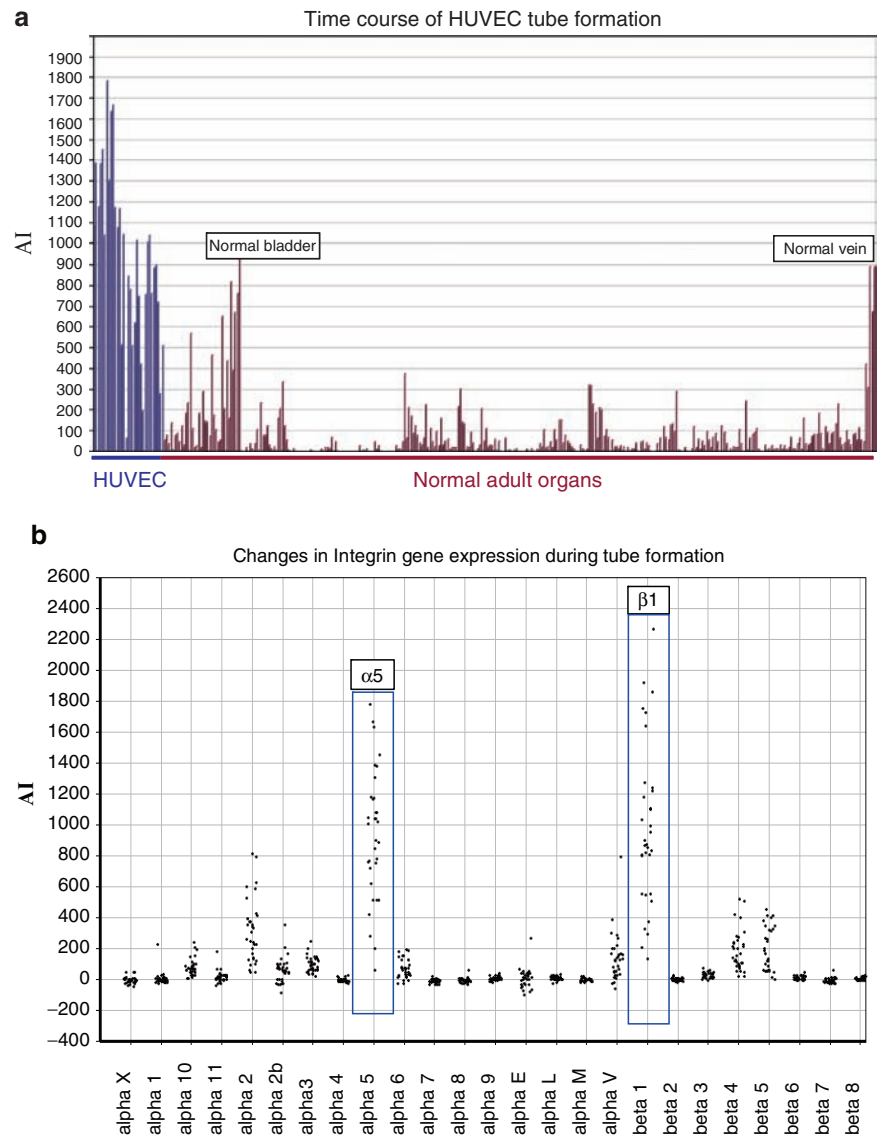


Fig. 1 **a** Time course of human umbilical vein endothelial cells (*HUVEC*) tube formation. **b** Changes in integrin gene expression during tube formation. **c** *HUVEC* grown to confluence or in log phase were removed with ethylenediaminetetraacetic acid (*EDTA*) and stained with the murine parent antibody of volociximab, IIA1 (*purple*), which is specific for integrin $\alpha 5\beta 1$ or a murine control IgG (*green outline*). Confluent, or resting, *HUVEC* expressed ~40% less $\alpha 5\beta 1$ than log phase, or proliferating, cells as measured by flow cytometry

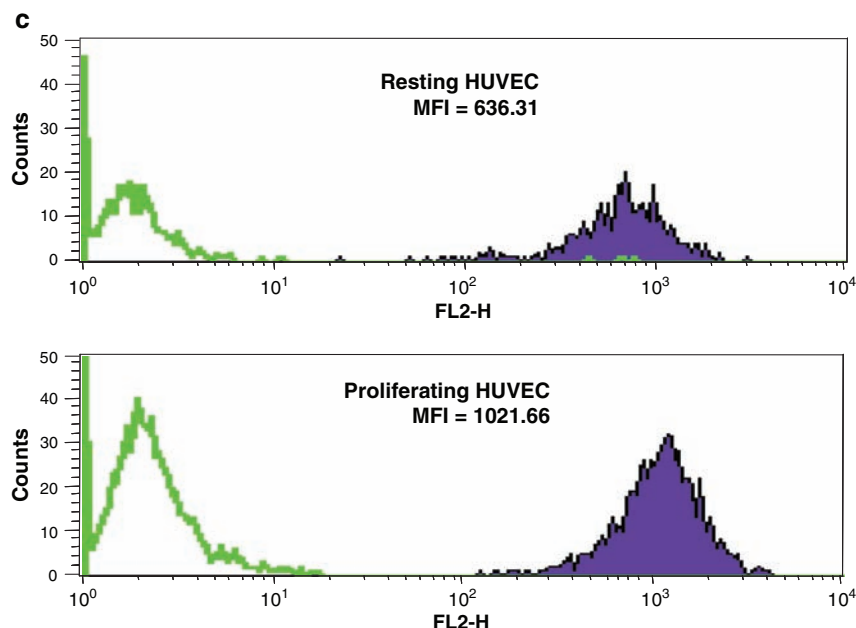


Fig. 1 (continued)

antibody IIA1) was found to inhibit HUVEC proliferation with an IC_{50} of ~ 0.4 nM ($0.06 \mu\text{g/ml}$). We also found that volociximab was consistently more effective at inhibiting HUVEC proliferation compared to HUMV833, even when VEGF was the only growth factor stimulus present (Fig. 2a). In the second assay (Fig. 2b), HUVEC were stimulated to form tubes in the presence of 20% human serum, VEGF and bFGF (100 ng/ml). Tube formation was visualized by fixation followed by staining with phalloidin-Alexa 488. In the presence of volociximab (or the parent antibody IIA1), tube formation was inhibited with an IC_{50} of 3 nM ($0.45 \mu\text{g/ml}$). Again, we noted that this direct effect on HUVEC under these conditions was more potent with volociximab compared to anti-VEGF. These data also suggest that $\alpha 5 \beta 1$ is independent of, and downstream of, the growth factor stimulus.

2.4 Selectivity of Volociximab

Volociximab (and the parent IIA1) has been tested for cross-reactivity to $\alpha 5 \beta 1$ from a variety of species. Functional antibodies against integrin targets such as Vitaxin (anti- $\alpha v \beta 3$) are usually specific for the human integrins, with limited cross-reactivity to corresponding integrins from other species. Volociximab is also highly selective

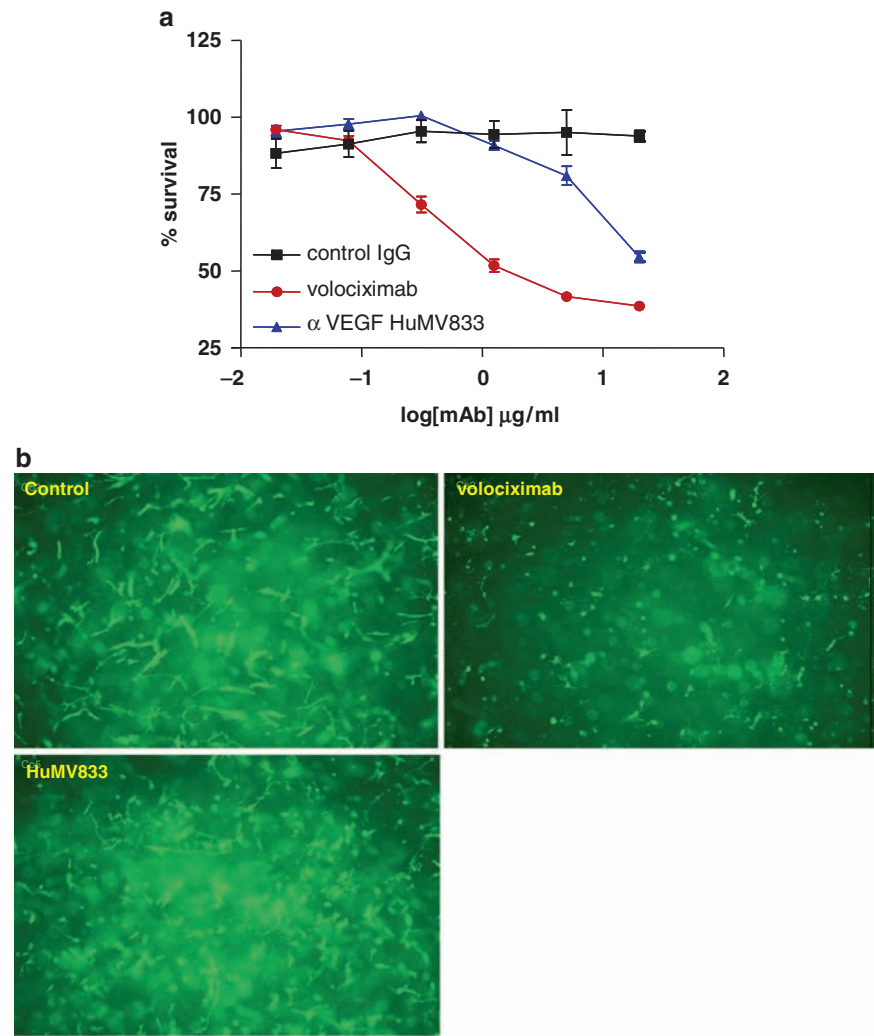


Fig. 2 **a** Human umbilical vein endothelial cells (*HUVEC*) were cultured on fibronectin in basic fibroblast growth factor (*bFGF*) and vascular endothelial growth factor (*VEGF*) containing serum-free medium in the presence of increasing concentrations of volociximab, HUMV833 or control IgG. After 4 days, viability was assessed by mean transit time (*MTT*) assay. **b** *HUVEC* were induced to form tubes within a three-dimensional fibrin matrix, in the presence of medium containing *bFGF*, *VEGF* and 20% human serum. Tubes were visualized by staining with phalloidin (green), followed by fluorescence microscopy. Antibodies were added at the start of the assay at 10 $\mu\text{g/ml}$ (See Color Plates)

for human $\alpha 5$ (Fig. 3). M200 was tested for cross-reactivity by ELISA to a number of human integrins ($\alpha 3\beta 1$, $\alpha \nu \beta 3$ and $\alpha \nu \beta 5$), and was found to be only recognize $\alpha 5\beta 1$ (Fig. 3a). Cross-species reactivity studies were conducted by immunohistochemistry (IHC) analysis of frozen cynomolgus and mouse tissue. Volociximab

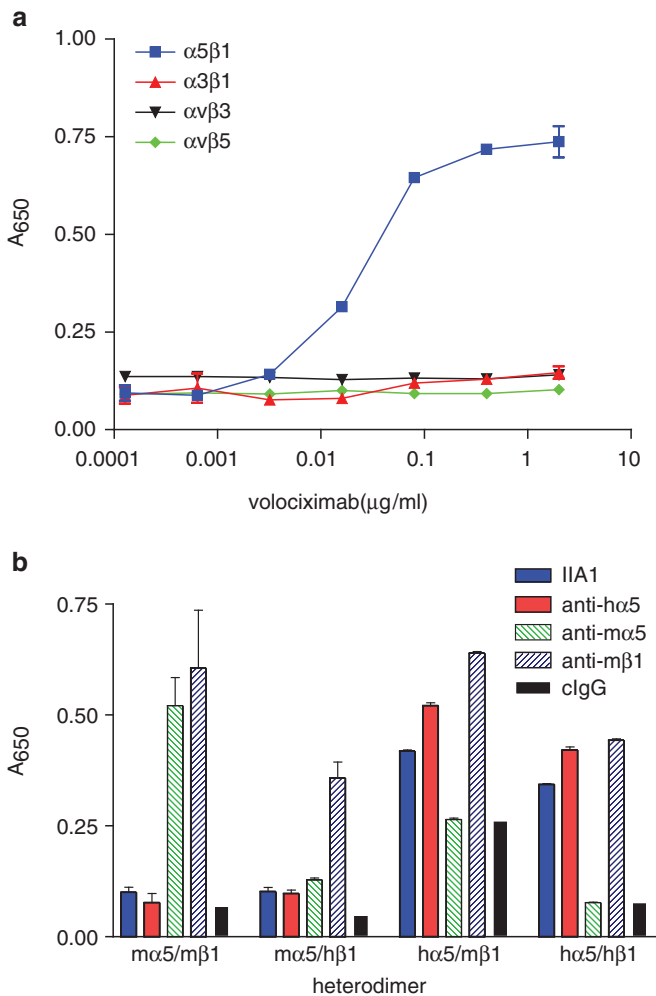


Fig. 3 **a** Specificity of volociximab for $\alpha 5 \beta 1$. **b** IIA1 requires for human $\alpha 5$ for binding

was found to detect the vasculature of cynomolgus samples by IHC, but no cross-reactivity to mouse $\alpha 5 \beta 1$ was detected (data not shown). Further, cloned mouse and human $\alpha 5$ and $\beta 1$ integrin extracellular domains were expressed as Fc fusion proteins. The parent antibody IIA1 was used to determine cross-reactivity to mouse $\alpha 5 \beta 1$, mouse $\alpha 5$ -human $\beta 1$ and human $\alpha 5$ -mouse $\beta 1$ hybrids. The recognition site for IIA1 was found to be dependent on the heterodimer of $\alpha 5 \beta 1$, with a strict requirement for the human $\alpha 5$ chain (Fig. 3b). Binding is permissive for the $\beta 1$ chain, since a mouse $\beta 1$ chain can support binding of volociximab.

2.5 *Effect of Volociximab on Angiogenesis in a Laser-Induced Model of CNV in Cynomolgus Monkeys*

Because of the species specificity of volociximab, mouse models of tumour formation were not informative. A limited number of models of angiogenesis are available in higher species. One such model is the choroidal neovascularization (CNV) model in monkeys. In this model, lesions are placed in the macula of the eye using a laser (26). The formation of neovasculature is evaluated weekly using fluorescein angiography. Anti-VEGF therapy has been shown to be efficacious in this model, indicating a role for VEGF in this scenario (27, 28). An affinity-matured Fab version of Avastin (Lucentis) has successfully conducted phase 3 clinical trials in the same indication. These data validate this monkey CNV model as one that reflects the process of angiogenesis, and has clinical relevance. We tested volociximab administered either intravitreally (300 µg/eye) or intravenously (5, 15 and 50 mg/kg). All the angiograms were evaluated and scored by a masked expert. Sample data are shown here (Fig. 4). Volociximab showed significant inhibition of CNV as assessed by fluorescein angiography. These experiments suggest that $\alpha 5\beta 1$ is important in the development of neoangiogenesis, and that volociximab is a potent inhibitor of this process.

2.6 *Expression of $\alpha 5\beta 1$ in Renal Cancer*

IHC using either volociximab or the parent mouse IIA1 antibody requires that the samples are frozen and not paraffin embedded, as the antibody only recognizes

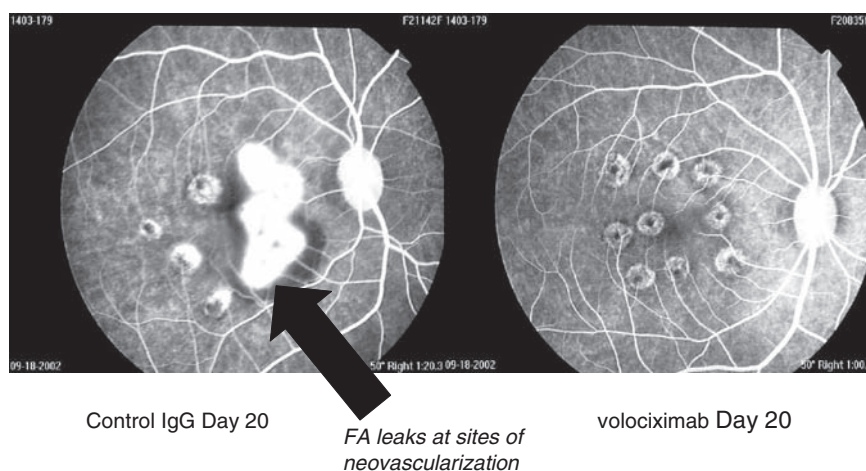


Fig. 4 Volociximab inhibits choroidal neovascularization (CNV) in cynomolgus eyes

$\alpha 5 \beta 1$ in its native state. In collaboration with the Mayo Clinic, we conducted an IHC campaign with volociximab on frozen renal cancer samples from patients with different grades of tumour. There were four clinical groups. Group A consisted of patients alive without disease following treatment with low-grade and low-stage RCC ($n = 23$). Group B consisted of patients who were alive with disease after presentation with metastases or with subsequent metastases ($n = 23$). Group C consisted of patients who had died of disease ($n = 25$), and Group D consisted of patients with RCC who had distal metastases ($n = 18$, included some lymph nodes). Sample data are shown (Fig. 5a). Each pathologic tissue assessed

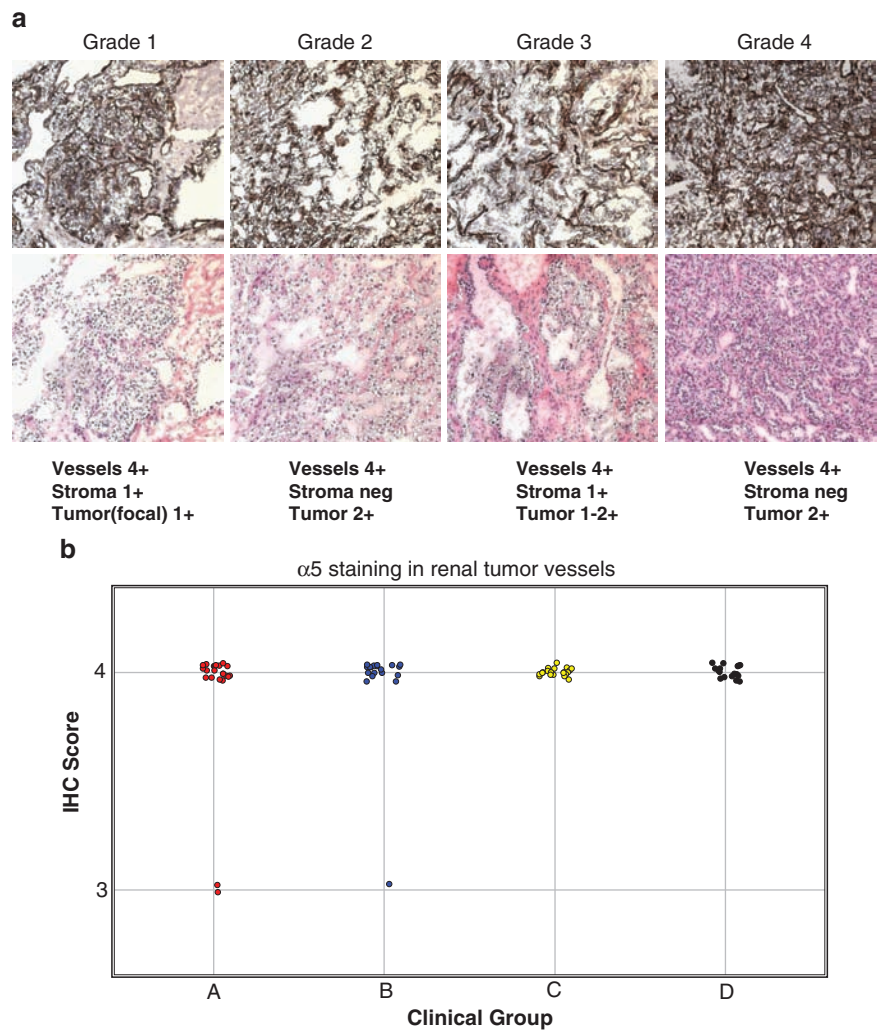


Fig. 5 **a** Immunohistochemistry (IHC) on frozen renal cell carcinoma (RCC) tissue with IIA1. **b** $\alpha 5$ staining in renal tumour vessels (See Color Plates)

was up-regulated in both the tumour cells and the tumour stroma as the disease progressed (Fig. 5b–d). A parallel assessment of annotated prostate cancer samples did not show such a correlation. In prostate cancer, the vasculature was positive for $\alpha 5 \beta 1$ (2–3+). However, the tumour epithelium and the tumour stroma did not express $\alpha 5 \beta 1$ to a significant degree, and no correlation was seen with Gleason grade. These data suggest that the up-regulation of $\alpha 5 \beta 1$ in RCC was specific to, and correlated with, the disease state.

2.7 Inhibition of Proliferation of RCC Cells In Vitro

In order to evaluate whether volociximab could affect the growth of RCC, we utilized a renal cancer cell line SW839 found to express $\alpha 5 \beta 1$ by flow cytometry (Fig. 6a). Cells were cultured in the presence of serum for 4 days with increasing concentrations of volociximab, or a control, non-functional mAb (known as VC5) that is specific for the $\alpha 5$ subunit of $\alpha 5 \beta 1$ (Fig. 6b). We found a statistically significant inhibition of the growth of the tumour cells in vitro, with a maximal inhibition of 30%, and an IC_{50} for inhibition at 6 nM (0.9 μ g/ml). These data suggest that volociximab, in addition to inhibiting the growth and development of endothelial cells, was able to inhibit tumour cell growth directly.

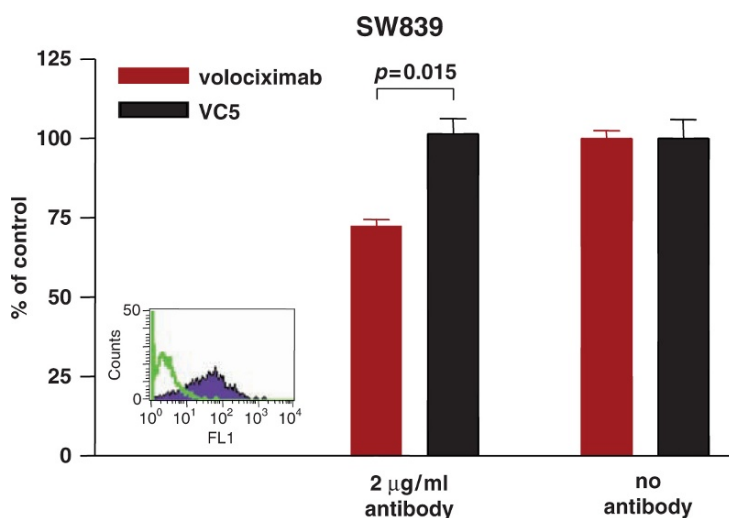


Fig. 6 A renal carcinoma cell line SW839 was cultured in serum-containing medium in the presence of increasing concentrations of volociximab or VC5, a non-function blocking anti- $\alpha 5$ antibody. After 4 days, viability was assessed by mean transit time (MTT) assay. Data shown are the comparison of the 2 mg/ml concentration. *Inset*: SW839 cells were removed with ethylenediaminetetraacetic acid (EDTA) and stained with the IIA1 (purple) or a murine control IgG (green outline)

Since volociximab does not cross-react with rodent $\alpha 5\beta 1$, standard xenograft models of disease are not suitable for evaluation of this therapeutic candidate. A mouse surrogate antibody with the properties of volociximab is currently unavailable, but we are in the process of developing an anti-mouse $\alpha 5\beta 1$ antibody to evaluate the role of this integrin on both the vasculature (mouse-derived) and the tumour cells (human-derived) to further investigate the biology of $\alpha 5\beta 1$ in in vivo models of disease.

3 Role of Platelets in Side Effects of Anti-Angiogenic Therapy

Data from the phase 3 clinical trial using the anti-angiogenic Avastin has shown an increase in the incidence of hypertension (29, 30). Of note was the increased morbidity due to heart attacks and stroke. Other anti-angiogenics targeting the VEGF pathway have also reported an increase in hypertension. The proposed mechanism for these incidents is hypothesized to be due to an inhibition of VEGF receptor-mediated production of endothelial nitric oxide (NO), a potent vasodilator. However, additional mechanisms may exist. Production of NO in endothelial cells is mediated by the activation of the arachidonic acid cascade, resulting in the production and release of prostacyclin or PGI_2 . PGI_2 plays an important role in maintaining platelets in the resting state. In the absence of PGI_2 , platelets can become activated to form a thrombus, particularly under conditions of high shear stress. Platelets themselves transport a number of growth factors including VEGF in their alpha granules. Platelet activation results in the release of alpha granule contents. It is conceivable that in the presence of drugs, such as bevacizumab, the removal of VEGF results in vasoconstriction and the resulting hypertension with the potential for platelet activation may explain the underlying biology of the clinical side effect of anti-VEGF therapy.

3.1 Role of $\alpha 5\beta 1$ in Platelet Function

Platelets also express a number of integrins (31, 32). The major protein on the platelet surface is the integrin $\alpha \text{IIb}\beta 3$, the fibrinogen receptor. In addition, platelets express the $\alpha 2\beta 1$, the $\alpha \text{v}\beta 3$ and the $\alpha 5\beta 1$ and target of volociximab, $\alpha 5\beta 1$. Integrin $\alpha 5\beta 1$ is not only the fibronectin receptor but is also functionally capable of ligating fibrinogen when activated. Thus, targeting $\alpha 5\beta 1$ could have effects in addition to the effects on angiogenesis and tumourigenesis.

Platelet function was investigated ex vivo in two ways using washed platelets prepared from fresh human blood. Agonists such as thrombin cause the inside-out activation of $\alpha \text{IIb}\beta 3$, rendering the platelet capable of binding soluble

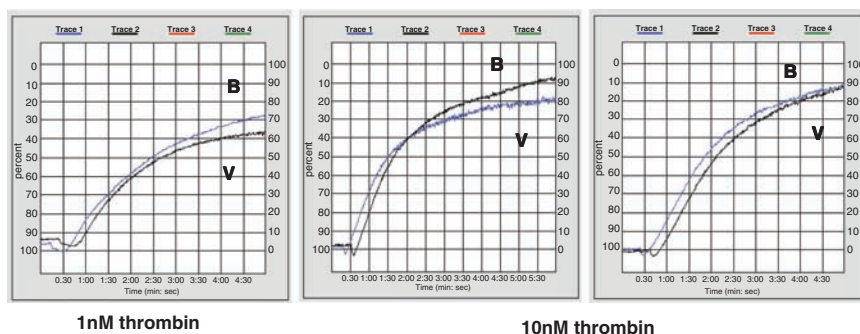


Fig. 7 Effect of volociximab on platelet aggregation

fibrinogen. Platelet activation by thrombin was assessed using a soluble fluorescein isothiocyanate (FITC)–fibrinogen binding assay that could be quantified by flow cytometry. Thrombin-induced platelet activation resulted in a robust and dose-dependent increase in the binding of FITC–fibrinogen. There was a trend towards a slight reduction in the maximal binding of FITC–fibrinogen in the presence of volociximab (data not shown). When thrombin-mediated platelet aggregation was measured by optical transmittance using an aggregometer, there was a trend to a small decrease in the maximal aggregation induced by a sub-optimal dose of thrombin (Fig. 7, panel 1). However, at high concentrations of thrombin, the data were more variable, with either a small difference or no difference observed (Fig. 7, panels 2 and 3). These data may suggest that upon platelet activation by agonists-like thrombin, there is activation of not only the integrin $\alpha\text{IIb}\beta 3$ but also of other minor integrins such as $\alpha 5\beta 1$. Volociximab can diminish the maximal response of platelets to such agonists by blocking the participation of $\alpha 5\beta 1$ in fibrinogen/fibrin binding, but only at sub-optimal doses of agonist. Thus, volociximab may have a benefit in pro-thrombotic conditions seen in diseases such as cancer.

4 Clinical Program with Volociximab

Volociximab is in early-stage clinical development. A phase 1 study with 21 patients with a variety of solid tumour types has been completed. Dose ranging was studied from 0.5 mg/kg to 15 mg/kg given weekly. No safety-related effects were seen at these doses and the maximum tolerated dose was not established. The best response seen in this small trial is stable disease. Six patients with stable disease after five doses of volociximab in the phase 1 study went on to receive additional weekly doses of volociximab at the same dose they received in phase 1. No adverse events associated with volociximab were noted with additional weekly dosing. Volociximab is being evaluated in several pilot phase 2 trials, including one in renal cancer.

5 Conclusions

Integrin $\alpha 5\beta 1$ is a novel target on endothelial cells that has been implicated in models of angiogenesis to play a critical role in the development of vasculature. We have also found that this target is over-expressed upon tumour epithelial cells upon disease progression in certain tumour types, such as renal cancer. A phase 1 clinical study has been completed without any complications due to treatment, and we are continuing to evaluate this novel, potent functional antibody, volociximab, as an anti-angiogenic therapy for the treatment of cancer.

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Carbonic Anhydrase IX: Biology and Clinical Approaches

Brian Shuch, Arie S. Beldegrun, and Robert A. Figlin

Abstract CAIX (carbonic anhydrase IX) is a member of the CA family of proteins and its expression is related to hypoxia. Expression observed in a wide variety of tumors is associated with poor histologic features and is an independent predictor of poor disease-specific survival in different malignancies. In clear cell renal cell carcinoma (RCC), its expression is related to alterations in the *vhl* gene. The majority of clear cell tumors demonstrate diffuse membrane expression of CAIX. Low CAIX expression in metastatic patients is a strong independent predictor of poor outcome. Response to (interleukin-2) IL-2-based systemic therapy appears to be associated with high CAIX expression. As CAIX is the best and most powerful diagnostic and predictor marker for clear cell RCC, those patients with clinico-pathologic predictors of response and high CAIX expression should be offered first-line IL-2 therapy.

Keywords CAIX • Clear cell RCC • Interleukin-2 response • Prognosis • G250

1 Introduction

The evolution of molecular biology and genetics has given clinicians insight into the behavior of renal cell carcinoma (RCC). Tissue-array-based technology has allowed the characterization of thousands of genes in RCC. Once specific genetic alterations are identified that correlate to poor clinical prognosis, these markers can be incorporated into new, more accurate models of RCC. By better understanding

R.A. Figlin(✉)

Department of Urology, David Geffen School of Medicine at University of California, Los Angeles, CA

Current Address: City of Hope National Medical Center, Duarte, CA

Table 1 Molecular markers with known prognostic significance in renal cell carcinoma (RCC)

Hypoxia inducible	Cell cycle regulators	Proliferation	Cellular adhesion	Other
CAIX	KI-67	p53	EpCAM	Geisolin
CAXII	PCNA	bci-2	EMA	CA-125
CXCR-4	Ag-NORs	PTEN	E-Cadherin	Vimentin
HIF-1A		Cyclin a	A-Catenin	CD44
VEGF		AKT	Cadherin-6	Androgen receptors
IGF-1		56 Kinase		Caveolin-1
		p27		VEGFR
				DNA-Ploidy

the genetic alterations that alter cell physiology, clinicians can tailor treatment. These tumor-specific treatment plans will hopefully be more effective at attacking the genotypic alterations with less systemic side effects. Currently, there are a variety of molecular targets that have been identified to be important in RCC prognosis and specific therapies created to combat these alterations. Molecular markers of known importance include hypoxia-inducible factors (HIFs), cell-cycle regulators, cellular proliferation mediators, cellular adhesion molecules, and a wide variety of other molecules (Table 1).

The discovery of one of these molecules (CAIX), could revolutionize the clinical management of RCC and represents one of the most exciting tumor markers in oncology. Current evidence supports its use as a biomarker, a prognostic marker for metastatic RCC, a marker of response to immunotherapy, a potential imaging modality to identify metastasis, and therapeutic target.

2 The Carbonic Anhydrase Family

Carbonic anhydrases (CA) belong to family of 15 zinc metalloproteins that catalyze the reversible hydration of CO_2 to HCO_3^- and H^+ (1, 2). The isoforms vary in location and cytosolic, membrane-bound, and mitochondrial forms have been described (3). Comparison of genetic sequences divided the CA enzymes into three families, alpha, beta, and gamma (4). Each isoform varies in structure but all maintain the essential zinc catalytic domain (5).

3 Discovery of CAIX

In the 1980s, a transmembrane protein was discovered in HeLa cervical cancer cell lines. The expression of this protein was correlated to tumorigenicity in HeLa-fibroblast hybrids and was not found in normal fibroblasts (6, 7). Pastorek later

isolated a cDNA clone coding for this protein, MN, and demonstrated it could induce in vitro malignant transformation of fibroblasts (8). Liao and colleagues later demonstrated MN to be a diagnostic biomarker of cervical cancer and believed it was important in oncogenesis (9). Opavsky and colleagues sequenced the MN gene and recognized its product as an additional member of the CA family (10).

While MN was being investigated, Oosterwijk and colleagues identified a murine monoclonal antibody (mAbG250) raised against a human RCC transmembrane protein, G250. This group utilized the G250 monoclonal antibody for use in mouse RCC xenografts and investigated its role in radioimmunotherapy. Grabmaier and colleagues later isolated and sequenced the cDNA encoding for G250 and determined that it is homologous to the MN/CAIX gene (11).

4 CAIX Function

To appreciate the function of CAIX, it is important to understand the local tumor microenvironment in RCC. CAIX may allow RCC to thrive in harsh hypoxic conditions that result from uncontrolled tumor growth.

4.1 *Biology of Hypoxic Cells*

Adequate tissue perfusion and oxygen delivery is essential for normal cellular hemostasis. The delicate balance between oxygen supply and demand is disrupted in neoplastic cells that demonstrate high metabolic demands. Oxygen tension in the tumor microenvironment is extremely variable in rapidly growing solid tumors. Vascular remodeling by angiogenesis may afford the tumor continued access to oxygen and energy supply. However, a critical threshold of tissue oxygenation is required for aerobic metabolism and oxidative phosphorylation. Once ATP production halts, active transport mechanisms fail and cells can no longer maintain cellular gradients. In the setting of hypoxia, cellular proliferation halts and the cell cycle arrests at the G1/S checkpoint. Hypoxia induces p53 and initiates apoptosis in normal and neoplastic cells (12, 13).

Because of inadequate tissue perfusion and cellular hypoxia, tumor cells frequently rely on high rates of glycolysis. Anaerobic metabolism is required during these periods of hypoxia to continue ATP production and survive. Anaerobic reduction in pyruvate generates lactic acid and acidosis can ensue. However, while lactic acid is important in acidosis, experiments with lactate dehydrogenase-deficient cell lines demonstrate hypoxia can cause extracellular acidification by other means (14).

As the oxygen tension rapidly fluctuates, tumors utilize glycolysis even in the presence of adequate oxygen levels. Intermittent hypoxia selects for cells that can constitutively upregulate anaerobic metabolism and have decreased apoptosis under hypoxic conditions (15).

The better understanding of RCC tumor biology has led to the discovery that hypoxia is an important regulator of gene expression. Besides altering the expression of individual genes, hypoxia alters cellular function on the posttranslational level (16). RCC tumor cells can adapt to nutritional depletion and flourish in the hypoxic environment.

4.2 Hypoxia and Acidosis-Induced Tumor Progression

Sustained tumor hypoxia can cause cellular changes resulting in a more aggressive phenotype (17, 18) Gatenby and Gawlinski describe an “acid-mediated tumor invasion model” (19). This “glycolytic phenotype” is postulated to result in the uniform development of invasive characteristics. Studies demonstrate that high glycolytic cell lines demonstrate increased cell proliferation and invasive potential (20). Hypoxia-driven tumor progression can lead to invasive growth and distant tumor metastasis (21–23). In cervical cancer, tumors with hypoxia demonstrate shorter overall and recurrence-free survival (24).

4.3 Resistance to Therapy

As early as the 1950s, it was evident that hypoxic solid tumors were more resistant to radiation (25). Resistance to radiation therapy is multifactorial. The presence of oxygen in target tissues generates free radical formation. DNA damage ensues which augments the effects of radiation therapy. Additionally, hypoxia stabilizes heat shock proteins and increases resistance to apoptosis both contributing to radio-resistance (26).

Hypoxia limits the response to chemotherapeutic agents (27). Several chemotherapeutic drugs require oxygen to function. Additionally, extracellular acidosis inhibits the delivery and effectiveness of certain drugs.

4.4 Tumor Hypoxia and CAIX

Wykoff and colleagues first demonstrated the importance of CAIX in tumor hypoxia. In various cell lines, CAIX mRNA expression increased under hypoxic conditions (28). Immunohistochemical analysis of several malignancies demonstrates increased CAIX expression in peri-necrotic areas. Staining with pimonidazole confirms the presence of hypoxia in both necrotic and peri-necrotic regions. While CAIX expression can be found in these regions of hypoxia, there is incomplete overlap with hypoxia in the pattern depending on tumor type. This indicates that additional mechanisms may be important for CAIX expression (28).

In von Hippel–Lindau (VHL)-defective clear cell RCC specimens, the expression pattern differs as CAIX is diffusely seen throughout the tumor. Wykoff and colleagues demonstrated immunohistologic localization of CAIX differs between

histologic subtypes of RCC (28). Analysis in papillary RCC demonstrated expression of CAIX only in peri-necrotic regions.

It is important to distinguish whether CAIX expression is a marker of hypoxia or allows adaptation to this environment. By inhibiting CAIX expression, the role the enzyme plays in response to hypoxia can be determined. Robertson and colleagues examined the survival of CAIX expressing cells under hypoxic conditions. Silencing CAIX expression with antisense *therapeutics*, cell survival under hypoxic conditions can be reduced by 50% (29). Thus, it appears that CAIX influences survival under hypoxic conditions.

4.5 CAIX and Acidosis

Ivanov demonstrated acidification of the tumor microenvironment using bicarbonate-buffered cell lines. The extent of acidification was correlated with the extent of CAIX expression (30, 31). Svastova and colleagues analyzed cells with positive CAIX expression and grew them in a bicarbonate-buffered media. Hypoxia leads to an extracellular increase in lactic acid and lowered pH. Acidification is not observed in cells transfected with CAIX deletion mutants (32). Additionally, a CAIX-selective inhibitor prevents extracellular acidification during hypoxia.

In RCC, the transmembrane CAIX *catalyzes* diffused CO₂ in the extracellular environment into bicarbonate and H⁺. This allows bicarbonate–chloride exchange to take place and buffer intracellular H⁺ produced from anaerobic glycolysis. Analysis of CA in cardiac myocytes demonstrates that CA augments extracellular transmembrane hydrogen ion exchange (33). In RCC, CAIX contributes to the regulation of cellular pH and to allow survival in hypoxic conditions (34).

4.6 Summary of Function

Based on the above findings, it appears that hypoxia regulates the activity of CAIX. CAIX facilitates transmembrane proton exchange to buffer intracellular pH and produce extracellular acidification. This process may allow tumor cells to maintain intracellular pH and homeostasis in the setting of hypoxia at the expense of extracellular acidification.

5 Regulation of CAIX

Many hypoxia-inducible genes are controlled by HIF-1, and its two subunits, HIF-1a and HIF-1b (34, 35). HIF-1A restores oxygen homeostasis by inducing glycolysis, erythropoiesis, and angiogenesis (36, 37). HIFs regulate the expression of CAIX in addition to vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and transforming growth factor alpha (TGF- α) (38) (Fig. 1).

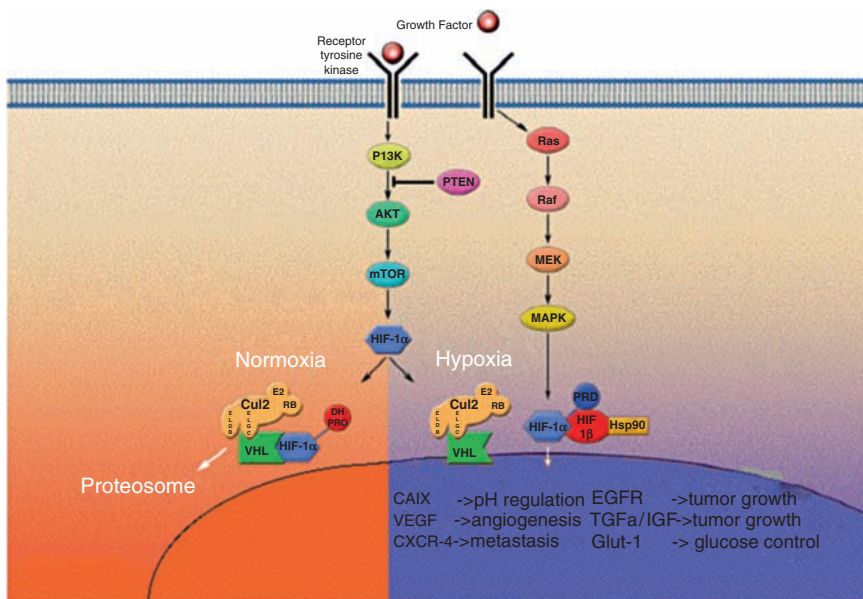


Fig. 1 Signal transduction and regulation of carbonic anhydrase IX (CAIX) pathway (*See Color Plates*)

HIF-1 α regulates CAIX expression and is controlled by the VHL suppressor protein (11). Under normal conditions, HIF-1 α and HIF-2 α bind to VHL and are marked for rapid proteasome degradation. Under hypoxic conditions, HIF-1 α and HIF-2 α are unbound and accumulate and lead to increased CAIX expression. Besides inducing gene expression, hypoxia can upregulate the activity of CAIX directly (39). Additionally, microenvironmental stressors can augment CAIX. Low levels of glucose and bicarbonate lead to increased CAIX expression in the presence of hypoxia (40).

Genetic alterations of VHL including mutation or hypermethylation lead to accumulation of HIF-1 α and HIF-2 α even in the absence of hypoxia (41, 42). In VHL-defective cell lines, HIF-1 α is constitutively active and CAIX expression is high. Restoring VHL function downregulates CAIX expression to normal levels (30).

CAIX expression is density-dependent in several cell lines with increased expression seen at higher cell density. This pathway requires activation of the PI3K pathway. Minimal levels of HIF-1 are required as demonstrated by the loss of density-dependent expression of CAIX in HIF-1 knockout cell lines (43).

6 CAIX Expression and RCC

In the absence of malignancy, CAIX expression is restricted to gastric mucosa and large bile ducts (44). CAIX has been demonstrated to be overexpressed in many malignancies including breast, lung, esophagus, stomach, biliary tree, colon,

bladder, and skin (31). In RCC, it has been shown to be present in various histologic subtypes. Liao and colleagues demonstrated the frequent expression of CAIX in all RCC subtypes except chromophobe RCC (45). Benign lesions (oncocytomas and adenomas) and normal renal tissue did not express CAIX.

Larger cohorts demonstrated the high expression of CAIX in clear cell RCC. Murakami and colleagues evaluated a cohort of nephrectomy specimens with reverse transcription- polymerase chain reaction (RT-PCR) and observed that 92% of clear cell RCC expressed CAIX (46). Bui later confirmed these findings in 321 patients with clear cell RCC. In this series, 94% of patients demonstrated immunohistochemical CAIX expression (47).

The high frequency of CAIX expression in clear cell RCC has prompted its use as a diagnostic marker. Renal biopsies have had a limited role in the management of renal tumors due to poor sensitivity. Li and colleagues recently reported the use of CAIX RT-PCR at diagnosing RCC. In 35 patients, fine needle aspiration of small renal tumors was performed before nephrectomy (48). The sensitivity and specificity of CAIX RT-PCR was 68% and 100%, respectively. CAIX's utility as a diagnostic biomarker may establish a role for biopsy in the workup of renal tumors.

7 CAIX and Clinical Behavior

7.1 Invasion and Metastasis in RCC

By altering the surrounding tumor microenvironment, CAIX may be an important marker of invasion in RCC. The presence of CAIX reduces cell–cell adhesion under hypoxic conditions (32). CAIX has been shown to reduce E-cadherin-mediated cell adhesion, a known marker of invasion (49). Inhibition of CAIX either by administration of acetazolamide or by antisense oligonucleotides can inhibit *in vitro* invasion in CAIX-positive RCC cell lines (50). Inhibition of invasion is more pronounced with other CA isoforms indicating that CAIX may be less vital to tumor invasion (29).

CAIX expression is decreased with advanced disease. Bui and colleagues examined CAIX expression in metastatic lesions (47). Fifteen patients had metastases resected at the time of nephrectomy and 80% demonstrated either equal or decreased expression compared to the primary tumor. Whether CAIX expression is associated with or is responsible for metastasis has yet to be determined.

7.2 CAIX and Prognosis

A relationship between CAIX expression and prognosis has been observed in various types of malignancies. Decreased CAIX expression predicts improved prognosis for with cervical, colorectal, and esophageal cancer (51–53). In contrast, increased CAIX expression is an independent predictor of worse survival in cervical, lung, and breast cancer (54, 55).

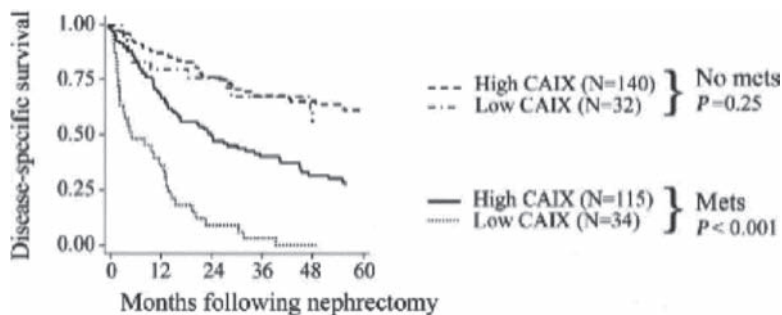


Fig. 2 Kaplan-Meier analysis of survival renal cell carcinoma (RCC) based on carbonic anhydrase IX (CAIX) expression and clinical progression. Reprinted with permission from Bui et al. (47)

Bui and colleagues analyzed the expression of CAIX in clear cell RCC to determine its role in prognosis. In their cohort of 321 tumor specimens 94% of stained positive for CAIX. Decreased expression was associated with advanced stage (47). A cutoff of 85% CAIX staining allowed prognostic risk stratification. Low CAIX staining was found to be an independent prognostic indicator of poor survival in patients with metastatic RCC (Fig. 2). In fact, CAIX status was greatest predictor of poor outcome with a hazard ratio (3, 10), almost double that of T stage, Eastern Cooperative Oncology Group performance status, or Fuhrman grade. For patients with localized disease, low CAIX expression was associated with poor prognosis but failed to reach statistical significance. Low CAIX expression was useful in identifying a subset of high-risk patients with localized RCC with poor outcome similar to that with metastatic disease. The authors suggested that CAIX status could be useful to select patients for adjuvant therapy.

Sandlund recently confirmed the prognostic role of CAIX in clear cell RCC. CAIX was found to be an independent predictor of improved disease-specific survival for patients with stage I–III but not for stage IV disease (56). This is opposite what had been previously reported that CAIX is an independent predictor only for stage IV disease (47). Important differences exist between studies including different CAIX expression stratification. Bui analyzed a larger number of metastatic specimens ($n = 150$) compared to a more limited analysis in this study ($n = 60$). Sandlund's patient cohort included many more high-risk patients including 81% with Fuhrman grade 3/4 compared to 41% in Bui's analysis. Additionally, Bui demonstrated that CAIX influences prognosis in high-risk M0 patients (T classification ≥ 3 or Fuhrman Grade ≥ 2). Perhaps Sandlund's analysis was able to capture the importance of CAIX in localized patients with the inclusion of a greater number of high-risk patients.

7.3 Response to Immunotherapy

Interleukin-2 (IL-2) therapy leads to a clinical response in a small number of patients and has significant associated morbidity and mortality. Determination of the clinical

features that dictate response is important to avoid inadvertent harm in patients unlikely to respond to therapy. Upton and colleagues attempted to evaluate the role of histology in the response to immunotherapy. A large cohort of patients with metastatic RCC treated with IL-2 had primary tumor specimens available for analysis. Of the 163 patients evaluated, the response rate was 21% (30/146) for patients with clear cell versus 6% (1/17) for patients with nonclear cell RCC (57). Additionally, the presence of greater than 50% alveolar features favored a clinical response to IL-2.

Bui and colleagues made an important observation regarding IL-2 response in 86 patients with metastatic RCC (47). Of this subset, 84% of patients had high expression of CAIX. It was noted that all complete responders to IL-2 immunotherapy, 8% of this cohort, included patients with high CAIX staining of the primary tumor. The overall response rate to IL-2 for patients with high and low CAIX expression was 27% and 14%, respectively. Based on these findings, it was suggested that CAIX expression aid the selection of patients for IL-2 or CAIX-targeted therapy.

Atkins and colleagues confirmed that high CAIX expression was associated with IL-2 response (Fig. 3). Patients with high CAIX expression were twice as likely to have a response to treatment and prolonged survival (58). These findings may explain why papillary and chromophobe subtypes, which express low or no levels of CAIX, respond poorly to IL-2 immunotherapy.

We reported preliminary results from a prospective CAIX study for patients with metastatic clear cell RCC. The response rate to IL-2 in patients displaying high

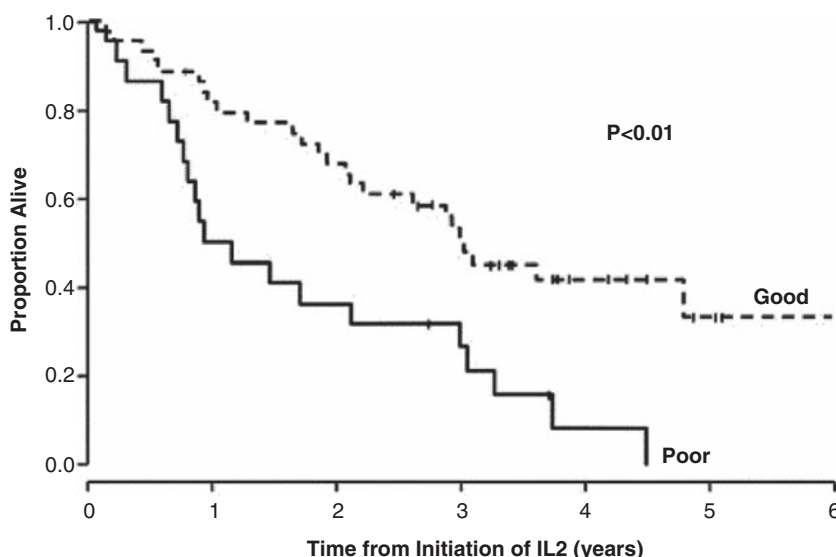


Fig. 3 Kaplan Meier survival after initiation of interleukin-2 (IL-2) therapy based on risk group. Good risk group consists of low and intermediate pathology plus high carbonic anhydrase IX (CAIX) expression. Poor risk group consists of high and intermediate pathology with low CAIX expression. Reprinted with permission from Atkins et al. (58)

CAIX expression was 37.5% (3/8), including two complete responders (59). With multiple therapeutic options now available to oncologists, we believe that patients with clinicopathologic predictors of response and high CAIX expression should be offered first-line IL-2-based therapy.

8 Targeting CAIX Pathway

As CAIX is a tumor-associated antigen with a limited expression in normal cells and a high frequency of expression in renal tumors, it serves as an excellent target for therapy. Recent studies demonstrate the presence of major histocompatibility complex (MHC) class I and class II epitopes encoded by CAIX are recognized by human T cell (60, 61). Additionally, the transmembrane location of this molecule makes it an ideal antigen for targeted immunotherapy. Both antibody and T cell-mediated approaches may be used to target CAIX.

8.1 CAIX Animal Model

An animal model is necessary to test the feasibility of targeted immunotherapy against RCC. An ideal model should involve an animal host with an intact immune system and a tumor cell line that expresses the tumor-associated antigen of interest. Recently, a murine RCC tumor model has been developed at University of California, Los Angeles (UCLA) that expresses CAIX. A retrovirus encoding the full-length human CAIX gene was used to transduce a murine RCC line, renal cancer (RENCA). The RENCA/CAIX line demonstrates nearly 100% CAIX expression. Balb/c mice injected with intravenous RENCA/CAIX tumor cells uniformly developed pulmonary metastasis (Fig. 4). Immunohistochemical analysis of pulmonary metastases confirmed the sustained expression of CAIX (Fig. 5). This tumor model will allow the continued development and testing of CAIX-directed, immune-based therapies.

8.2 G250 Monoclonal Antibody

Oosterwijk and colleagues developed a murine monoclonal antibody G250 raised against human RCC. This antibody reacts with a large proportion of RCC and is restricted to gastric mucosa and large bile ducts in normal tissues (44). Van Dijk and colleagues assessed the antibody's therapeutic efficacy in human RCC xenografts raised in nude mice. Treatment inhibited tumor growth and when combined with interferon alpha (IFN- α) and tumor necrosis factor alpha (TNF- α) a complete response was observed in 30% of mice. After 90 days, there was no evidence of recurrence (62).



Fig. 4 Pulmonary metastasis from renal cancer/carbonic anhydrase IX (*RENCA/CAIX*) murine model (See Color Plates)

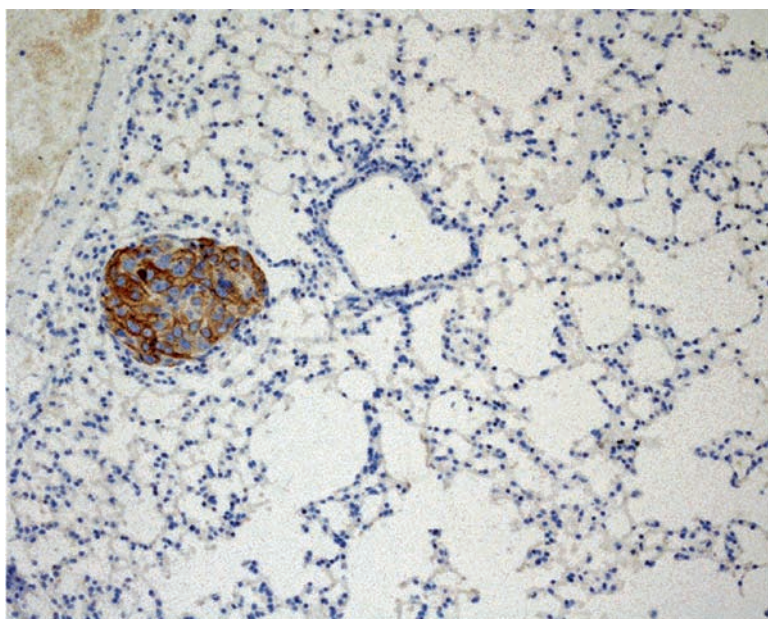


Fig. 5 CAIX expression in renal cancer/carbonic anhydrase (*RENCA/CAIX*) murine pulmonary metastasis (See Color Plates)

8.3 G250 Nuclear Imaging

Clinical trials with a radiolabeled G250 antibody demonstrated the efficacy of imaging primary and metastatic RCC. Oosterwijk reported radiolabeled G250 could identify metastatic lesions not seen on MRI, CT, or bone scan in 20% of patients. Tissue biopsies of 30 lesions confirmed the presence of RCC not seen by conventional imaging (63).

8.4 G250 Radioimmunotherapy

To enhance the antitumor efficacy of G250, the antibody was conjugated to ^{131}I (63–65). Several phase I trials assessed the safety and pharmacokinetics of ^{131}I -conjugated G250 with administered 1 week before nephrectomy. The antibody possesses excellent pharmacokinetics, is selective and specific, has extraordinarily high uptake, and requires a low dosage for tumor saturation (63–65).

A phase I/II radioimmunotherapy trial utilizing ^{131}I -G250 was performed at Memorial Sloan-Kettering Cancer Center (66). A total of 33 patients with metastatic clear cell RCC received increasing amounts of ^{131}I linked to 10 mg of G250. Hyperbilirubinemia lasting 2 weeks or less was observed at all doses. Transient reversible transaminitis was observed in 81% of patients. The maximal dose of ^{131}I used, 90 mCi/M², was determined by its incidence of hematopoietic toxicity with several patients demonstrating grade-III thrombocytopenia. In the phase II arm of the study, radioactivity was observed in all tumor sites 2 cm or greater by imaging scans performed 2 and 4 days after administration of ^{131}I -G250. No major responses were noted but an encouraging finding was over 50% of the patients had stable disease. Retreatment was precluded by the development of human antimouse antibodies in all patients 1 month postadministration (66).

8.5 Chimeric G250 Monoclonal Antibody

The need for reduced immunogenicity and multiple administrations lead to an engineered chimeric form of the G250 antibody (cG250). Combining the humanized Fc portion with the murine G250, cG250 was developed. Steffens and colleagues demonstrated that the specificity and affinity of cG250 was equivalent to its murine form (67). Only two of sixteen patients demonstrated an antichimeric antibody response which would allow multiple administration if needed.

Steffens and colleagues published the results of a phase I dose escalation study of ^{131}I -cG250 (67). Hematopoietic toxicity was used to determine the maximal therapeutic dosage. In this study, no hepatotoxicity was observed. The maximal therapeutic dosage for cG250 differed from the murine form due to the molecule's longer half-life.

A phase I/II trial has enrolled patients to receive two high doses of ^{131}I -cG250 separated by 3 months (68). Images obtained 2 weeks after cG250 administration demonstrate exceptional ^{131}I -cG250 retention. Toxicity is bone marrow related and is more severe with the second administration, suggestive of bone marrow exhaustion by the first high dose.

8.6 *Future of cG250 Radioimmunotherapy*

^{131}I is not an ideal radionuclide for radioimmunotherapy. Upon internalization of the antibody, ^{131}I is released from the tumor. As high-energy gamma photons are released to the surrounding tissue, there is a concern for radiation safety. Brouwers and colleagues demonstrate that Yttrium (^{90}Y) or lutetium (^{177}Lu) lead to a more homogeneous radiation distribution. These radionuclides can provide higher radiation doses to RCC and have demonstrated increased therapeutic efficacy in nude mice RCC xenografts (69).

8.7 *cG250 Antibody-Dependent Cellular Cytotoxicity*

Immune recognition of the bound Fc portion of cG250 can lead to antibody-dependent cellular toxicity (ADCC) (70). Bleumer and colleagues reported the results of a phase II trial demonstrating the effect of nonradiolabeled cG250 in advanced RCC (71). A total of 36 patients with metastatic clear cell RCC received weekly administration with cG250. The treatment was well tolerated and no grade III or IV toxicity was observed. The results were promising as the median survival was 15 months and one patient demonstrated a complete response.

Several studies have demonstrated the efficacy of cG250 at enhancing antibody dependent cellular cytotoxicity (ADCC) with concomitant use of cytokines in vitro (70, 72). Coadministration with IL-2, IFN- γ , IFN- 2α , and IFN- 2β resulted in augmentation of ADCC. However, only IL-2 and IFN-gamma provided sustained immunogenic response with IL-2 demonstrating the best results (72).

Bleumer and colleagues recently examined the effect of coadministration of weekly infusions of cG250 combined with a daily low-dose-IL-2 (71). A total of 35 patients underwent therapy and the regimen was well-tolerated with minimal toxicity. Radiologic assessment of metastasis was assessed with imaging performed at week 16. A durable clinical response was seen in eight patients (23%). ADCC was assessed with ^{51}Cr release assays and demonstrated no correlation with clinical response. The overall survival, a median of 22 months, was increased compared to that previously observed with cG250 monotherapy. The authors concluded that survival was at worst similar to that of IL-2 therapy alone but associated with a better toxicity profile.

A phase III trial is in progress to evaluate the effect of the WX-G250 antibody, Rencarex[®] (WILEX, Munich, Germany), as adjuvant therapy for patients with

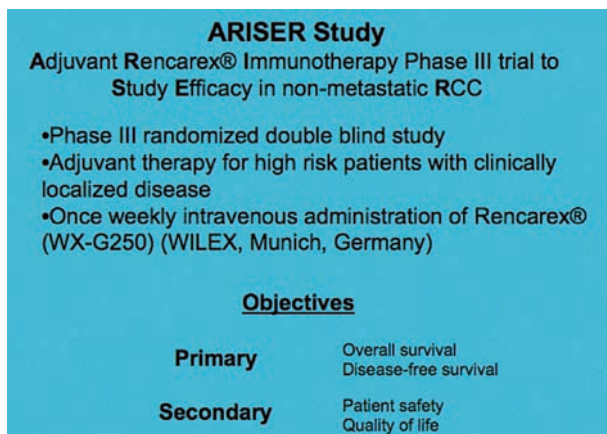


Fig. 6 ARISER study with WX-G250 antibody

high-risk localized disease (Fig. 6). The study, ARISER, is a randomized double-blind study that has accrued 600 patients to assess the affects on disease-free survival and overall survival. Additional analysis will be conducted to evaluate patient safety and quality of life.

8.8 Vaccine-Based Strategies

The ultimate form of immunotherapy would rely on the body's intrinsic immune system to destroy tumor cells and possess a minimal side effect profile. An alternative immunotherapy strategy to target cancer cells is dendritic cell-based rather than antibody-mediated. The development of a safe and reliable gene delivery vector that could reliably generate modified dendritic cells may be used to generate tumor vaccines. By efficiently presenting multiple tumor-associated antigen epitopes, these modified dendritic cells may promote antitumor activity. Additionally, the addition of an immunomodulatory protein could augment the cytotoxic immune response.

CAIX vaccine strategies are currently being developed for use in RCC. Tso and colleagues produced a fusion protein containing two immune activators, G250 and granulocyte/macrophage-colony stimulating factor (GM-CSF) (73). A fusion gene, G250-GM-CSF, was constructed using a baculovirus vector. Incubation of peripheral blood mononuclear cells with the fusion protein leads to the induction of dendritic cells and activates antitumor response by T-helper and CD8⁺ cells. The in vitro results with GM-CSF-G250 fusion protein are encouraging for RCC tumor vaccine clinical trials.

Mukouyama and colleagues utilized a different viral-mediated strategy to induce T cell response. An adenovirus containing the CAIX gene was used to transfect Peripheral blood monocytes (PMBC). Peripheral blood lymphocytes were incubated with the transfected monocytes and cytotoxic lymphocytes were raised (Fig. 7).

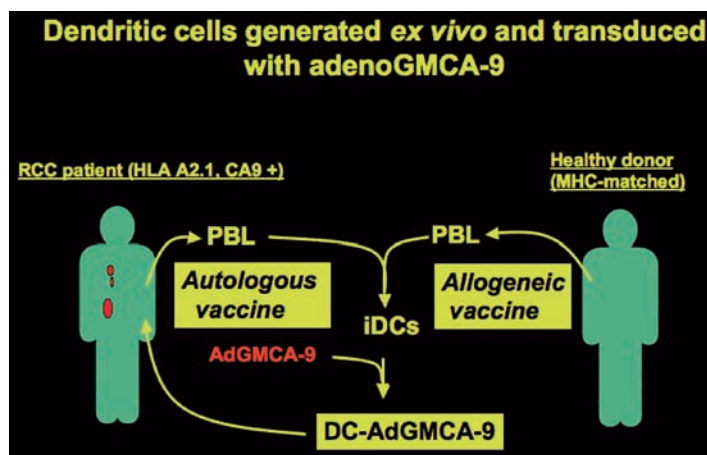


Fig. 7 Vaccine strategies for renal cell carcinoma (RCC) using a recombinant adenovirus encoding for carbonic anhydrase (CAIX) (Ad-GMCA-9) to target CAIX tumor cells (See Color Plates)

Administration to RCC cell lines expressing CAIX demonstrated that the generated cytotoxic lymphocytes could cause tumor lysis in vitro (74).

A phase I/II trial for adenovirus-CAIX-GM-CSF dendritic cell vaccination has been organized to assess its role as adjuvant therapy in metastatic RCC. Eligibility criteria include low or intermediate UCLA Integrated Staging System (UISS) category, high CAIX expression, metastatic disease while the prior to nephrectomy, and no prior treatments. End points will assess the safety profile and toxicity, maximal tolerated doses, as well as the clinical response rate.

9 Conclusions

CAIX is one of the most important molecular markers in kidney cancer to date. High CAIX expression in other malignancies is associated with hypoxia and appears to be a marker of poor prognosis. In clear cell RCC, however, its presence is linked to *vhl* mutation and high expression is an independent predictor of improved disease-specific survival in metastatic and possibly high-risk localized disease.

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Monoclonal Antibody G250 Recognizing Carbonic Anhydrase IX in Renal Cell Carcinoma: Biological and Clinical Studies

J. C. Oosterwijk-Wakka, Otto C. Boerman,
Peter F. A. Mulders, and Egbert Oosterwijk

Abstract Monoclonal antibody G250 (mAb G250) recognizes a determinant on carbonic anhydrase IX (G250/CAIX). CAIX is expressed by virtually all renal cell carcinomas of the clear cell type (ccRCC). This can be explained by a direct molecular link between CAIX gene expression and (one of the) the initiating mutational event(s) in ccRCC: inactivation of the von Hippel–Lindau gene product. The combination of restricted G250/CAIX expression in normal tissues, homogeneous G250/CAIX expression in RCC, and excellent targeting capability in animal models provided a solid basis for the initiation of the clinical evaluation of mAb G250 in patients with metastatic RCC (mRCC). These strategies include radioimmunotherapy (RIT) with different radiolabels as well as passive immunotherapy with the unmodified antibody in combination with interleukin-2 (IL-2) or interferon alpha (IFN- α)

Presurgical clinical trials with radiolabeled mAb G250 showed excellent tumor uptake of antibody, with a saturable hepatic uptake. After chimerization of the murine mAb G250, necessary to minimize immunogenicity, single dose RIT lead to occasional responses, and multiple-dose RIT demonstrated stabilization of disease in ~25% of the previously progressive patients. More potent radioisotopes such as ^{90}Y and ^{177}Lu are currently used to study whether the efficacy of mAb G250 RIT can be improved. Passive mAb G250 immunotherapy suggested a clinical benefit, and combination trials with either low-dose IL-2 or IFN- α showed some clinical benefit, but these results must be viewed with caution in view of the variability of this disease. Possibly, combination with small molecule drugs such as Sunitinib and Sorafenib might be useful in the treatment and clinical management of mRCC. Finally, mAb G250 imaging may become a useful tool for the preoperative identification of ccRCC of patients with suspect renal mass.

Keywords Monoclonal antibody • G250 • Immunotherapy

E. Oosterwijk (✉)
Department of Urology, Experimental Urology,
267, University Medical Center St Radboud,
Geert Grooteplein 28, 6525 GA Nijmegen, The Netherlands
e-mail: e.oosterwijk@uro.umcn.nl

1 Introduction

Since its inception, monoclonal antibody (mAb) technology has changed the clinical practice. Currently, these molecules with predefined specificity are coming of age and represent a new category of drugs in the fight against cancer. Also for renal cell carcinoma (RCC) numerous mAbs (1–4) have been described, but only one mAb (mAb G250) has been studied extensively in clinical settings. The antibody was isolated more than 20 years ago from the spleen of mice immunized with a fresh human RCC in an effort to isolate mAbs reactive with RCC-specific or RCC-associated antigens (5). Immunohistochemical analysis of renal tumors showed homogeneous expression in the vast majority (>80%) of primary RCC and also in metastatic RCC lesions. Initial specificity analysis on normal human tissues revealed cross-reactivity with gastric mucosal cells and large bile ducts. Subsequent in-depth fine-specificity analysis revealed reactivity with epithelial cells of the upper gastrointestinal tract and pancreas cells. Originally, no association with a particular histological RCC subtype was noted, but it has become clear that the antigen recognized by mAb G250 is almost ubiquitously expressed in clear cell RCC (ccRCC), the most prominent type of RCC ($\approx$ 80%). Analyses of non-RCC tumors revealed variable staining.

The general occurrence of the antigen recognized by mAb G250 in RCC and absence from normal kidney suggested that induction was inherently related to tumor development, possibly due to a common initiating event (5). Increased understanding of the initiating mutational events in the carcinogenesis of ccRCC and the molecular identification of the antigen has revealed a direct molecular link between these two. Elegant studies where families suffering from the von Hippel–Lindau (VHL) syndrome were studied demonstrated a direct link between this autosomally transmitted syndrome and loss of VHL function (6, 7). Members of these families develop numerous tumors, among others ccRCC. Indeed, further studies revealed that somatic inactivation of the *VHL* gene is common in sporadic ccRCC and this is now generally viewed as the initiating event in ccRCC development. The *VHL* gene product functions as a scavenger E3 ubiquitinase for the transcription factor hypoxia-inducible-factor (HIF) thereby preventing activation of genes dependent on HIF-1 α binding, such as vascular endothelial growth factor (VEGF), epidermal growth factor receptor (EGFR), and platelet-derived growth factor (PDGF) (8).

When the antigen was identified it was found to be identical to carbonic anhydrase IX (CAIX), a gene originally identified in HeLa cells (9, 10). Extensive molecular studies of the CAIX promoter region demonstrated that HIF-1 α binding was an absolute requirement for CAIX expression in ccRCC (11). Thus, the ubiquitous expression of the G250/CAIX antigen could be explained straightforward by non-functional VHL gene product in ccRCC (Fig. 1).

Although one might extrapolate that this implies that all metastatic sites derived from G250-positive ccRCC express G250/CAIX, this may not be the case; lower G250/CAIX expression levels in metastatic lesions relative to matched primary tumor specimens has been demonstrated (12). The loss of G250/CAIX expression is most likely a reflection of a more aggressive phenotype as tumors progress.

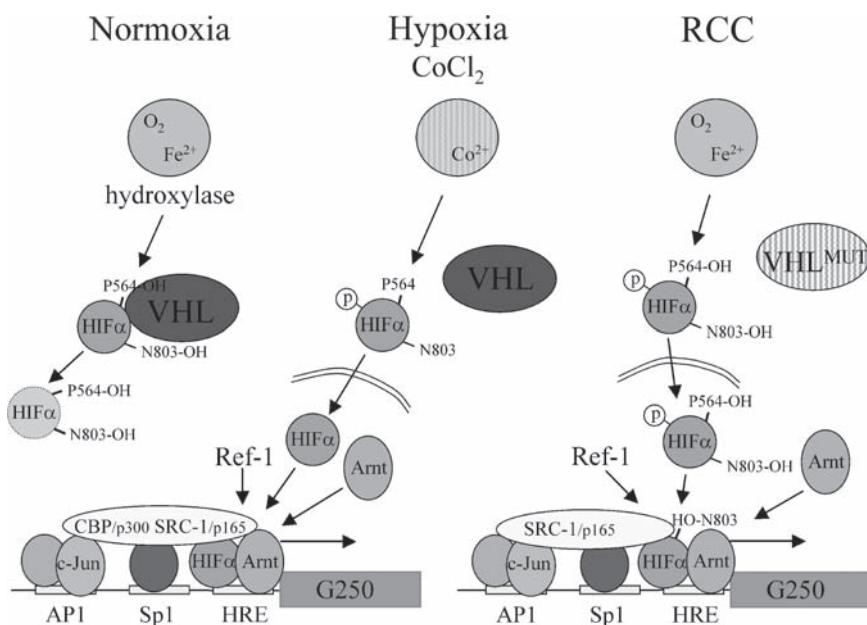


Fig. 1 Under normoxic conditions, the functional von Hippel-Lindau (VHL) protein binds hypoxia-inducible-factor-1 alpha (HIF-1 α) and degrades the protein. Under hypoxic conditions, HIF-1 α is not hydroxylated and therefore not able to bind to the functional VHL protein. Subsequently, HIF-1 α migrates to the nucleus and activates the G250 promoter leading to G250 protein expression. In clear cell renal cell carcinoma (ccRCC), VHL is mutated and therefore unable to bind to HIF-1 α and so, like in hypoxia, HIF-1 α migrates to the nucleus and activates the G250 promoter

Indeed, for high risk RCC patients, low G250/CAIX expression predicted a worse outcome similar to patients with metastatic disease (12).

Elucidation of the molecular pathway of G250 gene expression also readily explained the heterogeneous staining pattern in non-RCC tumors: this is most probably the consequence of local hypoxia, leading to HIF-1 α stabilization and subsequent G250/CAIX expression. In fact, G250/CAIX is now regarded as an appropriate substitute hypoxia marker in various tumor types (13).

To determine whether G250/CAIX could be a potential therapeutic target and a tumor marker, RCC samples were investigated by immunohistochemistry as well as by reverse transcriptase polymerase chain reaction (RT-PCR) analysis (14, 15). Immunohistochemistry demonstrated strong expression in 85–90% of RCC, with virtually all ccRCC expressing G250/CAIX. RT-PCR analyses of frozen specimens were almost identical to those for immunohistochemistry with clear detection of G250/CAIX mRNA in >90% RCC. Although high-grade and high-stage tumors exhibited significantly lower G250/CAIX expression than low-grade and low-stage tumors, a large proportion of tumors expressed mRNA and the G250/CAIX protein. Collectively, the results strongly suggested that G250/CAIX expression could be

used as diagnostic biomarker of RCC for detection of circulating tumor cells and might be used for targeted ccRCC-directed therapeutic approaches (13–18).

2 Targeted Therapy with mAb G250

MAB targeting depends on numerous factors, for example, (homogeneous) antigen expression, tumor perfusion, internalization rate, and expression in nontarget tissue. Indeed, a major caveat of mAb therapy in solid tumors is the poor perfusion rate given that diffusion is the main driving force of mAb uptake in antigen expressing tissues. The common view of RCC being a highly vascularized tumor, and thereby possibly a good target for mAb-guided therapy should be viewed with caution: high vascular density is not equivalent to high perfusion rates, which are required for optimal mAb delivery. Actually, the perfusion rate of RCC is lower than that of, for example, normal kidney tissue, and thus adequate delivery of mAb may be hampered, as is the case for all solid tumors.

Fundamentally, tumor therapy using mAbs can be mediated via, for example, effector cell mechanisms, complement activation, or conjugation to, for example, toxins, drugs, or radionuclides [for review, see Carter (19)]. Poor performance can be adjusted by the availability of established and emerging technologies to improve antibody performance (20), but obviously, adequate specificity is required. The caveat for mAb therapies relying on effector cell mechanisms is the necessity of antigen expression by all tumor cells. Antigen-negative tumor cells will escape and eventually lead to unwanted tumor recurrence and subsequent death of patients. Despite this disadvantage, many favor this form of passive immunotherapy, because administration of high doses of unconjugated, “naked” mAb is well tolerated without any side effects. For instance, trastuzumab (Herceptin®) a humanized version of a mouse mAb against Human Epidermal Growth Factor Receptor 2 (HER2) (21) is now used as first-line treatment and has been demonstrated to be a valuable adjuvant treatment for women with early breast cancer overexpressing HER2 (22).

Conjugated mAbs, particularly labeled with radionuclides, have been studied extensively for treatment of cancer. Radioimmunotherapy (RIT) of non-Hodgkin’s lymphomas is highly successful, but RIT in solid tumors has met limited success (23), for reasons discussed before. Radiolabeled mAbs have the advantage that they do not need to be internalized for cell killing and the antibody does not have to bind to every tumor cell for an efficient therapeutic effect. With radionuclides that can be used for therapy (α or β emitters such as ^{131}I , ^{90}Y , ^{212}Bi , ^{211}At , ^{186}Re , and ^{177}Lu) cell kill can occur at distances of up to 50 or more cell diameters. The majority of RIT studies have been performed with ^{131}I conjugated mAbs: it is readily available, cheap, with relatively easy labeling chemistry.

The use of a low diagnostic dose of radiolabeled conjugates allows for in situ tumor detection, preventing the inclusion of patients with antigen-negative tumors (poor mAb uptake) for high-dose therapy. Thus, unnecessary procedures, toxicity, and loss of quality of life can be precluded. It may permit patient

adjusted administration, although adequate dosimetric calculations are logistically demanding.

2.1 Clinical Studies with Radiolabeled Murine mAb G250

Before clinical studies were initiated, targeting studies were performed in various animal models (5, 24, 25) as well as in ex vivo perfusion experiments in tumor-bearing kidneys (26). Selective and extraordinary high uptake of murine mAb G250 (mG250) in antigen-positive tumor xenografts was observed (e.g., up to more than 100% of the injected dose per gram tumor tissue). The combination of restricted G250/CAIX expression in normal tissues, homogeneous G250/CAIX expression in RCC, and excellent targeting capability in animal models provided a solid basis for the initiation of the clinical evaluation of mG250 in patients with (metastatic) RCC.

2.1.1 ^{131}I -mG250, Phase I Dose Escalation

The first clinical study with mAb G250 concerned a phase I presurgical protein dose-escalating study of ^{131}I -labeled to escalating mass amounts of mG250 (0.2, 2, 10, 25, and 50 mg) conducted to determine tumor uptake and mAb distribution in patients suspect for RCC (27). Tumor uptake was clearly evident 2–3 days after mAb administration as judged by planar scintigraphy. At the lowest protein dose (0.2 and 2 mg mG250), liver uptake was observed, but the relative high liver uptake decreased at higher protein dose levels, suggesting saturation of G250 antigen sites by the mAb. At protein doses >2 mg, ^{131}I -labeled mG250 lead to excellent tumor imaging without visible uptake in nontumor tissues. Additionally, gamma camera images of $^{99\text{m}}\text{Tc}$ -human serum albumin, administered immediately before surgery, and ^{131}I -mG250 were clearly disparate, demonstrating that the images were not a reflection of blood pool, but of true selective antibody accumulation. Because radical nephrectomy was part of the clinical management of these patients, tumor tissue could be sampled extensively and examined ex vivo. Levels of mG250 accumulation reached levels of up to 0.1% of the injected dose per gram of tumor (%ID/g), these levels being among the highest reported in studies of solid tumors. Additionally, normal tissue uptake, actually limited to the liver, was saturable, encouraging future development of mG250 in RCC.

2.1.2 ^{131}I -mG250, Phase I/II RIT

Subsequently, a phase I/II RIT trial with ^{131}I -mG250 was initiated at Memorial Sloan-Kettering Cancer Center to determine its safety and possible efficacy (28). Cohorts of at least three patients received escalating amounts of ^{131}I (from 30 to 90 mCi/m²) labeled to 10 mg of mG250 as a single infusion; 33 patients were

treated. Since excellent targeting occurred in ccRCC, only progressive patients with measurable, histological proven ccRCC were eligible. Targeting to all known disease ≥ 2 cm in diameter was excellent, and in at least one patient additional metastatic sites (medistinal and bone), not recognized by other imaging technologies (CT) became visible. Hepatic toxicity was observed at activity dose levels ≥ 45 mCi/m², but the intensity was otherwise unrelated to the amount of radioactivity administered or the radiation dose absorbed to the liver. Furthermore, this toxicity was transient (symptoms usually resolved in 2 weeks) and not dose limiting. As in all RIT studies with radiolabeled antibodies, dose-limiting toxicity was hematopoietic, with the maximum tolerated dose (MTD) of activity determined to be 90 mCi of ¹³¹I/m². In the phase II arm of the trial, 15 patients received 90 mCi of ¹³¹I-mG250/m² to determine efficacy, but no major responses were noted.

The development of high titer human antimouse antibody (HAMA) in all patients precluded retreatment (28). The excellent targeting and the lack of significant nonhematopoietic toxicity demonstrated the potential of radiolabeled mG250 in the treatment of renal cancer. These characteristics in combination with the aim to apply multiple injections led to the development of chimeric mAb G250 (cG250) – a molecularly engineered antibody that was produced by replacing the constant region of the heavy and light chain of the murine antibody for the human parts of the molecule in the expectation that this would reduce immunogenicity.

2.2 Clinical Studies with Radiolabeled Chimeric mAb G250 (cG250)

2.2.1 ¹³¹I-cG250, Phase I Dose Escalation

Because chimerization of mG250 could lead to altered characteristics, a phase I protein dose-escalation trial of ¹³¹I-cG250 identical to the phase I mG250 protein dose-escalation trial was necessary (29). Sixteen patients with advanced RCC received a single IV infusion of 6 mCi ¹³¹I-cG250. All patients with an antigen-positive tumor (13) showed excellent targeting of radioactivity to all known metastatic sites. Tumor sampling was more extensive than in the mG250 trial, which allowed better assessment of determination of intratumoral uptake variation. Of four different tumors, whole tumor slices were cut into 1 cm³ cubes and in each cube the ¹³¹I-cG250 uptake was determined. Focally, the percentage of the injected dose per gram (%ID) was as high as 0.52%. Subsequent studies demonstrated that although high antigen expression was a prerequisite for high antibody uptake, regional differences within a tumor could not be explained by antigen expression alone. No consistent association with necrosis or vasculature was noted (30). ¹³¹I-cG250 uptake in nontumor tissues remained low. As was true for mG250, hepatic uptake was saturable at doses of ≥ 5 mg of cG250. Similar to mG250, previously undetected metastatic lesions (brain, bone, and soft tissue) were detected in three patients. Dosimetric analysis revealed that up to 48 cGy/mCi was guided to the primary

tumors. Selected “hot areas” within these tumors received up to 72 cGy/mCi. Most importantly, in only 2 of 15 patients low levels of human antichimeric antibody (HACA) were observed (29). Thus, chimerization of the antibody had indeed led to diminished immunogenicity, allowing multiple administrations. The other characteristics, particularly the outstanding targeting ability remained intact. The only change concerned a longer serum half-life ($t_{1/2}$ 69 h vs 47 h for mG250), which was anticipated based on the behavior of other chimerized mAbs.

2.2.2 ^{131}I -cG250 and ^{125}I -cG250, a Dual-Label Study

Since highly dynamic physiological factors may contribute to heterogeneous tumor uptake of antibodies as was observed with cG250, the impact of time on tumor uptake of cG250 was studied in a clinical setting. In this dual-label study, ten patients with a clinical diagnosis of primary RCC received two independent consecutive administrations of cG250, separated by 4 days. Postsurgery, the distribution of both administrations was mapped and analyzed to investigate the time dependence of heterogeneity of intratumoral antibody administration. The study demonstrated that cG250 distribution did not differ between different administrations, demonstrating that intrinsic tumor factors play a prominent role in intratumoral heterogeneity of antibody distribution (31). Thus, consistent “low uptake areas” may reflect less accessible areas for the mAb or intrinsically different tumor populations, for example, consisting of fast internalizing subpopulations, resulting in apparent low uptake areas, because internalized iodinated proteins are quickly degraded and the iodine is lost. Possibly, avid mAb uptake may have occurred. Initially, this explanation was felt to be improbable because ^{131}I -cG250 tumor retention in patients was in the order of weeks, suggesting very low internalization rates (28). However, follow-up animal experiments have shown that internalization may play an important role in the cG250 distribution (32, 33).

2.2.3 ^{131}I -cG250, Phase I Activity Dose-Escalation Study

Following the protein dose-escalation trial which established the most favorable protein dose, a phase I ^{131}I -cG250 activity dose escalation was performed to establish dose-limiting toxicity (34). Patients ($n = 12$) received an imaging dose (6 mCi of ^{131}I labeled to 5 mg of cG250), before being allowed to advance to high-dose ^{131}I -cG250. Only those patients showing targeting to tumor ($n = 8$) received the therapeutic infusion of ^{131}I -cG250 1 week later. Surprisingly, unlike mG250 RIT, hepatic toxicity was absent. This difference was attributed to saturation of the hepatic G250 antigen sites with the initial imaging dose of cG250, but this hypothesis has not been formally tested. Dose-limiting toxicity of cG250 was hematopoietic at 75 mCi ^{131}I -cG250/m², significantly lower than dose-limiting toxicity observed for the murine version. The MTD was therefore set at 60 mCi/m². Almost certainly, the extended serum half-life is responsible for the enhanced hematopoietic toxicity, since this

leads to extended radiation of the bone marrow compartment. Targeting of ^{131}I -cG250 to disease was outstanding in patients with positive diagnostic scans. Two out of eight patients showed an antitumor response; one SD for 3–6 months and one partial response (PR) >9 months. Both patients were treated at the 60 mCi/m^2 dose level. However, all other patients showed progression of disease. Clearly, to achieve more complete and lasting responses increased doses of radioactivity to the tumors are required. Several strategies have been explored.

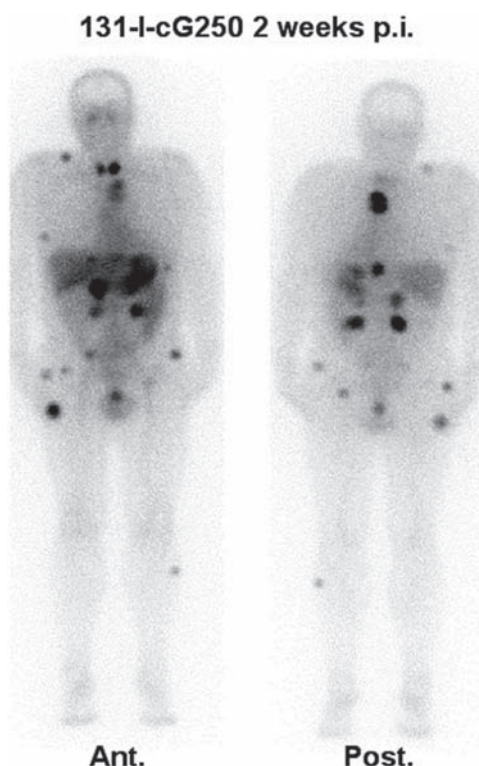
2.2.4 ^{131}I -cG250, Phase I RIT, Fractionated Dose

Fractionated RIT at lower cumulative amounts of radioactivity has been shown in animal models to be more effective than a single large amount and could be associated with a lower toxicity profile. This concept was tested in a phase I fractionated cG250 RIT trial based on whole-body radiation absorbed dose (35). The primary objective was to determine the maximum tolerated whole-body radiation-absorbed dose of fractionated ^{131}I -cG250 with dose escalation referred here to the escalation of average whole-body absorbed dose. Fifteen patients with measurable metastatic renal cancer were studied. For each treatment cycle, patients initially received a diagnostic dose consisting of 5 mg of cG250 antibody labeled with 5 mCi of ^{131}I . The first fraction in each cycle was 30 mCi of ^{131}I conjugated to 5 mg of antibody and subsequent fractions consisted of variable activities depending on the patient-specific whole-body clearance rates. Time between fractions was 2–3 days. Patients without evidence of disease progression were retreated after recovery from toxicity if there was no evidence of altered pharmacokinetics or serum HACA titers, for a total of not more than three treatments. The majority of patients tolerated repeated injections with no change in kinetics, confirming the lack of immunogenicity of the antibody construct. Similar to single dose cG250 RIT, targeting to known disease $\geq 2\text{ cm}$ in diameter was noted in all patients and dose-limiting toxicity was hematopoietic. In this logistically demanding fractionated administration regimen sparing of the hematopoietic system was not observed. Moreover, the total dose that could be delivered was low and efforts along these lines were abandoned.

2.2.5 ^{131}I -cG250, Phase I/II RIT, Two High Doses

In view of the absence of any clinical response in single dose cG250 RIT, patients with progressive metastatic RCC were treated with two high doses (at MTD) of ^{131}I -cG250, separated by 3 months to allow bone marrow recovery (36). Twenty-nine patients received a diagnostic dose of ^{131}I -cG250 to assess uptake in metastatic lesions and for dosimetric analysis before receiving a therapeutic dose of ^{131}I -cG250. Patients demonstrating adequate cG250 uptake at the diagnostic scan received a therapeutic dose of ^{131}I -cG250 at MTD (60 mCi/m^2) 1 week later. In the absence of grade 4 hematological toxicity, patients received a second cycle, consisting of a diagnostic infusion and a second high dose ^{131}I -cG250, escalated

Fig. 2 Whole-body scan of a patient with multiple renal cell carcinoma (RCC) metastases 2 weeks after infusion of 112 mCi ^{131}I -cG250. *Ant.*: anterior view; *Post.*: posterior view



from 30 mCi/m² to 45 mCi/m². An example of the excellent cG250 uptake is shown in Fig. 2. This image was obtained 2 weeks after 45 mCi/m² ^{131}I -cG250 administration and serves to show that ^{131}I -cG250 uptake and retention can be exceptional. In this multiple-dose ^{131}I -cG250 trial, MTD of the second RIT was 45 mCi/m², with bone marrow toxicity as dose-limiting toxicity. Of the 16 patients who completed the protocol at both MTDs, none demonstrated an objective response. Four previously progressive patients stabilized while on study (6 months), 11 stayed progressive and 1 patient was nonevaluable due to HACA formation. It is clear that in these end-stage patients with bulky disease efficacy was low because sufficiently high doses of ^{131}I could not be delivered to the tumor due to high toxicity. Since lesions <5 g were the only lesions that received radiation doses >50 Gy, it was suggested that future RIT with cG250 should aim at treatment of small-volume disease or should be used in an adjuvant setting (37).

As discussed earlier, radiolabeling with more potent radioisotopes such as ^{90}Y , ^{212}Bi , ^{211}At , ^{186}Re , and ^{177}Lu might be preferable, but the development has been hampered by more complex chemistry. Nevertheless, the impact of such radionuclides could be significant, also because some of these chelated radioisotopes are believed

to be retained in the lysosomes, enhancing efficacy. Although the internalization rate of cG250 was initially considered too low to be of importance, further analyses revealed that significantly higher cG250 uptake could be achieved when labeled with residualizing complexes. To investigate this phenomenon in detail in patients with RCC, a dual-label study was performed with cG250 labeled with the residualizing radionuclide (^{111}In) and nonresidualizing radionuclide ^{131}I . Both radiolabeled cG250 preparations were administered to the patients with metastatic RCC (5) enabling the direct comparison of quantitatively calculated activities in RCC metastases of a radioiodinated form of mAb cG250 with the calculated activities of cG250 labeled with ^{111}In within the same patient (38). A marked difference in tumor accumulation between the residualizing and nonresidualizing radiolabel was observed. More metastatic lesions were visualized in four of the five patients studied (47 lesions with ^{111}In vs 30 lesions with ^{131}I), which is in line with observations with other mAbs, but also quantitative analysis of the activity in 25 metastases demonstrated higher accumulation levels of ^{111}In -DTPA-cG250 compared with ^{131}I -cG250 in 20 out of 25 lesions.

Animal studies performed with cG250 labeled with ^{177}Lu , ^{90}Y , or ^{186}Re showed that ^{177}Lu -cG250, followed by ^{90}Y -cG250, ^{186}Re -cG250 and the least by ^{131}I -cG250, most effectively inhibited tumor growth. The preclinical RIT studies clearly showed the superiority of ^{177}Lu - and ^{90}Y -based RIT, in line with other studies (39). Supported by the dual-label study in patients subsequent studies have focused on the possibility to use ^{90}Y or ^{177}Lu .

2.2.6 ^{177}Lu -cG250, Phase I/II RIT

Currently, a phase I/II trial with ^{177}Lu -cG250 is performed at the University Medical Center Nijmegen, the Netherlands, paralleled by a trial with ^{90}Y -cG250 at Memorial Sloan Kettering Cancer Center, New York. Patients with progressive mRCC with proven ccRCC receive a diagnostic dose of ^{111}In -cG250 (5 mCi), to establish adequate tumor accumulation followed by a dose of ^{177}Lu -cG250 or ^{90}Y -cG250 1 week after ^{111}In -cG250 (Start dose 30 mCi/m²; increments of 10 mCi/m²; ≥ 3 patients/dose level). In the ^{177}Lu -cG250 trial, patients are eligible to receive a second and a third cycle, consisting of a diagnostic infusion and a second or third high-dose ^{177}Lu -cG250, at 75% of the dose level of the previous injection in the absence of grade 4 hematological toxicity. The predictive value of the ^{111}In -cG250 scout dose for ^{177}Lu -cG250 accumulation was demonstrated, that is, ^{111}In -cG250 and ^{177}Lu -cG250 images were superimposable (Fig. 3). ^{111}In -cG250/ ^{177}Lu -cG250 images merged to CT images showed perfect alignment, indicating specific tumor accumulation (Fig. 4). In one patient, grade IV toxicity has been observed at the 50 mCi/m² dose level. No significant toxicity has been observed in the other patients, also not after the second or third treatment cycle. The majority of patients responded by stabilization of disease (Fig. 5). In one patient (50 mCi/m² dose level), a PR has been documented. Dosimetric analyses indicated effective uptake after consecutive treatments.

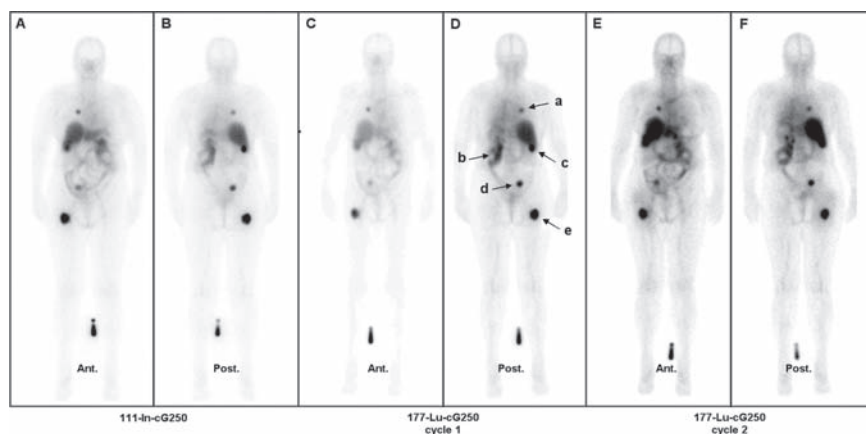


Fig. 3 Images of a patient injected with 5 mCi/m² ¹¹¹In-cG250 (a, b), 30 mCi/m² ¹⁷⁷Lu-cG250, first cycle (c, d), and second cycle (e, f) 7 days postinjection. Arrows point to computed tomography (CT)-confirmed tumor masses (a: pulmonary lesion, b: local recurrence of the kidney, c: kidney metastasis, d: sacrum metastasis, e: femur head metastasis). Please note complete concordance between ¹¹¹In-cG250 and between both ¹⁷⁷Lu-cG250 images, showing the predictive value of ¹¹¹In-cG250. Also, good tumor accumulation at the second treatment cycle is shown. Anterior images (a, c, e), posterior images (b, d, f)

The exquisite quality of the ¹³¹I-mG250 images, and the detection of previously nonrecognized lesions as observed in the first clinical study, appeared to warrant the development of mAb G250 as a diagnostic imaging agent. Indeed, the image quality of images acquired by radiolabeled mAb G250 has been outstanding in all clinical studies, regardless of the radiolabel used. In many primary patients with RCC included in phase I mAb G250 trials occult metastatic sites have been detected including lesions in the brain, soft tissue, and bone. Nevertheless, until recently the diagnostic possibilities of cG250 have not been pursued. In a recent study, the value of ¹²⁴I-cG250 (5 mCi, 10 mg) was evaluated in 26 patients with suspect renal masses. The use of ¹²⁴I allowed positron emission tomography (PET) imaging, enhancing the image quality. Indeed, positive images (defined as a tumor:kidney ratio >3:1) were obtained for 15/16 clear cell carcinomas (one patient received nonreactive antibody). All nine non-clear cell renal masses were negative, that is, the sensitivity of ¹²⁴I-cG250 PET for ccRCC was 94%, the negative predictive value 90%, specificity and positive predictive value were 100% (Divgi CR, *Lancet Oncology*, in press). Larger studies are necessary to confirm whether ¹²⁴I-cG250 imaging can play a role in the diagnosis of RCC. Secondly, other PET-imageable radionuclides such as ⁸⁹Zr, which are retained after internalization, are preferable above ¹²⁴I to preclude false negative images due to a fast internalizing tumor.

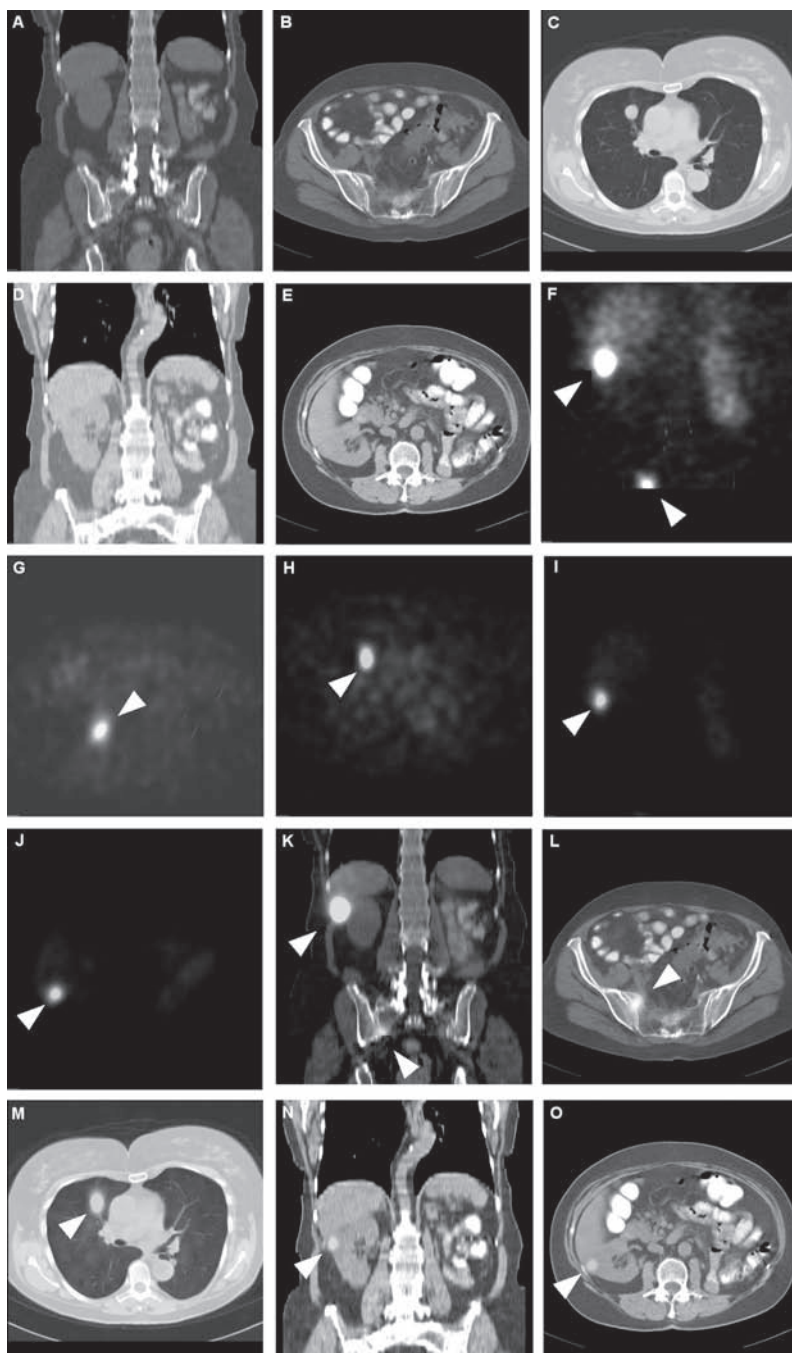


Fig. 4 Merged single photon emission computed tomography (*SPECT/CT*) images of patient shown in [Fig. 3](#) visualizing complete overlap between ^{177}Lu -cG250 uptake and CT. CT images shown in **a–e**, ^{177}Lu -cG250 images shown in **f–i** and merged images shown in **k–o**. Arrows point to kidney metastasis, coronal view (**f**, **k top**, **i**, **n**) and sagittal view (**e**, **o**), bone lesion, coronal view (**f**, **k bottom**), and sagittal view (**g**, **l**) and pulmonary lesion (**h**, **m**)

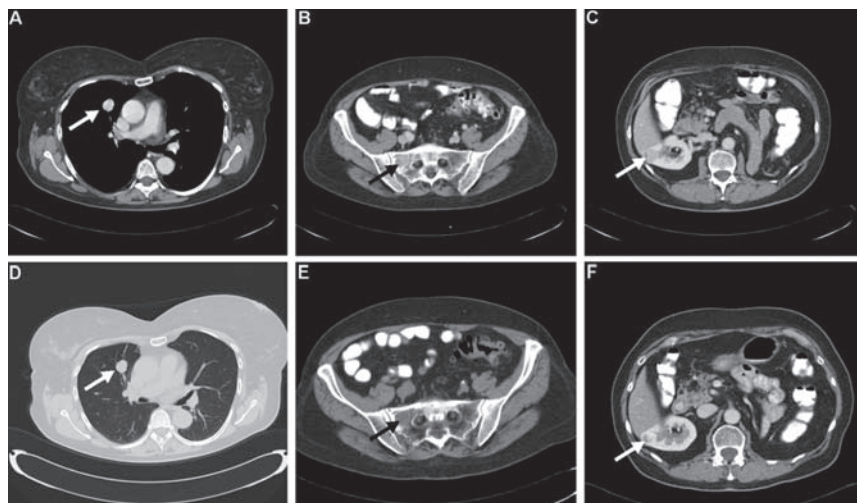


Fig. 5 Stabilization of formally progressive metastatic renal cell carcinoma (*mRCC*): computed topography (CT) scans of patient shown in Fig. 3. CT of baseline (*top*) and 12 weeks post second cycle (*bottom*) showing pulmonary lesion (*left*), sacrum (*middle*), and kidney metastasis (*right*)

2.3 Clinical Studies with Unmodified cG250

Apart from RIT studies, several trials with unmodified cG250 have been performed, despite the fact that cG250 demonstrated little antibody-dependent cellular cytotoxicity (ADCC) *in vitro* (40).

2.3.1 cG250, Phase II Immunotherapy (WX-G250)

In the first phase II study, 36 patients with advanced progressive RCC received weekly infusions (12 infusions, 50 mg cG250 per dose). Ten previously progressive patients achieved stabilization of disease for at least 6 months, suggesting a clinical benefit (41). Beyond the official observation period, one patient achieved a complete response more than 6 months after the start of cG250 treatment, and another patient experienced stabilization of disease. Both patients were free of disease progression for more than 1 year. The toxicity profile was favorable with only three patients with grade II toxicity possibly related to the study medication. Based on the good tolerability of the treatment with cG250 and the clinical benefit, further studies seemed warranted.

To improve activity of cG250-specific ADCC and the clinical response rate, combination with cytokines was explored (40, 42). Since interleukin-2 (IL-2) has been known to enhance ADCC of mAbs, the combination of this cytokine with cG250 was evaluated (40). Interferon gamma (IFN- γ) also enhanced the ADCC of cG250 throughout the study period but was not as effective as the IL-2 treatment.

IFN- α 2a and 2b increased cG250-mediated ADCC and K562 cytotoxicity for only 3 days. The potent and sustained immune effector activity observed with cG250 and cytokines in these in vitro studies suggested that the combination immunotherapy of cG250 with cytokines such as IL-2 might be advantageous in the treatment of RCC. Indeed, subsequent trials with naked cG250 have focused on the combination of cG250 with biological response modifiers.

2.3.2 cG250 and IL-2, Phase II Immunotherapy

In one trial, the effects of cG250 (20 mg cG250/dose) and low-dose IL-2 (1.8 MIU/day) plus periodic IL-2 pulsing (5.4 MIU/day) in patients with advanced progressive RCC was studied (43). A total of 35 patients with progressive ccRCC received weekly infusions of WX-G250 for 11 weeks combined with this IL-2 regimen. Patients were monitored for ADCC capacity. A durable clinical benefit was achieved in 8 of 35 patients (23%), including 3 with a PR and 5 with stabilization at >24 weeks. Mean survival was 22 months. In general, treatment was well tolerated with little toxicity. The number of effector cells (CD3⁺/CD16⁺/CD56⁺) increased during treatment but lytic capacity per cell did not increase and ADCC and clinical outcome did not correlate. With a substantial clinical benefit and a median survival of 22 months in patients with metastatic RCC who have progressive disease at study entry, combination therapy showed increased overall survival compared to cG250 monotherapy. Although it is possible that the favorable outcome was the direct consequence of the IL-2 regimen, this appears unlikely: clinical efficacy for low-dose IL-2 treatment has been described at IL-2 doses that were at least sixfold higher than the dose of IL-2 used in the current trial (44, 45). It seems more likely that the low-dose IL-2 regimen and cG250 acted in synergy. Survival was at least similar to that of currently used cytokine regimens but with a favorable toxicity profile.

2.3.3 cG250 and IFN, Phase II Immunotherapy

Additionally, the effects of cG250 combined with IFN- α was studied, based on in vitro observations that addition of IFNs upregulated G250/CAIX expression (42, 46). In a multicenter, open-label, prospective, single-arm phase I/II trial study, cG250 in combination with IFN- α 2a was studied in a total of 32 patients with stage IV RCC, nephrectomy for the primary tumor, in progression at study entry, and with measurable bidimensionally disease between ≥ 1 cm and ≤ 5 cm in diameter. Patients received 20 mg cG250 weekly for 3 months, combined with IFN- α 2a, 3 MIU, three times per week subcutaneously. In a preliminary analysis, 3 PR were described, 12 patients stabilized, and 11 patients progressed.

In summary, mAb G250 has shown remarkable targeting ability and cG250 PET imaging may play a role in the diagnosis of RCC. However, the effects of various approaches aimed at therapeutic intervention have not been very satisfactory. Some patients showed tumor shrinkage after RIT and the combination of

cG250 with cytokines showed some clinical effect. These results must be viewed with caution in view of the variability of the natural course of this disease. Placebo-controlled, multiple-arm trials would be necessary to provide definitive evidence that cG250 treatment can modulate the disease. Alternatively, cG250 might be useful in an adjuvant setting a hypothesis that is examined in a phase III trial with cG250 as an adjuvant therapy of patients with nonmetastatic RCC. The excellent targeting ability of cG250 might also be exploited to target potent molecules, for example, cytokines, drugs, to tumor cells, and to reduce systemic toxicity (47). Currently, a fusion protein consisting of cG250 merged with TNF is under investigation.

Finally, the introduction of several small-molecule drugs (e.g., Sunitinib, Sorafenib) is rapidly changing the treatment of metastatic RCC (48, 49). Significant responses (PR) have been described, and Sunitinib is now registered as first-line therapy for patients with ccRCC. Nevertheless, a substantial proportion of patients do not respond adequately, and possibly combination treatment aimed at different, nonrelated pathways may be advantageous. The future will show whether combination of mAb G250 and, for example, small-molecule drugs can be useful in the treatment and clinical management of mRCC.

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Chemokines in Renal Cell Carcinoma: Implications for Tumor Angiogenesis and Metastasis

Karen L. Reckamp, Robert A. Figlin, and Robert M. Strieter

Abstract Exploration of new agents and combinations involved in targeting novel pathways associated with tumor angiogenesis, invasion, and survival may lead to improvement in patient outcomes for renal cell carcinoma (RCC). Chemokines can potentiate tumor growth, metastasis, angiogenesis, and immune evasion through interactions with stromal cells and neoplastic cells. Chemokine ligand and receptor interactions stimulate signaling cascades that results in the transcription of target genes responsible for tumor cell invasion, motility, and survival. Further understanding of the mechanisms involved in chemokine-mediated angiogenesis and metastasis, and their interactions with other pathways important in RCC, may lead to more optimal therapeutic strategies in this disease. In addition, delineation of how chemokine ligands and receptors modulate the malignant phenotype will provide novel insights into RCC pathogenesis.

Keywords Renal cell carcinoma • Chemokines • Angiogenesis • Metastatic potential • Treatment

1 Introduction

The treatment of local renal cell carcinoma (RCC) is surgical resection, although metastatic RCC presents a therapeutic dilemma in that it does not respond to conventional chemotherapy or radiation therapy (1). Previous treatments for metastatic disease have focused on immunomodulating therapies. Prospective randomized trials of immunotherapy with high-dose interleukin-2 (IL-2) have demonstrated objective responses in 20% of patients with metastatic RCC, and evidence of ~5–10% durable remissions (2–6). Recently, with increased understanding of the molecular

K.L. Reckamp (✉)

Divisions of Medical Oncology and Experimental Therapeutics & Hematology
and Hematopoietic Transplantation, City of Hope and Beckman Research Institute,
1500 E. Duarte Road, MOB 1001, Duarte, CA 91010
e-mail: kreckamp@coh.org

pathogenesis of RCC and the importance of angiogenesis in the disease, targeted therapies have come to the forefront of treatment for metastatic RCC (7–12). The fact that the use of antiangiogenic therapies, molecular targets, and immunological targeting may be beneficial in the treatment of RCC supports the notion of the angiogenic and immunogenic nature of RCC. The immunogenic nature of RCC, the magnitude of tumor-associated angiogenesis, and the metastatic potential of RCC have led to the investigation of the factors that contribute to the pathobiology of this cancer. Chemokines are important for enhancing innate and adaptive immunity, regulating angiogenesis, and mediating tumor cell metastases and have been shown to have a direct impact on the biology of RCC. Therapeutically, targeting these features may lead to novel interventions and improved response to therapy in patients with RCC.

2 Chemokines and Chemokine Receptors

Chemokines are a family of 8–11 kDa proteins, subdivided on the basis of the position of the N-terminus cysteine residue. They are involved in leukocyte chemotaxis and activation, and have been associated with the regulation of angiogenesis and tumor cell invasion (13–15). Approximately 50 chemokines have been identified and are separated into four subgroups, α -chemokines (CXC), β -chemokines (CC), lymphotactin (C), and fractalkine (CXXXXC) (15, 16). Chemokine receptors are seven transmembrane, G protein-coupled receptors (GPCRs), and are expressed on leukocytes, stromal cells, epithelial cells, and tumor cells. Approximately 28 different chemokine receptors have been described, few are specific for one chemokine ligand, while others bind multiple chemokine ligands (16, 17). The CXC chemokines and their corresponding receptors are listed in [Table 1](#).

Chemokines have been shown to be produced by tumor cells, leukocytes, epithelial cells, stromal cells, and endothelial cells (18, 19). In addition, functional chemokine receptors have been found on endothelial cells, leukocytes, and transformed epithelial cells (20–22). Through interactions with stromal cells and neoplastic cells, chemokines can potentiate tumor growth, metastasis, angiogenesis, and immune evasion (23). Chemokine ligand and receptor interactions then stimulate signaling cascades that result in the transcription of target genes responsible for tumor cell invasion, motility, and survival (24). An essential element of chemokine signaling depends on the chemotactic gradient achieved. Chemokine gradients within the tumor microenvironment and target organs can determine the metastatic potential of a tumor (18). Immunotherapy and antibody treatments have been implicated in the upregulation of chemokines in RCC and other human cancers (25, 26), which may be important in the reduction of tumor burden.

3 Chemokines in Tumor Angiogenesis

RCC growth and metastases have been associated with primary tumor-associated angiogenesis resulting from the mutation or hypermethylation of the von Hippel-Lindau (VHL) gene with subsequent hypoxia-inducible factor (HIF) activation

Table 1 CXC chemokines (17, 18)

Systemic name	Human ligand	Receptor
CXCL1	GRO ^a α	CXCR2
CXCL2	GRO β	CXCR2
CXCL3	GRO γ	CXCR2
CXCL4 [*]	PF ^b 4	CXCR3
CXCL5	ENA ^c -78	CXCR2
CXCL6	GCP ^d -2	CXCR1, CXCR2
CXCL7	NAP ^e -2	CXCR2
CXCL8	IL-8	CXCR1, CXCR2
CXCL9 [*]	MIG ^f	CXCR3
CXCL10 [*]	IP ^g -10	CXCR3
CXCL11 [*]	I-TAC ^h	CXCR3
CXCL12	SDF-1 α/β	CXCR4
CXCL13	BCA ⁱ -1	CXCR5
CXCL14 [*]	BRAK	
CXCL15		
CXCL16	CXCL16	CXCR6

^aGRO, growth-regulated oncogene^bPF, platelet factor^cENA, epithelial neutrophil-activating peptide^dGCP, granulocyte chemotactic protein^eNAP, neutrophil-activating peptide^fMIG, monokine induced by IFN- γ ^gIP, interferon gamma-inducible protein^hI-TAC, interferon-inducible T cell alpha chemoattractantⁱBCA, B-cell-specific chemokine^{*}ELR negative chemokines

accompanied by the downstream effects of vascular endothelial growth factor (VEGF) induction (27, 28). Angiogenesis in the tumor environment is important for cancer growth, invasion, and metastasis. The balance between proangiogenic and antiangiogenic factors determines the overall angiogenesis in the tumor micro-environment. VEGF and its receptors (VEGFR-1–3) have been studied extensively in RCC as important components of net angiogenesis. Recent advances in antiangiogenic therapy have demonstrated benefit in a variety of tumor types including RCC (7–9, 12, 29, 30). Although VEGF has been the most extensively studied, other factors that regulate angiogenesis in both a positive and a negative manner are clearly involved in the process of angiogenesis in RCC. Targeting tumor immunity and angiogenesis through the inhibition of the chemokine receptor, CXCR2 and its ligands, or the upregulation of CXCR3, and its ligands may augment responses to therapy in RCC.

The CXC chemokines are important for enhancing immunity, regulating angiogenesis, and mediating tumor cell metastases. They are a cytokine family in which most members display either angiogenic or angiostatic activity. CXC chemokines have four highly conserved cysteine amino acid residues, with the first two cysteines separated by a nonconserved amino acid residue (15, 31–33). A second structural domain within the CXC chemokine family also dictates their functional

activity. The NH₂-terminus of several CXC chemokines contains three amino acid residues (Glu-Leu-Arg; "ELR" motif), which precedes the first cysteine amino acid residue of the primary structure of these cytokines (15, 31–33). Members that contain the "ELR" motif (ELR+) are equipotent angiogenic factors with basic fibroblast growth factor (bFGF) and VEGF (31). ELR + CXC chemokines promote angiogenesis through CXCR2, and this angiogenic activity has been shown to be inhibited with CXCR2 neutralizing antibodies (34). In contrast, members that lack the ELR motif (ELR-) and are interferon (IFN)-inducible, inhibit angiogenesis (31). The angiostatic members of the CXC chemokine family include, CXCL4, CXCL9, CXCL10, and CXCL11 (31, 35, 36). These IFN-inducible CXC chemokines act through their putative receptor, CXCR3, and promote cell-mediated immunity and inhibit angiogenesis. CXCR2 and CXCR3 and their ligands have been shown to be expressed in RCC tumors (37–39). Therefore, on both structural and functional levels the CXC chemokine family plays an integral role in the promotion or inhibition of angiogenesis relevant to RCC.

IFN-inducible CXC chemokines play a critical role in orchestrating Th1 cytokine-induced cell-mediated immunity via the recruitment of mononuclear cells expressing CXCR3 (15, 40–43). These same chemokines are potent inhibitors of angiogenesis. Thus, CXCR3 ligands represent the major chemoattractants for the recruitment of Th1 cells during cell-mediated immunity. CXCR3/CXCR3 ligands have been linked to Th1 cytokine-induced cell-mediated immunity and inhibition of angiogenesis, leading to suppression of tumor growth (44–46). Intratumoral injection of the CC chemokine, CCL21, that binds to CXCR3 and CCR7 in murine tumors induced potent antitumor responses, and evidence of a reduction in angiogenesis (44–46). To assess the importance of Th1 cytokines and CXCR3 ligands in mediating the effects of CCL21, depletion studies were performed with neutralizing anti-IFN- γ , anti-CXCL9, and anti-CXCL10 antibodies in the presence of CCL21 treatment (44). Depletion of CXCL9, CXCL10, and IFN- γ attenuated the antitumor effects of CCL21 (44), supporting the importance of CXCR3 ligand-dependent role in mediating the antitumor responses of CCL21. These results are similar to the previously reported study of IL-12-mediated regression of murine renal cancer (RENCA), where the antitumor effect of IL-12 was markedly attenuated when either CXCL9 or CXCL10 was depleted by specific neutralizing antibodies (47). These findings provide evidence that IFN-inducible CXC chemokines are important in the development of antitumor immunity and inhibition of angiogenesis relevant to RCC.

The integral role of CXC chemokines and receptors in the regulation of angiogenesis in metastatic RCC has been demonstrated in preclinical models. Mestas et al. recently described the importance of the ELR + CXC chemokines and their receptor, CXCR2, in RCC tumor growth and angiogenesis (37). Proangiogenic CXCR2 ligands were elevated in the plasma of patients with metastatic RCC, and CXCR2 was found to be expressed on endothelial cells of RCC tumors (37). Reduced tumor growth was seen in a CXCR2 knockout murine model, which correlated with decreased angiogenesis and increased tumor necrosis. They also demonstrated that the combined effect of systemic induction of CXCR3 expression

on circulating mononuclear cells by systemic IL-2 therapy in conjunction with concomitant intratumor spatial expression of CXCR3 ligands was essential to create an optimal CXCR3/CXCR3 ligand-dependent chemotactic gradient in renal cell tumors (37). The combined therapeutic intervention of systemic IL-2 and concomitant intratumor CXCR3 ligand (i.e., CXCL9) administration resulted in marked attenuation of tumor growth and tumor-associated angiogenesis (37).

Another study demonstrated that systemic priming with IL-2 could lead to a kinetic expression of chemokine receptor, CXCR3, from peripheral blood mononuclear cell (PBMC) of patients with metastatic RCC receiving standard therapy with high-dose IL-2 (48). IL-2 is the major agonist for the expression of CXCR3 on Th1 cells, which promote antitumor immune responses. Patients with metastatic clear cell RCC and normal controls had similar PBMC CXCR3 levels and peripheral blood angiostatic chemokines at baseline, but patients with metastatic RCC had elevated levels of CXCR2 angiogenic ligands (37, 48). CXCR3 ligands increased in response to high-dose IL-2 therapy and CXCR3 expression on circulating mononuclear cells of patients with metastatic RCC also increased. CXCR3 expression on CD4, CD8, and NK cells rose in response to high-dose IL-2 treatment. The induction of CXCR3 expression of PBMC was most pronounced in a patient with a complete response to therapy (48). Chemokines have been found to display pleiotropic activity, such as recruitment of specific subsets of leukocytes and regulation of angiogenesis (49). On the basis of the ability of IFN-inducible CXC chemokines (CXCL9, CXCL10, CXCL11) to promote Th1 immunity and inhibit angiogenesis, they have a potential biological role in promoting tumor regression in RCC.

Strieter et al. (50, 51) have coined the term, "immunoangiostasis," for their potential biological role in promoting tumor regression. A hypothesis is that immunoangiostasis can be optimized by first priming the systemic pool of PBMC to upregulate the expression of CXCR3 by using less than maximally tolerated systemic administration of IL-2. Second, increase the chemotactic gradient to target these cells to the tumor using a stimulus of local tumor overexpression of CXCR3 ligands (IFN-inducible CXC chemokines). The effect of this temporal and spatial therapy would enhance selective and specific extravasation of Th1 cells into the tumor, enhance Th1-mediated immunity in situ to tumor-associated antigens, further increase the expression of local IFNs, further augment the local expression of CXCR3 ligands, amplify further in situ Th1-mediated immunity, and at the same time promote CXCR3 ligand-related angiostasis – the concept of immunoangiostasis. While investigators have focused on CXCR3 and its ligands in tumor immunity, the missing link to ultimately improve this response was the mechanism to increase CXCR3 on PBMC through the concept of systemic priming with IL-2. In other words, overexpression of a CXCR3 ligand alone at the local tumor level will not be sufficient to recruit PBMC to the tumor unless they are primed and activated with upregulated expression of CXCR3. A recent study by Pan et al. describes this phenomenon in a murine RENCA model (51). Combined systemic IL-2 with intratumor CXCL9 (a CXCR3 ligand) led to a significant reduction in tumor growth and angiogenesis, compared with either therapy alone (51). Therefore, both components of the CXCR3/CXCR3 ligand biological axis must be optimized for

recruitment of mononuclear cells. Targeting the CXCR3/CXCR3 ligand biologic axis may be important in developing novel treatments of RCC. Furthermore, several studies have demonstrated a strong correlation between CXCR3 expression and survival (52) and metastatic potential (53), suggesting an additional role in tumor growth and invasion.

4 Chemokines in Tumor Metastasis

The mortality and morbidity of RCC is strongly associated with its high propensity to metastasize to specific organs. To explain the specific pattern of tumor metastases, it has been demonstrated that sites of metastases are determined not only by the characteristics of neoplastic cells but also by the microenvironment of the specific organ (54). In a similar manner to leukocyte trafficking, the target organs for metastatic events express constitutive levels of chemoattractants that mediate extravasation of tumor cells. Recently, extensive studies have suggested that chemokines may play a major role in mediating tumor metastasis (21, 55–58).

Different cancers are found to express several chemokine receptors, and their corresponding ligands are expressed at sites of tumor metastases (21, 57, 59, 60). However, CXCR4 appears to be the major chemokine receptor expressed on cancer cells (55, 56, 58). CXCR4 was originally discovered as the coreceptor for lymphotropic strains of HIV (61) and CXCL12 (stromal-derived factor-1, SDF-1) is its lone ligand (62). CXCL12 has been found to be secreted by bone marrow stromal cells and is important during embryogenesis for the colonization of bone marrow by hematopoietic stem cell (HSC) (63). It is also essential in adult life for retention/homing of HSCs (64). Both CXCL12^{-/-} and CXCR4^{-/-} mice die in utero with defects in heart, brain, and large vessel development (15, 65–68). The role of CXCL12/CXCR4 axis in organ-specific metastasis was initially suggested in breast cancer (21). Since then, CXCR4 expression has been reported in at least 23 epithelial, mesenchymal, and hematopoietic cancers, suggesting the importance of this ligand/receptor axis, in general in tumor metastasis (55, 56, 58, 69). In addition, studies have also suggested that CXCL12/CXCR4 may indirectly promote tumor metastases by mediating proliferation of tumor cells and enhancing tumor-associated angiogenesis (70–77). Therefore, further understanding the molecular mechanisms that are involved in the regulation of CXCR4 expression on tumor cells could lead to potential targets to modify the expression of CXCR4 and impact on metastases.

While CXCL12 is not an ELR+ CXC chemokine, CXCL12 via CXCR4 has been implicated in mediating angiogenesis (70, 74, 75, 78). This in turn has led to speculation that the predominant function of this ligand/receptor pair in tumorigenesis results from this perceived angiogenic capability. However, in order for the biological axis of CXCL12/CXCR4 to mediate RCC-associated angiogenesis, both the ligand and the receptor should be significantly present within the tumor. Schrader and colleagues (79) have demonstrated in both RCC cell lines and actual patient specimens of RCC that CXCR4 is predominantly expressed by the tumor cells, and its ligand CXCL12 was neither produced by the RCC cell lines nor was

it expressed at significant levels in patient specimens of RCC. These findings have also been supported by studies performed in human breast cancer and non-small cell lung cancer (NSCLC) cell lines and tumor specimens, in which CXCR4 was found to be predominately expressed on the tumor cells, and did not mediate tumor-associated angiogenesis *in vivo* in murine severe combined immunodeficiency (SCID) model systems of heterotopic or orthotopic human tumor growth (57, 80). In contrast, the studies demonstrated that when animals bearing breast or NSCLC tumors were treated with either neutralizing anti-CXCL12 or anti-CXCR4 antibodies, there was no change in the size of the primary tumor nor was there any evidence for a decline in primary tumor-associated angiogenesis (57); however, there was a marked attenuation of tumor metastases in an organ-specific manner (57, 80). These studies support the notion that CXCL12/CXCR4 biological axis mediates metastases of the tumor cells in an angiogenesis-independent manner.

CXCR4 expression is required for tumor metastasis to other organs, and CXCR4 activation by CXCL12 induces migration of neoplastic cells (81). CXCR4 expression has been correlated with the metastatic potential of multiple tumors, including RCC (21, 57, 69, 82–85). Müller and colleagues provided initial evidence linking CXCL12/CXCR4 biological axis to breast cancer metastasis to specific organs (21), which was confirmed in non-small lung cancer (57). More recent studies have suggested that CXCR4 is expressed on various other cancer cells and its expression stimulated migration of cancer cells toward a CXCL12 gradient established in specific target organs for metastases (55, 56, 58). Furthermore, elevated CXCR4 expression was detected in several human RCC cell lines and tumor samples, while only minimal CXCR4 expression was detected in normal kidney tissues (79). These findings suggested that the CXCL12/CXCR4 biological axis may be a critical determinant for the metastatic potential of RCC.

A recent study by Pan et al. demonstrated that CXCR4 expression was markedly increased on circulating pan-cytokeratin+ cells of patients with metastatic RCC, as compared to normal control subjects, suggesting that these cells were comparable with circulating malignant cells (82). The extremely low levels of pan-cytokeratin+ cells in normal subjects may have been due to expression of pan-cytokeratin marker by bone marrow-derived stem cell subpopulations which has previously been described (86–88). When the cells from patients with metastatic RCC were examined for expression of CXCR4, more than 90% of them were found to be CXCR4 positive (82). These findings suggest that CXCR4 is a predominant biomarker on pan-cytokeratin+ cells in the circulation of patients with metastatic RCC and the presence of CXCR4 expression on these cells may directly correlate with metastatic RCC.

5 Regulation of Chemokines

The most common mutated gene in RCC is the VHL tumor suppressor gene that results in overexpression of HIF-1 α and HIF-2 α . Although studies have primarily focused on this defect related to the overexpression of VEGF, it is now apparent that other genes may play a role in mediating the metastatic potential of RCC.

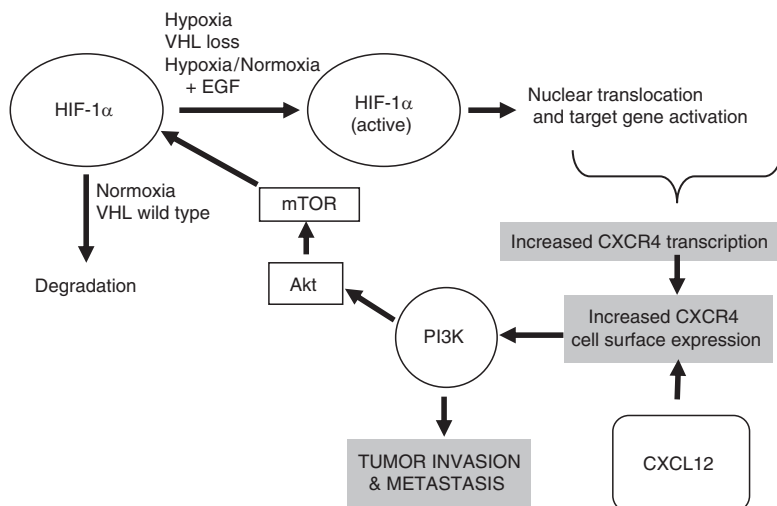


Fig. 1 von Hippel-Lindau (*VHL*), hypoxia, and renal cell carcinoma (*RCC*) tumorigenesis. In *RCC*, the hypoxia-induced pathway is linked to *VHL* loss. Accumulation of hypoxia-inducible factor (*HIF*) results in upregulation of genes and proteins involved in *RCC* tumorigenesis, including *CXCR4*. Endothelial growth factor (*EGF*) is also capable of upregulating *CXCR4* expression through the phosphatidylinositol 3 (*PI3*)-kinase, Akt, mammalian target of rapamycin (*mTOR*), and *HIF-1α* pathway

Recent findings have linked *HIF-1α* and the expression of *CXCR4* on *RCC* (Fig. 1). Hypoxia and more specifically *HIF-1α* has been found to be a critical transcription factor for gene expression of *CXCR4* (89–91). Moreover, *VHL* can negatively regulate the expression of *CXCR4*, owing to its capacity to target *HIF-1α* for degradation under normoxic conditions (89, 90). This process may be suppressed under hypoxic conditions in cells allowing *HIF-1α*-dependent induction of *CXCR4* expression (89, 90). These findings suggest that *HIF-1α/VHL* may play a significant role in regulating the expression of *CXCR4* on tumor cells, and further understanding this molecular mechanistic link between *HIF-1α* and *CXCR4* expression may allow the discovery of a novel means to intervene and impact on reducing metastatic *RCC*.

Ceradini et al. demonstrated that *SDF-1/CXCL12* was regulated by *HIF-1* in endothelial cells, increasing migration of circulating *CXCR4*-positive cells to areas of ischemic tissue. Blocking *CXCL12* or *CXCR4* inhibited the recruitment of these cells to sites of regenerating tissue (92). Hypoxia, and particularly *HIF-1α*, has also been shown to regulate the expression of *CXCR4* in *RCC* (90, 93, 94). Recent studies suggest that the loss or functional inactivation of the protein product of *VHL* resulted in persistent activation of *HIF-1α* and a dramatic increase in *CXCR4* expression due to loss of its ability to target *HIF-1* for degradation by 26S proteasome (90, 93, 94). In addition, a recent study demonstrated that either knocking down *VHL* expression in human *RCC* cells or exposing these cells to hypoxic

conditions lead to markedly increased expression of CXCR4 mRNA and protein (82). Metastases of human RCC cells was increased in an organ-specific manner to adrenal glands, buffy coat, bone marrow, brain, kidney, spleen, liver, and lung of mice bearing human RCC VHL knockdown tumors, as compared to those bearing human RCC wild-type tumors (82). Most of these organs have been found to express elevated levels of CXCL12 in SCID mice in previous studies (21, 57). Furthermore, this study showed that the upregulation of CXCR4 expression in RCC induced by these conditions was mediated by the direct physical interaction between the HIF-1 transcriptional factor and its specific binding sites on the CXCR4 promoter region (82), which is probably the hypoxia response element (HRE) as suggested by previous studies (93, 95, 96). Finally, neutralization of CXCL12 inhibited metastasis of RCC to target organs expressing high levels of CXCL12. These studies establish a link between the VHL/HIF-1 α axes to CXCL12/CXCR4-mediated organ-specific metastases of human RCC.

Regulation of CXCR4 expression and the metastatic potential of tumors involve the interaction of multiple signaling pathways. The upregulation of CXCR4 expression has been described to be mediated through the mitogen-activated protein kinase (MAPK) (MEK)/extracellular signal regulated kinase-1/2 (ERK) signaling pathway and nuclear factor (NF)- κ B activation in prostate cancer cells (97). Findings in lung cancer demonstrate that the combination of hypoxia and activation of the epidermal growth factor receptor (EGFR) tyrosine kinase domain lead to additive to synergistic upregulation of CXCR4 expression via HIF-1 α (96). Moreover, EGFR activation in the presence of hypoxia further augments CXCR4 expression. The EGFR is a receptor tyrosine kinase that activates phosphatidylinositol 3 (PI3)-kinase and subsequent downstream targets including Akt and *mammalian target of rapamycin* (mTOR). In the presence of either PI3-kinase inhibitors or mTOR inhibitors, activation of HIF-1 α and increases in CXCR4 expression were blocked. HIF-1 directly transactivated CXCR4 gene expression. Inhibitors of PI3-kinase also prevented chemotaxis of tumor cells in response to CXCL12. These studies suggest that the PI3-kinase pathway abrogates increased expression of CXCR4 induced by hypoxia and the EGFR, and CXCL12-mediated chemotaxis (96). EGFR activation/signaling and exposure to hypoxia, unlike the VHL mutation, are known to be shared by many cancers. Therefore, these findings suggest that tyrosine kinase receptor activation and/or a response to hypoxia leading to increased HIF-1 α is critical for the regulation of the expression of CXCR4, and may represent a general scheme in tumor metastasis.

6 Targeting Chemokines

Chemokines and their receptors are often upregulated in a number of human malignancies, and are involved in tumor growth, angiogenesis, and invasion. Inhibition of chemokine receptors through the use of small molecules or antibodies may be important in decreasing tumor angiogenesis and metastasis. Utilizing the mechanism

of chemokine receptor antagonism to treat tumors depends on the upregulation of the specific chemokine receptors, such as CXCR4 in the presence of hypoxia or VHL loss, and CXCR2 in response to angiogenic signals. Therefore, analysis of chemokine receptors in the tumor environment could potentiate the efficacy of modalities as therapy in RCC (16).

6.1 CXCR2 Antagonists

Attempts to block CXCR2 and inhibit tumor cell angiogenesis have been successful in a multiple tumor models (37, 98, 99). In a RCC CXCR2 knockout model, tumors developed increased necrosis and decreased tumor-associated angiogenesis when compared to CXCR2 wild-type RCC (37). The CXCR2 small-molecule antagonist, SB 225002, also demonstrated activity by inhibiting cell proliferation in an esophageal carcinoma cell line (99). Substance P analogues (SPA), which are broad-spectrum GPCR antagonists, have potential antitumorigenic activities. SPA have been evaluated for their ability to inhibit CXCR2-mediated Ca²⁺ mobilization (98). In a pancreatic cancer model, SPA inhibited tumor growth through a dual mechanism involving both antiproliferative and antiangiogenic mechanisms (98). Blocking the CXCR2/CXCR2 ligand biologic axis provides an innovative approach to regulating angiogenesis and tumor growth in RCC.

6.2 CXCR4 Antagonists

Strategies to attenuate the CXCL12/CXCR4 biological axis may potentially be employed as a new antimetastatic strategy to inhibit the metastatic potential of RCC. In fact, small-molecule antagonists to CXCR4 and RNA interference strategies have already been developed to inhibit the binding of lymphotropic strains of HIV to CXCR4 on T cells (100–102). Multiple CXCL12/CXCR4 antagonists are under evaluation for their safety and efficacy in various tumors (Table 2) (16).

Table 2 CXCR4 antagonists

Antagonist	Type	Reference
AMD3100	Bicyclam derivative	(22, 102–109)
T140	Peptide	(110, 111)
ALX40-4C	Peptide	(102)
T22	Peptide	(112)
TN14003	Peptide	(111, 113, 114)
TC14012	Peptide	(111)
4F-bTE	Peptide	(115, 116)

AMD3100 has been studied most extensively in human tumors. It is a bicyclam derivative, and is a selective CXCR4 antagonist, but may act as a partial agonist at high doses (22, 102–104). AMD3100 inhibited the growth and invasion properties of ovarian cancer cell lines (22). The compound was well tolerated in healthy volunteers resulting in only mild, transient toxicities (104). T140 is a 14-residue polypeptide that was downsized from a naturally occurring horseshoe crab self-defense peptide. T140 analogs are peptidic CXCR4 antagonists composed of 14 amino acid residues that were previously developed as anti-HIV agents having inhibitory activity against HIV-entry through its coreceptor, CXCR4 (110). T140 was shown to inhibit SDF-1-induced migration of human breast cancer cells, leukemia T cells, and human umbilical vein endothelial cells, and decreased the formation of pulmonary metastasis in mice. T140 also demonstrates weak, partial antagonism at high concentrations (102). Another CXCR4 antagonist, ALX40-4C, is an inverse agonist to CXCR4 that does not induce signaling through CXCR4, and may prevent partial agonist activity seen with other CXCR4 antagonists (102).

Other modalities that have been studied in cancer treatment used to block CXCR4 include antisense cDNA and siRNA duplexes. Antisense CXCR4 cDNA transfected into metastatic nasopharyngeal carcinoma cells inhibited the expression of CXCR4 and SDF-1-induced cell migration, and decrease the capacity of the tumor to metastasize to the lung and lymph nodes of athymic mice (69). In addition, siRNA duplexes of CXCR4 have been successful in blocking the migration of breast cancer metastasis (117). Furthermore, inhibition of chemokine signaling pathways (e.g., PI3K, mTOR, HIF, ERK) with small-molecule inhibitors has shown promise as a therapeutic option for regulating RCC growth and metastasis (10, 11, 16). Thus, blocking the CXCL12/CXCR4 biological axis may have a significant impact on the propensity for RCC to invade and metastasize and therefore, the future of treatment for the disease.

7 Conclusions

Chemokines and their receptors have been implicated in regulating RCC growth, angiogenesis, and metastases. There is increasing evidence for the involvement of multiple chemokine pathways in the diverse process of renal cell tumorigenesis and invasion. The inhibition of angiogenesis, either directly through VEGF and its receptors or indirectly through signaling pathways, has shown efficacy in advanced RCC, primarily of the clear cell histology. The importance of these agents and combining them with modulators of tumor immunity or invasion may lead to improvements in the treatment of RCC. Clinical trials studying the potential role of chemokine inhibition will help to determine mechanisms of chemokine-induced angiogenesis, growth, and metastasis in RCC. In addition, delineation of how genes and proteins regulate chemokine activity and modulate the malignant phenotype will provide novel insights in kidney cancer pathogenesis.

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PI3K/Akt/mTOR Pathway: A Growth and Proliferation Pathway

Daniel Cho, James W. Mier, and Micheal B. Atkins

Abstract The phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt)/mammalian target of rapamycin (mTOR) pathway regulates numerous cellular processes such as growth, proliferation, cell cycle progression, motility, adhesion, and angiogenesis, and appears to be constitutively active in a majority of renal cell carcinoma (RCC). The integrity of the pathway is clearly vital to the survival and growth of RCC as pharmacologic inhibition of PI3K or Akt induces apoptosis in RCC tumor cells and tumor regression in vivo. These observations suggest that the PI3K/Akt/mTOR pathway may be an attractive target for drug development in the treatment of RCC. The recently demonstrated clinical efficacy of inhibitors of mTOR supports this hypothesis and demonstrates the relevance of this pathway in RCC. As Akt activates numerous kinases, transcription factors and other proteins associated with cell growth and survival in addition to mTOR, it is possible even greater clinical responses may be achieved with agents that disrupt the PI3K/Akt/mTOR pathway upstream of mTOR. Concurrent with the development of inhibitors of PI3K or Akt for clinical application are efforts to identify predictive biomarkers of response to agents targeting elements of the PI3K/Akt/mTOR pathway so as to develop more individualized patient selection strategies. In this chapter, we will review the molecular biology of the PI3K/Akt/mTOR pathway, its relevance to RCC, and its potential as a therapeutic target in RCC.

Keywords PI3K inhibitors • Hyperactivation of Akt/PI3K • Akt activity • Temsirolimus • RCC

D. Cho (✉)
Beth Israel Deaconess Medical Center, Boston, MA
e-mail: dcho1@bidmc.harvard.edu

1 Molecular Biology of PI3K/Akt/mTOR Pathway

1.1 PI3K-Akt Activation

The PI3K pathway is one of the most frequently altered signaling pathways in cancer, with mutations of various elements of the pathway estimated to be present in up to 30% of all human cancers (1). PI3K acts mainly through its executor, Akt, to regulate numerous anabolic processes essential for cell cycle progression and survival. PI3K catalyzes the phosphorylation of inositol phospholipids at the 3' position, a modification that results in the recruitment of a discrete subset of serine/threonine kinases to the plasma membrane through an interaction between their pleckstrin homology (PH) domain and the negatively charged phospholipids. Among the kinases recruited to the membrane under such circumstances are the various Akt isoforms (Akt1, Akt2, and Akt3). As shown in Fig. 1, the activation of PI3K typically occurs as a result of growth factors binding to receptor tyrosine kinases, but can also occur via the small G-protein ras as well as through G-protein coupled receptors such as CXCR4. This activation of PI3K results in the generation of phosphatidylinositol 3,4,5-triphosphate (PIP3). PIP3 binds directly to the PH domain of Akt and mediates its localization to the cell membrane where it is then activated by phosphorylation at two sites, threonine 308 and serine 473. The binding of PIP3 to the PH domain results in a conformational change in Akt which allows the

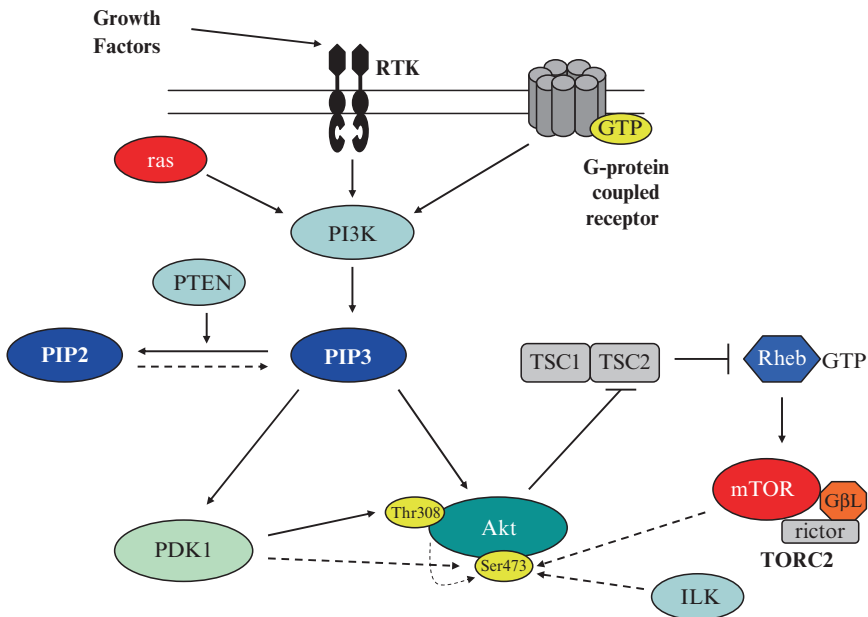


Fig. 1 Activation of Akt

phosphorylation of threonine 308 by phosphoinositide dependent kinase-1 (PDK-1), another kinase recruited to the membrane by PI3K activation (2). The mechanism by which the serine 473 is phosphorylated remains controversial. Serine 473 was originally thought to be an autophosphorylation site (3). However, recent evidence has suggested that the serine 473 can also be phosphorylated by an assortment of kinases including integrin linked kinase (ILK) (4), PDK1 (5), and TOR complex 2 (TORC2), a complex consisting of mTOR and rapamycin-insensitive companion of mTOR (rictor) (6). Regardless of the mechanism, phosphorylation at both of these residues is necessary for optimal activation of Akt and assessment of such phosphorylation by western blot or immunohistochemistry can serve as a proxy for Akt activity in tumor cells.

1.2 Hyperactivation of PI3K/Akt in Tumors

Hyperactivation of Akt and its downstream elements has been shown in a wide variety of solid and hematologic cancers, including RCC (7, 8). Many mechanisms have been proposed to account for enhanced Akt activation in cancer. These include PI3K activation due to autocrine or paracrine loops involving receptor tyrosine kinases (9, 10), enhanced PI3K signaling due to activation of ras or the presence of ras mutations (11), overexpression of growth factor receptors such as epidermal growth factor receptor (12), gene amplification of *Akt2* and *PIK3CA* (a gene which encodes the p110 α catalytic subunit of PI3K) (13, 14), and deletion or inactivation of PTEN. Although somatic activating mutations in growth factor receptors (15) and the p110 α catalytic subunit of PI3K (16) have been described in some cancers, they have not been reported in clear cell RCC.

PTEN is perhaps the most studied and well understood regulator of Akt activity. PTEN regulates the PI3K-Akt pathway by tightly controlling the levels of PIP3. The PTEN protein is a dual specificity protein and lipid phosphatase whose activity reduces the intracellular levels of PIP3 by dephosphorylation to form phosphatidylinositol-4,5-bisphosphate (PIP2) (17). The balance between PI3K and PTEN activity largely determines the intracellular levels of PIP3 and downstream activity of Akt. Deletion or inactivation of PTEN in tumors has been shown to result in constitutive activation of Akt and other downstream targets including mTOR (18, 19). Germline mutations in *PTEN* have been found in a number of inherited cancer syndromes, including Cowden's disease and Bannayan-Zonana syndrome. Somatic mutations in *PTEN* have been described in a high percentage of glioblastoma, breast, prostate, endometrial, and ovarian cancers and *PTEN* is now believed to be second most commonly mutated tumor suppressor in humans after *p53* (20). Interestingly, cancer cells with either *PTEN* mutations or diminished *PTEN* expression have been shown to be more susceptible to the effects of inhibitors of mTOR and PI3K in vitro (21, 22).

Although *PTEN* mutations are infrequent in RCC, *PTEN* gene expression has been shown to be downmodulated in a majority of RCC presumably through epigenetic

silencing (23, 24). Diminished *PTEN* expression has also been correlated with Akt activation in RCC tumor specimens (25). Not surprisingly, lack of *PTEN* expression has been shown to be an independent negative prognostic factor for disease-specific survival in patients with metastatic RCC (26).

1.3 Consequences of Akt Activation

Once activated, Akt regulates the function of a broad array of proteins involved in cell growth, proliferation, motility, adhesion, neovascularization, and cell death (27). There is consensus in the literature that Akt activation in a tumor cell is decidedly prosurvival. Consistent with numerous observations in other solid tumors, higher expression of phosphorylated (activated) Akt in RCC tumor specimens has been linked to more aggressive tumor behavior and worse clinical outcomes (28). Furthermore, Akt activation has also been shown to promote resistance to both cytotoxic chemotherapies and radiation in various tumor types (29, 30). Akt activation is proposed to mediate these effects through a variety of mechanisms, including modulation of tumor cell susceptibility to apoptosis and promotion of cell cycle progression (Fig. 2).

Akt appears to effect apoptosis susceptibility by regulating the activity of both apoptotic proteins and transcription factors. Akt directly phosphorylates several proapoptotic proteins, including procaspase 9, the bcl-2 family member BAD, and apoptosis signal regulating kinase-1 (ASK1), resulting in their inactivation (31–33). Phosphorylation of the *p53* binding partner and E3 ubiquitin ligase MDM-2 by Akt causes its translocation to the nucleus where it downregulates *p53*, bypassing an apoptotic checkpoint (34). Akt also appears to phosphorylate and differentially regulate transcriptional factors controlling expression of apoptotic genes, negatively regulating factors promoting expression of death associated genes and positively

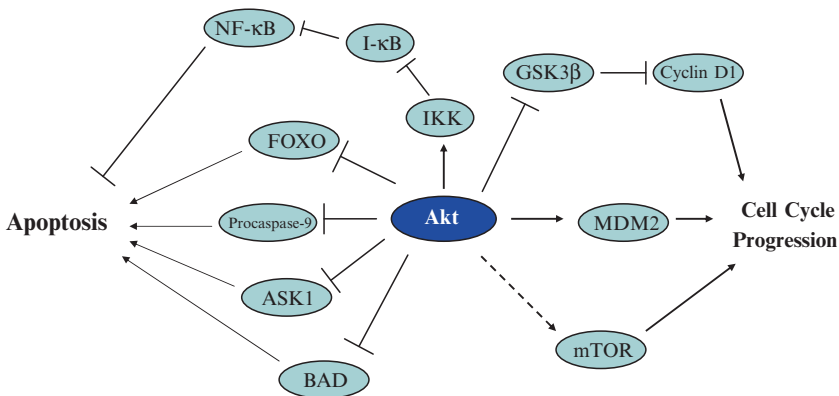


Fig. 2 Prosurvival activity of Akt

regulating genes promoting survival. For example, phosphorylation by Akt of members of the Forkhead (*FOXO*) family of transcription factors, which normally reside in the nucleus and promote expression of proapoptotic genes such as *Fas-ligand*, results in their exportation to the cytoplasm where they are sequestered by 14-3-3 proteins (35). In contrast, Akt activity enhances the activity of NF- κ B, a transcription factor promoting the expression of prosurvival genes such as the caspase inhibitors *c-IAP-1* and *c-IAP-2*. Akt has been shown to phosphorylate the I- κ B kinase IKK, resulting in the degradation of I- κ B and subsequent activation of NF- κ B (36). Akt activation has also been shown to enhance expression of the antiapoptotic gene *bcl-2* through phosphorylation of cyclic AMP response binding protein (CREB) (37). Thus, the phosphorylation of several known substrates by Akt increases cellular resistance to programmed cell death.

In addition to its prosurvival effects, Akt also promotes tumor proliferation by enhancing progression through the cell cycle. Several Akt-regulated proteins appear to modulate the activity of cyclin-dependent kinases (CDKs) which in turn inactivate retinoblastoma protein (RB) and allow progression through the G1-S checkpoint. Perhaps the most important example of this cell cycle promoting activity of Akt is the modulation of cyclin D1 levels, which are elevated in many human cancers. Akt affects cyclin D1 levels through suppression of glycogen synthase kinase 3 β (GSK3 β). Originally named for its ability to phosphorylate glycogen synthase and inhibit glycogen synthesis, GSK3 β has subsequently been found to modulate a diverse array of processes including cell structure, metabolism, and apoptosis (38). Phosphorylation of GSK3 β at its serine 9 position by Akt leads to the inactivation of GSK. GSK3 β has been shown to phosphorylate cyclin D1 and promote its proteasomal degradation (39). GSK3 β is also known to phosphorylate other cell cycle regulatory proteins such as c-Myc and cyclin E1 as well as transcription factors governing cell fate such as c-Jun, β -catenin, GLI, and Notch. In general, phosphorylation of these substrates by GSK3 β promotes their degradation. Hence, by inhibiting GSK3 β activity, Akt activation promotes the activity of a number of cell cycle regulatory proteins and transcription factors, the net effect of which appears to be to promote cell cycle progression and growth.

1.4 mTOR in Growth and Proliferation

Another critical pathway by which Akt promotes cell growth and survival is by the activation of mTOR. mTOR is a highly conserved serine/threonine kinase that regulates cell growth and metabolism in response to environmental factors. As shown in Fig. 3, Akt directly phosphorylates tuberous sclerosis complex 2 (TSC2, tuberin) resulting in the inhibition of its GTPase-activating protein (GAP) activity toward the small GTPase ras homolog enriched in brain (Rheb) (40, 41). Relief of this GAP activity enhances the GTP loading of Rheb, promoting its activity. Rheb has been shown to bind directly to the kinase domain of mTOR and activate mTOR kinase activity in a GTP-dependent manner (41).

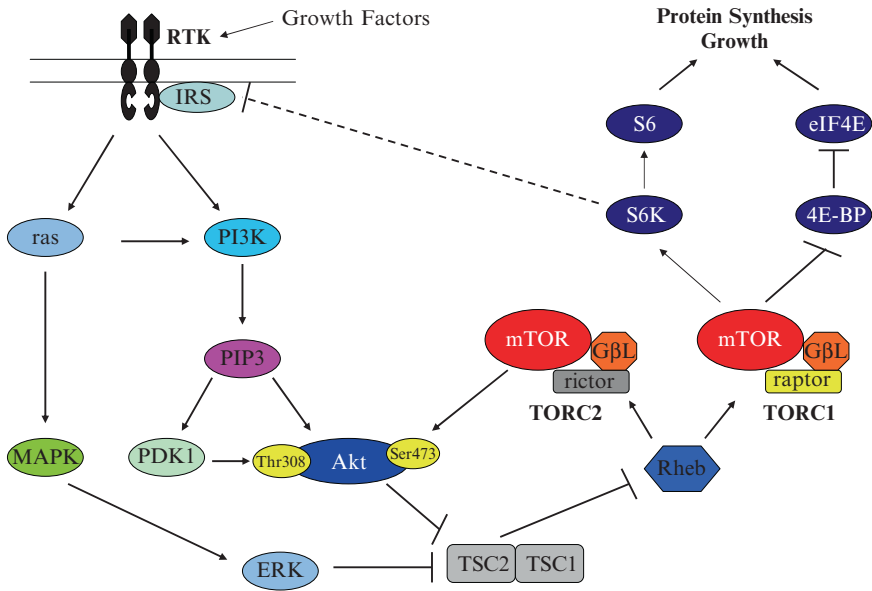


Fig. 3 Regulation and feedback loops of the Akt pathway

Once activated, mTOR executes its biologic functions as a critical component of two distinct complexes, TORC1 and TORC2, which have differential sensitivities to rapamycin. TORC1, which includes mTOR, LST8 (GβL), and raptor (regulatory protein associated with mTOR), is inhibited by rapamycin whereas TORC2, which includes mTOR, LST8, and rictor, is insensitive to rapamycin. Only recently discovered, TORC2 is currently believed to regulate cell polarity and spatial growth (42). TORC1 executes most of the biologic functions traditionally attributed to mTOR, acting through its downstream effectors, the eukaryotic translation initiation factor 4E binding protein (4E-BP) and the 40S ribosomal protein p70 S6 kinase (S6K), to stimulate protein synthesis and entrance into G1 phase of the cell cycle. The primary substrate of S6K, the S6 ribosomal protein, has been shown to have an important role in determining cell size whereas phosphorylation of 4E-BP by mTOR results in activation of cap-dependent translation of nuclear mRNA by releasing inhibition of eukaryotic translation initiation factor 4E (eIF4E).

Given its central role in governing cell growth and proliferation, TORC1 activity is responsive to many intra- and extracellular stimuli including those mediated through Akt. The upregulation of TORC1 by the PI3K-Akt pathway occurs typically in response to growth factors. However, TORC1 can also be regulated by levels of nutrients, energy, and cellular stress. Depletion of nutrients, typically in the form of amino acids, has been shown to diminish mTOR activation through a yet unclear mechanism (43). Low cellular energy characterized by low levels of ATP and high levels of AMP also inhibits mTOR activity through the AMP-dependent activation of AMP-activated protein kinase (AMPK). In contrast to Akt, phosphorylation of TSC2 by AMPK enhances its GAP activity, resulting in diminished Rheb

GTP binding and TORC1 activity (44). AMPK bound to AMP is phosphorylated and activated by the tumor suppressor LKB1 (45). Dominant mutations in LKB1 are responsible for Peutz-Jeghers syndrome, an inherited cancer disorder. TORC1 activity can also respond to cellular stress through a variety of additional mechanisms. Hypoxia has been shown to stimulate the REDD proteins through upregulation of hypoxia inducible factor-1 (HIF-1). REDD has been shown to act upstream of TSC2 complex to inhibit TORC1 activity (46). DNA damage results in the activation of p53 which has been shown to downmodulate TORC1 by activating the AMPK-TSC2 pathway (47). Thus, TORC1 activity is responsive to a number of environmental stimuli that determine whether a cell should undergo anabolic (ATP consuming) processes such as protein synthesis.

In addition to these environmental stimuli and PI3K-Akt signaling, TORC1 activity can also be modulated by other intracellular signaling pathways acting through TSC2. Extracellular-signal regulated protein kinase (ERK) has been shown to phosphorylate TSC2 and inhibit its activity (48). Similarly, the p90 S6 Kinase has been shown to phosphorylate TSC2, resulting in activation of TORC1 activity (49). The confluence of so many modulating factors highlights the fact that regulation of TORC1 activity is actually a complex interplay of various signaling pathways responding to both extra- and intracellular stimuli. Aberrations in any limb of this regulatory system in a tumor cell can potentially result in inappropriate activation of cell growth and proliferation.

Although activation of mTOR (TORC1) has been found in a large number of RCC tumor specimens, in most cases the driving force behind its activation is unclear (8). In addition to stimulation of growth and proliferation, activation of the mTOR pathway may be of particular relevance to RCC because it has been shown to increase *hypoxia inducible factor-1 α* (*HIF-1 α*) gene expression, both at the levels of messenger RNA (mRNA) translation and protein stabilization (50, 51). Inappropriate accumulation of *HIF-1 α* and *HIF-2 α* as a result of biallelic alterations in the *von Hippel-Lindau* (*VHL*) gene observed in the majority of clear cell RCC is believed to be a critical step in RCC tumorigenesis (52, 53). The mechanism by which mTOR regulates HIF levels remains unclear. The mRNAs for both *HIF-1 α* and *HIF-2 α* have been shown to contain 5' terminal oligopyrimidine (TOP) tracts (54). It has been proposed that translation of mRNA containing 5'-TOP sequences is stabilized by TORC1 signaling through S6-kinase and its substrate S6 ribosomal protein. Regardless of the mechanism, inhibition of TORC1 by temsirolimus (an ester of rapamycin) has been shown to reduce expression of *HIF-1 α* and *HIF-2 α* under both normoxic and hypoxic conditions in mouse xenograft models and this effect remains a potential contributor to the clinical response observed in RCC (55).

1.5 Regulatory Loops in the PI3K/Akt/mTOR Pathway

Regulation of the PI3K/Akt/mTOR pathway is made even more complicated by the presence of several regulatory feedback loops (Fig. 3). Some of these interactions may have significant implications on how the PI3K/Akt/mTOR pathway responds

to the pharmacologic modulation of various signaling elements. Activation of mTOR has been shown to result in attenuation of PI3K signaling by stimulating phosphorylation and inactivation of IRS (insulin response sequence) proteins by the mTOR target p70S6K (56). In contrast, pharmacologic inhibition of TORC1 activity by rapamycin appears to have differing consequences depending upon the chronicity of inhibition. The immediate effect of inhibition of TORC1 activity by rapamycin appears to be to drive the formation of TORC2. As TORC2 is known to phosphorylate Akt at the serine 473 position, inhibition of TORC1 would therefore result in Akt activation and presumed restimulation of TORC1 activity (6). In contrast, long-term administration of rapamycin is believed to lead to diminished Akt activation through the activation of S6K by PDK1 (57). S6K activated in this fashion can then downmodulate Akt activation through its activities against the IRS proteins and PI3K. The p70S6K has also been found to phosphorylate mTOR at the threonine 2446 and serine 2448 positions (58). It remains unclear if this phosphorylation is a positive or negative regulatory process. Thus, although the PI3K/Akt/mTOR pathway has traditionally been depicted as linear, there is clearly complex interplay between the various signaling elements.

In summary, PI3K, Akt, and mTOR kinases form a complex pathway which regulates a diverse array of cellular functions, most prominently cell growth and proliferation. Akt phosphorylates a wide spectrum of substrates resulting in the undermining of apoptosis, promotion of cell cycle progression, and resistance to cytotoxic chemotherapy and radiation. mTOR kinase, activated by Akt, promotes cell growth through stimulation of metabolism and protein synthesis. Aberrant activation of various elements of this pathway has been observed in RCC and correlated with worse clinical outcomes (28). The therapeutic potential of targeting this pathway in RCC is just beginning to be explored.

2 Clinical Activity of mTOR Inhibitors

Although the benefit of mTOR inhibitors in RCC was initially discovered empirically, subsequent correlative studies have highlighted the relevance of this pathway in RCC. Although there are many mTOR inhibitors in development, only two, temsirolimus and everolimus, have advanced to phase 2 testing and beyond. Similar to rapamycin, both temsirolimus and everolimus bind with high affinity to the cytoplasmic protein FK506 binding protein-12. This resulting complex interacts with and prevents activation of mTOR (59).

2.1 *Temsirolimus in RCC*

Temsirolimus has demonstrated antitumor activity and increased progression-free and overall survival (OS) in patients with renal cancer. In a phase 2 trial in patients

with advanced RCC, patients were randomized to 3 different doses of temsirolimus at 250, 75, or 25 mg given intravenously once weekly (60). There were 111 patients enrolled and 110 patients received therapy: 36 at 25 mg/m², 38 at 75 mg/m², and 36 at 250 mg/m². Most patients (91%) had received at least one prior therapy, and 51% had two or more prior therapies. Overall, 85% had received prior IL-2 and 45% had received prior interferon- α (IFN- α). Patients also had extensive metastatic disease, with 83% of patients having more than two sites of metastasis. Sixty-five percent of patients were Eastern Cooperative Oncology Group (ECOG) performance status 1 and 35% were ECOG performance status 0. Tumor assessments were made at baseline and 8-week intervals. Response was defined using standard bidimensional measurements in accordance with World Health Organization criteria for complete response (CR), partial response (PR), and stable disease (SD). In addition, minor response (MR) was defined as a 25% or more decrease but less than 50% decrease in the sum of the two greatest perpendicular dimensions of all measurable lesions.

Although the objective response rate was only 7%, 26% of patients experienced MRs and another 17% of patients had SD that lasted six or more months. The median time to tumor progression and median survival for the study patients as a whole were 5.8 months and 15.0 months, respectively. The most frequently occurring adverse events of all grades were maculopapular rash (76%), mucositis (70%), asthenia (50%), and nausea (43%). The most frequently occurring grade 3 or 4 adverse events were hyperglycemia (17%), hypophosphatemia (13%), anemia (9%), and hypertriglyceridemia (6%). Neither toxicity nor efficacy was significantly influenced by temsirolimus dose level. In addition, six patients (five at the 75-mg dose level) developed a nonspecific pneumonitis during therapy. Treatment was held and four patients were able to resume therapy, with two developing recurrent pneumonitis.

Patients in intermediate or poor-risk groups based on the clinical criteria used by Memorial Sloan-Kettering Cancer Institute (MSKCI) for a first-line metastatic renal cancer population were believed to particularly benefit from temsirolimus therapy with a 1.6- to 1.7-fold longer median survival than the historic control of patients treated with IFN first-line (61). Because there was no difference in clinical response between dose levels, a temsirolimus dose of 25 mg intravenously weekly was selected for exploration in future single-agent trials.

On the basis of promising preclinical data suggesting synergy, a subsequent phase 1/2 study was conducted combining temsirolimus with IFN- α (62). The maximum tolerated dose was determined to be 15 mg of temsirolimus administered intravenously weekly with 6 million units of IFN administered subcutaneously thrice weekly. Clinical activity was seen in 41% of all patients, including 29% of patients treated at the maximum tolerated dose (MTD). Median time to progression (TTP) for all patients was 9.1 months, and 28% of patients continued receiving treatment for more than 12 months.

Following this phase 1/2 trial, a randomized three-arm Phase 3 trial comparing temsirolimus versus IFN alone versus the combination of temsirolimus and IFN was performed and preliminary analysis was recently reported (63). Overall, 626 previously untreated patients with metastatic RCC and poor risk features were

enrolled and randomized in a 1:1:1 fashion to receive either IFN up to 18MU subcutaneously three times per week (207 patients), temsirolimus 25 mg IV weekly (209 patients), or the combination of temsirolimus 15 mg IV weekly and IFN 6MU subcutaneously three times per week (210 patients). Poor risk features were defined as having at least three of six prognostic factors including (1) serum lactate dehydrogenase > 1.5 times the ULN; (2) hemoglobin less than the lower limit of normal; (3) corrected serum calcium > 10mg/dL; (4) time from initial RCC diagnosis to randomization < 1 year; (5) Karnofsky performance score 60 to 70; and (6) multiple organ sites of metastases. Randomization was stratified according to geographic treatment location and presence or absence of prior nephrectomy. Patients were required to have histologically proven RCC but were predominantly (80%) of clear cell histology. The primary endpoint of the trial was OS of all patients randomized. Secondary endpoints included progression free survival (PFS), objective response rate by RECIST criteria, and clinical benefit as defined proportion of patients with SD lasting at least 24 weeks or objective response. Tumor evaluations were conducted at 8-week intervals.

At the time of analysis, 442 deaths had occurred. The OS of patients treated with temsirolimus alone was statistically longer than those treated with IFN alone (10.9 vs. 7.3 months; 0.73 hazard ratio, $p = 0.0069$). There was no statistical difference between patients treated with IFN alone and the combination of IFN and temsirolimus (OS 8.4 months; $p = 0.70$). Progression-free survival was significantly longer for patients treated with temsirolimus and the combination of temsirolimus and IFN (5.5 and 4.9 months, respectively) than for those treated with IFN alone (3.1 months; $p < 0.007$). Objective response rates for the IFN, temsirolimus, and combination groups were 4.8%, 8.6%, and 8.1%, respectively, and were not significantly different. The proportion of patients with clinical benefit was significantly greater in patients treated with temsirolimus alone or in combination with IFN (32% and 28%, respectively) compared with IFN alone (16%; $p = 0.002$).

Although all the treatment arms were well tolerated, the proportion of patients experiencing any grade 3 or 4 adverse event was significantly lower in temsirolimus alone arm (67%) compared with the IFN alone and combination arms (78% and 87%, respectively; $p = 0.02$). The most common treatment-related adverse events attributed to temsirolimus were asthenia, rash, anemia, nausea, dyspnea, diarrhea, peripheral edema, hyperlipidemia, and hyperglycemia. Hyperglycemia, hypercholesterolemia, and hyperlipidemia were greater in the temsirolimus-containing arms, likely reflecting the metabolic consequences of mTOR inhibition. Asthenia was the most common adverse event and was more common in the IFN-containing arms compared with temsirolimus alone.

The higher proportion of adverse events in the IFN-containing arms was also associated with more frequent dose delays and dose reductions as compared with temsirolimus alone, resulting in different mean dose densities for the three treatment groups. The mean weekly dose of temsirolimus in the temsirolimus-alone arm was 23 mg (92% of planned dose intensity) as compared with 11 mg per week in the combination arm (73% of planned dose intensity). This difference in temsirolimus dose has been cited as a potential reason that the combination of

temsirolimus and IFN did not result in an improved OS compared with IFN alone. Regardless, temsirolimus is the first molecularly targeted agent to demonstrate an OS benefit in patients with advanced RCC and will now be further investigated in numerous clinical settings.

Several trials are underway combining temsirolimus with VEGF-targeted therapies. A phase 1/2 trial of temsirolimus in combination with sunitinib in patients with advanced RCC has begun accrual to the phase 1 portion. The efficacy of temsirolimus in combination with sorafenib and in combination with bevacizumab will be investigated as part a large randomized phase 2 trial (BeST Trial) through the Eastern Collaborative Oncology Group. As hypoxia-induced upregulation of HIF as a result of angiogenesis inhibition has been proposed as a potential mechanism of resistance of VEGF-targeted therapies, trials to assess the efficacy of temsirolimus in the setting of VEGF-targeted therapy failure are also underway. Finally, the efficacy of temsirolimus in patients with nonclear cell RCC will be actively investigated.

2.2 *Everolimus in RCC*

In addition to temsirolimus, other inhibitors of mTOR are at various stages of development. Preliminary results from a phase 2 trial of everolimus (RAD001), an oral derivative of rapamycin, in patients with metastatic RCC were recently reported (64). Primary endpoints included TTP, response rate, toxicity, and changes in metabolic imaging using CT-PET. RAD001 was given at a daily dose of 10 mg orally without interruption. At the time of presentation, 28 patients had been enrolled and treated. Twenty patients had received prior therapy, the majority with cytokine-based regimens, and 82% of patients had an ECOG performance status of 0. The objective response rate was 28% and the median TTP had not yet been reached but was already greater than 6 months. Treatment related adverse events to date included mucositis, skin rash, pneumonitis, hypophosphatemia, hyperglycemia, thrombocytopenia, anemia, and elevated liver function tests. Everolimus is currently entering further exploration in RCC both as a single-agent and in combination with other targeted agents. In particular, a large randomized phase 3 comparing everolimus to placebo in patients refractory to VEGFR targeted tyrosine kinase inhibitors has been initiated.

2.3 *Patient Selection Opportunities*

Efforts have proceeded to identify potential molecular predictive biomarkers for response or clinical benefit to temsirolimus in the hopes of further defining the appropriate treatment population. Similar studies in patients treated with immunotherapy have identified certain histologic features and carbonic anhydrase IX (CAIX)

expression as promising predictive biomarkers for IL-2 therapy (65). Efforts to identify predictive biomarkers for response to temsirolimus have been directed by observations from preclinical and in vitro experiments. As discussed earlier, pre-clinical studies have suggested that cells deficient in PTEN and with high levels of Akt activation are more sensitive to the effects of temsirolimus in vitro (21, 22). Additionally, a recent study shows that loss of *VHL* may sensitize RCC cells to temsirolimus in both in vitro and mouse xenograft models, suggesting that the high HIF levels seen with defective proteasomal degradation are also in part dependent on intact translation and are therefore susceptible to mTOR inhibition (55).

These potential predictive biomarkers are being explored in human RCC tumor tissue samples to determine if expression of these markers can predict for response to mTOR inhibitors. Preliminary efforts from a cohort of patients treated on the randomized phase 2 trial of temsirolimus suggest that, in contrast to patients treated with IL-2 therapy, CAIX expression or pathology risk group do not correlate with response to temsirolimus (66). However, similar to IL-2, patients with very low CAIX expression do not appear to respond to temsirolimus, and median survival is longer in patients with high CAIX-expressing tumors. Additional analyses suggest that patients whose tumors express low levels of the upstream modulator of mTOR activity, phospho-AKT, or the down-stream target of mTOR activation, phospho-S6 ribosomal protein, may be more unlikely to respond to temsirolimus (66). High phospho-S6 expression frequency or intensity, in particular, appeared to be associated with clinical benefit, freedom from disease progression, and prolonged survival in patients treated with temsirolimus. Although these results require independent validation, they suggest that analysis of phospho-S6 expression might help select patients who could benefit from mTOR inhibitor therapy. Additional studies to explore the relationship between temsirolimus response and tumor expression of *PTEN* and *VHL* mutational status are currently under way and could yield additional predictive biomarkers for mTOR therapy.

It is possible that patients who benefit from mTOR inhibitors may be distinct from the group of patients who benefit from VEGF-targeted therapies. Interestingly, a post hoc analysis of the randomized phase 3 trial of temsirolimus demonstrated that in patients ≤ 65 years of age and with nonclear cell histology, median OS and PFS were longer in the temsirolimus-alone arm (OS: 11.6 months; PFS: 7 months) as compared with IFN-alone (OS: 4.3 months; PFS: 1.8 months) (67). The clinical benefit observed in nonclear cell RCC with functional VHL suggests that the mechanism of action of temsirolimus may be mediated through a non-VHL-HIF dependent pathway. Characterization of the molecular features of these clear cell and nonclear cell RCC responding to temsirolimus may lead to the identification of novel predictive biomarkers.

In addition to pretreatment predictors of response, efforts are underway to identify early predictors of response during therapy. Given that inhibition of mTOR is believed to result in attenuation of glycolysis (68), most of these efforts have focused on FDG-PET scanning. Decreases in FDG-PET avidity of tumors during therapy with mTOR inhibitors have been shown to predict for tumor responses, both in animal models and in patients with sarcoma (55, 69). The ability of

FDG-PET to serve as an early predictor of response prior to standard CT imaging is being actively investigated in numerous clinical trials and is the primary endpoint of the phase 2 trial everolimus in patients with advanced RCC.

3 Future Directions for the PI3K/Akt/mTOR Pathway in RCC

3.1 Therapeutic Potential of Targeting the PI3K-Akt Pathway

The promising clinical activity of inhibitors of mTOR in RCC has highlighted the therapeutic potential of this pathway and its upstream regulators. As discussed earlier, inhibition of mTOR has in some cases been associated in Akt activation through a feedback loop involving TORC2. Akt activation in this situation could potentially promote cell survival and undermine the potential benefits of mTOR inhibition. Similarly, S6K has been shown to downregulate PI3K signaling through its activities against the IRS proteins. Inhibition of S6K induced by mTOR inhibition may result in activation of PI3K signaling. While these signaling events have yet to be studied rigorously in human subjects, the theoretical ramifications of these feedback loops highlights the potential benefits of targeting upstream of mTOR where such feedback effects could be diminished or muffled.

Although few agents with inhibitory activity against PI3K or Akt have advanced to clinical testing, several agents are available for laboratory investigation. Recently, pharmacologic inhibition of PI3K/Akt signaling in RCC has been shown to induce tumor cell apoptosis both in vitro and in vivo (70). Inhibition of PI3K/Akt signaling by PI3K inhibitors LY294002 and wortmannin resulted in significant reduction in cell proliferation and induction of tumor cell apoptosis by both TUNEL and propidium iodide staining in RCC cell lines (786-O). Treatment of nude mice bearing RCC xenografts derived from the 786-O cells with LY294002 resulted in up to 50% reduction in tumor size. PI3K/Akt signaling disruption was verified by showing reduction of phospho-Akt (serine 473) expression by both immunohistochemistry and western blot analysis. The reduction in tumor size was associated with increased apoptosis by TUNEL staining. Interestingly, despite the decrease in tumor size and clear demonstration of tumor cell apoptosis, tumor neovascularization was increased. Akt1 (the predominant Akt isoform in endothelial cells) has recently been shown to regulate endothelial cell response to VEGF and Akt1-/- knockout mice demonstrate increased neovascularization and enhanced endothelial cell proliferation in response to VEGF (71). Thus, while the net effect of inhibition of PI3K/Akt signaling appears to reduce tumor size in xenograft models by causing tumor cell apoptosis, these agents may increase angiogenesis, suggesting that combination with VEGF-targeted therapies may be of interest. Regardless, these experiments strongly suggest that inhibition of PI3K-Akt pathway is a viable therapeutic approach in RCC.

3.2 *Agents in Development*

Several agents with inhibitory effects against PI3K or Akt signaling are in clinical development, although few have advanced as far a phase 2 clinical trials. The diverse biologic targets and mechanisms of action of these agents are reviewed in more detail elsewhere (72) but range from reconstitution of PTEN via gene therapy, small molecule inhibitors of the p110 catalytic subunit of PI3K, inhibitors of PDK1 (competitive binding to ATP domain, inhibition of PDK1 catalytic domain), and lipid-based and small-molecule inhibitors of Akt. Unfortunately, existing PI3K inhibitors LY294002 and wortmannin have many characteristics felt to be unfavorable to clinical development, including poor bioavailability, high toxicity, and poor specificity.

One agent with promising preliminary clinical activity entering phase 2 testing in patients with advanced RCC is perifosine, an oral alkylphospholipid which is believed to inhibit Akt activation by preventing its translocation to the cell membrane. Perifosine has been shown to inhibit the growth of a wide variety of solid tumors associated with inhibition of Akt activity and has been well tolerated in early clinical trials (73, 74). Several patients with RCC have experienced tumor regressions. Based on the promising activity seen in patients with metastatic RCC in early phase 1–2 trials, perifosine will soon be evaluated in a phase 2 trial in patients with advanced RCC who have failed the FDA-approved tyrosine kinase inhibitors sorafenib or sunitinib.

As other agents with inhibitory effects against PI3K-Akt will likely soon follow perifosine into clinical testing in RCC, it will be interesting to observe the potential effects on angiogenesis. As discussed earlier, inhibition of Akt in preclinical animal models appears to have actually enhanced angiogenesis through increasing endothelial responsiveness to VEGF. Given the potential elevations of VEGF associated with VHL loss in clear cell RCC, enhanced angiogenesis may undermine potential clinical benefits of Akt inhibition. Clinical trials of any agent inhibiting Akt signaling in RCC should include correlative studies to investigate this possibility. The potential additive effects of combining an inhibitor of Akt signaling with an inhibitor of VEGF signaling is additionally worthy of further exploration. Separate phase 1 trials of perifosine in combination with sorafenib and sunitinib are already underway.

4 Conclusion

The poor clinical outcomes associated with surrogates of PI3K/Akt/mTOR pathway activation in RCC appear to confirm the theoretical molecular benefits that activation of this pathway confers on tumor growth and proliferation. The clinical activity of inhibitors of mTOR in patients with advanced RCC and the efficacy of inhibitors of PI3K/Akt signaling in preclinical studies in RCC highlight the therapeutic potential of this pathway. As agents with more specific inhibitory effects upstream of mTOR against PI3K or Akt signaling enter clinical development, the

Table 1 Current trials with agents targeting PI3K/Akt/mTOR pathway in RCC

Trial	Sponsor, sites	Status
Phase 1 temsirolimus + bevacizumab	CTEP, Mayo Clinic	Ongoing
Phase 1 temsirolimus + sorafenib	CTEP, San Antonio	Ongoing
Phase 1/2 temsirolimus + sunitinib	MSKCC, FCCC, DF/HCC	Ongoing
Phase 2 temsirolimus + sorafenib, phase 2 temsirolimus + bevacizumab (Arms of ECOG 2804 BeST trial)	CTEP, ECOG	To open Spring 2007
Phase 3 trial of temsirolimus + sorafenib versus sorafenib alone in sunitinib failures	Wyeth	In development
Phase 2 temsirolimus in nonclear cell histology RCC	Wyeth	In development
Phase 3 RAD001 versus placebo	Novartis	Ongoing
Phase 2 RAD001 PET study	Novartis, University of Chicago, DF/HCC	Ongoing
Phase 2 RAD001 + PTK787	Novartis, Duke	Ongoing
Phase 2 perifosine following TKI failure	Keryx, DF/HCC, Cleveland Clinic, UPENN, City of Hope	Ongoing

therapeutic value of inhibiting this pathway in RCC will be able to be validated clinically. Table 1 notes some of the current or upcoming trials of interest using agents targeting the PI3K/Akt/mTOR pathway. In the meantime, efforts must continue to identify factors that allow further selection of patients likely to derive significant benefit from mTOR inhibitors. It is possible that many of these factors will have similar predictive value in agents that target upstream of mTOR. These efforts will be critical as these agents move into more widespread use, likely given either sequentially or in combination with VEGF-targeted therapies, so as to allow the direction of these therapies to the most appropriate patients.

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EGFR and HER2: Relevance in Renal Cell Carcinoma

Eric Jonasch and Cheryl Lyn Walker

Abstract With the improvement in our understanding of the molecular biology of renal cell carcinoma (RCC), a number of targeted therapeutics have become available for treatment of this disease, both FDA approved and in the experimental realm. The epidermal growth factor receptor (EGFR) family of molecules is an attractive target in RCC due to their overexpression on RCC tissue, and the ready availability of agents that target the pathway. This chapter summarizes the molecular biological information available for EGFR in RCC, preclinical work with blocking agents, and the clinical data on use of EGFR targeting strategies in RCC.

Keywords Renal cell carcinoma • ErbB • Targeted therapy • Resistance • Signaling

1 ErbB Receptor Family in RCC

The ErbB family of receptor tyrosine kinases is composed of the epidermal growth factor (EGF) receptor (HER1/ErbB1), HER2/neu (ErbB2), HER3 (ErbB3), and HER4 (ErbB4) (1, 2). In addition to multiple receptors, several different ligands have been identified for the ErbB receptors including EGF, transforming growth factor alpha (TGF- α), heparin-binding EGF (HB-EGF), and amphiregulin (3). In the absence of ligand, ErbB receptors reside within the membrane as inactive monomers. In the presence of ligand, these receptors form homo- and heterodimers and become active via transphosphorylation of the intracellular carboxy tail of the receptor. These interactions are facilitated by the extracellular “dimerization loop” of the receptor as well as by interactions between the transmembrane domains of the liganded receptors. Further oligomerization of the receptors ensues resulting in the formation of higher-order aggregates, which may form “signaling platforms” within the plasma membrane (1).

E. Jonasch (✉)

Department of Genitourinary Medical Oncology, MD Anderson Cancer Center,
University of Texas, 1515 Holcombe Dr, Houston, TX 77030
e-mail: jonasch@mdanderson.org

While ErbB1 is activated by its ligands, EGF and TGF- α , HER2/ErbB2 is a ligandless coreceptor for other members of the ErbB family, and is the preferred dimerization partner for ErbB1, 3, and 4 (4, 5). In contrast to other ErbB family members, HER2/ErbB2 does not require ligand for activation, as its extracellular domain has a fixed conformation that resembles the ligand-activated state of the other ErbB receptors (6). It is able to form both active homodimers in cells overexpressing HER2/ErbB2 (7) and cause an increase in the activity of other ErbB family members with which it dimerizes via increased basal phosphorylation and inhibition of receptor degradation (8–11). In contrast to other ErbB family members, the ErbB3 receptor lacks intrinsic tyrosine kinase activity. However, ErbB3 contains multiple docking sites for phosphoinositide-3 kinase (PI3K) (see below) and when it is phosphorylated in heterodimers with other ErbB family members is a more potent activator of PI3K (12, 13).

As shown in Fig. 1, dimerization of ErbB monomers activates their intrinsic tyrosine kinase activity. As a result of transphosphorylation of ErbB homo- and heterodimers, several signal transduction pathways within the cell become activated [for review, see Yarden and Silwowski (2)]. PI3K/AKT signaling is activated via docking of the Src homology 2 (SH2) domain of the p85 regulatory subunit of PI3K

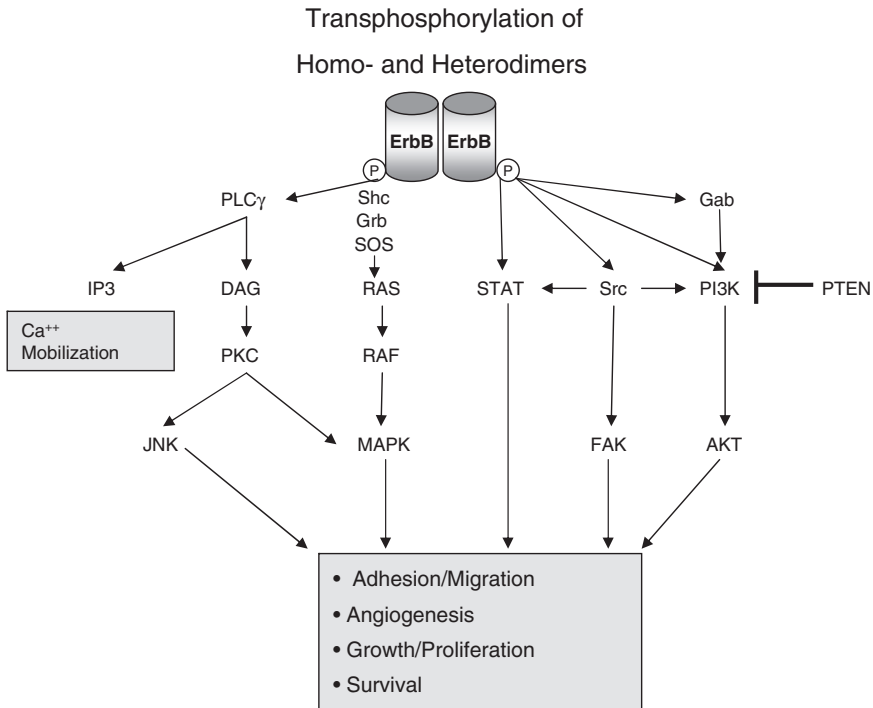


Fig. 1 Transphosphorylation of homo- and heterodimers

to the receptor (in the case of ErbB2, 3, and 4) or via the adaptor Gab1 (in the case of ErbB1) (14). Docking of the Shc and Grb adaptors to the phosphorylated ErbB receptor acts as scaffolds to recruit SOS, which brings SOS in proximity to its target Ras to activate the RAS/RAF/MAPK signaling cascade (15, 16). Activation of RAS/RAF/MAPK is an invariant feature of all activated ErbB receptors and PI3K/AKT signaling is a downstream target of most active ErbB dimers (2). In addition to these signaling pathways, signal transducers and activators of transcription (STAT) activation occurs as a result of ErbB receptor activation [reviewed in Yu and Jove (17)]. c-Src is also activated by ErbB dimers, phosphorylating, and activating focal adhesion kinase (FAK) and contributing to both PI3K and STAT activation. Finally, phospholipase C is also activated by ErbB receptors, leading to increases in both diacylglycerol (DAG) and inositol trisphosphate (IP_3), resulting in activation of MAPK and the stress kinase JNK via PKC and mobilization of Ca^{2+} stores. As a consequence of activation of these different signal transduction pathways, ErbB receptor signaling modulates angiogenesis, adhesion and migration, cell growth, proliferation, and survival.

Several mechanisms underlie aberrant ErbB signaling in cancer: inappropriate ligand expression, receptor amplification/overexpression, and mutational receptor activation. In RCC, overexpression of TGF- α and the resultant autocrine loop is a consistent feature of clear cell RCC (18–25) due to the overexpression of hypoxia-inducible factor (HIF) that occurs in these tumors. HIF is a transcription factor that becomes stabilized in clear cell RCC when the von Hippel–Lindau (VHL) tumor suppressor gene is lost [reviewed in Kaelin (26)], leading to upregulation of HIF targets including TGF- α (27–29). TGF- α production by RCC may also have paracrine effects, as stromal and endothelial cells can express ErbB receptors, resulting in receptor activation and/or induction of vascular endothelial growth factor (VEGF) and other angiogenic factors (30–33).

With regard to receptor overexpression/amplification, elevated expression of ErbB1 has been frequently noted in RCC (23, 25, 34–42). HER2/ErbB2 expression in RCC is less well characterized, with conflicting data in the literature as to a potential role for this member of the ErbB family in this disease. Data suggesting that HER2/ErbB2 expression is decreased in RCC (34–36), increased (37–39) or expressed in a subset of RCC (40–43) have been reported, with some differences likely due to technical issues, such as the use of antibodies, which recognize the intracellular versus the extracellular portion of HER2/ErbB2. It has also been suggested that HER2/ErbB2 expression may correlate with tumor type and origin within the renal nephron, with collecting duct and Bellini duct tumors and oncocytoma > chromophobe > papillary > clear cell tumors being positive for HER2/ErbB2 expression (38, 43, 44). In addition to these data on primary tumors, the Caki-2 RCC-derived cell line has been reported to be HER2/ErbB2 positive (45), although expression of ErbB2, 3, and 4 was reported to be undetectable by Western analysis in Caki-2, ACHN, A498, and several other RCC-derived cell lines (46). Mutations in ErbB family members such as those that occur in other tumor types (i.e., lung cancer) and which correlate with response to targeted therapy have not been reported in RCC to date.

2 ErB Receptor Blockade: Strategies

Several different molecules have been developed to block ErB signaling. The two major strategies have been to engineer small-molecule inhibitors of Erb1/EGF receptor signaling, and to generate inhibitory antibodies against the extracellular domain of the EGF receptor. The following section describes the key agents, their preclinical development, their putative mechanism of action, and specific information on their preclinical efficacy in RCC models.

2.1 *Cetuximab (C225)*

Mendelsohn and colleagues demonstrated the inhibitory effect of murine monoclonal antibodies against EGF receptor signaling. These antibodies were prepared using EGF receptor protein from human A431 epidermoid carcinoma cells as an immunogen. They demonstrated the inhibitory effect of these antibodies on EGF binding and tyrosine kinase activity in an in vitro system (47, 48). One of these antibodies, antibody 225, was later tested in several preclinical models confirming its anti-EGF receptor activity. The 225 antibody was chimerized with human IgG1 in its constant region (designated c225, and later, cetuximab). Cetuximab efficacy was compared to the native 225 against established A431 human skin squamous cell carcinoma tumor xenografts in nude mice. These experiments indicated that cetuximab was more effective than 225 in inhibiting tumor growth in this model (49).

Prewett and colleagues investigated the effects of cetuximab on human RCC cell lines. Cetuximab inhibited DNA synthesis of cultured A498, Caki-1, SK-RC-4, SK-RC-29, and SW839 RCC cells in a dose-dependent manner. Cetuximab inhibited exogenous ligand-stimulated tyrosine phosphorylation of EGF receptor on RCC cells. Mice treated with cetuximab in a Caki-1 ascites xenograft model showed a significant increase in survival. Cetuximab also inhibited the growth of subcutaneous SK-RC-29 xenografts in a dose-dependent manner, and inhibited the growth and metastasis of RCC tumors growing orthotopically in the renal subcapsule of nude mice. Histological examination of RCC tumors from mice treated with cetuximab showed a substantial decrease in proliferating cell nuclear antigen staining and an increase in tumor cell apoptosis.

A subsequent study by Perera and colleagues demonstrated that in vitro treatment of clear cell RCC-derived cell lines lacking VHL resulted in only a modest decrease in growth rate. In contrast, nonclear cell RCC-derived cell lines that retained VHL responded significantly to cetuximab treatment. Transfection of VHL into VHL-negative RCC cell lines restored responsiveness to cetuximab, indicating that VHL was required for effective EGF receptor-blockade (50). These were the first preclinical data to suggest a possible lack of efficacy of anti-EGF receptor monotherapy in a VHL-negative genetic background.

2.2 *Panitumumab (ABX-EGF)*

Panitumumab is a high-affinity, human monoclonal antibody that binds the EGF receptor and prevents ligand binding. The antibody was generated using a murine human chimeric immune “Xenomouse” system. A panel of human IgG2 anti-EGF receptor monoclonal antibodies was generated by immunizing the XenoMouse IgG2 strain with A431 cells. A total of 70 EGF receptor-specific hybridomas were established from five fusions. Among these, at least 15 were neutralizing antibodies. One of these, ABX-EGF, later renamed panitumumab-bound EGF receptor with high affinity, blocked the binding of both EGF and TGF- α to the receptor, and inhibited EGF-activated EGF receptor tyrosine phosphorylation and tumor cell activation. Panitumumab did not activate the EGF receptor tyrosine kinase. Upon binding to the receptor, panitumumab caused EGF receptor internalization in tumor cells (51, 52).

Panitumumab treatment led to significant growth inhibition of multiple tumor xenografts, including SK-RC-29, BxPC-3, IGROVI, PC3, HS766T, and HT-29 (51).

In an experiment assessing the association between receptor number and response, panitumumab treatment led to significant growth inhibition of tumors expressing 1,7000 or more EGF receptors per cell. In contrast, the growth of tumors expressing 1,1000 or fewer EGF receptors per cell was unaffected by the panitumumab treatment. Panitumumab had no effect at all on the EGF receptor-negative tumor SW70, supporting the potential predictive value of EGF receptor staining in the clinical setting (51).

It has been suggested that panitumumab may trigger the downregulation of EGF receptor expression by triggering receptor internalization, induction of apoptosis triggered by blocking EGF receptor signaling pathways and induction of cell cycle arrest, and inhibition of angiogenesis (53).

2.3 *Small Molecule Inhibitors*

2.3.1 *Gefitinib*

The screening of a compound library using an EGF enzyme prepared from A431 cells identified a series of potent ($IC_{50} < 1 \mu M$) and selective quinazoline enzyme inhibitors (54). Of the compounds discovered, Gefitinib (ZD1839), a substituted anilinoquinazoline, was not the most potent compound, but still selectively inhibited EGF-stimulated tumor cell growth with an IC_{50} of 0.054 M (55). Importantly, gefitinib achieved sustained in vivo blood levels (56). Gefitinib was found to have good oral bioavailability and inhibited the growth of a broad range of human solid tumor xenografts in a dose-dependent manner (range 12.5–200 mg/kg, po once daily) with marked regressions seen in some tumors (56).

Gefitinib was shown to block EGF-stimulated EGF receptor autophosphorylation in tumor cells in an in vitro system, and to inhibit growth of EGF receptor expressing

tumors in xenograft models (55). In athymic nude mice bearing A431-derived xenografts, PO treatment once a day with gefitinib (from day 7 after implantation for 3 weeks) inhibited tumor growth in a dose-dependent manner. Gefitinib also inhibited the growth of A549 lung and Du145 prostate tumor xenografts in a dose-dependent manner. Dose-dependent growth inhibition was also observed in colon (HCT15, HT29, LoVo), and squamous (KB) tumor xenografts (55).

A study assessing the efficacy and effect of gefitinib in combination with paclitaxel in a variety of human RCC cell lines revealed that gefitinib was able to induce apoptosis only in combination with paclitaxel (57). Furthermore, gefitinib blocked the paclitaxel-induced activation of the MAPK pathway, and downregulated BCL-2 protein expression, in an AKT-independent fashion (57).

Gemmill and colleagues demonstrated that combined EGF receptor and mammalian target of rapamycin (mTOR) inhibition synergistically impaired growth in a VHL-dependent manner. Gefitinib blocked ERK1/2 phosphorylation specifically in wild-type VHL cells. As in the study with cetuximab by Perera et al. (50), the absence of a consistent response to gefitinib was noted in VHL mutated cells. The reason for this lack of response is poorly understood, but once again leads one to be cautious about the application of EGF receptor blocking agents in the clinical treatment of clear cell RCC, which usually has an inactivated VHL gene.

2.3.2 Erlotinib

Induction of apoptosis and cell cycle arrest was shown by Erlotinib (OSI-774), [6,7-bis(2-methoxy-ethoxy)-quinazolin-4-yl-(3-ethynylphenyl)amine, a quinazoline derivative which reversibly inhibited the kinase activity of purified EGF receptor with an IC_{50} of 2 nM, and inhibited autophosphorylation in intact cells, with an IC_{50} of 20 nM [Moyer (58)#2307]. Erlotinib was shown to block the cell cycle in G1, resulting in accumulation of p27 (59). Erlotinib also induced apoptosis in vitro (58) and demonstrated activity against various human tumor xenografts in vivo, including DiFi (58), HN5, and A431 (59). Resolution of the crystal structure of EGF receptor bound to erlotinib confirmed the binding of drug to the intracellular kinase domain (60).

No published data are available describing the antitumor effect of erlotinib in RCC models.

2.3.3 Lapatinib

Lapatinib is an oral dual tyrosine kinase inhibitor that targets EGF receptor and HER2. In vitro cell growth assays of lapatinib on the EGF receptor overexpression HN5 and A431, and the HER2 overexpressing BT474, and the EGF receptor and HER2 overexpressing N87 cell lines demonstrated IC_{50} values for growth inhibition of less than 0.16 μ M. The average selectivity for the tumor cells versus a control human foreskin fibroblast cell line was 100-fold. Lapatinib was shown to be

effective in murine xenografts of HN5 and BT474, and inhibition of EGF receptor and HER2 receptor autophosphorylation and phosphorylation of a downstream effector molecule, AKT, was demonstrated in tumor tissue taken from both models. There was no effect on the amount of EGF receptor or HER2 protein expressed in these samples. Further in vitro study demonstrated both growth arrest and cell death occurred in HN5 and BT474 lines (61, 62).

3 Clinical Experience with ErbB Receptor-Targeted Therapy in RCC

Because of the consistent demonstration of ErbB receptor upregulation in human RCC specimens and RCC cell lines, and the presence of preclinical data suggesting potential benefit, a number of clinical trials were performed using a variety of ErbB blocking agents in advanced RCC. As a whole, this strategy has not resulted in consistent clinical benefit. The lapatinib study described below indicates that proper patient preselection will be necessary to achieve any improvement in outcome in patients with RCC with ErbB blocking agents.

3.1 *Cetuximab Studies*

A phase II clinical trial using cetuximab in patients with treatment-naïve RCC was reported in 2003. Fifty-five patients with metastatic RCC received single-agent cetuximab administered by intravenous infusion at a loading dose of 400 or 500 mg/m², followed by weekly maintenance doses at 250 mg/m². None of the patients treated with cetuximab achieved either a complete or partial response. The median time to progression (TTP) was 57 days (Motzer 2003#2252). This compared unfavorably to a median progression-free survival (PFS) of 4.7 months seen in patients treated with interferon alpha, and even more unfavorably to the VEGF blocking agents, including sorafenib, bevacizumab, and sunitinib (63–66).

3.2 *Panitumumab*

An 88 patient study in previously treated patients with metastatic RCC assessed the efficacy toxicity, immunogenicity, pharmacokinetics, and pharmacodynamics of 8 weekly infusions of panitumumab. Patients were treated with panitumumab doses of 1.0, 1.5, 2.0, or 2.5 mg/kg weekly. The study demonstrated 5 tumor responses, including 3 partial responses and 2 minor responses, and disease stabilization occurred at 8 weeks in 44 patients (67). The median PFS was 100 days. EGF receptor immunostaining was performed on 76 tumor biopsy specimens, and 69 of these (91%) scored positive.

3.3 *Gefitinib Studies*

Drucker et al. published their experience with gefitinib in 18 patients with metastatic RCC. Eleven of twelve evaluable tumor specimens stained positive for the EGF receptor. Patients received gefitinib at 500 mg per day by mouth, with dose reduction permitted. Treatment with gefitinib did not result in any complete or partial responses, and 13 patients (81%) had progression of disease within 4 months of start of therapy. VEGF and basic fibroblast growth factor (bFGF) serum levels were tested before starting therapy and every 3 months on therapy, but there was no correlation between pretreatment level and TTP. The authors concluded that at the dose and schedule used in this trial, the lack of antitumor activity associated with gefitinib does not support further study in patients with metastatic RCC (68).

In late 2004, Dawson et al. published their experience with gefitinib in RCC. Twenty-one patients were enrolled. Patients had received a median of one prior therapy. The best response was stable disease in 8 patients (38%). Median PFS was 2.7 months, and median overall survival (OS) was 8.3 months (69). EGF receptor analysis and corresponding best response were assessed. In 19 of the 20 patients with adequate tissue for EGF receptor analysis, the tumor specimens stained positive for EGF receptor. One patient's submitted tissue was inadequate for staining. There was no correlation between the intensity of EGF receptor staining (0 or 1 vs 2 or 3) and having stable versus progressive disease.

Jermann et al. published a phase II, open-label study of gefitinib in patients with locally advanced, metastatic, or relapsed RCC. Twenty-eight patients were enrolled. Patients received oral gefitinib 500 mg/day. No responses were recorded, but stable disease seen but 14 patients (53.8%). Median TTP was 110, and median OS was 303 days. Baseline tumor biopsies were analyzed immunohistochemically for EGF receptor expression. Ninety-one percent of tumor biopsies had at least 70% of tumor cells expressing membrane EGF receptor (70).

3.4 *Erlotinib Studies*

The combination of bevacizumab and erlotinib was reported in a paper by Hainsworth et al. in November 2005. Sixty-three patients with metastatic clear cell RCC were treated with the anti-VEGF antibody bevacizumab 10 mg/kg intravenously every 2 weeks and erlotinib 150 mg po daily. The majority of these individuals had not received prior systemic therapy. Fifteen (25%) of 59 assessable patients had objective responses to treatment, and an additional 36 patients (61%) had stable disease after 8 weeks of treatment. Only eight patients' (14%) disease had progressed at this time point. The median and 1-year PFS were 11 months and 43%, respectively. After a median follow-up of 15 months, median survival has not been reached; survival at 18 months was 60% (64).

These impressive data were followed up by a 104 patient phase II study randomizing patients with untreated metastatic RCC between bevacizumab and

bevacizumab plus erlotinib, administered at 150 mg by po daily. Eligibility criteria included previously untreated metastatic RCC with >50% clear cell histology. All patients received bevacizumab with either erlotinib 150 mg po daily or placebo until disease progression or unacceptable toxicity. Primary end points included objective response rate (ORR) and PFS. Median survival duration was not reached. Patients who received bevacizumab alone had a PFS of 8.5 months, and those who received both agents had a PFS of 9.9 months. The difference was not statistically different, and there was no survival difference between arms [Bukowski (97)#2314]. The conclusion from these two studies is that erlotinib did not add a significant PFS or OS benefit to bevacizumab therapy.

3.5 Lapatinib

A phase III study for patients with advanced RCC of any histology who had failed first-line cytokine therapy was recently reported in abstract form. Patients were randomized to receive oral lapatinib 1,250 mg OD or hormonal therapy with meg-estrol acetate. A total of 417 patients were randomized. The primary efficacy end point was TTP, with secondary end points of OS. At the time of analysis, median TTP was 15.3 weeks for lapatinib and 15.4 weeks for medroxyprogesterone [hazard ratio (HR) = 0.94; $p = 0.60$], and median OS was 46.9 weeks for lapatinib versus 43.1 weeks for medroxyprogesterone (HR = 0.88; $p = 0.29$) (71).

All patients had tumor assessed for EGF receptor expression by immunohistochemistry. In the 241 patients whose tumors had a high level of EGF receptor expression (3+ by immunohistochemistry, IHC), median TTP was 15.1 weeks for lapatinib versus 10.9 weeks for medroxyprogesterone (HR = 0.76; $p = 0.06$), and median OS was 46.0 weeks for lapatinib versus 37.9 weeks for medroxyprogesterone (HR = 0.69; $p = 0.02$) (71). Although the survival benefit in the high EGF receptor expressing subgroup who received lapatinib was statistically significant, it was numerically small and is of questionable clinical significance. Nevertheless, this study indicates that any future work with Erb family blocking agents in RCC will likely need to be done with prospective assessment of receptor levels to choose the individuals most likely to benefit from therapy.

4 Future Perspectives: Patient Selection and Mechanisms of Resistance

RCC provides a clear example of the clinical and preclinical challenges facing us as we target pathways and molecules associated with carcinogenesis. Understanding the molecular basis for acquired and intrinsic resistance to targeted EGFR/HER2 therapy can aid in patient selection and enhance the success of clinical trials. In this regard, while evidence is still accumulating on the use of EGFR/HER2 therapy

in RCC, and the clinical efficacy to date has not been significant, much has been learned from targeted therapy in clinical trials for other types of cancer.

Of central importance is the concept of “oncogene addiction” (72, 73), which postulates that the dependence of tumors on certain oncogenic alterations for the maintenance of the malignant phenotype makes targeted therapy to these specific alterations an especially effective form of therapy. There is ample evidence for addiction to several ErbB family members in breast (HER2/ErbB2) and lung (EGFR/ErbB1) cancer. In these tumors, targeting of specific ErbB family members with drugs such as trastuzumab (HER2/ErbB2) or gefitinib/erlotinib (EGFR/ErbB1) may have enhanced therapeutic efficacy (72). In addition, cancer cells whose growth is driven by ErbB family members often depend on coupling of ErbB receptors with ErbB3 to activate PI3K/AKT signaling (see above) and promote the malignant phenotype. This suggests that ErbB3 overexpression may identify a subset of tumors dependent on ErbB signaling and may predict responsiveness to targeted ErbB therapy, such as gefitinib, which has been observed in the clinic in patients with non-small cell lung cancer (NSCLC) (74). The relevance of ErbB3 overexpression in RCC has yet to be determined, and may be worthy of further study.

There is conflicting evidence that oncogenic signaling via EGF receptor/ErbB1 is involved in HIF-mediated transformation of VHL-null RCC. As proposed by Hahn and Weinberg (75), TGF- α expression and activation of EGF receptor signaling would fulfill two of the six essential characteristics of cancer cells: decreased dependence on exogenous growth factors (i.e., growth autonomy) and promotion of angiogenesis required for growth and metastasis. In RCC, recent evidence indicates that TGF- α /EGFR signaling may be obligatory for the malignant phenotype in VHL-null cells (76). In this study, RNA knockdown of the EGF receptor resulting in inhibition of EGF receptor signaling was able to inhibit the growth of VHL-null RCC in vitro and in vivo, phenocopying the effect of HIF-2 α silencing or reintroduction of VHL. In direct contrast, the absence of functional VHL appeared detrimental to response in cell lines treated with cetuximab (50). Thus, the importance of EGFR signaling in maintaining a malignant phenotype in a VHL-null background is unresolved. Clearly, in the clinical arena, use of EGFR inhibitors as monotherapy patients with clear cell RCC has not shown promise (67–70). The possibility that these agents are effective in nonclear cell, non-VHL mutated histologies is currently being evaluated in several phase II clinical trials.

The efficacy of therapy targeted against ErbB receptors may be modulated by other alterations that occur in RCC, such as defects in the PTEN tumor suppressor (77–80). As shown in Fig. 1, PTEN opposes the action of PI3K, and loss of PTEN function can result in constitutive activation of PI3K/AKT signaling downstream of ErbB receptors (81–84). PTEN-deficient cell lines have been shown to be gefitinib-resistant, presumably because they continue to express activated AKT even in the presence of EGFR inhibition (85). Mutations in PTEN are rare in RCC, although PTEN expression is frequently reduced in these tumors (80, 86) and correlates with increased AKT activity (87). This suggests that with regard to patient selection, individuals with both VHL and PTEN alterations may more resistant to EGF receptor-targeted therapy, but may benefit from combination therapy targeting both EGF receptor and

mTOR signaling, which is activated downstream of AKT. mTOR inhibitors are being evaluated in the clinic for RCC (see accompanying of chapter this volume) and have shown promise. Unfortunately, combination therapy with EGF receptor-targeted therapy (gefitinib) and an mTOR inhibitor (rapamycin) has shown synergistic growth inhibition in RCC cell lines, but only in the presence of wild-type VHL (46). These preclinical data provide an appropriate cautionary note for investigators who choose to combine targeted agents without a clear understanding of the operative signaling pathways.

ErbB-targeted therapy may also contribute to inhibition of endothelial cell growth and tumor angiogenesis, as VEGF and TGF- α expressed by tumor cells can have paracrine effects on tumor-associated vasculature (88–91). This suggests that tumor-associated endothelial cells may also be targetable by ErbB inhibitors (92–94). Elevated VEGF expression is associated with resistance to targeted EGF receptor therapy in several tumor types (95, 96) suggesting that in clear cell RCC, HIF-dependent VEGF could override the antiangiogenic effect of EGFR inhibition. Therefore, in RCC, combination therapy targeting both ErbB receptors and VEGF receptors may be beneficial. As the randomized study of bevacizumab plus or minus erlotinib in patients with metastatic RCC shows synergy between VEGF and EGFR blockade does not appear to exist, at least using the agents at the chosen dosages (97).

The identification of predictors of response to ErbB-targeted therapy has in many cases been problematic. For trastuzumab, overexpression of HER2 is predictive of response, but for EGFR/ErbB1 targeted therapy, no reliable biomarkers of response have been identified (98–104). While EGF receptor mutations have been shown to predict patient response to gefitinib in NSCLC (105–109), EGF receptor mutations have not been identified in other tumor types that respond to EGFR-targeted therapy (110). In RCC, the only clinical data that provide predictive data come from the randomized study of lapatinib, where patients whose tumors showed high EGFR expression demonstrated a survival benefit if they received lapatinib versus placebo (71).

5 Summary

Targeting the ErbB family in RCC has not enjoyed the clinical success of vascular targeting strategies in clear cell RCC. Preclinical data provide some evidence of ErbB dependence and sensitivity to EGFR modulation in RCC, although inconsistent data arise when agents designed to target the ErbB receptors are used in vitro and in animal models, with some data suggesting an antagonistic effect of VHL mutation on ErbB receptor blockade.

Clinical data consistently demonstrate the lack of efficacy of anti-EGFR monotherapy when administered to a predominantly clear cell RCC population. Even when combined with VEGF blocking agents, no consistent evidence exists that EGFR blocking agents can modulate clear cell RCC biology. The only evidence of

a relationship between biomarker expression and efficacy comes from a subgroup analysis of a randomized study comparing an agent that blocks EGFR and Her2 to placebo in previously treated patients.

Future directions in RCC research with ErbB blocking agents should include investigation of histologies that are not dependent on VHL mutation. Several phase II studies should be reported in the near future shedding light on the clinical outcome of such a strategy.

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Proteasome–NFκB Signaling Pathway: Relevance in RCC

Jorge A. Garcia, Susan A.J. Vaziri, and Ram Ganapathi

Abstract In kidney cancer, the von Hippel–Lindau protein (pVHL) is an integral part of an E3 ubiquitin ligase complex that targets the degradation of hypoxia inducible factor-1 α by the 26 S proteasome under normal oxygen levels. Further, the 26 S proteasome has been shown to play a major physiological role in apoptosis, primarily by regulating the cellular level of p53 and NFκB. Our studies in a panel of wild-type or mutant VHL clear cell renal cell carcinoma (RCC) cell lines demonstrated that VHL status was not associated with cytotoxic response to bortezomib (PS-341) treatment. Cytotoxicity was correlated with downregulation of proteasome activity, survivin expression and induction of p21 expression. Downregulation of p53 expression by siRNA led to attenuation of PS-341 effects, survivin downregulation, and p21 induction, suggesting that cellular effects are p53-dependent. These results suggest that in vitro, the antiproliferative effects of PS-341 in RCC cells are p53-dependent. Despite these data, two multi-institutional phase II studies evaluating the clinical activity and safety of the proteasome inhibitor bortezomib (PS-341) in RCC have failed to demonstrate the clinical utility of this agent in RCC. Since the proteasome pathway remains of importance in RCC biology, future studies understanding the relationship between pVHL, the proteasome complex, and NFκB could be helpful in designing clinical trials pursuing dual inhibition of vascular endothelial growth factor and proteasome signaling pathways.

Keywords Ubiquitin–proteasome system • Proteasome pathway in RCC • Proteasome inhibition

J.A. Garcia (✉)

Departments of Solid Tumor Oncology and Urology, Cleveland Clinic Taussig Cancer Center, Glickman Urological and Kidney Institute, 9500 Euclid Avenue, Cleveland, OH 44195
e-mail: garciaj4@ccf.org

1 Introduction

The ubiquitin–proteasome complex is a multicatalytic protease complex that functions as the principal nonlysosomal proteolytic system in all eukaryotic cells (1). This system is in charge of degrading regulatory proteins and their inhibitors and, thus it is critical for signal transduction, transcriptional regulation, response to stress, and control of receptor function (2–4). Dysregulation of this pathway has been shown to alter immune surveillance and promote tumor progression and drug resistance (5–7). The importance of the ubiquitin–proteasome system in cell regulation is apparent since aberrations of this system are found in malignancies, immune and inflammatory diseases, genetic diseases, and neurodegenerative diseases (8, 9).

Several of the proteins known to be regulated by the proteasome pathway include the inhibitor of nuclear factor κ B (NF κ B; I κ B), the tumor suppressor p53, the cyclin-dependent kinase inhibitors p21 and p27, and the proapoptotic protein Bax. Among them, NF κ B is required to maintain cell viability through the transcription of inhibitors of apoptosis, in response to environmental stress or cytotoxic agents (8, 9). Moreover, NF κ B has also been implicated in controlling the cell surface expression of adhesion molecules involved in tumor progression, metastasis, and angiogenesis (10–12).

Substantial constitutive NF κ B activation has been observed in several renal cell carcinoma (RCC) cell lines and the frequency of constitutive NF κ B activation is significantly greater in locally advanced and metastatic cases compared with localized cases of RCC (13–15). Similarly, the resistance to chemotherapy frequently observed in advanced RCC may indeed be mediated by NF κ B activation which leads to overexpression of genes that augment intracellular glutathione levels that eventually lead to multidrug resistance (16).

To date, several proteasome inhibitors have been developed and tested in the clinic (17). Among those, bortezomib (Velcade; Millennium Pharmaceuticals, Inc., Cambridge, MA; formerly PS-341) is the only available proteasome inhibitor in the market after its recent US Food and Drug Administration (FDA) approval for the treatment of patients with multiple myeloma (MM) (18). In this chapter, we discuss the biological rationale for targeting the proteasome pathway in RCC, preclinical studies on mechanisms that govern the antiproliferative effects of proteasome inhibitors in RCC, and the current status of clinical trials utilizing proteasome inhibitors in this disease.

2 Molecular Aspects of the Proteasome Pathway

The ubiquitin–proteasome system is comprised of ubiquitin, a three-enzyme ubiquitination complex, the intracellular protein ubiquitination targets, and the proteasome that is the organelle of protein degradation. The first step in the pathway is the addition of a small polyubiquitinated tag to proteins destined for destruction. This is a highly regulated process where specific proteins can be targeted for degradation by controlling the affinity of the ubiquitin to a given substrate (19, 20). The second

step is the conjugation of several ubiquitin molecules, a process that is catalyzed by three enzymes that act in concert: E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme), and a substrate-specific E3 (ubiquitin-protein ligase). As seen in Fig. 1, the final step is degradation of identified ubiquitinated proteins by the intracellular proteasome complex. Subsequently, the derived peptides are further degraded into individual amino acids by downstream cytosolic proteases (21).

The 26S proteasome, the site for ATP-dependent degradation of ubiquitin-tagged proteins, is a large organelle composed of two major subunits, the core 20S catalytic complex and the 19S regulatory complex. While the 20S catalytic component contains multiple proteolytic sites with chymotryptic, tryptic, and peptidylglutamyl-like activities, the 19S regulatory component contains multiple ATPases and a binding site for ubiquitin concatemers (22). This unit acts as the proteasome gate keeper, thus limiting the number of tagged proteins entering the system for degradation. In fact, cleavage products in the proteasome complex average six to ten amino acids in length, and eventual hydrolysis to individual amino acids occurs in the cytosol (23).

Dysregulated proteins that are targeted for degradation and potentially important in cancer include p53, p27, cyclins D1, E and B, MHC-1 restricted, IκB, an inhibitor of NFκB, and transcription factors of AP-1 family (24). Among these molecules (Table 1), activation of NFκB by proteolysis represents one of the most

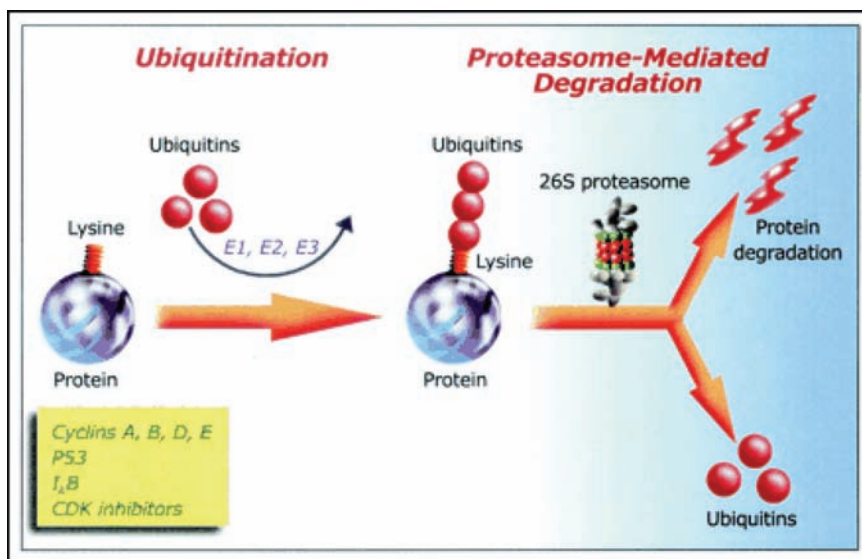


Fig. 1 Ubiquitin–proteasome pathway processing. First is the addition of polyubiquitinated tails to specific lysine moieties on the protein destined for destruction (ubiquitination). This involves three enzymes, ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin ligase (E3). Ubiquitinated proteins are degraded by the intracellular 26S proteasome to short peptides, followed by a release of free and reusable ubiquitin molecules. Adapted from Rajkumar et al. (6)

Table 1 Selected potential targets affected by inhibition of the ubiquitin–proteasome system

Proteins	Effects of proteasome inhibition
Topoisomerase II α	DNA disruption
Cyclins	Induction of apoptosis by overexpression of cyclin A, B, D, and E
Bax	Induction of apoptosis by overcoming Bcl-2 overexpression
p53	Induction of apoptosis by upregulation of p21 and Bax
C-myc, N-myc	Unknown
I κ B	Inhibition of NF κ B activity, resulting in growth inhibition, apoptosis and decrease expression of angiogenic cytokines, and adhesion molecules
Cyclin-dependent kinase inhibitors (p21, p27)	Induction of G1-S cell cycle arrest and apoptosis

critical steps shown to be related with tumor growth. Although initially considered a mediator of immune and inflammatory responses by its ability to induce expression of genes encoding cytokines, cytokine receptors, and cell-adhesion molecules, recent data suggest that NF κ B plays a role in cellular growth, angiogenesis, and migration (25, 26). Several reports in fact have shown overexpression of NF κ B in a variety of tumor cell lines grown both in vitro and in vivo (27–29).

The active form of NF κ B is a heterodimeric molecule consisting of a p65 and a p50 subunit, which is present in the cytosol as an inactive precursor (p105). Activation of NF κ B occurs through cytoplasmic degradation of I κ B coupled with the degradation and direct phosphorylation of the p65–p50 subunit (Fig. 2). It is well established that NF κ B complexes with I κ Bs are transcriptionally inactive in the cytoplasm. The activation of NF κ B allowing its translocation to the nucleus is dependent on the degradation of the inhibitory protein I κ B α by the proteasome following its phosphorylation at serine 32/serine 36 and ubiquitination (30, 31).

Using both in vitro and in vivo models, Orlowski and Baldwin (32) have suggested that while in early oncogenesis, NF κ B protects against transformation-associated apoptosis, in most late-stage tumor cells, NF κ B is clearly not the only survival factor because its inhibition does not induce apoptosis in vitro. Instead, NF κ B can induce transcription of angiogenic genes that mediate metastases and migration (33, 34). Correspondingly, NF κ B can also contribute to cell progression by transcriptionally upregulating cyclin D1 with subsequent hyperphosphorylation of the tumor suppressor protein Rb (35).

3 Importance of the Proteasome–NF κ B Pathway in RCC

Induction of functional defects in host T lymphocytes by tumor is thought to be critical in immune evasion by solid malignancies, especially RCC (36). Thus, the role that NF κ B plays in these subsets of cells has become the major focus of research. In fact, it is well established that defective activation of the transcription factor

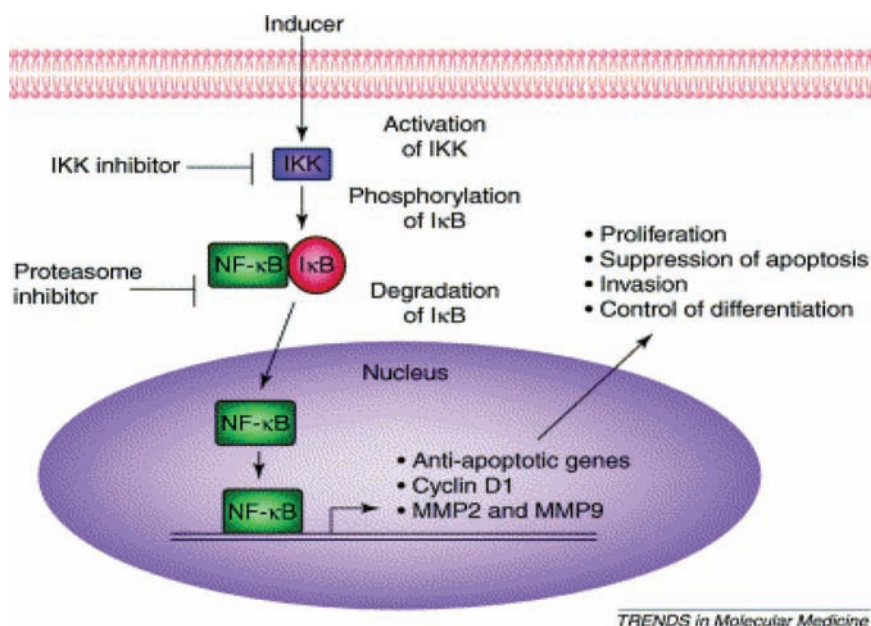


Fig. 2 Proposed model for the intracellular activation of NFκB. Inducers of NFκB activate IκB kinase (*IKK*) leading to phosphorylation of IκB. Phosphorylated IκB is degraded by the ubiquitin–proteasome system (*UPS*) allowing the accumulation of NFκB in the nucleus. In the nucleus, NFκB stimulates the transcription of genes associated with oncogenesis, suppression of apoptosis, angiogenesis, migration, and invasion. IKK inhibitors block NFκB activation through suppression of IκB phosphorylation. Similarly, proteasome inhibitors block IκB degradation. This figure has been reproduced with permission from Orlowski and Baldwin (32)

NFκB occurs in both tumor-bearing mice and patients with RCC (37, 38). Increased activation of NFκB may be responsible for the clonal selection of RCC through the production of inflammatory cytokines, which provide autocrine growth and selective survival. Additionally, inhibition of the constitutive activation of NFκB in RCC cell lines leads to the induction of apoptosis. In T cells, NFκB also appears to induce genes that protect T cells from apoptosis (39). In animal models, tumor progression has been associated with reduced NFκB activation and IκB-dependent gene expression; however, the cause of NFκB suppression in patients with cancer is not known. Using blood samples from T cell-deficient RCC patients, our group has demonstrated the inherited defects in NFκB activation in T cells may be indeed mediated by tumor or tumor-derived soluble products (40). Impaired NFκB activation has also been reported in tumor-infiltrating T lymphocytes, perhaps to a much greater degree than seen in peripheral T cells (41). Studies conducted by our group also suggest that tumor-derived gangliosides are capable of inducing apoptosis via inhibition of NFκB (42). Additional studies have shown that suppression of NFκB activity may also be due to impaired phosphorylation and degradation of its cytoplasmic inhibitor IκB, although the mechanism leading to this alteration was unknown (43).

Other studies have also suggested that the mechanism by which renal tumors inhibit NF κ B activation *in vitro* and *in vivo* is by inducing the degradation of RelA and p50 proteins. The degradation of RelA/p50 results in decreased DNA-binding activity and impaired expression of NF κ B-regulated antiapoptotic proteins. In addition, loss of RelA is a critical event in tumor-induced apoptosis of T cells (44).

In RCC cells lines, von Hippel–Lindau tumor suppressor protein (pVHL) appears to facilitate TNF- α -induced cytotoxicity through downregulation of NF κ B activity and subsequent reduction of the antiapoptotic protein production (45). The association between the pVHL and the RCC has been previously reported and it is reviewed elsewhere (46, 47). In RCC, pVHL normally ubiquitinates hypoxia-inducible factor (HIF) and thereby targets HIF for proteasomal degradation (48). Interestingly, NF κ B stabilizes the level of HIF- α protein (49–51). More relevant to the current working biology in RCC is the ability of NF κ B to induce overexpression of genes regulating the expression of vascular endothelial growth factor (VEGF) (52), urokinase plasminogen activator (53), and multiple cell adhesion molecules such as ICAM-1 (54, 55).

4 Preclinical Models Evaluating Proteasome Inhibition in RCC

Proteasome inhibitors have received considerable attention as tools to characterize cell regulatory events (56–59). The cellular effects of proteasome inhibitors and potential mechanisms involved in promoting apoptosis have been discussed in a number of recent reviews (5–7, 60). Therefore, our review will be restricted to the effects of NF κ B-related activation and potential mechanisms that regulate the induction of apoptosis in clear cell RCC.

Our early studies on proteasome inhibition were focused on inhibitors of topoisomerase (topo) I and II that activate NF κ B in human nonsmall cell lung carcinoma (NSCLC) cells. The rationale for these studies was to exploit the effects of proteasome inhibition on preventing activation of NF κ B and enhance the apoptotic effects of inhibitors. These studies in three separate model systems of human NSCLC suggested that NF κ B-dependent pathways may not be a useful target to manipulate the therapeutic activity of topo inhibitors (61). Specifically, pretreatment with the proteasome inhibitor MG-132 followed by the topo inhibitors SN-38 or etoposide reduced rather than enhanced the apoptosis at 4 h following treatment. Since the treatment with MG-132 and topo inhibitors inhibited and activated NF κ B, respectively, as predicted, the mechanism for this anomalous finding was further analyzed by stably expressing a dominant negative form of I κ B α that prevents activation of NF κ B (62). Unfortunately, this strategy also failed to enhance the apoptotic effects of topo inhibitors, and the conclusion from these studies with further experimentation was that treatment with topo inhibitors followed by the proteasome inhibitor led to maximal effects on promoting apoptosis and reducing cell survival in human NSCLC by mechanism(s) that were independent of NF κ B.

Since the 26S proteasome can play a major physiological role in apoptosis by regulating the cellular level of p53 and other target proteins, including p21 and p27, manipulation of proteasomal activity to affect these targets can be employed to alter apoptosis (58, 63). In our subsequent study, we evaluated the mechanistic basis for the sequence-dependent effect of proteasomal inactivation on DNA damage-induced apoptosis (64). Our results demonstrated the requirement of p53 in potentiation of apoptosis by the proteasome inhibitor, bortezomib (PS-341, Velcade®), following posttreatment with PS-341 after DNA damage induced by SN-38. Under normal physiological conditions, p53 is maintained at low steady-state levels by 26S proteasome degradation following ubiquitination by MDM2, an E3 ubiquitin ligase. Following DNA damage, p53 undergoes stabilization via posttranslational modification and activates a number of different signaling pathways involved in cell cycle arrest, DNA repair, or apoptosis. However, the precise mechanism for p53-mediated apoptosis is poorly defined. Induction of p21 is a key mechanism by which p53 arrests cells in the G₁ and/or G₂ phase of the cell cycle (63). The p53-dependent sensitization of DNA damage-induced apoptosis by posttreatment with PS-341 was accompanied by persistent inhibition of proteasome activity and increased cytosolic accumulation of p53, including higher molecular weight forms likely representing ubiquitinated species. In contrast, pretreatment with PS-341 followed by treatment with SN-38 (PS-341 $\rightarrow$ SN-38), which leads to an antagonistic interaction, results in transient inhibition of proteasome activity and accumulation of significantly lower levels of p53 localized primarily to the nucleus. Whereas cells treated with PS-341 $\rightarrow$ SN-38 undergo G₂ + M cell cycle arrest, cells treated with SN-38 $\rightarrow$ PS-341 exhibit a decreased G₂ + M block with a concomitant increase in the sub-G₁ population. Decreased accumulation of cells in the G₂ + M phase of the cell cycle in SN-38 $\rightarrow$ PS-341-treated cells compared with PS-341 $\rightarrow$ SN-38-treated cells correlated with enhanced apoptosis and reduced expression of two p53-modulated proteins, 14-3-3 σ and survivin, both of which play critical roles in regulating G₂ + M progression and apoptosis. The functional role of 14-3-3 σ or survivin in regulating the divergent function of p53 in response to SN-38 $\rightarrow$ PS-341 and PS-341 $\rightarrow$ SN-38 treatment in inducing apoptosis versus G₂ + M arrest/DNA repair, respectively, was confirmed by targeted downregulation of these proteins. These results provided insights into the mechanisms by which inhibition of proteasome activity modulates DNA damage-induced apoptosis via a p53-dependent pathway.

Since p53 is generally wild type in RCC, we were interested in elucidating the mechanism of action of PS-341 in a panel of RCC lines with and without mutations in the VHL gene (65). Five clear cell renal cancer cell lines were treated with PS-341. CAKI-1 was the only cell line harboring wild-type VHL, three cell lines had mutations in the VHL gene and in one cell line RC-13, the promoter of VHL was methylated. Following treatment with PS-341 for 30 min, cell counts determined 7 days later revealed a wide range of cytotoxic response (Table 2) with no clear pattern of antiproliferative effects based on VHL status, suggesting that VHL may not play a key role in PS-341-mediated apoptosis.

Two lines, RC-13 and RC-26B, both VHL deficient due to promoter methylation and VHL sequence mutation, respectively, were selected for further evaluation

Table 2 Antiproliferative effects of PS-341 in clear cell RCC

		Bortezomib (PS-341)				
		50nM	100nM	250nM	500nM	1,000nM
Cell type	VHL	Cell count (control, %)				
RC-13	MT	100 ± 7	97.4 ± 6	95.2 ± 17.5	48 ± 22.8	10.5 ± 0.2
RC-26B	MT	74.6 ± 17.3	62.5 ± 9.6	21.9 ± 2.3	2.1 ± 0.4	0.1 ± 0.2
RC-28	MT	73.7 ± 13.6	75.3 ± 4.7	40.6 ± 7.5	18.6 ± 14.8	3.8 ± 1.2
CAKI-1	WT	88.0 ± 10.7	76.3 ± 11.7	65.5 ± 18.0	41.2 ± 14.4	16.9 ± 8.3
786-0	MT	84.5 ± 6.0	74.3 ± 11.3	24.8 ± 22.0	10.2 ± 14.0	0.8 ± 1.1

Note: Cell counts were determined 7 days posttreatment

Table 3 Effect of treatment with PS-341 (Bortezomib) on proteasome activity in RC-13 and RC-26B cells

					P-value for time comparisons		
		4h		24h		48h	
	Cell line	N	Mean ± SD	N	Mean ± SD	N	Mean ± SD
100nM	RC-13	2	25.1 ± 1.1	3	67.3 ± 3.7	2	75.7 ± 11.5
	RC-26B	3	26.2 ± 15.5	3	24.1 ± 13.2	3	17.4 ± 12.9
	P-value		0.94		0.009*		0.005*
250nM	RC-13	3	13.1 ± 3.4	3	51.0 ± 7.9	3	67.9 ± 7.0
	RC-26B	3	12.2 ± 12.0	3	9.3 ± 3.7	3	9.2 ± 3.7
	P-value		0.85		<0.001*		<0.001*
500nM	RC-13	3	9.4 ± 7.9	3	28.1 ± 14.9	3	45.8 ± 10.7
	RC-26B	3	18.3 ± 8.0	3	12.9 ± 5.3	3	7.2 ± 6.0
	P-value		0.28		0.09		0.001*

*Significant (*P* < 0.05; not adjusted for multiple comparisons)
Cells were treated with indicated concentrations of PS-341 for 30 min, washed and reincubated in drug-free medium

because they demonstrated the largest differential response to PS-341 treatment in our panel. VHL inactivity in these lines was demonstrated with the lack of pVHL expression and elevated pHIF1α expression under normoxic conditions. As shown in Table 3, the differential response to PS-341 in these cell lines was found to be associated with the duration of proteasome inhibition, that was elicited as a persistent decrease in proteasome activity to 25% of untreated control in RC-26B beginning at 4h posttreatment and continuing out to 48h, while in contrast, RC-13 cells demonstrated an initial similar decrease in proteasome activity at 4h posttreatment, followed by a PS-341 dose-dependent recovery in activity observed at 24 and 48h.

On the basis of the results from other ongoing studies in human NSCLC and colon carcinoma suggesting that downregulation of the antiapoptotic protein survivin, which is regulated by p53 (62), is linked to PS-341-induced apoptosis (64), we subsequently determined protein levels of survivin and p21 in RC-13 and RC-26B cells treated for 30min with 100 or 250nM PS-341 and reincubated in drug-free medium for 24 and 48h. The data in Fig. 3 demonstrate that unlike RC-13 cells, significant downregulation of survivin and upregulation of p21 protein occur

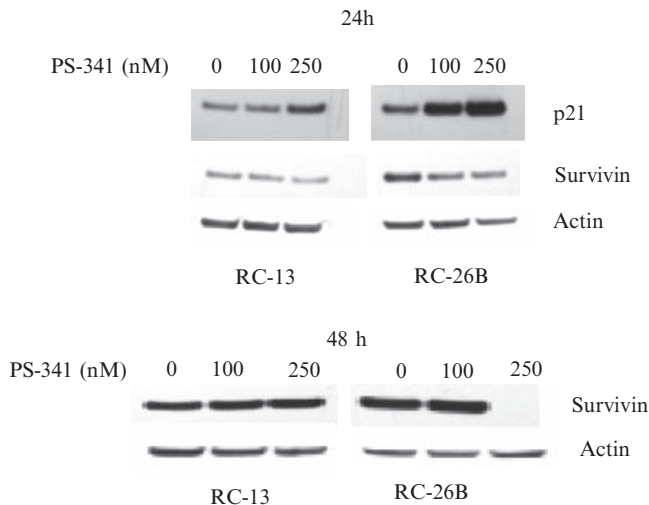


Fig. 3 Differential response of RC-13 and RC-26B cells to PS-341 is dependent on downregulation of survivin protein and induction of p21 protein

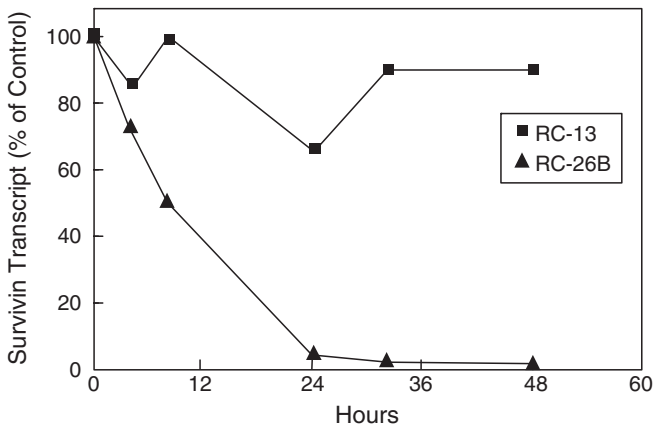


Fig. 4 Differential response of RC-26B versus RC-13 cells to PS-341 is dependent on downregulation of survivin transcript

in RC-26B cells treated with PS-341. The induction of p21 protein was also found to be consistent with the markedly higher accumulation of cells in the $G_2 + M$ fraction in RC-26B (40%) compared with RC-13 (9%) at 24 h following treatment with 250 nM PS-341. Using primers specific for survivin and β_2 -microglobulin as the internal control, the effect of PS-341 treatment on survivin transcript levels by real time PCR (Fig. 4) demonstrates that following PS-341 treatment there is persistent and significant downregulation of survivin transcript levels in RC-26B compared with RC-13 cells.

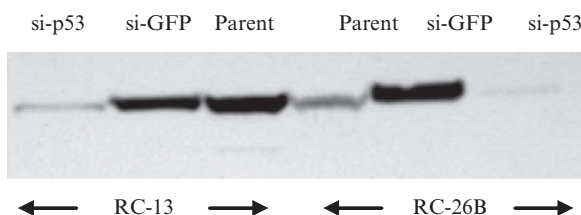


Fig. 5 Immunoblot analysis of p53 protein levels in parent RC-13 and RC-26B cells and in these cells selected for stable expression of control si-RNA (si-GFP) and si-RNA targeted to p53 (si-p53)

Table 4 Effect of downregulation of p53 protein in RC-26B cells on cytotoxic response to PS-341

	RC-26B (si-p53)	RC-26B (si-GFP)	
	Control (%)		
PS-341 (nM)	Cell count	Cell count	<i>P</i> -value
50	100.0 ± 6.6	86.8 ± 4.6	0.04
100	92.8 ± 15.9	58.5 ± 7.4	0.03
250	55.1 ± 22.5	6.7 ± 2.9	0.02
500	6.6 ± 5.2	2.3 ± 2.8	0.28

Cell were treated with indicated concentrations of PS-341 for 30 min, washed, resuspended in drug-free medium, and cell counts determined 7 days later. Data is the mean ± SD from replicate experiments

To test the functional role of p53 in mediating the apoptotic effects of PS-341, RC-26B and RC-13 cells were selected for stable expression of si-RNA directed to p53 (18, 19). Si-RNA directed to green fluorescent protein (GFP) was used as the control. As shown in Fig. 5, p53 protein levels in the si-p53 cells are markedly downregulated compared to si-GFP-transfected (control or parental cells) RC-13 or RC-2GB.

Using these cells, we tested the cytotoxic effects of PS-341, and the data in Table 4 clearly demonstrate that downregulation of p53 protein in the RC-26B (si-p53) cells leads to a 2- to 12-fold decrease in the growth inhibitory effects of PS-341 compared with the 26B-siGFP line, suggesting a functional role for p53. In contrast, the cytotoxic effects did not significantly differ in the RC-13 si-p53 lines compared with the RC-13 si-GFP cells (Table 5).

These results suggest that p53 regulates PS-341-mediated cytotoxicity in clear cell renal carcinoma cells. This is also supported by results from Ling et al. (66) who demonstrated that p53 may be involved in PS-341-induced G₂ + M phase arrest and apoptosis in lung cancer cell lines. Further, results with stable downregulation of p53 in RC-26B and the attenuated PS-341 response, but not in the RC-13 resistant line suggest that p53 may be important in cells that are sensitive to the antiproliferative effects of PSA-341.

Overall, our results demonstrate that p53 is a key player in regulating the anti-proliferative effects of PS-341 in clear cell RCC. The role of p53 appears to be in promoting apoptosis by downregulating expression of survivin.

Table 5 Effect of downregulation of p53 protein in RC-13 cells on cytotoxic response to PS-341

	RC-13 (si-p53)	RC-13 (si-GFP)	
	Control (%)		
PS-341 (nM)	Cell count	Cell count	<i>P</i> -value
50	93.7 ± 9.1	84.7 ± 8.6	0.28
100	80.1 ± 3.2	80.9 ± 9.9	0.91
250	77.4 ± 16.8	37.1 ± 19.5	0.054
500	36.2 ± 32.9	11.2 ± 4.1	0.26

Cell were treated with indicated concentrations of PS-341 for 30 min, washed, resuspended in drug-free medium, and cell counts determined 7 days later. Data is the mean ± SD from replicate experiments

5 Proteasome Inhibition in Advanced RCC: Clinical Data

To date, several reversible and irreversible 20S/26S inhibitors have been developed (67). Among those, bortezomib (Velcade; Millennium Pharmaceuticals, Inc., Cambridge, MA; formerly PS-341) a boronic acid dipeptide, is a unique reversible inhibitor of the proteasome pathway by inhibiting the chymotryptic activity of the proteasome resulting in the attenuation of the degradation of cell cycle regulatory proteins (58). In vitro studies show that the cytotoxic effects of bortezomib are independent of p53 status, and do not overlap with the activity of other cytotoxic agents (58). Various preclinical/animal models suggest that bortezomib’s ability to induce apoptosis is by inhibiting TNF-α-induced NFκB activation through inhibition if IκB phosphorylation and by causing G₂ + M phase arrest with induction of p21 and/or stabilization of p53 in a cell type-specific manner (5, 68).

Two phase I trials evaluated the safety and activity of bortezomib in patients with refractory malignancies. The first trial evaluated 27 patients with advanced refractory hematological malignancies. The maximum-tolerated dose was 1.04 mg/m² given on days 1, 4, 8, 11, 15, 18, 22, and 25 followed by a 2-week rest period (6-week cycle). Among the treated patients, significant clinical activity was observed in those with MM (69). In the trial by Aghajanian et al. (70), bortezomib was studied in 43 patients with advanced solid tumors. In this trial, the maximum-tolerated dose was 1.56 mg/m² and the main dose-limiting toxicities were diarrhea and neuropathy. Dosing and schedule was similar to the other phase I study, with treatments given twice weekly for 2 weeks (on days 1, 4, 8, and 11), followed by 1 week of rest. Subsequent phase II and III studies leading to the regulatory approval by the FDA were conducted in patients with MM. These studies have been well described before and are reviewed elsewhere (6, 18, 71).

Based on the preclinical work demonstrating the importance of the proteasome/ NFκB pathway in RCC, two phase II trials evaluating the activity of bortezomib in advanced cytokine-refractory RCC patients were conducted. In the trial by the

University of Chicago group (72), 21 patients with advanced RCC received 1.5 mg/m² of bortezomib administered intravenously twice weekly for 2 weeks every 21 days. In the absence of grade 3 to 4 toxicities, dose escalation to 1.7 mg/m² was allowed. The primary endpoint of this study was response and safety. Patients were evaluated weekly for toxicity, graded according to the National Cancer Institute Common Toxicity Criteria (73). Disease response was performed every three cycles using Response Evaluation Criteria in Solid Tumors committee (RECIST) criteria (74). To assess proteasome inhibition, investigators also randomly assigned patients to tumor core biopsies either before the first dose or after the third cycle of bortezomib. Only 21 of 23 enrolled patients received therapy. The overall response rate was 5% (95% CI, 0.05–9.5%), with one partial response. An additional six patients (28%) had stable disease for a median of 12 weeks (range, 9–18 weeks). Among all patients, 86% completed three or more cycles of therapy, whereas 14% experienced clinical disease progression before three cycles. The study was discontinued in the first stage after observing one single response. With regards to the correlative studies proposed, inadequate timing and insufficient sample numbers preclude conclusions regarding whether the dose and schedule was sufficient to cause proteasome inhibition within the tumor tissue.

With a similar design, Kondagunta et al. (75) evaluated 37 patients with advanced RCC. Similar to the Chicago experience, almost half of the patients had received no systemic treatment for their disease. Patients received 1.5 mg/m² of bortezomib twice weekly for 2 consecutive weeks followed by a 1-week rest period. The last 12 patients on trial received a lower dose of bortezomib (1.3 mg/m²) due to the fact that more than 50% of patients enrolled on the first stage of the study required dose reductions secondary to toxicities. Of the 37 patients assessable for response, 4 (11%; 95% CI, 3–25%) achieved a RECIST defined partial response where as 14 (38%; 95% CI, 23–55%) had stable disease. The median time to progression was 1.4 months (95% CI, 1.2–3.4 months). The 6-month progression-free survival rate was 24% (95% CI, 11–38%).

Similar to the MM studies, the most common toxicities encountered in the two RCC phase II trails included Grade 1–2 fatigue, sensory neuropathy, nausea, dyspnea, constipation, anemia, thrombocytopenia, and transaminitis. While in the Chicago study, three patients developed grade 4 toxicities (arthralgia, diarrhea, and vomiting), in the study by Kondagunta et al., no grade 4 toxicities were observed.

6 Summary

Despite of the biological rationale for targeting the proteasome pathway in RCC, two well-conducted phase II studies evaluating the activity of bortezomib, a selective reversible inhibitor of the proteasome complex, have failed to demonstrate the clinical utility of this agent in this epithelial neoplasm. Although partial responses were observed in both trials, when compared with other contemporary agents currently available for the treatment of RCC, bortezomib's antitumor activity in RCC

is quite limited. Although this pathway remains of importance in RCC biology, further testing of proteasome inhibitors in RCC should only be pursued in the context of a clinical trial utilizing biological markers capable of predicting response. With the understanding of the relationship between pVHL, the proteasome complex and NFκB, trials pursuing dual inhibition of VEGF and proteasome signaling pathways may be of interest.

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The Role of Hepatocyte Growth Factor Pathway Signaling in Renal Cell Carcinoma

Benedetta Peruzzi, Jean-Baptiste Lattouf, and Donald P. Bottaro

Abstract The urgent need for effective therapies for patients with advanced renal cell carcinoma (RCC), fewer than 20% of whom will survive more than 2 years, has led to the identification of critical genetic determinants and associated molecular pathways contributing to RCC oncogenesis, progression, and spread. Among the signaling pathways dysregulated in RCC is that of hepatocyte growth factor (HGF), which through the cell surface receptor tyrosine kinase, c-Met, stimulates proliferation, motility, and morphogenesis. Germ line missense mutations in the tyrosine kinase domain c-Met are associated with hereditary papillary renal carcinoma (HPRC) type 1, while somatic mutations and frequent trisomy of chromosome 7 implicate pathway involvement in sporadic papillary RCC. In addition, loss of the *VHL* tumor suppressor gene results in the derepression of an embryonic HGF-driven phenotype likely to contribute to tumor invasiveness and metastasis in clear cell RCC. Our knowledge of HGF/c-Met signaling has enabled rapid progress in characterizing its contributions to RCC and in laying the framework for the development of novel anticancer therapeutics. A better understanding of how HGF/c-Met signaling is integrated with other oncogenic pathways in RCC should aid the development of combinatorial treatment strategies, and help predict potential adverse effects of long-term pathway blockade.

Keywords Hepatocytegrowthfactor • c-Met • Renalcellcarcinoma • Oncogenic mechanisms • Cancer drug development

1 Introduction

Over 200,000 cases of kidney cancer are diagnosed each year worldwide, claiming more than 100,000 lives (1). Despite significant advances in the development of immunologic therapies for this disease, there is still no effective therapy for the

D.P. Bottaro (✉)

Urologic Oncology Branch, Center for Cancer Research, National Cancer Institute,
National Institutes of Health, 10 Center Drive MSC 1107, Bethesda, MD 20892
e-mail: dbottaro@helix.nih.gov

majority of patients with advanced renal cell carcinoma (RCC) (1, 2). Four main RCC subtypes with distinct histologies are currently recognized: clear cell, papillary, chromophobe, and oncocytoma. Papillary RCC is further subclassified into types 1 and 2 based on additional clinical, histological, and genetic criteria (2). Rare, inherited forms of RCC exist which characteristically present with one or two of these histological subtypes; the study of these familial diseases has facilitated the identification of their underlying genetic defects, and helped forge mechanistic links with sporadic RCC types with similar histologies (2). For two of the most prevalent RCC subtypes, clear cell and papillary type 1, these mechanistic links strongly implicate the hepatocyte growth factor (HGF) signaling pathway in oncogenesis, tumor progression, and metastasis.

HGF is a plasminogen-like protein with mitogenic, motogenic, and morphogenic activities (3, 4). HGF is typically produced by cells of mesenchymal origin and acts in a paracrine manner on a variety of cellular targets including epithelial and endothelial cells, hematopoietic cells, neurons, melanocytes, as well as hepatocytes (3, 4). HGF is essential for early embryonic development and also contributes to organogenesis in liver, lung, kidney, and other tissues (5). The cell surface receptor for HGF is the transmembrane tyrosine kinase (TK) encoded by the *c-Met* protooncogene (6). The *Met* oncogene was isolated originally from a chemically mutagenized human osteogenic sarcoma cell line; its transforming activity was due to gene rearrangement where sequences from the translocated promoter region (*TPR*) locus on chromosome 1 fused to sequences from the *Met* locus on chromosome 7 (*TPR-Met*) (7). Subsequent isolation of the full-length *c-Met* coding sequence revealed structural features of a membrane spanning receptor TK (7). The identification of HGF as the natural ligand for the *c-Met* protein and the identity of scatter factor and HGF united a collection of findings demonstrating that a single receptor transduced all HGF biological activities (3). Consistent with its relationship with HGF, *c-Met* is widely expressed early in development, deletion of the gene is lethal in mice, and widespread expression persists throughout adulthood (5, 8).

2 The HGF/c-Met Signaling Pathway: An Overview

Upon HGF binding, *c-Met* is autophosphorylated on two tyrosine residues (Y1234 and Y1235) within the activation loop of the TK domain which significantly enhance kinase activity, while phosphorylation on two tyrosine residues near the C-terminus (Y1349 and Y1356) form a multifunctional docking site that recruits a collection of intracellular adapters containing Src homology-2 (SH2) domains and other specific receptor recognition motifs that transmit signals further downstream (7, 9). Among the adapter proteins and direct kinase substrates thus far implicated in *c-Met* signaling are Grb2, Gab1, phosphatidylinositol 3-kinase (PI3K), phospholipase C- γ , Shc, Src, Shp2, Ship1, and STAT3 (7). Gab 1 and Grb2

in particular connect larger networks of adaptor proteins involved in signaling, presumably contributing to HGF pleiotropism (3, 7). The direct binding of Grb2 to c-Met through Y1356 links the receptor to the Ras/MAPK pathway regulating cell cycle progression (3, 7, 9). Gab1–c-Met interactions initiate branching morphogenesis in several epithelial and vascular endothelial cell types. Gab1 is also highly phosphorylated by c-Met, resulting in the recruitment of PI3K, contributing in turn to cell cycle progression, protection from apoptosis, as well as increased cell motility (7–9). Among the many genes upregulated by this pathway is that of the receptor itself (9), creating the potential for c-Met overexpression in otherwise normal target cells through persistent ligand stimulation; in fact, c-Met overexpression is widely observed in cancers of epithelial origin (3).

HGF/c-Met signaling is implicated in a wide variety of human malignancies including colon, gastric, bladder, breast, kidney, liver, lung, head and neck, thyroid and prostate cancers, sarcomas, hematological malignancies, melanoma, and central nervous system tumors (3). Through paracrine signaling, overexpression of ligand and/or receptor, autocrine loop formation, and/or receptor mutation and gene rearrangement, this signaling pathway can enhance tumor cell growth, proliferation, survival, motility, and invasion (3, 7, 9). Inappropriate c-Met signaling in disease can resemble developmental transitions between epithelial and mesenchymal cell types normally regulated by HGF. Importantly, the pathway initiates a program of cell dissociation and increased cell motility coupled with increased protease production that has been shown to promote cellular invasion through extracellular matrices and that closely resembles tumor metastasis in vivo (9). In addition, pathway activation in vascular cells stimulates tumor angiogenesis, facilitating tumor growth for cancers that are growth limited by hypoxia and promoting tumor metastasis (9). Hypoxia alone upregulates c-Met expression and enhances HGF signaling in cultured cells and mouse tumor models (9).

3 The HGF/c-Met Signaling Pathway in Kidney

3.1 HGF Signaling in Kidney Development

The critical roles of HGF and c-Met in embryonic development were first demonstrated in mice by targeted disruption of each gene; these animals displayed placental defects, defective somite migration, stunted liver and limb muscle development, and death in utero (5, 10). HGF promotes the development of tubular structures in organs such as mammary gland and kidney (11). Proper kidney development depends on the multicellular process of branching morphogenesis. During the metanephric phase of kidney development, nephrogenesis is initiated by ingrowth of the Wolffian duct-derived ureteric bud into the presumptive kidney mesenchyme (11, 12). In response to signals from the ureter, mesenchymal cells condense, aggregate into pretubular clusters, and undergo an epithelial conversion generating a simple tubule.

This tubule then undergoes morphogenesis and is transformed into the excretory system of the kidney. The nephron epithelial tube gives rise to the branched collecting duct system, while the surrounding metanephric mesenchyme undergoes mesenchymal–epithelial transition to form the proximal parts of the nephron (11, 12). The coordinated exchange of signals in both directions between the growing buds of the epithelium and the mesenchyme that they are invading is critical. Several soluble factors act in a complementary fashion either as pro- or antitubulogenic regulators, including members of the fibroblast growth factor, transforming growth factor- β , and Wnt families as well as glial-derived neurotrophic factor, epidermal growth factor, and HGF (11, 12).

The HGF-driven intracellular signaling events in mesenchymal–epithelial transitions during nephrogenesis presumably resemble those defined using cultured renal epithelial cell models of branching morphogenesis. In that context, the recruitment of Gab1 and Grb2 to c-Met activates SOS1, contributing to Ras–MAP kinase pathway activation, adherens junction disassembly, cell motility, and proliferation (13). Reorganization of the actin cytoskeleton, which is required for observed cell shape changes, is regulated by the Rho family of small GTPases, activated downstream of PI3K and Ras (13, 14). Rac1 and cdc42 regulate actin polymerization at the cell periphery resulting in the extension of lamellipodia that are essential for cell migration and filopodia that precede de novo tubulogenesis in vitro (13, 14). In contrast, RhoA acting via its downstream effector Rho-associated kinase stimulates myosin light chain phosphorylation and regulates actin stress fiber formation and cell contractility (14). Thus, a coordinated activation and deactivation of Rac and Rho is required for cell shape change and migration (11, 13, 14). HGF stimulation also results in the tyrosyl phosphorylation of β -catenin, inducing its dissociation from E-cadherin in adherens junctions, contributing to junction breakdown and freeing β -catenin for nuclear translocation and transcriptional activation (8).

3.2 HGF Signaling in Renal Homeostasis

HGF and c-Met expression persist in the adult kidney, but striking changes occur in the quality and magnitude of the response of renal epithelial cells to HGF stimulation on completion of normal development. Morphogenic and proliferative responses are minimized. While the role of HGF in adult renal physiology is not yet fully understood, the kidney is an important source of circulating HGF in adults, and HGF is an endogenous renoprotective factor with potent antifibrotic activity (15, 16). HGF has been shown to protect adult kidney tissue from acute toxicity and ischemic stress (15). Endogenous HGF levels are elevated in kidneys exposed to long-term stress, and HGF counteracts TGF- β signaling associated with renal fibrosis, a major cause of chronic renal failure (15–17). At the cellular level, these tissue protective effects are most likely to be mediated through HGF-driven cell survival pathways and pathways that control extracellular matrix composition and turnover (15–17).

3.3 *Dysregulated HGF Signaling in RCC*

Most of the intracellular mediators and pathways activated by c-Met persist through development into adulthood, and it is unclear which signals are modified or silenced to provide a homeostatic, as opposed to developmental or pathological, HGF response. Given the functional similarities between tumorigenesis and epithelial–mesenchymal transitions at the cellular level, the loss of such signal attenuation mechanisms are likely to contribute to tumorigenesis, invasiveness, and metastasis. Among the four main RCC subtypes, an oncogenic role of HGF/c-Met signaling has been firmly established for hereditary papillary renal carcinoma (HPRC) type 1, where inherited missense mutations in the *Met* gene were first found; similar somatic mutations were also found in a small subset (13%) of sporadic PRC tumor samples (18–21). The biochemical and biological impact of these *Met* mutants have been investigated in several model systems, confirming their suspected oncogenic potential, as described in greater detail below (22–28). A growing body of evidence also supports HGF/c-Met pathway involvement in clear cell RCC, where loss of *von Hippel–Lindau* (*VHL*) tumor suppressor gene function occurs in familial and most sporadic cases (2). *VHL* loss results in the aberrant expression of genes that control cell proliferation, invasion, and angiogenesis (2).

3.3.1 HGF/c-Met Pathway Activation in HPRC Type 1 and Sporadic PRC

Several missense mutations in *Met* have been identified in individuals with PRC, HPRC type 1, in other human cancers, as well as in cancer cell lines (21). Schmidt et al. first reported nucleotide changes in exons 17, 18, and 19 in the germ lines of HPRC families and also in a subset of sporadic PRCs (18). Five germ line mutations and four somatic mutations were localized to the c-Met TK domain (18). Of the five germ line mutations found, D1246H and D1246N were located in the codon homologous to a naturally occurring mutation in c-kit, which is responsible for systemic mastocytosis in humans (18). Another mutation, M1268T, was homologous in position and residue change to the human *Ret* proto-oncogene codon mutated in multiple endocrine neoplasia (MEN) type 2B and sporadic medullary carcinoma of the thyroid gland (18). Later studies revealed a germ line mutation in exon 16 of (H1112R), which significantly enhanced focus formation when ectopically expressed in NIH3T3 cells, and V1110I, a mutation also found in the homologous codon (V157I) of chicken *c-erbB*, where it triggers the sarcomagenic potential of the *v-erbB* oncogene (19, 20, 29).

The biochemical and biological impact of these MET mutants were first investigated in NIH3T3 cell transfectants (22, 23, 30). Mutant c-Met receptors displayed increased levels of tyrosyl autophosphorylation relative to wild-type (WT) receptors, as well as greater TK activity toward an exogenous substrate (22, 30). Cells expressing mutant receptors acquired focus-forming activity in monolayer

culture, and the ability to form tumors in athymic nude mice, in contrast to weak tumorigenicity displayed by WT c-Met in the same context (22, 23, 30). Mutant receptors showed increased cell motility relative to WT, as well as increased intracellular activation of the Ras-Raf-MEK-ERK signaling pathway (23, 24, 30). Transgenic mice harboring the PRC mutant c-Met constructs under the control of a metallothionein promoter developed metastatic mammary carcinoma, confirming that these *Met* mutations were oncogenic (22, 23, 30).

Later analysis of an extended panel of tumor samples included the complete sequencing of exons 5 and 7 in the extracellular domain, exon 13 encoding the transmembrane domain, and exons 14–20 encoding the bulk of the intracellular portion of the receptor (19). These studies showed that *Met* mutations occur in only a small proportion (13%) of sporadic PRC, which is noteworthy in light of prior reports of highly frequent (95%) trisomy of chromosome 7 in this disease (31). A detailed study of trisomy 7 in HPRC showed that duplication of the mutant *Met* allele occurred in all of the 16 tumor samples analyzed, suggesting that having two copies of the mutant allele conferred a proliferative advantage to the affected tumor cells (32). While this potential mechanism of selective overexpression of mutant c-Met can be viewed as providing a “second hit” leading to tumorigenesis, the prevalence of trisomy 7 in sporadic PRC indicates that most PRC tumors display trisomy 7 in the absence of *Met* mutations (31, 32). Whether the potentially increased dose of *Met* and/or HGF genes, both located on chromosome 7, confers a selective advantage in the absence of mutation is an intriguing hypothesis that warrants further investigation.

Several studies have addressed in detail the mechanisms by which PRC-associated c-Met mutations act at the cellular and molecular levels. Bardelli et al. showed that the M1268T mutation changed substrate preference in vitro, using a panel of peptides differentially phosphorylated by epidermal growth factor receptor (EGFR), Src, or Abl; M1268T acquired a preference similar to that displayed by the homologous *Ret* mutation characteristic of MEN 2B (24). When expressed in NIH3T3 cells, the mutations Y1248H, D1246H/N, and M1268T showed constitutive association with the key intracellular effector Gab1 (24). Similar to signaling by WT c-Met, the link to Gab1 and other effectors required phosphorylation of the C-terminal docking sites, as did other indices of cell transformation such as growth in soft agar (24). Thus, the oncogenicity of c-Met mutants is mediated by many of the same receptor-proximal intracellular effectors involved in WT c-Met signaling, suggesting that interruption of key receptor–effector interactions at the C-terminal docking sites remains a viable strategy for blocking mutant c-Met signaling (24).

Building upon prior studies, Giordano et al. hypothesized that different mutations may contribute to disease pathogenesis through distinct molecular pathways downstream of c-Met (25). When ectopically expressed in NIH3T3 cells or the murine liver oval cell line MLP 29, the *Met* PRC mutants fell into two functional groups: M1268T and D1246H possessed enhanced receptor kinase activity, stimulated increased Ras pathway activation, and transformed cells in focus formation assays (25). Mutations L1213V and Y1248C, in contrast, displayed lower kinase activity, Ras pathway activation, and focus-forming ability, but were more effective

in PI3K pathway activation, protecting cells from apoptosis, sustaining soft agar colony formation, and promoting matrix invasion (25). All of these effects were enhanced on addition of HGF (25).

The role of ligand binding in the oncogenic potential of PRC-associated c-Met mutations was investigated further by Michieli et al. using cultured epithelial cells, which typically do not express HGF (26). c-Met mutants reconstituted in MDCK epithelial cells required exogenously added ligand for colony formation in soft agar (26). c-Met mutations reconstituted in truncated receptor constructs lacking most of the extracellular domain failed to induce focus formation, and M1268T reconstituted in this context was transforming only on addition of a receptor-ligating monoclonal antibody (26). Soft agar colony formation by NIH3T3 cells bearing c-Met M1268T could be blocked by coexpression of a soluble c-Met extracellular domain, an uncleavable form of HGF, or the HGF competitive antagonist HGF/NK4 (26). Together these results revealed that ligand binding may contribute significantly to oncogenesis associated with PRC *Met* mutations. Ligand dependence may explain why patients with germ line *Met* mutations exhibit only kidney cancer, as the kidney is an abundant source of HGF, as well as urokinase, an important activator of immature HGF. Michieli et al. speculated that the long-term combination of ligand, ligand activator, and highly responsive target cells may render these otherwise benign receptor mutations “regionally” oncogenic (26).

In the first study designed to predict how PRC-associated c-Met mutations might alter catalytic function, Miller et al. aligned the TK domain of c-Met with that of the insulin receptor by computer modeling (27). The results showed that certain HPRC type 1 mutations could disrupt the normal mechanism of TK autoinhibition, thereby stabilizing the active form of the receptor (27). In the unphosphorylated form of the WT receptor, residues in the activation loop of the TK domain normally block access to ATP and to peptide substrates, while phosphorylation of specific tyrosine residues leads to stabilization of the open, active conformation (27). Notably, M1268T and Y1248C/D/H were predicted to stabilize the open, active TK conformation. Mutation of Y1248 to the more hydrophilic residues C, D, or H might also stabilize the active TK conformation by rendering the site resistant to phosphatase action (27). Overall, these findings predicted that mutant c-Met forms are more easily activated than WT c-Met, and more likely to remain active, but did not that clearly eliminate the need for an initiator of kinase activation such as ligand binding or other environmental cue.

In a study complementary to that of Miller et al., Chiara et al. later compared the autophosphorylation events in WT and mutant c-Met receptors expressed in cultured cells using phosphorylation-site-specific antibodies, and proposed that mutant receptors possessed a lower threshold for kinase activation (28). HGF binding to WT c-Met triggers autophosphorylation of Y1235 and Y1234 in the TK activation loop; substitution of F for Y at either position severely impairs kinase function, suggesting that phosphorylation at both sites is required for kinase activation (28). Unlike WT c-Met, the D1246H/N and M1268T c-Met mutants did not undergo Y1234 phosphorylation, and were not catalytically impaired by F substitutions at that site (28). Thus, these mutants were not constitutively active, but mutation

overcame the normal requirement for a second phosphorylation step leading to kinase activation (28). Importantly, the apparent need for ligand activation of HPRC and PRC associated c-Met mutant forms suggests that therapeutic strategies aimed at ligand blockade remain viable possibilities for these patient populations.

3.3.2 HGF/c-Met Signaling in Clear Cell RCC

VHL syndrome is an autosomal dominant hereditary neoplastic disorder (3, 33, 34). VHL-associated clear cell RCC tumors are malignant and frequently metastatic (3, 34). Defects in the *VHL* tumor suppressor gene, which is located on the short arm of chromosome 3 (3p25-26), lead to VHL syndrome and also occur in the majority of sporadic clear cell RCC cases (3, 35). Reconstitution of WT *VHL* expression in RCC-derived cells regulates tumorigenesis in mice, confirming a critical role for *VHL* in RCC (36). The VHL protein (pVHL) is part of an E3 ubiquitin ligase complex that targets hypoxia-inducible factors for polyubiquitination and proteosomal degradation (37). During hypoxia or when pVHL function is lost, hypoxia-inducible factors accumulate and cause broad changes in gene expression that are potentially important in oncogenesis (34, 37, 38). Cultured *VHL*-negative RCC cells also acquire an abnormal response to HGF, manifested as matrix degradation, increased cell motility, matrix invasion, and morphogenesis (39). These HGF-driven activities are abolished when WT *VHL* expression is reconstituted in RCC cells, directly linking loss of *VHL* function to an invasive tumor phenotype (39).

Investigating the molecular mechanism by which RCC cells acquire an invasive response to HGF, Peruzzi et al. hypothesized that *VHL* loss in clear cell RCC might promote oncogenic signaling downstream of c-Met (40). Among the known intracellular mediators of HGF signaling with oncogenic potential is β -catenin, which links cadherins to the actin cytoskeleton and also functions as a gene transactivator (41–44). β -catenin and E-cadherin are initially expressed during renal development, specifically upon transition of the mesenchyme surrounding the branching ureteric buds to the epithelium that will form the tubules of the nephron (45). As described above, this mesenchymal to epithelial transition and ensuing tubule formation involves several Wnt family members acting in an autocrine manner (12, 46), as well as HGF acting in a paracrine mode (47). Dysregulated β -catenin signaling in the adult is potently oncogenic: mutations in the genes encoding APC or β -catenin are frequent in colorectal cancer (48). Both types of mutation allow β -catenin to bypass APC-mediated ubiquitination and proteosomal degradation and it is now known that cytoplasmic β -catenin can be stabilized by a variety of genetic defects (48).

Using several RCC cell models, Peruzzi et al. found that HGF stimulated β -catenin tyrosyl phosphorylation, adherens junction disruption, cytoplasmic β -catenin accumulation, and reporter gene transactivation (40). These activities were repressed when *VHL* expression was reconstituted ectopically (40). Expression of a ubiquitination-resistant β -catenin mutant specifically restored HGF-stimulated invasion and morphogenesis in *VHL*-transfected RCC cells, while *VHL* gene silencing in non-RCC renal epithelial cells phenotypically mimicked *VHL* loss in RCC (40). Finally,

HGF-driven invasiveness was blocked by the expression of a dominant negative mutant of Tcf, reinforcing the conclusion that in RCC cells, *VHL* loss enables HGF-driven oncogenic β -catenin signaling (40). These findings identify β -catenin as a potential target for drug development for *VHL*-negative clear cell RCC.

Independent of its role in HGF/c-Met signaling, the widespread involvement of β -catenin in human cancers has prompted several other investigations of its potential dysregulation in RCC. Initial studies suggest that activating mutations in *β -catenin* are rare in RCC tumors (49–51), and no inactivating mutations in *APC* or *axin* have been reported in RCC (52, 53). Oncogenic β -catenin signaling can also be initiated through aberrant Wnt stimulation or loss of negative repressors of Wnt signaling, such as members of the Dkk family. A recent study demonstrated the striking downregulation of REIC/Dkk-3 in 15 of 17 (88%) RCC tumor samples (54). The persistent expression of Wnt family members from kidney development through adulthood (5, 12, 55) suggests that loss of this potential Wnt inhibitor combined with the frequent loss of *VHL* function in clear cell RCC could contribute significantly to tumorigenesis, invasion, and metastasis.

4 Cancer Drug Development: Targeting the HGF/c-Met Pathway

Our present understanding of oncogenesis mediated by c-Met signaling supports at least three avenues of therapeutic development: antagonism of ligand–receptor interaction, inhibition of TK catalytic activity, and blockade of receptor–effector interactions (56). In addition, combinations of conventional and c-Met targeted therapies may offer promise for specific cancers (57).

Antagonism of ligand binding is a logical therapeutic strategy for a majority of carcinomas where paracrine HGF signaling and c-Met overexpression result in aberrant pathway activation, including PRC and clear cell RCC. A collection of structure–function studies, including the early discovery that a naturally occurring truncated HGF variant, HGF/NK2, was a specific competitive mitogenic antagonist, led the development of HGF/NK4, a larger, more antagonistic HGF fragment (58), and to an uncleavable form of pro-HGF (59), both of which block tumor growth and metastasis in animal models. Similarly, the early development of a c-Met ectodomain–IgG fusion protein with HGF–neutralizing activity preceded the engineering of a soluble c-Met ectodomain fragments with pathway neutralizing and antitumor activities (60, 61). Neutralizing mouse monoclonal antibodies against human HGF have also been shown to be effective antitumor agents in animal models (62–64). A fully human monoclonal antibody with HGF–neutralizing and antitumor properties is now in human clinical trials (65).

Recent successes in the treatment of cancers using TK inhibitors strongly support the potential efficacy this therapeutic strategy for targeting c-Met in RCC. Early work with the staurosporine-like alkaloid K252a showed that it could inhibit c-Met autophosphorylation, MAPK and Akt activation, and revert the transforming

potential of the *TPR-Met* oncogene (66). More selective and potent synthetic inhibitors of c-Met ATP binding have been developed and tested in various model systems (67–74). Of these, the compounds SU11274 and PHA665752 displayed a minimum of 50-fold selectivity for c-Met relative to several other TKs, potentially blocked HGF-stimulated activities in cultured cells, and tumorigenicity in c-Met-driven xenograft models (71). Analysis of SU11274 using cells expressing HPRC-associated *Met* mutants revealed interesting differences in sensitivity (70), and gastric cancer cells with *Met* gene amplification displayed significantly increased sensitivity to PHA665752 (74), suggesting that knowledge of genetic alterations should help predict the efficacy of c-Met TK inhibitors for specific patient groups.

The requirement of the C-terminal docking site for WT or mutant c-Met transforming activity in cultured cells (24, 25) and the known roles of intracellular effectors including Gab1, PI3K, Grb2, Shc, and STAT3 in cell transformation (4, 7) suggest that targeting one or more of these interactions could effectively disrupt c-Met-driven oncogenesis. Knowledge of the unique structure of the Grb2 SH2 domain provided the basis for the development of small synthetic Grb2 selective binding antagonists (75). Further refinement of these early structures has yielded compounds that block HGF-stimulated cell motility, matrix invasion, and morphogenesis in normal and tumor-derived cultured cells, as well as vascular endothelial cells, at low nanomolar concentrations (76). Beyond effector targeting, compounds that block HSP90–client interactions, such as geldanamycin (77), also potentially block c-Met oncogenic signaling (78, 79). Human clinical trials of geldanamycin-related compounds are under way for a variety of cancers where the c-Met pathway is active, including RCC.

While the potential efficacy of HGF/c-Met targeted drugs for treating subtypes of RCC as single agents is promising, combining agents such as geldanamycin that attenuate receptor supply with inhibitors of other critical receptor functions could lower the effective dose of each, reducing drug toxicity as well as the emergence of drug resistant mutations. Improving our understanding of the molecular basis of oncogenic HGF/c-Met signaling in RCC should facilitate the development of other combinatorial treatment strategies, and help overcome other challenges facing drug development, such as identifying patients most likely to benefit from HGF/c-Met targeted therapeutics, assessing drug activities in tumor tissues, and predicting the potential toxicity of long-term pathway blockade.

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Smac/DIABLO: A Proapoptotic Molecular Target in Renal Cell Cancer

Yoichi Mizutani, Akihiro Kawauchi, Benjamin Bonavida,
and Tsuneharu Miki

Abstract Renal cell carcinoma (RCC) accounts for about 2% of all cancer cases worldwide. Metastatic disease is often present at time of diagnosis of RCC, and its poor prognosis is determined by its poor response to chemotherapy and radiotherapy. Immunotherapy including interleukin-2 and interferon- α is relatively effective against metastatic RCC; however, the response rate is 15–20%. Chemotherapy, immunotherapy and radiotherapy have been shown to mediate their cytotoxic and antitumor effects by inducing cell death by apoptosis. The poor response of RCC to conventional therapies may be due in large part to the development of resistance to cell death by apoptosis through the modification of apoptosis regulatory gene products. Such genes and their products may also have important utility as diagnostic/prognostic markers and therapeutic targets in this disease. However, few genes of this nature have been found to date in RCC.

This chapter introduces second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI (Smac/DIABLO), a gene product of significant prognostic and therapeutic significance in RCC. Smac/DIABLO was recently identified as a protein that is released from mitochondria in response to apoptotic stimuli and promotes apoptosis by antagonizing inhibitor of apoptosis proteins. Smac/DIABLO is a new biomarker for RCC. The level of expression of Smac/DIABLO and its biological activity determine in large part the pathogenesis and fate of RCC and its response to antitumor cytotoxic agents, including chemotherapy, immunotherapy, etc. In addition, Smac/DIABLO offers a promising target for therapy by agents that regulate its expression and stabilization. Such agents, when used in combination with subtoxic doses of chemotherapy and/or immunotherapy, may be very effective in killing the resistant RCC cells and improve survival. Thus, the level of expression of Smac/DIABLO in RCC has implications in the pathogenesis, diagnosis, and the development of new treatment modalities.

Y. Mizutani (✉)

Department of Urology, Kyoto Prefectural University of Medicine,
Kawaramachi-Hirokoji, Kamigyo-ku, Kyoto 602-8566, Japan
e-mail: ymizutan@koto.kpu-m.ac.jp

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1 Introduction

Cytotoxic chemotherapy, an integral part of the therapeutic approach for a lot of solid and non-solid tumors, has shown little or no antitumor activity against renal cell carcinoma (RCC) and has played no role in either an adjuvant or a neo-adjuvant support therapy (1). Immunotherapy, including interleukin-2 and interferon- α , is relatively effective against metastatic RCC and the overall response rate of immunotherapy and/or chemotherapy has gradually increased. However, the response rate is 15–20% and metastases and recurrences of RCC remain major problems in the treatment of RCC (2). Therefore, new therapeutic approaches are urgently needed for the treatment of RCC patients.

Historically and currently, clinical factors have been used as prognostic markers for patients with RCC. Recent advances have been achieved in an attempt to understand the pathogenesis, behavior and molecular biology of RCC with the objective of developing methods for early diagnosis, better prognosis and improved survival for RCC patients. Although numerous factors have been studied, few of them maintained independent significance in terms of overall survival. Moreover, several reported results are controversial from one report to another. There are no known molecular or cytogenetic tumor markers identified that can help diagnosis, manage or confirm RCC remission, progression or relapse. For example, nuclear morphometry was studied using parameters of size, shape and chromatin structure in nuclei. For some authors, nuclear morphometry is an independent prognostic factor (3, 4), whereas for others, none of these provided independent prognostic information (5). Proliferation cell nuclear antigen (PCNA) is reported by some, whereby a low PCNA index (less than 10%) is an independent, positive predictor of disease and free survival, but not overall survival (6), whereas for others, it is associated with good survival (7, 8). Ki-67 identifies an antigen detected in G1 phase, increases during S phase and rapidly decreases after mitosis. The Ki-67 index is correlated with the histological grade and stage (5, 9). The expression of multi-drug resistance 1 (MDR-1) is low in RCC compared to normal kidney tissue and has been associated with short disease free survival and poor overall survival (10). For some authors, MDR-1 expression showed no predictive value (11). The tumor suppressor gene p53 and mutations of p53 have been associated with prognosis (12), while for others, p53 mutations carried no predictive value (13). Angiogenesis has been considered by some as a highly predictive prognostic factor (14), and for others, it does not demonstrate an independent prognostic factor (13, 15). The contribution of aneuploid DNA content as a prognostic factor continues to be debated (16). Thus, diagnostic/prognostic markers and therapeutic targets in RCC are necessary.

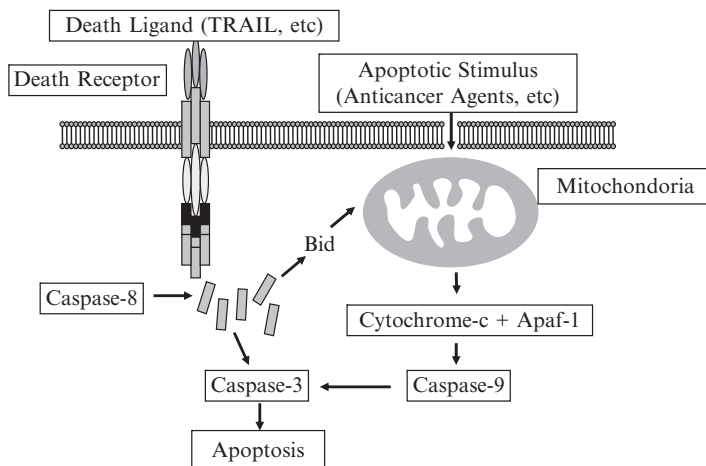


Fig. 1 Apoptosis signaling pathway

2 Smac/DIABLO in the Apoptotic Pathway

Recent advances in apoptosis have revealed two major apoptotic signaling pathways (Fig. 1). One is initiated from the death receptors [example: tumor necrosis factor receptor 1 (TNFR1), Fas, death receptor 4 (DR4) and DR5] and the other via the activation of the mitochondria and release of factors such as cytochrome c, inducer of apoptosis factor (IAF), endonuclease G, mitochondrial apoptotic serine protease (OMI) and second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI (Smac/DIABLO). The activation of caspases are required for the induction of final apoptotic phenotype. There is also a caspase-independent apoptotic pathway induced by IAF. The mitochondria are present in almost all eukaryote species. Besides being guardians of survival, mitochondria also harbor toxic molecules in the inter-membrane spaces (17). These death-inducing proteins, although encoded in the nucleus of eukaryotic cells, are stored in the mitochondria. Upon apoptotic stimulation (example: drugs, stress, cytotoxic cells and antibodies), several mitochondria proteins are released, such as cytochrome c, Smac/DIABLO and OMI that contribute to the apoptotic stimulus. Cytochrome c serves as the apoptotic inducer, whereas Smac/DIABLO neutralizes the inhibition of the activation of caspase 3 and 9 (18). The presence of cytochrome c in the cytoplasm leads to the formation of an intracellular death-inducing complex named apoptosome. An apoptosome is a protein complex consisting of cytochrome c, apoptotic protease activation factor 1 (Apaf-1), pro-caspase 9 and other regulatory proteins such as inhibitor of apoptosis proteins (IAPs). Cytochrome c activates caspase 9 in the apoptosome and leads to activation of caspases 3, 7, 8 and downstream activation of cell death. Smac/DIABLO and OMI function as inhibitors of IAPs, a family of cellular caspase inhibitors. However, Smac/DIABLO and OMI

also provide caspase-independent cell death by unknown mechanisms. The regulation of caspases occurs at the level of their activation. Members of the IAP family counteract both the activation and the activity of caspases. IAPs are characterized by the presence of one or more baculovirus IAP repeats (BIR) of BIR domains, of about 70–100 amino acids and contain a C-terminal RING domain. The RING domain of IAPs displays ubiquitin ligase activity which allows its target proteins for proteasomal degradation. Target proteins include caspase 3, Smac/DIABLO and IAPs themselves. Inhibition of caspases by IAPs is thought to be through BIR domains (Figs. 2 and 3). The antiapoptotic activity of IAPs is counteracted by proapoptotic IAP-binding proteins, namely Smac/DIABLO and OMI. These two proteins share binding for residues IAP-binding motif at their N-end terminals.

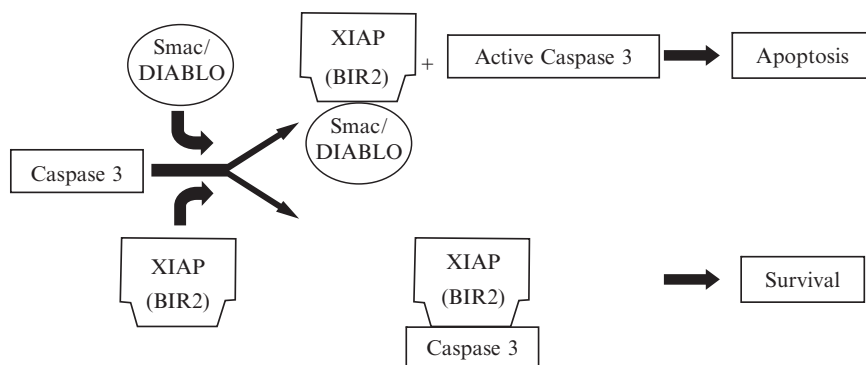


Fig. 2 Activation and inhibition of caspase 3 by second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI (*Smac/DIABLO*) and X-linked inhibitor of apoptosis protein (*XIAP*)

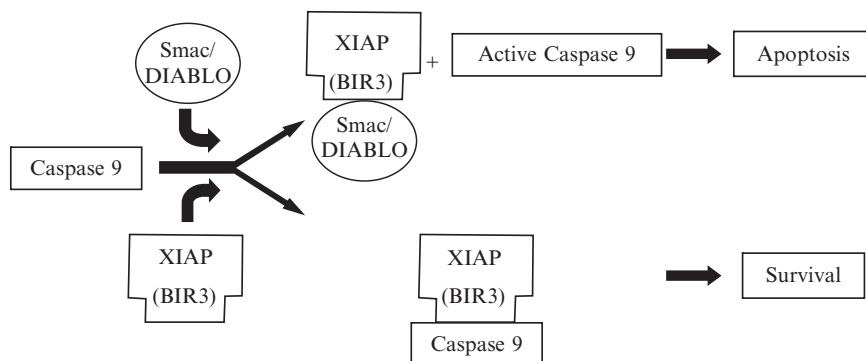


Fig. 3 Activation and inhibition of caspase 9 by second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI (*Smac/DIABLO*) and X-linked inhibitor of apoptosis protein (*XIAP*)

Table 1 Expression of Smac/DIABLO in normal tissues

High Smac/DIABLO expression	Low Smac/DIABLO expression
Heart	Brain
Liver	Lung
Spleen	Thymus
Pancreas	Small intestine
Kidney	Colon
Prostate	Leukocyte
Testis	Skeletal muscle
Ovary	Placenta

Smac/DIABLO second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pl

Smac/DIABLO was identified as a protein that can stimulate caspase 3 activation in cell extract (19). DIABLO was described independently by Verhagen AM, et al. (20). Smac/DIABLO is expressed in most adult human tissues, including kidney, prostate and testis, at different levels (Table 1). It is synthesized in the nucleus as a precursor protein of 239 amino acid residues and amino terminal mitochondrial leader sequence (MLS). Upon mitochondrial import, the MLS is removed by proteolysis, exposing the IAP-binding motif (IBM) at the N-terminus of mature Smac/DIABLO, which is present as dimers. Through this IBM (Ala-Val-Pro-Ile), Smac/DIABLO is able to bind X-linked IAP (XIAP), cIAP-1 and cIAP-2, and survivin. One way by which Smac/DIABLO promotes caspase activation is by displacing caspase 9 coupled with XIAP (Fig. 3). This inhibitory function of Smac/DIABLO relies on IBM. However, IAPs are able to fight back Smac/DIABLO. The RING domain present in IAPs possesses ubiquitin ligase activity and may contribute to the antiapoptotic function of IAPs by targeting Smac/DIABLO for proteasome-mediated degradation.

Smac 3, a Smac-spliced variant, contains an IBM and is released in the cytosol (21). Smac β , a sliced variant of Smac, lacks the mitochondrial targeting sequence and does not bind IAP yet sensitizes to apoptosis (22).

3 Smac/DIABLO Expression as a Prognostic Marker for RCC (23)

Smac/DIABLO expression was detected in all normal kidney specimens (Fig. 4). Smac/DIABLO is poorly expressed in RCC compared to normal kidney (Fig. 5). Smac/DIABLO expression in clear cell RCC was similar to that in papillary RCC (Fig. 6). The level of Smac/DIABLO expression in RCC compared to normal kidney was low (Fig. 7). Smac/DIABLO expression inversely correlated with the stage progression and the increase of the histologic grade of RCC (Figs. 5 and 7). A recent study by Yoo et al. (24) has reported analysis of archival tissues of carcinoma and sarcoma by immunohistochemical analysis for the expression of Smac/DIABLO. Smac/DIABLO

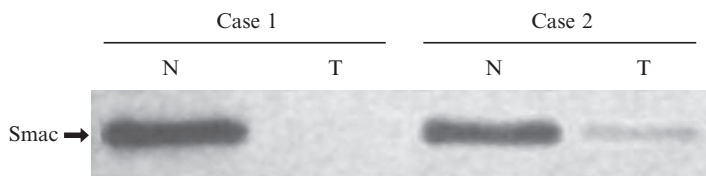


Fig. 4 Expression of second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI (*Smac/DIABLO*) in renal cell carcinoma (RCC) and the normal kidney. Expression of Smac/DIABLO was measured by western blotting. *N* normal kidney, *T* renal cell carcinoma

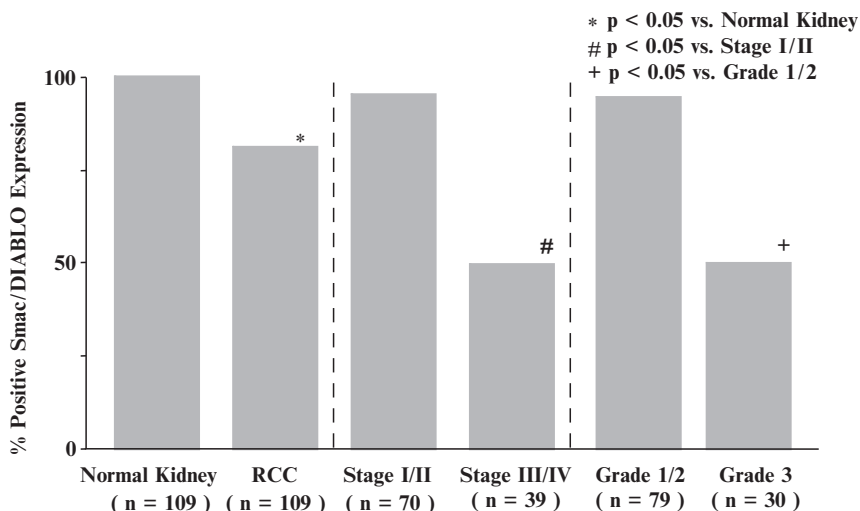


Fig. 5 Expression of second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI (*Smac/DIABLO*) in renal cell carcinoma (RCC). Expression of Smac/DIABLO was measured by western blotting

expression was observed in 62% of carcinoma and 22% of sarcoma. The level of Smac/DIABLO expression varied depending on the individual tumor. For instance, two out of ten prostate carcinoma were positive for Smac/DIABLO whereas the remaining eight were negative. Normal tissues adjacent to the cancer showed various degrees of Smac/DIABLO expression. However, in this report, there were no data on the expression of Smac/DIABLO in RCC. Our studies and those of Yoo et al. (24), demonstrating that the expression level of Smac/DIABLO in carcinoma and sarcoma is significantly different from the corresponding non-cancerous tissues, strongly suggest that Smac/DIABLO might play a role in the development of cancer.

Patients with RCC with positive Smac/DIABLO expression had a longer disease-specific survival, compared to those with negative expression in the 5-year

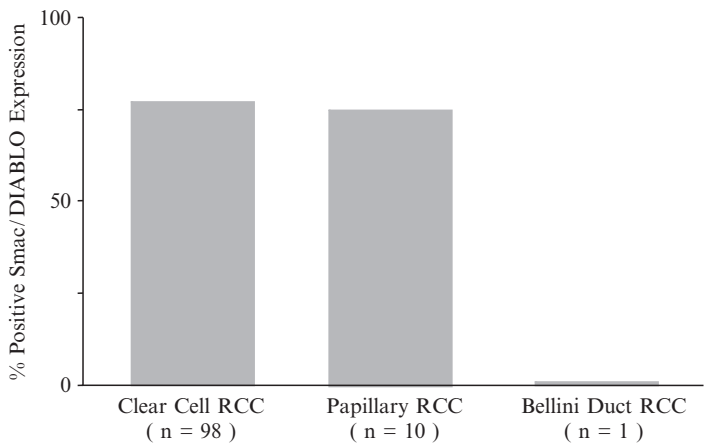


Fig. 6 Expression of second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI (*Smac/DIABLO*) in renal cell carcinoma (*RCC*). Expression of *Smac/DIABLO* was measured by western blotting

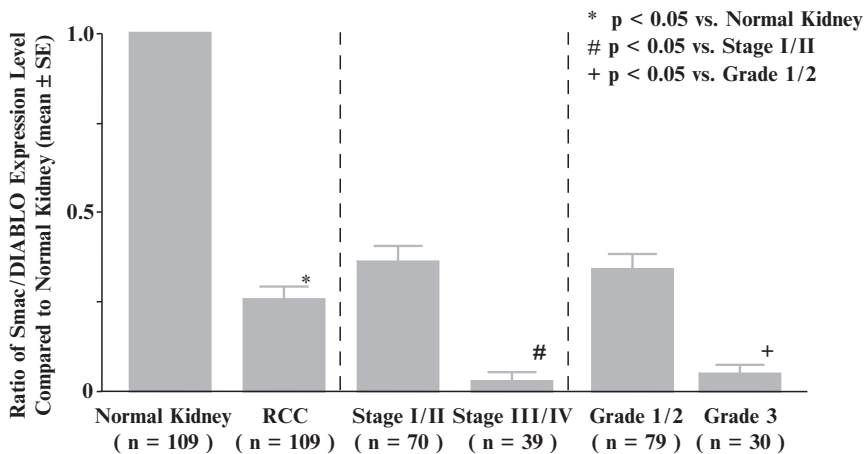


Fig. 7 Expression of second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI (*Smac/DIABLO*) in renal cell carcinoma (*RCC*). Expression of *Smac/DIABLO* was measured by western blotting

follow-up (Fig. 8). In addition, the dramatic post-operative disease-specific survival advantage for *Smac/DIABLO*-positive stage III/IV RCCs is also observed (Fig. 9). Thus, the level of *Smac/DIABLO* expression in RCC may be a prognostic indicator, and the absence of *Smac/DIABLO* predicted worse prognosis in post-operative cases.

Patients' tumor tissues or biopsy can be analyzed by more than one method for the detection and quantitation of *Smac/DIABLO*. For immunohistochemistry, the

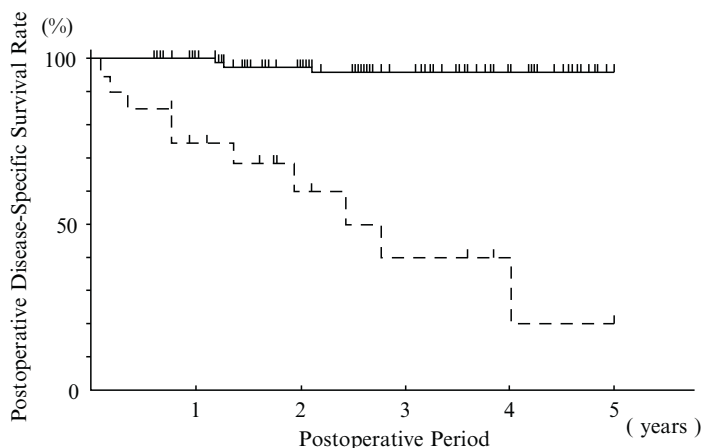


Fig. 8 Relationship between second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI (*Smac/DIABLO*) expression and postoperative disease-specific survival in patients with renal cell carcinoma (RCC). —: 89 patients with positive *Smac/DIABLO* expression. - - - -: 20 patients with negative *Smac/DIABLO* expression

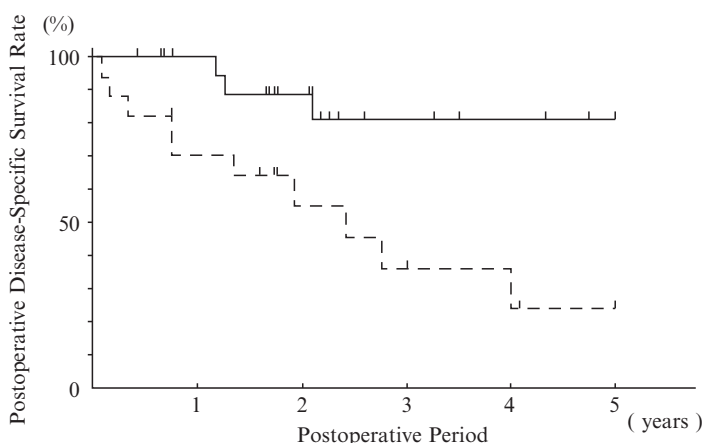


Fig. 9 Relationship between second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI (*Smac/DIABLO*) expression and postoperative disease-specific survival in patients with stage III/IV RCC. —: 22 patients with positive *Smac/DIABLO* expression. - - - -: 17 patients with negative *Smac/DIABLO* expression

level of *Smac/DIABLO* expression will be analyzed and quantitated using standard techniques. Another method is to isolate mRNA or proteins from the tissue sample and perform reverse transcriptase-polymerase chain reaction (RT-PCR) and western blotting for protein transcription and expression, respectively. In addition, with minimal tumor tissue, one can use microlaser micro-dissection to isolate a few cells for RNA extraction and then run RT-PCR. Other methods for protein levels

can be used, such as flow cytometry and HPLC. In all of these methods, one will determine the relative expression of Smac/DIABLO in comparison with the level of autologous normal kidney tissues or control normal tissues. The low or negative expression of Smac/DIABLO will indicate a poor prognosis. Also, poor expression should suggest the type of therapy to be used which may be either independent of Smac/DIABLO expression and/or the use of agents to upregulate Smac/DIABLO expression. In addition, combination of markers such as Smac/DIABLO and IAP levels may be useful as combination prognostic markers.

4 Smac/DIABLO as a Proapoptotic Molecular Target for RCC

RCC cell lines have been described to be resistant to apoptosis inducing stimuli. A set of cell lines derived from human RCC almost completely lacked the expression of caspase 3 and further expressed other caspases at low levels (25). Such alteration might contribute to RCC development. A recent study by Gerhard MC, et al. (26) examined the functional competence of the apoptosome in RCC cell lines and RCC fresh tissues. They found that the apoptosome is structurally and functionally intact in both RCC cell lines and primary RCC by the criteria of adding exogenous cytochrome c. These findings suggested that the apoptosome may not be directly involved in resistance. Their study, however, did not examine the activation of the apoptosome and apoptosis by intrinsic cytochrome c and the role of Smac/DIABLO in the activation. The interaction of the apoptosome with low expression of cytochrome c or Smac/DIABLO may not be sufficient to trigger the apoptosome. Low expression of Smac/DIABLO with a possibly intact apoptosome may be associated with resistance and suggests the therapeutic effect of overexpressing Smac/DIABLO in the reversal of resistance (23). Therefore, the down-regulation of Smac/DIABLO expression in RCC compared to the normal kidney identifies Smac/DIABLO as a molecular therapeutic target (Figs. 4, 5 and 7). The transfection with Smac/DIABLO cDNA had no effect on the growth of RCC cell lines. However, overexpression of Smac/DIABLO in RCC by transfection resulted in high sensitivity to TNF-related apoptosis-inducing ligand (TRAIL)/cisplatin-mediated killing (23, 27). The endogenous low level of Smac/DIABLO in RCC may not be adequate to neutralize the antiapoptotic mechanism regulated by IAPs. Thus, immunotherapy/chemotherapy in combination with Smac/DIABLO agonists may be a promising strategy against RCC (Table 2). The agonists used to induce Smac/DIABLO, namely sensitizing agents, can be small organic molecules, sense oligonucleotides, peptides, chemicals, biologicals, gene transfection, etc., all of which can upregulate the expression of Smac/DIABLO. In addition, proteasome inhibitors and inhibitors of ubiquitination to prevent the degradation of Smac/DIABLO will also be important sensitizing agents. The N-terminal tetrapeptide of Smac/DIABLO has been shown to mimic Smac/DIABLO and sensitize the cells to drugs and TRAIL-induced apoptosis (28). Arnt et al. (29) found

Table 2 Enhanced sensitivity of RCC to cytokines and anticancer agents by Smac/DIABLO agonists

Smac/DIABLO agonists	Cytokines, anticancer agents
Smac/DIABLO	TRAIL, anti-Fas antibody
N-terminal peptide	Doxorubicin, Cisplatin
	Etoposide, Paclitaxel
Small molecule	TRAIL, TNF- α
Smac/DIABLO	
Transfection of Smac/DIABLO gene	TRAIL
	Cisplatin
<i>Smac/DIABLO</i> second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pl, RCC renal cell carcinoma	

that the first four amino acids of Smac/DIABLO increased apoptosis in cell lines treated with paclitaxel, etoposide, camptothecin and doxorubicine. Thus, based on changes in Smac/DIABLO expression patterns, one can tailor specific therapies to RCC patients. Especially, RCC patients with low expression of Smac/DIABLO will be best suited for treatment with the combination therapies.

5 Concluding Remarks

Smac/DIABLO could be used as a prognostic parameter in patients with RCC. Because of the absence of prognostic factors for RCC, this finding should provide the incentive to extend these studies for validation as a prognostic factor for RCC. In addition, since Smac/DIABLO is also a target for therapeutic intervention, the generation of agents that can upregulate Smac/DIABLO or prevents its degradation will be interested as a novel sensitizing therapeutic. This sensitizing therapeutic can be used in combination with cytotoxic agents and override the resistance of RCC to both drugs and immunotherapy. However, further studies are needed to determine the regulatory effects of Smac/DIABLO in RCC.

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EphA2: A Novel Target in Renal Cell Carcinoma

Mayumi Kawabe, Christopher J. Herrem, James H. Finke,
and Walter J. Storkus

Abstract Eph receptors and their ligands, ephrins, are known to play important roles in organ development (formation of tissue boundaries, neural crest cell migration, axon guidance) and angiogenesis. In particular, EphA2 has recently attracted interest in the field of cancer research. Many observations, including our own, have demonstrated that most cancers, such as renal cell carcinoma (RCC), overexpress the EphA2 protein. EphA2 overexpression/dysregulation is associated with carcinogenesis, metastasis, and poor clinical prognosis. Indeed EphA2 is not just a marker of metastatic potential, but its overexpression is directly linked to an aggressive tumor phenotype. As a consequence, EphA2 represents a potential target for therapeutic intervention in the setting of EphA2+ cancer histologies, with several agents being developed with clinical intent. Several strategies can be contemplated in this regard, including: (1) selected promotion of EphA2 degradation or to reduce EphA2 expression and signaling (via the application of agonistic antibody, ephrin-A1 Fc fusion protein, siRNA against EphA2, or protein tyrosine phosphatases (PTP) that regulate EphA2 expression or specific EphA2 kinase inhibitors); (2) antagonism of EphA2 receptor–ligand binding (by provision of mimetic peptides or EphA2 Fc fusion protein); and/or (3) vaccination against EphA2 (using specific peptide-, protein-, or gene-based methods) to elicit specific T-cell- or Ig-mediated immunity. In this chapter, we will discuss the basic immunobiology of tumor-associated EphA2 and potential therapeutic interventions directed against EphA2 that may yield clinical benefit in the setting of (renal cell) cancer.

Keywords RTK (receptor tyrosine kinase) • CD8+ T lymphocytes • Proteasome • Combinational therapy • Major histocompatibility complex

W.J. Storkus (✉)

Departments of Dermatology and Immunology, University of Pittsburgh School of Medicine,
W1041.2 Biomedical Sciences Tower, Pittsburgh, PA 15213
e-mail: storkuswj@upmc.edu

1 EphA2 Overview

1.1 *Eph Receptor and Ephrin Ligands*

The Eph family of molecules contains 16 members, comprising the largest known cohort of receptor tyrosine kinases (RTK) (1) (for nomenclature, refer to http://eph-nomenclature.med.harvard.edu/cell_letter.html). Based on sequence similarity and binding affinities, two classes of receptors, EphA and EphB, and two classes of corresponding ligands, ephrin-A and ephrin-B, have been defined. The EphA receptor family consists of ten members (EphA1–EphA10), and primarily bind six ephrin-A ligands (ephrin-A1–ephrin-A6). Ephrin-A ligands are attached to the cell surface via glycosylphosphatidylinositol anchors and tend to bind only EphA receptors. EphB receptor family consists of six members (EphB1–EphB6), which primarily bind three ephrin-B transmembrane ligands (ephrin-B1–ephrin-B3). However, Eph–ephrin binding is promiscuous in some cases, as there is some crossover in the interactions between Eph receptors and ephrin ligands. For instance, Ephrin-B molecules bind with low affinity to EphA4 whereas ephrin-A5 is known to interact with EphB2 (2, 3).

Like other RTK, Eph receptors are type 1 transmembrane proteins (4). The extracellular N-terminus of these molecules consists of a highly conserved ligand-binding region, followed by a cysteine-rich region, and two membrane-proximal fibronectin Type-III repeats. The intracellular region of Eph receptors is characterized by a juxtamembrane segment containing two major autophosphorylation sites, followed by a conserved kinase domain, which phosphorylates secondary adapter proteins and can also phosphorylate Eph receptors themselves (5). The C-terminal region contains a sterile α -motif (SAM), and a PDZ-binding domain. The PDZ domain may be involved in receptor oligomerization (6), facilitating the binding of adapter proteins or mediating the receptor dimerization (7). The binding of Eph receptors to their corresponding ephrin ligands occurs at a 1:1 stoichiometric ratio, with the complex forming a circularized heterotetramer (8).

Eph receptors were originally defined based on their involvement in various embryonic developmental processes, such as formation of tissue boundaries, neural crest cell migration, axon guidance, and vascular system organization (9–14). However the developing embryo is not the only area where the Eph–ephrin system exerts its biologic influence. Eph–ephrin binding and subsequent signaling regulates the attachment, shape and motility of adult cells due to modulation of integrins/adhesion molecules on the cell surface and/or reorganization of the cell cytoskeleton (15–22). Furthermore, some Eph receptors (in particular EphA2), have also been shown to play an important role(s) in neo-angiogenesis in normal adult tissues, as well as tumors (23–27).

1.2 *EphA2*

One Eph receptor, EphA2, is of particular interest due to recent evidence suggesting it may play a role in the development and progression of cancer. The EphA2 gene is located on human chromosome 1 and encodes a 130-kDa Type-1 glycoprotein.

EphA2 binds to ephrin-A1, -A3, -A4, and -A5, but does not require ligand binding for its enzymatic activity (25). Along with other Eph/ephrin family members, EphA2 is involved in the organization of the developing nervous system (28) and vasculature (29), and is even expressed in embryonic stem cells (30). In normal adult tissues, EphA2 is expressed at low levels on a broad range of epithelial tissues (31), where it is principally localized to sites of cell-to-cell contact, and may play a role in contact inhibition of cell growth/migration that is critical for the organization and formation of epithelial layers in EphA2+ tissues (22, 32). EphA2 is also expressed by endothelial cells where it contributes to normal tissue (and tumor) neovascularization in the adult (23). More recently, EphA2 expression on Langerhans- and interstitial-type dendritic cells (DCs) has been reported, suggesting the possible role(s) of EphA2 in the localization and networking functions of Langerhans cells in the epithelium, as well as their ability to traffick and stimulate T cells under the appropriate activating conditions (33–35).

2 Regulation of EphA2 Expression

2.1 *EphA2 Expression and Life Cycle in Nontransformed Tissues*

In normal adult tissues, EphA2 is expressed at low levels on a diverse array of epithelial tissues, where it may stably bind to its ligand, ephrin-A1 (22), which is anchored to the surface of neighboring cells (36, 37). In contrast, malignant cells express high levels of EphA2 protein, but these only poorly bind ligand (22, 38). The EphA2 gene is expressed most highly in tissues that contain a high proportion of epithelial cells (e.g., skin, small intestine, lung, and ovary), with somewhat lower expression levels typically observed in kidney, brain, spleen, and submaxillary gland, and very low expression levels noted for heart, skeletal muscle, liver, testes, and thymus. These results diverge from studies of EphA2 protein expression, where strong expression has been reported for epithelial cells of kidney and lung, with weaker expression suggested for liver, small intestine, and skin in the rat (31). Our own studies using Western blot analysis of mouse organ lysates showed that moderate levels of EphA2 protein were expressed in the spleen, liver, and lung, whereas no detectable expression was noted for brain, heart, and skeletal muscle tissues (39). The observed disconnect between mRNA and protein level assessments in the various tissues likely reflects differences in posttranslational stability (i.e., the “lifecycle” of EphA2 protein in a given organ system).

In general, binding of ligand to the RTK promotes RTK dimerization/oligomerization and phosphorylation, creating binding sites for adaptor proteins in order to relay biological signals. During this process, the RTK protein is ubiquitinated by Cbl family ubiquitin-protein ligases. C-cbl contains an internal SH2 domain that binds to phosphorylated RTKs (pRTKs) and a ubiquitin E3-ligase responsible for the addition of ubiquitin molecules to pRTKs (40, 41). The ubiqu-

ubiquitinated RTK is internalized via clathrin-coated pits, forming a sorting endosome (42, 43), which is then targeted toward a lysosomal compartment for proteolytic degradation. However, this process is reversible, and dephosphorylated and/or nonubiquitinated receptors may be recycled to the cell surface where their function may be temporally extended (42, 44, 45). RTK signaling may also be modulated reversibly by the action of specific PTPs, as well as irreversibly by lysosomal degradation (42).

The RTK EphA2 seems to have a similar destiny upon ligand binding. Using ephrinA1-Fc fusion protein or EphA2-specific antibody as ligand agonists, it has been shown that (1) cell surface EphA2 is internalized and degraded in response to ligand- or antibody-mediated stimulation, (2) EphA2 interacts with the c-Cbl adaptor protein in a stimulation-dependent manner, and (3) the proteasome serves as the major degrader of internalized EphA2 protein (46–49). Overall, these results suggest that cellular levels of EphA2 protein are sensitive to turnover based on ligand-induced activation, as well as relative levels of “regulatory” protein interactions (i.e., Cbl, PTPs, etc.) that dictate the fate of internalized EphA2 molecules.

Of note, one striking difference between EphA2 and most other RTKs is that the enzymatic activity associated with EphA2 is not dependent on ligand-binding or receptor autophosphorylation, likely due to the lack of an “activation loop” tyrosine in the EphA2 cytoplasmic domain (22, 48, 50). Therefore, in the absence of ligand-binding signals, EphA2 exhibits constitutively active enzyme activity and the protein is not degraded. Such dysregulation may result in the “accumulation” of EphA2 protein (overexpression) and progressive buildup of quantitative EphA2 signaling (supporting tumorigenesis, etc.) (38).

2.2 *EphA2 and PTPs*

Tyrosine phosphorylation of RTKs in both resting and ligand-activated cells are modulated by a dynamic equilibrium of tyrosine kinase and PTP activities. With regard to the latter class of enzymes, the PTP superfamily has approximately 70 members. These can be classified into four major categories based on enzyme function, structure, and sequence: (1) tyrosine-specific phosphatases, (2) VH1-like dual specificity PTPs, (3) cdc25, and (4) low molecular weight (LMW) phosphatases (51).

Of these, low molecular weight-protein tyrosine phosphatases (LMW-PTP) have been reported to play a major role in acting on pEphA2 as a substrate (52–54). LMW-PTPs are a group of 18 kDa cytosolic enzymes that are constitutively expressed by most cell types (55). Under normal conditions, LMW-PTP removes the phosphate groups on cytoplasmic tyrosine residues that are initially added to EphA2 as a consequence of agonist ligand binding. This dephosphorylation event is necessary for the homeostatic regulation of secondary signals that are generated upon ligand binding. Indeed, LMW-PTP is reported to negatively regulate the ephrinA1-mediated repulsive response, cell proliferation, cell adhesion

and spreading, and the formation of retraction fibers, by its dephosphorylation of EphA2, suggesting LMW-PTP acts as a terminator of ephrinA1 signaling through EphA2 kinase activity (52).

Interestingly, LMW-PTP has been reported to be overexpressed in several cancer types (53, 54, 56). This is consistent with additional reports that tumor EphA2 is largely nonphosphorylated, while it is prominently tyrosine phosphorylated in nontransformed cells (22, 38, 50). Hence, high levels of EphA2 protein are coordinately observed in cells that overexpress LMW-PTP. This is a logical outcome since elevated levels of LMW-PTP would be expected to be associated with decreased levels of pEphA2, leading to enhanced recycle of EphA2 protein versus degradation in cancer cells.

Another PTP reported to be involved in pEphA2 dephosphorylation is the SH2-domain-containing PTP2 (SHP2), which may regulate changes in cell morphology that occur as a consequence of EphA2 activation (21, 27). And more recently, SHIP2 (Src homology 2 domain-containing phosphoinositide 5-phosphatase 2) has also been reported to be recruited to activated EphA2 via a heterotypic SAM–SAM domain interaction, leading to the inhibition of EphA2 internalization (57). The relative hierarchy of LMW-PTP versus SHP2 versus SHIP2 in regulating EphA2 phosphorylation status and EphA2 accumulation has not been comprehensively investigated in normal cells or tumor cells to date.

2.3 Genetic Regulation of EphA2

Several stimuli appear competent to efficiently upregulate *EphA2* gene transcription. Most interestingly, p53, known as guardian of the genome or gatekeeper for growth and division, has been shown to upregulate EphA2 transcription (58–61). Upregulated expression of p53 family members (p53, p73, and p63) increased both EphA2 transcript and protein levels, and a p53-binding site located within the *EphA2* promoter was defined and shown to be responsive to wild-type p53, p73, and p63, but not mutant p53 or p73 (61). This turned out not to be a consensus p53-binding site, but rather a novel ten-bp perfect palindromic decanucleotide (GTGACGTCAC) binding site in the EphA2 promoter region (58). While at face value, it might appear strange that wild-type p53 upregulates EphA2 transcription, it is important to note that levels of p53 are upregulated as a consequence of stabilization by mutant p53 protein in many forms of cancer (62, 63), hence p53-facilitated EphA2 expression could be a very logical conclusion in cancer cells.

EphA2 transcription is also enhanced as a result of activating the Ras-Raf-MAPK (MAPK stands for mitogen-activated protein kinase) signaling pathway. Increased EphA2 protein level was observed previously in Ras-transformed mammary epithelial cells (50), with more direct evidence demonstrated by Macrae et al. (64), with Raf activation shown to stimulate both EphA2 mRNA and protein expression. They also demonstrated that EphA2 is a direct transcriptional target of the Ras-Raf-MAPK pathway and that ligand-stimulated EphA2 attenuates the

growth factor-induced activation of Ras, suggesting a negative feedback loop is created that impacts Ras activity (64). It is noteworthy that ligand binding itself has been shown to upregulate EphA2 mRNA via the MAPK pathway (65).

Various stimuli/stressors that invoke the Ras-Raf-MAPK signaling cascade are potent inducers of EphA2 expression. IL-1 β , IL-2, EGF, which are known to activate Ras, stimulated expression of EphA2 (66). Deoxycolic acid, a well-known constituent of bile acid and cancer promoter, upregulates EphA2, at least partially through activation of the MAPK pathway (67). Hypertonic stress and urea-associated stress increase Ras activity in renal epithelial cells (68), in concert with enhancing EphA2 mRNA and protein expression (69). Thus, EphA2 expression in the renal medulla may represent an adaptive response to hypertonicity or exposure to urea. In renal ischemia-reperfusion injury, EphA2 mRNA may be increased as a consequence of src kinase activation (70). In addition, systemic injection of lipopolysaccharide (a MAPK activator via TLR2/4 stimulation) increased EphA2 mRNA expression in the liver (71).

Negative regulators of EphA2 expression have also been identified, with the most notable examples being signaling through the estrogen receptor and *c-myc* (72). Exposure of nontransformed breast epithelial cells to physiological levels of estradiol was sufficient to silence EphA2 expression. Levels of EphA2 expression in breast cancer cells were also shown to be inversely related to estrogen receptor expression, suggesting that loss of hormone sensitivity may override this repressive mechanism and contribute to widespread overexpression of EphA2.

3 Signaling Through EphA2

3.1 *Mitogen-Activated Protein Kinase*

The MAPK pathway is a signal transduction pathway that couples intracellular responses to the binding of growth factors to their corresponding cell surface receptors. Given the complexity of this signaling pathway, it is perhaps not surprising that investigations of how EphA2 receptor ligation impacts MAPK pathway activation have proven equivocal. Pratt et al. demonstrated that ligand stimulation of EphA2 activates MAPK/ERK in breast cancer cells and that pEphA2 interacts with the PTB and SH2 domains of SHC (65, 73). Another study also reported that ligand-mediated activation of EphA2 activated MAPK/ERK pathway and increased cell proliferation of glioblastoma cells (74). In contrast, Miao et al. reported that EphA2 activation via ligand-binding potentially inhibited the Ras/ERK signaling cascade in fibroblasts, endothelial cells, as well as normal and transformed epithelial cells. Moreover, EphA2 activation was shown to antagonize Ras/ERK activation in response to Epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), and platelet-derived growth factor (PDGF) in concert with reduced cellular proliferation (75). More recently, this same group showed that ephrin-A1 stimulation of wild-type, but not EphA2-null, keratino-

cytes attenuates ERK1/2 activation (76). Similarly, the attenuation of VEGF-induced MAPK pathway activation by ligand-stimulated EphA2 has been confirmed by other groups in analyses of breast cancer or retinal endothelial cells (64, 77). While these discrepancies may be simply due to differences in the cell type analyzed (76), more investigations are clearly needed to further clarify the impact of EphA2 stimulation on MAPK activity.

3.2 Focal-Adhesion Kinase

Focal-adhesion kinase (FAK), a cytoplasmic kinase localized to sites of cellular adhesion (i.e., where cells make contact with the ECM via integrins, etc.), is a major phosphoprotein species associated with changes in both the actin and microtubule cytoskeleton in adhering cells (78–80). Phosphorylated FAK exhibits stronger kinase activity versus unphosphorylated FAK and appears important in recruiting signaling and adaptor proteins important to the strength of cellular adhesion to matrix. Miao et al. reported that activation of EphA2 results in the dephosphorylation of pFAK, which in turn suppresses integrin function (21). They showed that EphA2 is constitutively (physically) associated with FAK in resting cells, and that upon stimulation with the ligand ephrin-A1, the protein tyrosine phosphatase SHP2 is recruited to EphA2, followed by the rapid dephosphorylation of FAK (within two minutes), and dissociation of the FAK–EphA2 complex. As a result, integrins assume an inactive conformation, with cell spreading, migration, and integrin-mediated adhesion coordinately inhibited. In contrast, Carter et al. showed that EphA2 activation by ephrinA1 induces FAK phosphorylation and cell adhesion and actin cytoskeletal assembly in fibroblasts (81). These apparent discrepancies may be simply the result of when FAK phosphorylation status was evaluated after EphA2 activation (i.e., FAK may be quickly dephosphorylated, then rephosphorylated at later time points). When studies of steady-state cells were performed using EphA2 siRNA or EphA2 transduction procedures, EphA2 silencing induced FAK dephosphorylation and reduced cellular invasiveness (82). On the other hand, ectopic EphA2 overexpression in cells results in increased FAK phosphorylation; however, after 1 h of ephrin-A1 ligation, FAK phosphorylation is reduced and the invasive phenotype is attenuated (83).

4 Role of EphA2 in Cancer

4.1 Overexpression of EphA2 in Cancer

Overexpression of EphA2 has been observed in an ever-expanding range of cancer types (26, 27). For example, EphA2 has been reported to be overexpressed at levels 10–100 times greater in metastatic prostate carcinoma cells when compared to noninvasive prostate epithelial cells (84). EphA2 protein is also expressed in

greater amounts in malignant mammary tissue when compared to benign mammary epithelia (38, 72). Similarly, EphA2 is vastly overexpressed in aggressive MUM-2B melanoma cells when compared to poorly invasive MUM-2C melanoma cells (85), and EphA2 mRNA levels were significantly higher in cell lines derived from distant metastases versus primary melanomas (86). PANC1, a poorly differentiated pancreatic adenocarcinoma that is highly invasive, overexpresses EphA2 when compared to the noninvasive Capan-2 pancreatic cancer cell line (82). In renal cell carcinoma (RCC), we have reported that EphA2 is expressed in metastatic RCC cell lines to a degree exceeding that for primary RCC cell lines, a trend recapitulated when analyzing freshly resected RCC clinical specimens (87). More recently, we compared RCC and normal adjacent kidney tissues and found that EphA2 expression varied between different tumors and that RCC lesions expressing higher levels of EphA2 were significantly higher in grade and tended to be larger, more-vascularized tumors (88).

Given such strong evidence supporting an association between EphA2 overexpression and tumor aggressiveness and metastatic potential, one would predict that the degree and distribution of EphA2 protein expression by primary tumors may prove predictive of clinical outcome in patients with EphA2+ tumors. Indeed, it has been recently suggested that the quantitative levels of EphA2 expressed by lung cancer cells or esophageal squamous cell carcinoma might provide useful prognostic information with regard to the metastatic potential of human carcinomas in situ and the clinical course of cancer patients (46, 89, 90). Consistent with this hypothesis, our group has also shown that the degree of EphA2 overexpression by biopsied RCC tissues (versus normal matched autologous kidney tissue) is predictive of short-term (<1 year) versus longer-term (≥ 1 year) disease-free interval, as well as overall survival (88). Among the RCC patients evaluated, individuals with tumors exhibiting the lowest levels of EphA2 expression were more likely to remain disease-free for a longer period of time after surgery, whereas those patients with tumors expressing high levels of EphA2 relapsed quickly and tended to survive for a shorter period of time (88).

4.2 Mechanisms of EphA2 Overexpression in Cancer

The dominant mechanism by which EphA2 is overexpressed and consequently promotes metastasis has not yet been conclusively elucidated. Several different possibilities impacting cellular expression of EphA2 include gene amplification, decreased rates of protein degradation, and increased or stabilized mRNA transcription/translation. An attractive current hypothesis associated with EphA2 overexpression by tumor cells involves the disruption of EphA2 homeostatic protein degradation.

In order for EphA2 to be properly degraded, it needs to become phosphorylated and any disruption of this process may result in the accumulation of cell surface EphA2 molecules. As we discussed above, LMW-PTP, which preferentially

dephosphorylates pEphA2, is overexpressed in several cancers (53, 54, 56), and tumor cell-expressed EphA2 protein is typically not phosphorylated (22, 38, 50). Overexpression of LMW-PTP by tumor cells is associated with decreased levels of pEphA2 and enhanced levels of EphA2 due to enhanced protein stability.

Additional/alternative mechanism(s) linked to elevated EphA2 expression may involve changes observed for E-cadherin expression in cancer cells. E-cadherin is a major protein involved in cellular adhesion, and is localized in adherens junctions formed between adjoining cells in tissues (91, 92). In order to mediate proper signaling, EphA2 needs to bind its corresponding ligand (i.e., ephrin-A1) on adjacent cells, and in the cancer setting, this ability appears to be disrupted. Notably, EphA2+ tumors that lose expression of E-cadherin tend to express the highest levels of EphA2 protein and EphA2 is no longer restricted to sites of cell-to-cell contact. Instead, EphA2 adopts a diffuse expression pattern throughout the cell (22). Consistent with this notion are observations made by Hess et al. investigating the transient knockout of E-cadherin (93). In this study, the authors found that E-cadherin and EphA2 were typically colocalized in cell–cell adhesion junctions, and that E-cadherin regulated EphA2 expression by modulating its ability to interact with, and become activated by, ligand ephrin-A1 (93). Thus E-cadherin serves as a “rheostat” in controlling EphA2 expression at the cell surface, with an inverse correlation reported between E-cadherin and EphA2 expression in bladder carcinoma (94).

Furthermore, as we have discussed in Genetic Regulation of EphA2, mutated/decreased levels of p53 and decreased levels of estrogen receptor may contribute to tumor cell overexpression of EphA2. Indeed, an analysis of ovarian cell lines and human ovarian cancers showed that EphA2 overexpression occurred in 91% of tumors with p53 null mutations versus only 68% in tumors with wild-type or missense mutations ($p = 0.027$) (95), suggesting the possible role of p53 in EphA2 accumulation in cancer cells.

4.3 Role(s) of EphA2 in Tumorigenesis

The role(s) of RTKs in carcinogenesis have been increasingly characterized over the last decade, in particular, with recent research demonstrating that tumor cell EphA2 expression levels may correlate with disease progression to metastasis (46, 88–90). Metastasis is the process whereby cancerous cells leave their tissue of origin, invade the extracellular matrix, and traffic via either the lymphatics or the bloodstream to distal tissue sites. Metastatic disease is the most advanced and deadly type of cancer as it tends to be lethal and is extremely hard to treat.

A foundational study linking EphA2 and tumorigenesis was performed by Zelinski et al. (38), in which enforced overexpression of EphA2 (via transfection of specific cDNA) was shown to be sufficient to promote transformation of mammary epithelial cells, allowing them to form invasive tumors/metastases in immunocompromised (athymic) mice. EphA2 cDNA-transduced cells exhibited defects in adhesion, EphA2

subcellular distribution, and decreased P-Tyr content of EphA2, all of which were reminiscent of the phenotype observed for metastatic breast cancer cells (22). The noted transforming capacity of EphA2 overexpression was related to the inability of transgenic EphA2 to interact appropriately with its natural ligand ephrin-A1; however, artificial stimulation of EphA2 was able to reverse the growth and invasiveness of EphA2-transformed cells in this model. This provides promise that similar manipulations may be effective in the clinical management of EphA2+ cancers.

In addition, enforced overexpression of the EphA2-associated PTP, LMW-PTP, was found to be sufficient to transform normal epithelial cells *in vitro* (54), with LMW-PTP acting as a positive regulator of tumor onset and progression in *in vivo* animal models. This supports the oncogenic potential of LMW-PTP, likely via its ability to efficiently dephosphorylate EphA2, leading to EphA2 accumulation (53). Since tyrosine phosphorylation of EphA2 is not necessary for its intrinsic enzymatic activity, nonphosphorylated, overexpressed EphA2 may serve as a potent oncoprotein (22, 38, 48, 53).

While the detailed mechanism(s) by which overexpressed, hypophosphorylated EphA2 promotes malignant transformation remain poorly defined, one may speculate that these impact cell adhesion properties [i.e., disruption of E-cadherin and integrin binding (21, 83, 93)]. Additionally, since EphA2 activation via ligand binding may inhibit the MAPK pathway (important for cellular responses to growth factors), the state of EphA2 hypophosphorylation in cancer cells may be linked to enhanced growth potential (64, 75–77), although this may be somewhat controversial. When taken together, it is likely that cellular overexpression/hypophosphorylation of EphA2 results in an abnormal distribution of this RTK, disruption of cell-to-cell contacts, and an enhancement in cell-to-extracellular matrix attachment, yielding cells with increased motility and invasive properties.

4.4 Angiogenesis

As tumors increase in size, they require more nutrients and oxygen for their continued and sustained growth. The inability of progressive tumors to gain access to a new blood supply puts the tumor at risk of reaching growth maxima, above which anoxia and necrosis are likely to occur. Hence, the successful tumor microenvironment is conducive to neoangiogenesis (96). Establishment of neovascular vessels also provides primary solid tumors portals through which they may metastasize and eventually colonize distant sites. Indeed, neoangiogenesis has been correlated with metastasis and poor prognosis in several cancers, including breast and pancreatic carcinomas (97, 98).

Many factors including EphA2 and its ligands have been implicated in the process of angiogenesis. EphA2 along with its ligand ephrin-A1 are expressed in growing neovessels found in breast tumors and sarcomas (99), and EphA2-deficient endothelial cells fail to become incorporated into nascent tumor microvessels *in vivo* (100). While the ephrin-A1 ligand is expressed in both tumor and normal vascular endothelial cells, EphA2 appears to be differentially expressed by tumor-associated vascular

endothelial cells (101). Notably, EphA2 overexpression in both tumor cells and tumor-associated endothelial cells has been linked to higher lesional vascularity/angiogenesis and poor clinical outcome in the setting of renal and ovarian carcinoma (88, 102). Soluble EphA2 receptor, which antagonizes EphA2-mediated signaling, inhibits VEGF-mediated and ephrin-A1-mediated angiogenesis, and can antagonize tumor neoangiogenesis (103, 104) and growth in vivo (101, 105, 106). Thus, therapeutic agents directed toward antagonizing the expression/function of EphA2 have two potential clinically meaningful target cell types: EphA2+ tumor cells themselves and EphA2+ tumor-associated neovessels.

5 Therapeutic Approaches Targeting EphA2

5.1 Interventions Targeting EphA2 Activation/degradation

5.1.1 Anti-EphA2 Antibodies

Carles-Kinch et al. were the first to report that an agonist anti-EphA2 antibody could reverse the aggressive phenotype of EphA2+ tumors (48). They generated monoclonal antibodies to extracellular domain of EphA2, and found that a subset of EphA2 monoclonal antibodies (mAb) induced EphA2 phosphorylation, followed by the internalization and destruction of EphA2 protein. Notably, monoclonal antibodies identified a conformationally distinct determinant that was differentially expressed by tumor cell-expressed EphA2 versus normal cell-expressed EphA2. These mAbs were effective in prohibiting human tumor growth in xenograft tumor models, in concert with decreased EphA2 protein expression levels and an increased apoptotic index in treated tumor lesions (48, 107, 108). Given their specificity for EphA2 expressed on malignant cells, such antibodies have significant potential to be clinically beneficial, without overt concerns for untoward toxicity directed toward normal EphA2+ tissues. In addition, combinational therapies implementing EphA2 agonistic antibody and chemotherapeutic drugs (paclitaxel or tamoxifen) may be readily envisioned, as this regimen yielded superior tumor growth inhibition, when compared to either single agent alone, in preclinical models (109, 110). Such results may be dependent on the relative dominance of EphA2-dependent oncogenic processes in tumor cells, however, since a contradictory report suggests that at least in some models, agonist anti-EphA2 Abs have little impact on tumor growth (111).

5.1.2 Ephrin-A1 Fc

Ephrin-A1 Fc is a dimerized ephrin-A1 fused to human immunoglobulin G (IgG) Fc. In vitro experiments suggest that EphA2 ligation by ephrin A1-Fc results in EphA2 phosphorylation, and consequent degradation of this RTK in concert with reduced tumor growth (83, 112). To investigate the impact of sustained ephrin-A1 delivery on tumor cells in vivo, adenovirus expressing secreted forms of ephrin-A1 Fc has

also been investigated (113). Noblitt et al. showed that adenoviral delivery of ephrin-A1 Fc (i.e., rAd.ephrin-A1) into breast cancer cells increases the degree of EphA2 activation and degradation, along with inhibited tumor growth in vitro. Furthermore, they demonstrated that intratumoral injection of rAd.ephrin-A1 limited human tumor growth in xenograft models (113, 114). With regard to their potential clinical utility, one concern in using ephrin-A1 Fc (protein or gene constructs) as a therapeutic agent reflects the fact that ephrin-A1 serves as a ligand for multiple Eph receptors (i.e., EphA4, EphA5, EphA6, and EphA7 in addition to EphA2), which may increase chances of unanticipated toxicities. While no gross toxicities were noted in the reported xenograft model (113), further safety studies prior to clinical consideration would appear warranted.

5.1.3 Peptide Mimetics

Koolpe et al. employed a phage display approach and identified two peptides that bind selectively to the extracellular domains of EphA2 and antagonize ephrin ligand binding (115). One of these peptides stimulates EphA2 tyrosine phosphorylation and signaling, which indicates a potential therapeutic use of this peptide, based on analogy to similar effects mediated by either agonist anti-EphA2 mAb or ephrin-A1 Fc. In addition, they found that this peptide targets the delivery phage particles to EphA2+ cells, suggesting potential therapeutic value in selectively delivering therapeutic agents to EphA2+ tumor sites (115).

5.2 Interventions Targeting EphA2 Ligands

Soluble EphA2-Fc, a chimeric receptor of EphA2 fused with an IgG Fc fragment, antagonizes EphA2 signaling and has been found to inhibit VEGF-mediated and ephrin-A1-mediated angiogenesis. Local or systemic administration of soluble EphA2 inhibits tumor angiogenesis, growth (101, 105), and even metastasis (106) in vivo tumor models. Interestingly, VEGF induces ephrin-A1 expression, which in turn activates EphA2-dependent angiogenesis (104). Hence, soluble EphA2 would be anticipated to suppress tumor-associated VEGF-, but not basic fibroblast growth factor-, induced angiogenesis (104). With regard to safety concerns, soluble EphA2 significantly inhibits pathological retinal angiogenesis without affecting normal intraretinal vessels (103), suggesting that this agent may not inflict untoward toxicity on normal EphA2+ tissues.

5.3 Gene Silencing by siRNA

Since most human cancers, including RCC, overexpress EphA2, EphA2 gene silencing is a plausible therapeutic strategy to reverse malignancy. A limited cohort of recent studies suggest that application of EphA2 siRNA suppresses EphA2 protein

expression and cellular invasiveness, and that systemic administration of EphA2 siRNA retards tumor growth and inhibits metastasis in vivo (82, 111, 116, 117) via the induction of tumor cell apoptosis mediated via caspase 9 activation (117).

In addition to targeting the EphA2 gene, one may consider targeting PTPs linked to EphA2 expression/function, that is LMW-PTP, SHP2. By reducing the overexpressed levels of these PTPs in tumor cells, one might anticipate the normalization of pEphA2 levels and consequent EphA2 protein degradation. Our own preliminary data demonstrated that LMW-PTP silencing with siRNA reduced EphA2 protein expression of metastatic RCC cells (preparing for publication), suggesting the possibility for an alternative therapeutic method.

5.4 *EphA2-Based Vaccines*

Seven human leukocyte antigen (HLA)-A2-binding and three HLA-DR4-binding peptides derived from EphA2 have been identified by our group and by Alves et al. (87, 118). These peptides are immunogenic, being recognized by CD8+ or CD4+ T cells generated from normal donors or cancer patients bearing the appropriate HLA types, and by CD8+ T cells developed in HLA-A*0201-transgenic HHD mice (87, 118). In all cases, T-cell lines and clones produced using EphA2 peptides as a stimulus also recognized EphA2+, HLA-matched tumor cell lines, including RCC. This supports the natural processing and MHC presentation of these epitopes on the cell surface of tumor cells in a manner that allows for effector T-cell reactivity [i.e., interferon- γ (IFN- γ) production, cytotoxicity]. Such EphA2-specific T cells have been identified in the peripheral blood of patients with RCC (87), prostate cancer (118), or glioma (119, 120), suggesting that these responses may be naturally primed during cancer progression. In RCC patients, we observed that anti-EphA2 CD4+ and CD8+ T cells were most pronounced in patients that had no evidence of disease after therapy or that were long-term survivors in the face of limited tumor load (87). In the anti-EphA2-reactive CD4+ T-cell compartment, we noted that T cells isolated from patients with active disease were more prone to produce Th2- or Treg-associated cytokines, such as IL-4, IL-5, and TGF- β , while patients without disease, or those with better clinical outcomes, were more biased toward Th1-type cytokine production (i.e., IFN- γ). The lack of Th1-type T cells reacting against EphA2 in RCC patients with active disease may reflect the high frequency of proapoptotic T cell in these populations (W.J.S., unpublished data). This may suggest that therapeutic/protective RCC (or other EphA2+ cancer types) vaccines may need to expand, appropriately polarize (i.e., Type-1), and protect anti-EphA2 T cells against tumor-induced immune deviation and death.

In this regard, active vaccination against EphA2 in order to elicit and sustain specific T cells is a logical endpoint that would be anticipated to provide clinical benefit in RCC (cancer) patients. Given the various cancer vaccine formats (peptide-, protein-, or gene-based) that are currently being investigated for their relative

efficacy in both preclinical models and clinical trials, this aspect of a potential EphA2-based vaccine will not be discussed here [for reviews see (121, 122)]. However, regardless of whether an EphA2 peptide, recombinant protein, or gene vector (plasmid, viral, or bacterial) is contemplated as a therapeutic agent in the setting of EphA2+ cancers (including RCC), their application in the context of a DC-based vaccination would be attractive, given the ability of the this antigen presenting cell type to prime, polarize, and extend the survival of antigen-specific T cells (123, 124). Indeed, Hatano et al. recently demonstrated that DC pulsed with murine EphA2 peptide epitopes effectively elicit specific CTL responses in vivo that are capable of inhibit syngenic tumor progression in C57BL/6 mice (39). While there remains a theoretical concern that vaccination with EphA2-derived peptides may induce pathologic autoimmune reactions in normal EphA2+ tissues (i.e., lung, spleen, kidney, and liver), these organs were not infiltrated by T cells, nor was tissue pathology observed in vaccinated animals (39). This may reflect greater densities of EphA2 epitopes presented on the surface of tumor cells versus normal tissues, with T cells exhibiting moderate avidity only able to functionally respond to tumor cells. Under such conditions, while flirting with potential autoimmune toxicities that warrant further scrutiny, this type of vaccine may ultimately prove both safe for clinical effective.

5.5 Combination Therapies Targeting EphA2

In the above sections, we have described potential therapeutic means by which the growth contributions of tumor EphA2 may be antagonized (use of agonist ligand-Fc fusion proteins, mAbs, or siRNA silencing to reduce EphA2 expression and use of peptide mimetics or soluble EphA2 to limit EphA2-mediated signals into tumor cells) or targeted for immune intervention (vaccines). Since these strategies involve largely nonoverlapping operational pathways, one would envision that may be combined with an expectation of superior efficacy. We have recently observed that ephrinA1-Fc and anti-EphA2 mAb promote the proteasomal degradation of tumor cell-expressed EphA2 protein that ultimately results in the improved ability of anti-EphA2 CD8+ T cells to recognize and kill cancer cells (125). Combination of (DC-based) EphA2 vaccines to enhance circulating levels of anti-EphA2 T cells in (RCC) cancer patients with such agonist agents would be expected to facilitate T-cell recognition of EphA2+ tumor cells (or their tumor-associated vasculature) in vivo. Other drugs known to affect tumor cell levels of receptor tyrosine kinases, such as the HSP90 inhibitor geldanamycin [or its derivatives (126)], might also be expected to work well in combination with active vaccination. Furthermore, to limit the aggressiveness of tumors during such treatments, one might also consider combinations that include EphA2 antagonists or agents such as VEGF or PTP inhibitors that would be anticipated to reduce tumor-supportive signals associated with ephrin-A1 or EphA2 overexpression, respectively, by tumor cells or their associated vascular endothelial cells.

6 Conclusions

EphA2 is a receptor tyrosine kinase that is commonly overexpressed in most human cancers, including RCC. This protein is associated with aggressive disease that is characterized by a higher degree of vascularity and metastasis, in accordance with a poorer clinical prognosis. Translational studies clearly suggest that the antagonism of EphA2 expression/function in cancer cells reduces tumor aggressiveness, providing support for the evaluation of these inhibitory agents in clinical trials for patients with RCC. Additionally, EphA2 may be targeted in active vaccination (single modality or combinational) protocols in order to elicit specific T-cell-mediated immunity that is competent to slow tumor growth or mediate the regression of disease.

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Restoring Host Antitumoral Immunity: How Coregulatory Molecules Are Changing the Approach to the Management of Renal Cell Carcinoma

Brant A. Inman, Xavier Frigola, Haidong Dong, James C. Yang,
and Eugene D. Kwon

Abbreviations APC: Antigen-presenting cell; CD: Cluster of differentiation; DC: Dendritic cell; IFN: Interferon; IL: Interleukin; MHC: Major histocompatibility complex; NK: Natural killer cell; RCC: Renal cell carcinoma; SMAC: Supramolecular activation complex; T_C : Cytotoxic T lymphocyte; TCR: T-cell receptor; T_H : Helper T lymphocyte; TNF: Tumor necrosis factor; T_{reg} : Regulatory T lymphocyte.

Abstract Renal cell carcinoma (RCC) is a tumor whose past is filled with failed treatments and unpreventable patient death. Fortunately, science is progressing at an ever increasing rate and novel discoveries are bringing new possibilities for patients with RCC. The future of RCC treatment is bright, and we believe that immunotherapy will realize much of its potential within the next decade.

In this chapter, we discuss one of the newest discoveries in RCC tumor immunology: T-cell coinhibition. This chapter will first introduce key concepts of T-cell function and tumor immunology that are necessary for a good understanding of how coinhibition works. We then describe some of the key defects in immunity that are present in RCC. Finally, we propose a model to explain why certain renal tumors are eliminated by the immune system and others are not.

Keywords Costimulation • Coinhibition • Tumor immunology • Immunoediting • Immunotherapy • T lymphocyte • Renal cell carcinoma

1 A Brief Introduction to Tumor Immunology

Human cells are continuously dividing, differentiating, and dying: acts that are orchestrated by a highly redundant bureaucracy of cellular control mechanisms that are in place to ensure the stability of growth in bodily tissues. Like all large bureaucracies, the body's safeguards occasionally fail, allowing individual cells to

E.D. Kwon (✉)

Department of Urology, Mayo Clinic, Rochester, MN 55905

e-mail: kwon.eugene@mayo.edu

escape the control mechanisms that normally govern their existence, and permitting such cells to proliferate or to acquire specialized features that are inappropriate for the tissue to which they belong. Often these rogue cancer cells become unstable and die on their own. Occasionally, however, such cancer cells persist and require higher levels of intervention to permit the body to rid itself of its pariah.

All the host factors that determine whether a cancer cell survives or perishes are not known, though the immune system does appear to be very important. In this section, we will explore some of the ways that the immune system is involved in tumor identification and removal. Based on these principles, we will later build a case for the existence of peripheral mechanisms of immune dysfunction, principally involving lymphocytes and tumor cells, that contribute to the progression and immune evasion of tumors such as renal cell carcinoma (RCC).

1.1 *Central Tolerance*

The immune system can be classified into two different components: the *innate immune system* that responds nonspecifically to a variety of antigens and the *adaptive immune system* that is responsible for deliberate and antigen-specific immune attacks. The principal effector cells of the adaptive immune system are lymphocytes that have antigen-specific surface receptors. These receptors are stochastically generated by early rearrangements in the lymphocyte receptor genes, a process that can result in the formation of an autoreactive receptor that could potentially be a cause of autoimmunity (1–4). A key feature of the adaptive immune system is its ability to identify and destroy lymphocytes, early in their development, that possess autoreactive cell surface receptors. *Central tolerance* is the name given to this process because it occurs centrally in the thymus for T lymphocytes (aka T cells) and in the bone marrow for B lymphocytes (aka B cells). Central tolerance was first demonstrated in twin calves (5), but has now been fairly well elucidated in humans, particularly in the context of organ transplantation. In the case of T lymphocytes, central tolerance is a complicated process that involves two major checkpoints: the *β -selection checkpoint* where the β chain rearrangement of the T-cell receptor (TCR) is tested for functionality, and the *TCR $\alpha\beta$ checkpoint* that occurs after α -chain rearrangement and where the new $\alpha\beta$ -TCR is tested for self-major histocompatibility complex (MHC) restriction (*positive selection*) and self-antigen tolerance (*negative selection*) (6). A slightly different process occurs in the bone marrow for B lymphocytes. The end result of central tolerance is the formation of a population of B and T cells with a large spectrum of receptors, designed to detect foreign antigens and to be tolerant to self antigens.

1.2 *Peripheral Tolerance*

Lymphocytes with autoreactive antigen receptors occasionally slip into the bloodstream prior to being inactivated by the process of central tolerance. Another problem is that not all the body's antigens are present in the thymus and bone

marrow during lymphocyte development and certain antigens are only expressed at specific time points in human development. All of these idiosyncrasies can lead to the presence of defective lymphocytes in the circulation, and this has the potential to cause autoimmune disease. Fortunately, tolerance can be induced outside the thymus and bone marrow to correct these problems. *Peripheral tolerance*—tolerance induced at any peripheral site where a lymphocyte can encounter antigen—can be initiated in number of circumstances (7). In fact, in order for a T cell to respond to a particular antigen, it must pass three critical tests (not unlike committing to marriage). First, several TCRs on a particular T cell must bind avidly and specifically to MHC–antigen complexes on an antigen-presenting cell (APC), demonstrating the desire for a relationship (8, 9). Second, costimulatory molecules on the APC must bind to their cognate receptors on the T cell, effectively confirming that the offer of mutual stimulation has been accepted (10). Finally, a permissive biochemical environment must be present for the courtship to proceed expeditiously (i.e., the father-in-law must agree!) (11). A problem at any one of these steps can produce a tolerizing signal that leads to one of two basic fates: death of the T cell via apoptosis or the induction of the unresponsive state of T-cell anergy (12–16). These events can result in the inactivation or death of an entire family of T-cell clones that express a particular TCR.

Other mechanisms of peripheral tolerance exist that are different from those described above. *Immunological ignorance*, for instance, refers to the situation where the T cell is not activated to destroy its target antigen simply because it never encounters enough of that antigen on the surface of an APC to become activated (17, 18). Immunological ignorance therefore appears to be an antigen presentation problem and not the result of apoptosis or anergy. At the opposite end of the spectrum is *clonal exhaustion* (aka replicative senescence), a process whereby T cells that are continuously exposed to an abundance of antigen replicate so frequently and use up so many metabolic resources that they experience “burnout” or exhaustion (19–22). Some groups have been able to demonstrate that T cells can be rescued from exhaustion by targeting some of the coinhibitory pathways that will be discussed below (22).

1.3 Regulatory T Cells

A specialized subpopulation of T cells—known as *regulatory T cells*—has been described for its ability to cause antigen-specific immunosuppression (23). Regulatory T cells (T_{reg} cells) are defined by their cell surface proteins and intracellular transcription factors and are capable of preventing other immune cells (including T cells) from becoming activated and proliferating in response to antigen. It is thought that T_{reg} cells are important in preventing overexuberant immune responses to antigen.

There are several types of regulatory T cells (T_{reg} cells) and (24–27), although a complete discussion of their characteristics is beyond the scope of this chapter, we would like to familiarize readers new to this subject to a few important points.

T cells were first shown to suppress immune responses in the 1970s, and they were called *suppressor T cells* (28, 29). The failure to identify antigen-specific factors and unique identifying molecules that caused immune suppression led to stagnation of this field. Interest was rekindled in the mid-1990s when Sakaguchi *et al.* demonstrated that a small population of CD4+ T cells that coexpressed the interleukin-2 (IL-2) receptor α -chain (CD25) could control autoreactive CD4+ T cells *in vivo* (30). *In vitro* murine studies have since confirmed that certain CD4+ CD25+ T cells can function as potent inhibitors of T-cell-mediated immunity (31), subsequent studies have revealed a similar CD4+ CD25+ T-cell population in humans (32, 33). Our current understanding now suggests that T_{reg} cells constitute a family of specialized T cells that can be divided into two broad subsets: natural and adaptive. The natural T_{reg} cell population develops as a separate T-cell lineage in the thymus (27, 34). These cells are antigen-specific and populate in the periphery to guard against autoimmune reactions (27, 34). The second subset of “adaptive” T_{reg} cells appears to develop from mature, peripheral CD4+ T cells in response to tissue-specific or foreign antigens, and mediate regulation through the release of soluble cytokines (IL-10). A major problem with the study of T_{reg} cells has been the lack of specific cell markers that distinguish them from other lymphocytes. Recently, the ability to characterize T_{reg} cells was improved with the identification of the transcription factor Foxp3, which has been demonstrated to be a critical component of natural T_{reg} cell generation and function (35). Murine and human natural CD4+ CD25+ T_{reg} cells express high levels of FoxP3 compared to other lymphocytes, permitting its use as a relatively specific marker.

Although research into the impact of regulatory T cells on human cancer has been limited, several key studies have emerged in recent years demonstrating their importance. Cancer patients appear to have increased numbers of both peripherally circulating and tumor-associated CD4+ CD25+ T_{reg} cells (36, 37). Murine studies have shown that antibody-mediated depletion of CD25+ cells can enhance tumor immunity and rejection (38, 39). Curiel *et al.* were the first to demonstrate a direct link between T_{reg} cells and human tumor immunopathogenesis by showing that tumor-infiltrating T_{reg} cells block tumor-antigen specific effector T-cell responses and positively correlate with poorer prognosis (40). Work in our laboratory also suggests that T_{reg} cells also play an important role in RCC (unpublished results).

1.4 Immune Surveillance

It has been nearly 100 years since Paul Ehrlich first suggested that the immune system plays an active role in finding and eliminating newly developing cancer cells (41). It was almost 50 years later when Burnet and Thomas revived this notion and coined the term “*immune surveillance*” (42, 43). The implication of immune surveillance is that the immune system is on constant alert and actively seeks out and destroys malignant cells as they are formed. Although

immune surveillance was deemed an attractive proposal in the 1950s and 1960s, establishing its existence *in vivo* proved to be a difficult matter. A series of experiments conducted in the 1970s provided early evidence that immune surveillance might not actually exist (44–46). Specifically, several groups reported that tumors do not arise more frequently in athymic nude mice that lack an intact immune system compared with immunocompetent mice—a finding that ultimately led to abandonment of the immune surveillance hypothesis in the late 1970s. Several observations even suggested that the immune system could be involved in promoting cancer development (47–49). Auspiciously, a few stalwart proponents of the immune surveillance theory persisted, and new knowledge regarding natural killer (NK) cells (50, 51), $\gamma\delta$ -T lymphocytes (52, 53), perforin (54), interferons (55, 56), and other components of the immune system, rekindled interest in immune surveillance theory.

1.5 Immunoediting

The theory of immune surveillance is currently undergoing a phase of rebirth and is emerging in a more complex form than originally envisioned (57). The term “*immunoediting*” has been proposed to stress the duality of immune surveillance—it is both a host-protecting and a tumor-sculpting process (56, 58). Dunn et al. have published a series of enlightening articles in which they put forward a three-part model of cancer immunoediting that encompasses tumor elimination, host-tumor equilibrium, and tumor escape (58–62). They argue that cancer immunoediting is a broad process that involves both adaptive and innate immunity and could lead to either tumor rejection or tumor tolerance. This model echoes ideas initially put forth by Stutman over 20 years ago (63). Much of what we will discuss in this chapter conforms nicely with this paradigm.

1.6 T-Cell Activation

As part of the maturation and selection processes occurring in the thymus, a T cell is committed to one of two lineages: the CD4+ T helper cell lineage (T_H) which is MHC class II (MHC_{II}) restricted or the CD8+ cytotoxic T-cell (T_C) lineage which is MHC class I (MHC_I) restricted. It is important to note that antigen receptors of both lineages are similar in that they can bind to any MHC molecule (class I or class II) that presents appropriate antigen. It is in fact the coreceptors, CD4 and CD8, that determine the MHC specificity of the T cell because they bind exclusively to MHC_{II} and MHC_I , respectively.

Once T cells have matured in the thymus, T_H and T_C cells are released into the bloodstream in search of antigen. Although mature, these newly minted and emigrated T cells are naïve in the sense that they have never encountered specific

antigen for which their TCRs were constructed to recognize. The fate of the naive T cell is, in turn, governed by two determinants: the type of coreceptor it expresses and the nature of its first contact with its antigen. As mentioned in the section on tolerance, the relationship between the T cell and the antigen-bearing APC is a demanding one, and even minor deviations from the normal sequence of T-cell activation can result in death or anergy for the T cell. A superficial awareness of the events required for T-cell activation is, therefore, critical to understanding how cancers such as RCC can trick the immune system into a state of unresponsiveness.

1.7 *Signal 1: Antigen Presentation to the T Cell*

The number of cells required for effective presentation of antigen to a naive T cell depends on the specific T-cell lineage in question. For naive T_H cells, activation requires the presence of only one other cell: a mature dendritic cell (DC) with antigen-loaded MHC_{II} . In distinction, the activation of a T_C cell is much more demanding and requires a target cell, an APC with antigen-loaded MHC_I , and frequently an antigen-specific effector T_H cell to provide cytokine support. Activation of a T cell therefore is not a chance occurrence but rather requires an highly orchestrated effort. This will be further discussed below.

Tumor antigens are secreted, shed, and sloughed by cancer cells throughout their lifecycle. Some of these antigens are *tumor-specific* because they are mutated forms of normal cellular proteins or are viral oncoproteins that are found only in cancer cells. Other tumor antigens are *tumor-associated* which simply implies that although they are not specific to the cancer cell, they are produced in an abnormal and dysregulated manner within the tumor, as typically revealed using biochemical methods. Host scavenger cells, usually of dendritic or macrophage–monocyte lineage, will pick up the tumor antigens and carry them into draining lymph node in proximity to the tumor. These scavengers are usually attracted to the tumor site by various chemoattractants, such as chemokines secreted by the tumor cells or the host cells that are being disrupted by the tumor. Once in the draining lymph node, antigen will be presented by APCs on both MHC class I and II molecules for recognition by specific TCRs that can mediate T-cell activation. It should be pointed out that most tumor-associated antigens will not generally elicit an immune response because the T cell will be tolerized against these antigens due to the development of central or peripheral tolerance. Tumor-specific antigens, on the other hand, typically maintain the potential to trigger an immune response in the appropriate setting.

For the T cell to be activated by an MHC-bound target antigen, several things must occur. First, *cell adhesion molecules*, often with the integrin and selectin family of molecules, must be present on both the APC and the T cell. These cell adhesion molecules serve two main purposes: to steer the T cell toward the APC and to help these two cells remain in contact long enough to engage as many TCRs

on the T cell as possible in order to elicit activation. It has been estimated that it can take as long as 30 hours and involve the binding of roughly ~100 MHC–antigen complexes to ~20 000 TCRs, in order to activate a T cell (64). Second, the formation of an *immunological synapse* must occur. The formation of the immunological synapse can be visualized with microscopy as a 3-dimensional triple-ringed structure called the *supramolecular activation complex* (SMAC) (65). The SMAC is formed due to alterations in the actin cytoskeletons of both the T cell and the APC, and has been shown to be comprised of TCRs, MHC–antigen complexes, the CD4 and CD8 coreceptors, and enzyme-bearing plasma membrane lipid rafts, all of which are required to elicit T-cell activation (66). Finally, after binding the right MHC–antigen combination, the TCR is phosphorylated in its intracellular domain and an appropriate signal is directed into the T cell for processing. This signal results in the immediate commencement of a process in which the TCR is internalized and downregulated. A race thus begun with the remaining TCRs within the SMAC competing against the internalization process to determine the fate of the T cell. If enough TCRs manage to bind to MHC–antigen complexes, the T cell survives to subsequently seek out “Signal 2”. If the internalization process dominates and the TCRs fail to bind enough MHC–antigen complexes, the T cell aborts activation. This control or threshold mechanism ensures that an overexuberant T-cell response does not result every time a T cell encounters its cognate antigen.

1.8 Signal 2: Costimulation and Coinhibition

After receipt of the first signal (Signal 1) which is generated by occupancy of the antigen-specific TCR by appropriate MHC–antigen complex, the T cell must still receive a second “confirmatory” signal (Signal 2) in order to avert the induction of T-cell anergy or apoptosis. The molecules that provide this second signal to the T cell are termed *costimulatory molecules*, and these molecules are numerous and varied in their characteristics (Table 1) (67). Only certain of these molecules (i.e., CD28) provide the T cell with the *costimulatory* signal that is necessary to optimize activation/proliferation, whereas others (i.e., CTLA-4) do exactly the opposite, acting as *coinhibitors to downregulate T-cell responses*. Interestingly, some costimulatory molecules can be either stimulatory or inhibitory, depending on the intensity of their signal and the environment or context in which the signaling process takes place (68, 69).

The principal costimulatory molecule is *CD28*, a receptor that is constitutively expressed on the surface of nearly all CD4+ T_H cells and the majority of CD8+ T_C cells (10, 67, 70). CD28 has moderate affinity for its cognate ligands, *B7-1* and *B7-2*, that are found on the surface of the APC. When both the TCR and CD28 bind to their corresponding ligands simultaneously (MHC–antigen and B7-1 or B7-2, respectively), these receptors generate converging intracellular signals that act synergistically in order to activate the T cell’s replicative machinery and

Table 1 Major costimulatory molecules and their actions

	Receptors (T cell)	Ligands (APC)	Major cellular effects
B7/CD28 Family	CD28	B7-1 (CD80)	–Constitutively expressed on most T cells –T-cell activation and proliferation (↑ IL-2 and IL-2R)
		B7-2 (CD86)	–Differentiation (↑ IL-1, IL-4, IL-5, IL-6, IL-10, TNF, IFN-γ) –Block apoptosis (↑ Bcl-xL) –↓ TCR signaling threshold
	CTLA-4 (CD152)	B7-1 (CD80)	–Induced 48–72 hours after TCR signaling (Signal 1) –Competes with CD28 for its ligands
		B7-2 (CD86)	–Blocks T-cell activation and proliferation (↓ IL-2) –Cell cycle arrest
	PD-1	PD-L1 (B7-H1)	–Blocks T-cell activation and proliferation (↓ IL-2)
		PD-L2 (B7-DC)	–Cell cycle arrest
	ICOS	ICOSL (B7-H2)	–Inducible molecule found only on activated T cells –B-cell development and germinal center formation (↑ IL-4, IL-10, CD40L) –T _H 2 differentiation of T _H cells (↑ IL-4, IL-10)
	?	B7-H3	–Blocks T-cell activation and proliferation (T _H 1 > T _H 2)
	?	B7-H4	–Blocks T-cell activation and proliferation (↓ IL-2, IL-4, IL-10, IFN-γ) –Cell cycle arrest
			–Blocks T-cell activation and proliferation
TNF/TNFR Family	BTLA	HVEM	–T-cell activation and proliferation
	CD40L	CD40	–T _C -cell activation and proliferation –T _C > T _H
	4-1BB (CD137)	4-1BBL	–T-cell activation and proliferation –T _H > T _C
	OX40	OX40L	–T-cell activation and proliferation –2° > 1° immune responses (favors immune memory)
	CD27	CD70	–T-cell activation and proliferation
	HVEM	LIGHT	–T-cell activation and proliferation

secretory apparatus (Fig. 1a). A second consequence of costimulation, however, is progressive migration of a coinhibitory receptor, called *CTLA-4*, from the subcellular Golgi compartment to the T-cell plasma membrane. This progression occurs over a 48-hour interval following the generation of Signal 2. Surface *CTLA-4* is normally not expressed to any significant degree on the membranes of inactive

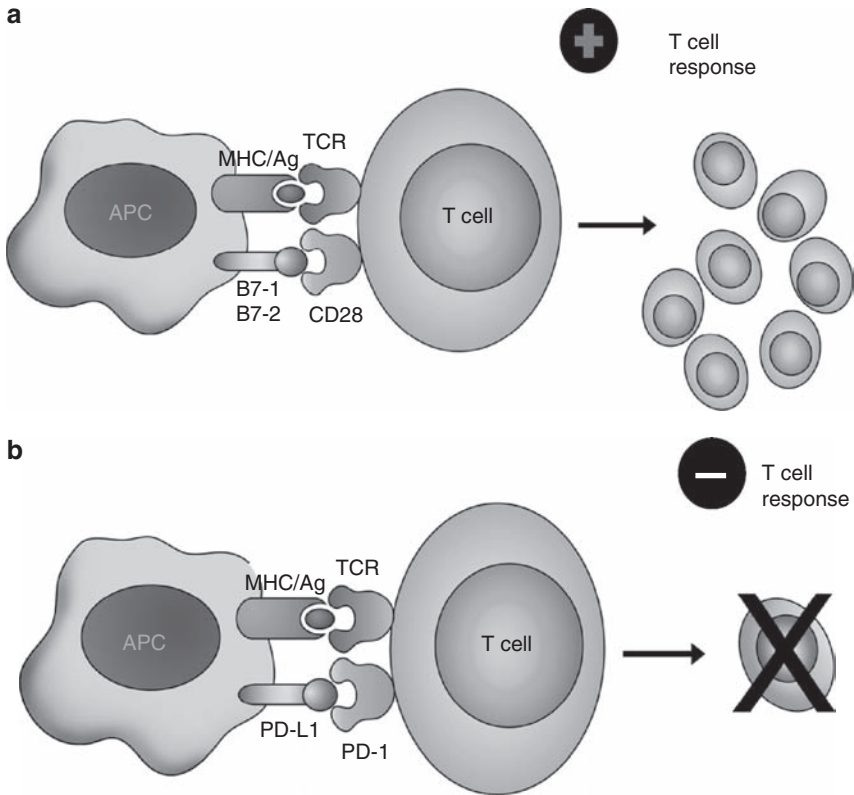


Fig. 1 (a) Costimulation with B7-1 or B7.2 induces T-cell activation. (b) Coinhibition with PD-L1 induces T-cell anergy or apoptosis

T cells, but when expressed CTLA-4 has a 10- 20-fold higher affinity for B7-1 and B7-2 than CD28. Not only does CTLA-4 compete ravenously with CD28 for its ligands but it also sends a completely different signal into the T cell, instructing it to turn off its secretory apparatus and replicative machinery. Therefore, the newly activated T cell has to constantly reconcile two contradictory signals: an early and positive activation/proliferation signal emanating from CD28, and a delayed inhibitory deactivation/nonproliferation signal emanating from CTLA-4. It is currently believed that this duality may act to protect against exaggerated and deleterious immune responses (i.e., autoimmunity). Finally, it should be recognized that the traditional lock-and-key concept of receptors (signal-receiving molecule) and ligands (signal-inducing molecule) may be an oversimplification in the case of B7-1 and B7-2. In fact, recent evidence suggests that when B7-1 and B7-2 bind to their receptors, signals are generated in both directions: into the APC as well as into the T cell (71–73). This indicates that both the APC and the T cell are ultimately

affected by the binding of B7-1/B7-2 to CD28/CTLA-4, a fact that likely has consequences for both cells.

More recent years have brought the discovery of several new costimulatory molecules that have added complexity to the understanding of T-cell activation and deactivation. Some of these costimulatory molecules are part of the B7/CD28 family (e.g., B7-H1, B7-DC, ICOSL, B7-H3, B7-H4), while others are members of the tumor necrosis factor receptor (TNF/TNFR) family [e.g., CD27, CD40L, OX40, HVEM (herpes virus entry mediator), and 4-1BB] (67, 74–76). In general, members of the TNFR family act in a similar fashion to CD28, stimulating T-cell proliferation and cytokine secretion. In contrast, the physiological actions of the newest B7 family members are not so easily summarized and we will therefore detail them individually.

Inducible costimulator (ICOS) is a receptor whose expression is induced onto the surface of the T cell following binding of either the TCR to MHC–antigen complex or the CD28 to B7 (77–79). ICOS binds to a specific ligand called *ICOSL* (aka ICOS ligand, B7-H2, B7h, B7RP-1) that is constitutively expressed in lymphoid tissue (80–84). Although the ICOS/ICOSL pathway has been shown to provide positive costimulation of the T cell, it is definitely distinct from the CD28/B7 pathway in several aspects (80, 85). For instance, the ICOS/ICOSL pathway does not appear to mediate the immediate release of IL-2 from the T cell, but it is clearly involved in the differentiation of T_H cell subtypes (discussed further later) and provides help to B cells (77, 86–91).

Programmed death 1 (PD-1) is another receptor found on T cells and numerous other cell types, including thymocytes, B cells, monocytes, endothelial cells, and NKT cells (92, 93). An overwhelming amount of evidence suggests that PD-1 signals inhibit T-cell activation and proliferation, although some studies have suggested that it may have a stimulatory capacity as well (Fig. 1b) (94–98). PD-1 has two known ligands, *PD-L1* (aka B7-H1) and *PD-L2* (aka B7-DC) (94, 95, 99) that differ markedly in both their affinity for PD-1 and their expression patterns. PD-L2 appears to have a higher affinity for the PD-1 receptor than PD-L1, and its expression is preferentially induced on DCs and macrophages by the presence of IL-4 (100–102). In distinction, PD-L1 is expressed on T cells, B cells, and macrophages in response to inflammatory cytokines such as IFN- γ (101–103). Generally speaking, T_H1 responses favor PD-L1 expression whereas T_H2 responses favor PD-L2 expression. PD-L1 also differs from PD-L2 in that it can be found at low levels in other tissues unrelated to the immune system (101), it mediates the induction of T-cell apoptosis (104), and it appears to play a major role in regulating levels of inflammation and autoimmunity (103, 105, 106). The differences in the physiological actions observed between PD-L1 and PD-L2 can be rationalized in four ways: (1) the two molecules probably interact in different ways with the PD-1 receptor (107), (2) there are probably other here-to-fore unidentified receptors other than PD-1 that engage these ligands (108, 109), (3) bidirectional signaling may cause changes in the APC–T cell interaction that confer ligand-specific outcome, and (4) PD-1's immunoreceptor tyrosine-based

switch motif may interact with different phosphatases within the T cell, which implies that PD-1 signaling inside of the T cell may vary based on the state of T-cell activation (110–113).

B- and T-lymphocyte attenuator (aka BTLA) is a receptor found on B cells and T cells that has many structural and functional similarities to PD-1, including inhibition of T-cell activation (114, 115). Due to its similarities with PD-1, it was originally assumed that BTLA would bind to a member of B7 family of ligands, and B7-H4 was initially proposed as the most likely candidate (114, 116, 117). However, an interesting series of recent articles has shown that BTLA's ligand is, in fact, not a member of the CD28/B7 family but a member of the TNF/TNFR family HVEM (118–121). This discovery is important because it demonstrates that cross talk exists between structurally dissimilar families of costimulatory molecules, and that costimulatory and coinhibitory signals can be competitive, and perhaps even coordinated, through a single receptor.

B7-H3 (aka B7RP-2) is a ligand found on APCs and other cell types that binds to an unknown counter-receptor on the T cell that is distinct from CD28, CTLA-4, ICOS, PD-1, and BTLA (122, 123). Studies evaluating the precise physiological role of B7-H3 are conflicting. While some groups report evidence suggesting that B7-H3 has positive costimulatory properties (123, 124), other groups have observed the exact opposite (125–127). Feasible explanations for this discrepancy are the presence of functionally distinct B7-H3 splice variants and the possibility of antagonistic B7-H3 receptors (128). Alternatively, the function of B7-H3 may differ according to the context or experimental conditions in which it is being studied. Like PD-L1, inflammatory cytokines such as IFN- γ induce B7-H3 expression on macrophages and monocytes (123). Certain DC subtypes may constitutively express B7-H3, but this is of unknown significance (129).

B7-H4 (aka B7S1, B7x) is similar to B7-H3 in that it is found on a wide variety of tissues (including APCs) and binds to an unidentified receptor on T cells (130). Unlike B7-H3, less controversy exists as to the function of B7-H4: it inhibits the activation and proliferation of T cells (117, 130, 131). B7-H4 appears to bind a receptor that is present on activated, but not naive T cells (117, 131). Contrary to early suggestions, BTLA does not appear to be the B7-H4 receptor (118). Nevertheless, our understanding of the function of B7-H4 is still in a relative state of infancy, and further studies will be required to confirm that B7-H4 is indeed a potent inhibitory coregulator of T-cell activation and function.

In summary, there are two large families of costimulatory molecules: the TNF/TNFR family and the B7/CD28 family (Fig. 2). TNF/TNFR costimulatory molecules tend to activate the T cell and induce its proliferation, each in a slightly different way. The B7/CD28 family is heterogenous: CD28 and ICOS are predominantly positive costimulators whereas CTLA-4, PD-1, BTLA, B7-H4 (and its unidentified cognate receptor) are predominantly coinhibitory molecules. B7-H3, and to some degree PD-1 when bound by PD-L1, can assume a stimulatory or inhibitory role depending on the circumstances in which the T cell is activated.

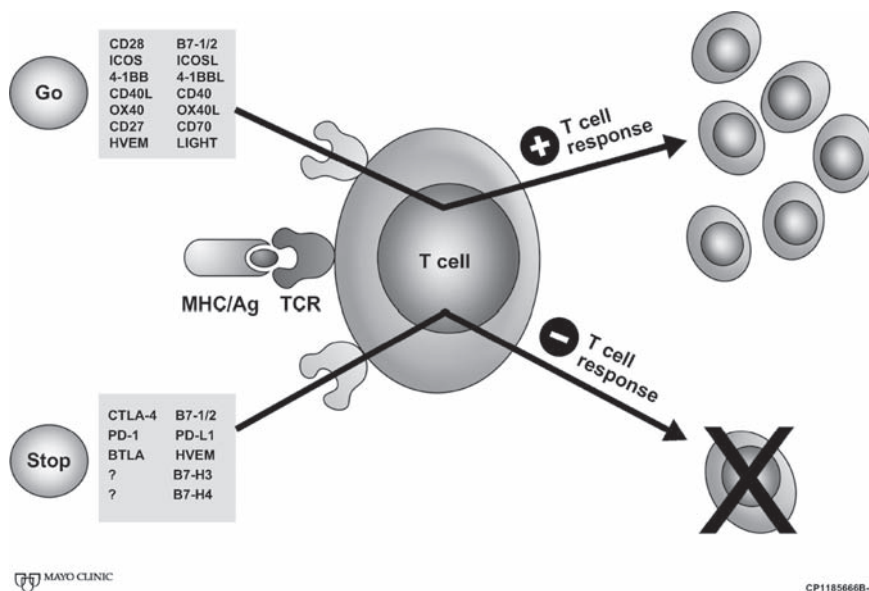


Fig. 2 A multitude of costimulatory and coinhibitory receptor-ligand pairs influences T-cell function

1.9 Signal 3: Cytokine Release

After receiving Signals 1 and 2, the newly activated T cell is ready to commence transcribing many genes whose protein products are required to permit the T cell to divide and differentiate into a clonal family of antigen-specific effectors and memory T cells. Some of these genes are activated *immediately* (within minutes) whereas others are activated later and are considered either *early* (hours) or *delayed* (days) genes. Cytokines are critical to process of T-cell activation and differentiation. The key cytokines involved appear to be IL-2, IL-4, IL-7, IL-12, and IL-15 (132–134).

Perhaps the most important cytokine that is transcribed immediately following Signals 1 and 2 is *IL-2* (135). IL-2 can act in an autocrine (stimulate itself) or paracrine (stimulate others nearby) fashion to initiate and sustain T-cell proliferation. The paracrine component is particularly important for CD8+ T_C cells because they tend to have difficulty generating enough IL-2 to induce and sustain their own proliferation. Thus, CD8+ T_C cells often require paracrine IL-2 help from activated CD4+ T_H cells in order to achieve maximal activation and function. Although IL-2 stimulates the proliferation of all T-cell subsets (i.e., CD4+ T_H cells, CD8+ T_C cells, and $\gamma\delta$ -T cells), it tends to have its most pronounced effect on T_H cells. IL-2 is also crucial for the development of self-tolerance (136). The receptor for IL-2, IL-2 receptor, in resting T cells consist of two different chains (β - and γ -chains). Upon activation, there is transcription and assembly of a third chain (α -chain, CD25) which multiplies its affinity by 10,000-fold (137). Conversely, the constitutive

expression of CD25 (CD25^{high}) on an inactivated T cell is indicative of an inhibitory regulatory T-cell phenotype (30, 138). Some regulatory T cells have also been demonstrated to express a transcription factor called FoxP3 (24, 139).

Interleukin-4 (IL-4) is involved in the differentiation of T_H cells into specific subsets, in particular the T_H2 subset (140). T_H2 cells are important for mediating immune responses directed against extracellular pathogens, such as bacteria and parasites, and to ensure that B cells are empowered to produce appropriate isotypes of antibody (141).

Interleukin-7 (IL-7) is a key regulator of early lymphocyte development in the bone marrow and thymus (142). It is involved in triggering TCR gene rearrangement and facilitates the development of a distinct type of TCR, the $\gamma\delta$ -TCR (143, 144). Memory T cells are also induced and maintained by IL-7.

Interleukin-12 (IL-12) induces differentiation of T_H cells into T_H1 subset (145). T_H1 cells are vital for combating intracellular pathogens, such as viruses and intracellular bacteria. Whereas IL-4 favors the generation of humoral immunity (antibody responses), IL-12 favors the induction of cell-mediated immunity. IL-12 antagonizes IL-4 and inhibits the differentiation of T_H2 cells (141).

Interleukin-15 (IL-15) is critical for the formation of NK cells and it is also very important for lymphocyte recirculation in the body as well as homing of cells to the lymph nodes. IL-15 is involved in promoting the differentiation of effector T_C cells (i.e., cytotoxic T cells, CTLs) and CD8+ memory T cells (134).

2 Leukocyte Dysfunction in RCC

There is an abundance of research that suggests that the immune system is often impaired and dysfunctional in patients with cancer (146). Certain cancers, such as RCC, seem to elicit more prominent defects in immunity than other tumors. In this section, we discuss the evidence supporting that the host immune system may be compromised in the context of RCC.

2.1 Tumor-Infiltrating Leukocytes

If the immune system can target and kill tumor cells, it seems logical that tumors filled with leukocytes and lymphocytes would be less likely to progress than tumors without such tumor-infiltrating immune cells—something akin to trying to escape from a room full of assassins who are singular expertise is to kill you. Consistent with this notion, many researchers have shown the presence of intratumoral mononuclear immune cells within histological tumor sections – portend an unfavorable prognosis. Specific tumors for which this has been the case include: breast cancer (147–149), lung cancer (150, 151), colorectal cancer (152–156), stomach cancer (157–161), esophageal cancer (162–164), gallbladder cancer (152, 165), malignant melanoma (166–169), neuroblastoma

(170, 171), cancers of the head and neck (172), prostate cancer (173), testis cancer (174–178), bladder cancer (179–184), and kidney cancer (185). Unfortunately, the story is also obfuscated by a number of manuscripts that have reported opposing findings pertaining to in breast cancer (186–188), malignant melanoma (189, 190), lung cancer (191, 192), bladder cancer (193), and RCC (194–198).

Why are these results so divergent and why do certain tumors appear to be associated with “bad” immune responses while others do not? While there is no simple response to these questions, part of the answer may certainly be explained by the fact that not all tumor-infiltrating immune cells are the same in terms of composition and states of activation. In fact, a critical first step in understanding the immunobiology of a given tumor typically arises from analyses of what populations and subsets of immune cells are actually infiltrating the tumor. Although the pathologist can distinguish many cell types with considerable accuracy, a microscopic view of events remains overly superficial. Routine histology simply cannot distinguish between many related cell types (e.g., NK, NKT, and T cells), nor can a basic microscopic analysis reveal the functional state of an immune cell (i.e., activated vs anergic T cell). Thus, to secure a more sophisticated view of the intratumoral immune microenvironment, specialized techniques are often required including immunohistochemistry, flow cytometry, cell culture, and functional assays. Immune cell subset studies of this nature have been conducted in RCC and are discussed briefly in the subsections to follow.

Another reason why studies may diverge in their interpretation of the importance of tumor-infiltrating immune cells is the frequent lack of thoroughness in controlling for other recognized prognostic factors. Studies that employ sound methodology, adjusting for confounding predictors (such as tumor grade and stage), are less likely to reveal artifactual prognostic associations with a novel predictive marker than uncontrolled studies. In the specific case of RCC, we are aware of 13 published prognostic tools for predicting survival (199–211), all of which were rigorously designed, and none of which incorporated the histological presence of tumor-infiltrating lymphocytes as part of their scoring system. Unfortunately, none of these prognostic systems included levels of tumor infiltration by immune cells as a covariate during the modeling process. At present, we are aware of only one large and adequately controlled study that has assessed the importance of tumor-infiltrating immune cells within RCC tumors (198). This study demonstrates that mononuclear cell infiltration in histological RCC sections is associated with poorer cancer-specific survival, even after controlling for all other important and well-described prognostic factors.

2.2 *T Lymphocytes in RCC*

Clarifying the role of T cells in RCC has been the focus of much research over the years. It has been clearly shown that the T cells infiltrating an RCC tumors differ markedly from T cells observed circulating within the peripheral bloods of corresponding

patients. For instance, although the absolute quantity of tumor-infiltrating T cells observed in RCC specimens increases in parallel with both tumor stage and grade (197), the relative proportion of activated intratumoral T cells actually diminishes (212). This suggests that although aggressive RCCs recruit many T cells to the tumor site, these tumors are somehow capable of preventing the T cells from being activated (to be discussed later). It has also been shown that T cells infiltrating RCC tumors frequently exhibit dysfunctional TCRs (213–216).

If we now only consider the specific CD8+ T-cell subset, it has been shown that the quantity of tumor-infiltrating CD8+ T cells increases with severity of tumor grade and tumor proliferative activity (195, 212, 217). Some groups have even shown that a high level of intratumoral CD8+ T cells predicts an increased probability of RCC recurrence (197), a poor response to IFN- α immunotherapy (185), and a shorter cancer-specific survival (185, 195). In distinction, one group noted that an increased quantity of intratumoral CD8+ T cells predicted a more favorable outcome when infiltrating T cells were actively proliferating (195). Taken together, these data suggest that the mere presence of CD8+ T cells within an RCC tumor is insufficient to produce an antitumoral immune response. Rather, it appears that CD8+ T cells need to be appropriately activated in order to effectively mediate tumor regression; a fact that makes intuitive sense. These data also raise the specter of tumor-related factors that prevent CD8+ T cells from being fully activated as well as the possible existence of regulatory/inhibitory CD8+ T-cell subsets (218–220).

In the case of the CD4+ T-cell subset, the results pertaining to RCC are much more heterogeneous. While some researchers have found increased CD4+ T cells in high-grade and high-stage tumors (194, 221), others have reported the opposite (217). One group showed that the response to IFN- α immunotherapy tended to be better in patients with high intratumoral CD4+ cell counts and, perhaps, thereby improving survival (185). Contrarily, another group reported that RCC-specific survival was diminished in patients with high intratumoral CD4+ T-cell counts (194). Clearly, not all CD4+ T cells are the same. An interesting and relevant finding in other experimental and clinical tumors has been the observation that CD4+ CD25+ regulatory T cells may encompass one entity that inhibits CD8+ effector T-cell function (222–225).

2.3 NK Cells in RCC

NK cells comprise an important fraction of the immune cells that infiltrate RCCs and are typically of the CD16^{bright} cytotoxic-effector variety (212, 226, 227). The more the NK cells that are found in an RCC, the more active these NK cells appear to be (227). The number of tumor-infiltrating NK cells may decrease as the tumor grade increases, possibly indicating that aggressive tumors tend to attract less NK cells (212). We are aware of no study that has shown NK cells to be of prognostic value in RCC, though such studies do exist for other cancers (150, 156, 161, 228–230).

2.4 *Neutrophils, Monocytes, and Macrophages in RCC*

An awareness has slowly developed that inflammation and the innate immune system play an important role in cancer development (231–233). In the case of RCC, markers of inflammation have long been known to predict a poor outcome (234–236). A few clinical trials have also shown that high levels of circulating or tumor-infiltrating inflammatory cells such as neutrophils and macrophages predict a poor response to IL-2 immunotherapy (200, 237, 238). Interestingly, one group showed that a drop in monocytes occurring during IL-2 therapy predicted a favorable treatment response (239). One explanation of why such inflammatory cells might impair antitumoral immunity pertains to the interaction of such cells with T cells (240). It has been speculated that part of the dysfunctional phenotype of intratumoral NK and T cells may result from oxidative stress or other forms of damage directly caused by products secreted by activate tumor-associated neutrophils and macrophages cells (237). Prompted by such speculation, clinical trials combining IL-2 and histamine (an inhibitor of reactive oxygen species formation and release) have been conducted as one experimental form of treatment for advanced RCC, but, to date, these trials have not shown a clear benefit resulting from the addition of histamine to the treatment regimen (241).

3 Clinical Aspects of Costimulation and Coinhibition in RCC

As previously discussed, the B7 family of molecules interact with a large array of positive and negative modulatory receptors on T cells (Table 1). Although most of these molecules have not been systematically evaluated in RCC, the few that have been studied have revealed some interesting results. In this section, we will contrast the roles of costimulation and coinhibition in the specific context of RCC.

3.1 *Classic Pathways: B7-1/2, CD28, and CTLA-4*

It has been known for some time that positive costimulation has significant effects on RCC-associated immunity. The first costimulatory interaction discovered was that of B7-1 and B7.2 with their receptor CD28 to provide a positive second signal necessary for T-cell activation (MHC presenting an antigenic epitope to the TCR being the first signal). This typically is thought to occur within a lymph node at the interface between an APC and the T cell. Much effort has been expended in attempting to genetically induce tumors to perform this function in lieu of professional APCs after some animal models suggested the strategy could induce antitumor activity. For instance, several groups have shown that transfecting RCC cells to constitutively

express the costimulatory ligands, B7-1 and B7-2, result in significantly amplified antitumoral immune responses by the host (242–247). Difficulty with culturing and genetically modifying autologous tumor lines for clinical vaccination has led many to abandon this approach, though it has recently been exploited in a phase I clinical trial that tested genetically modified RCC cells expressing high levels of B7-1 as a tumor vaccine coadministered with IL-2 (248, 249). The efficacy of this regimen remains to be assessed in phase II/III clinical trials. It has furthermore been observed that a high level of B7-1 expression in patients with metastatic RCC predicts a favorable response to nephrectomy and IL-2 immunotherapy (250). Interestingly, IL-2 immunotherapy appears to cause RCC tumors to upregulate their expression of B7-1 (251, 252). Intuitively, the receptor promoting B7-1- and B7-2-mediated T-cell activation in RCC should be CD28 because this receptor is instrumental in facilitating T-cell activation and stimulatory function, whereas the other cognate receptor for B7-1 and B7-2, CTLA-4, is generally inhibitory. Indeed, laboratory results have confirmed that stimulation of RCC-associated T cells with anti-CD28 stimulating antibody amplifies the antitumoral immune response (253). A phase I clinical trial has also shown that CD28-activated T cells can serve as a form of adoptive T-cell therapy for RCC patients (254).

Another effort that has reached clinical trials is the use of recombinant pox viruses engineered to express defined tumor antigens such as CEA or PSA to which are added costimulatory molecules such as B7-1. A recent report of one such clinical trial describes patients with metastatic CEA-expressing tumors who received pox viruses expressing CEA along with a “triad of costimulatory molecules,” including B7-1 (255). Of the 58 patients studied, no patient generated CEA-reactive T-cell frequencies over 1/13,000 and there was only one clinical response (by postmortem examination after accidental death rather than measurement criteria). Similar findings were reported for a pox virus expressing PSA and B7-1 in patients with prostate cancer (256).

Up to this point, there has been little therapeutic success to suggest that these B7-1/2 approaches have created an “improved APC” or that significant antitumor T-cell responses are induced; indeed, only anecdotal tumor regressions have been seen. Nevertheless, other preclinical and clinical data indicate that the B7 family of ligands and their respective receptors can play a decisive role in the immune recognition and rejection of human cancer. The most compelling data are from clinical trials of antibodies that block the inhibitory receptor CTLA-4, which can interact with either B7-1 or B7.2 to turn off the immune response (Fig. 3a). CTLA-4 is an inhibitory receptor on T cells which is induced by activation, shows prolonged expression, and binds its ligands with high affinity (257). Its opposing receptor, CD28, is a positive costimulator of T cells, is constitutively expressed by resting T cells, decreases with activation, and binds CD80 and CD86 with lower affinity than CTLA-4 (258). One study has reported that RCC patients harbor increased levels of CTLA-4 positive lymphocytes and that these lymphocytes are phenotypically and functionally consistent with regulatory T cells (37). Interestingly, those patients that responded to IL-2 immunotherapy in this study were also patients in which CTLA-4 positive T_{Reg} cell levels declined during treatment. In animals, genetic

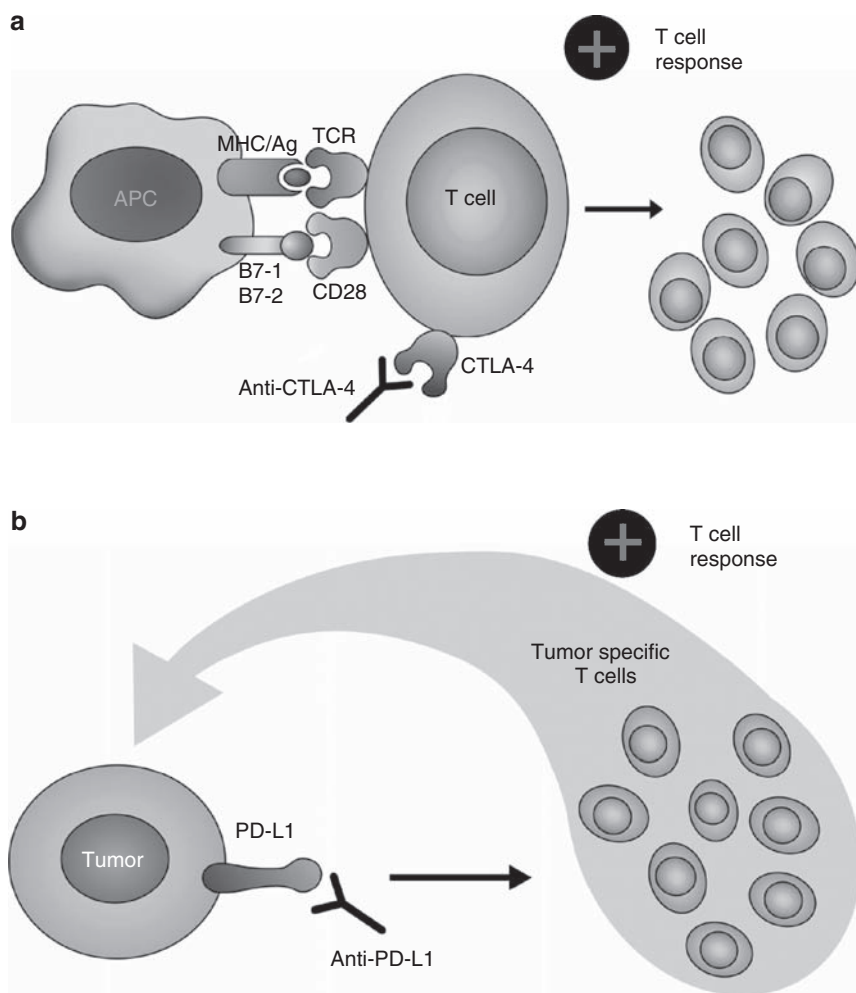


Fig. 3 Potential strategies for therapeutic targeting of coinhibitory molecules. (a) Monoclonal antibody blockade of CTLA-4 on the T cell. (b) Monoclonal antibody blockade of PD-L1 on the tumor cell or T cell

“knockouts” for CTLA-4 die of unrestrained lymphoproliferation at a young age and antibody blockade of murine CTLA-4 can enhance antitumor activity in some tumor models (259, 260). Therefore, two companies developed anti-CTLA-4 antibodies for clinical testing. Ipilimumab (Medarex, Inc., Princeton NJ) is an IgG1 raised in mice genetically modified to contain the human immunoglobulin locus. Therefore, it is a fully human IgG1 that reacts with human CTLA-4. Tivolumab (CP-675206; Pfizer, Inc., New York, NY) is an IgG2 human antibody made with similar technology. While a tivolumab clinical phase I/II trial noted no responses in four patients with RCC (261), ipilimumab has induced durable tumor

regressions in a phase II trial in patients with metastatic renal cancer (262). Both agents have induced regressions in patients with metastatic melanoma, and in both cases, interesting associations of tumor responses and autoimmune phenomena have been seen (261, 263).

In a phase II trial performed by the Surgery Branch of the National Cancer Institute, patients with measurable metastatic renal cancer received ipilimumab every 3 weeks in one of two dose regimens. Twenty one initial patients received 3 mg/kg as their initial dose and then 1 mg/kg for all subsequent doses. The second group received 3 mg/kg for all doses. Among the patients in the initial lower dose cohort, all of whom had previous IL-2 therapy, only three patients had significant toxicity. In all three, there was colitis, presenting with diarrhea, and in one of the three, arthritis and dermatitis also occurred. That patient received a total of five doses of antibody and had the only tumor regression seen in this cohort, with a partial regression of lung, adrenal, and epicardial metastases. This partial response by RECIST criteria lasted 18 months, an isolated relapse in femur then was irradiated and no further progression occurred for an additional 13 months. In a subsequent cohort of 40 patients who all received 3 mg/kg of ipilimumab every 3 weeks, there were 6 partial responses for an overall response rate of 15% (Fig. 4). Most toxicities in these patients seemed immune mediated and were presumed to be of autoimmune origin. Patients developed enteritis with diarrhea ($N=14$), hypophysitis with

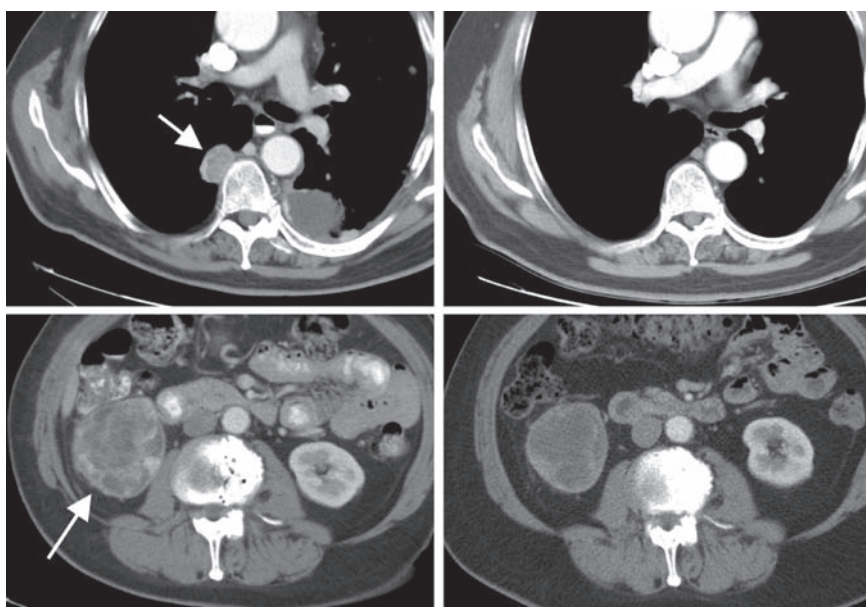


Fig. 4 Patient with primary renal tumor in place and extensive pulmonary and pleural disease treated with ipilimumab monotherapy. Durable partial response of more than 20 months was seen which included regression of the primary renal tumor (left, pretreatment; right, follow-up at 20 months)

hypopituitarism ($N=2$), primary adrenal insufficiency, and aseptic meningitis with CSF lymphocytosis ($N=1$ each). There was a highly significant association between these immune-mediated toxicities and tumor regression. Analyzing all patients in both cohorts together, the response rate in patients with autoimmune toxicity was 35% (7 of 20), while there were no responses among the 41 patients without these toxicities ($p=0.0002$). The importance of the association between autoimmunity and tumor response is not clear, though some have speculated that an impairment in regulatory T-cell function may be at fault. Although T-regulatory cells constitutively express high levels of CTLA-4, conflicting data have been reported on the effect of anti-CTLA-4 antibodies on this population of inhibitory T cells (264, 265).

Taking a different approach, a superagonist monoclonal antibody against the positive coreceptor CD28, called TGN1412, was tested in a phase I trial (266). The first six subjects given the lowest dose suffered immediate adverse symptoms followed by major multiorgan toxicity. Although the events and mechanism of toxicity are still unclear, many of the reported toxicities were reminiscent of a cytokine “storm,” again suggesting that manipulating costimulatory pathways can have potent systemic effects. Notably, for ipilimumab and perhaps for TGN1412, the preclinical and in vitro effects did not truly anticipate the in vivo effects seen on clinical testing. In mice, although genetic deletion of CTLA-4 had potent effects, antibody blockade in mature animals had quite modest antitumor impact and provoked little to no autoimmunity. Therefore, clinical testing of specific agents may be the only reliable way to eventually assess the benefits and risks of some of these manipulations. Combinations of modulators of costimulation and other agents which might provide a more focused or directed cognate antitumor response are likely to be explored next. Initial experience combining ipilimumab and minimal determinant peptide vaccines in patients with melanoma have thus far not demonstrated enhanced vaccine responses occurring with the addition of ipilimumab, but further work is needed (267). A trial testing the empirical combination of ipilimumab and IL-2 in patients with melanoma also may have had a modest additive effect at best, but again this combination lacked a tumor-specific immune stimulus (268). Ultimately, it may be necessary to better understand the immunological mechanisms leading to tumor regression with ipilimumab monotherapy in order to design effective strategies combining this agent with other immunotherapies.

3.2 Novel Pathways: Other Members of the B7/CD28 Family

In view of the activity of anti-CTLA-4 in patients with melanoma and renal cancer and even more impressive murine data on other similar T-cell-modulating receptors (269), there is great interest in pursuing clinical testing of additional agents which manipulate these other coreceptors. Recently, some intriguing data pertaining to the importance of PD-L1 (B7-H1) expression by RCC tumors has been published that suggests that this coinhibitor may be a good target for immunotherapy. Specifically, it has been reported that increased tumor level expression of the T-cell coinhibitor

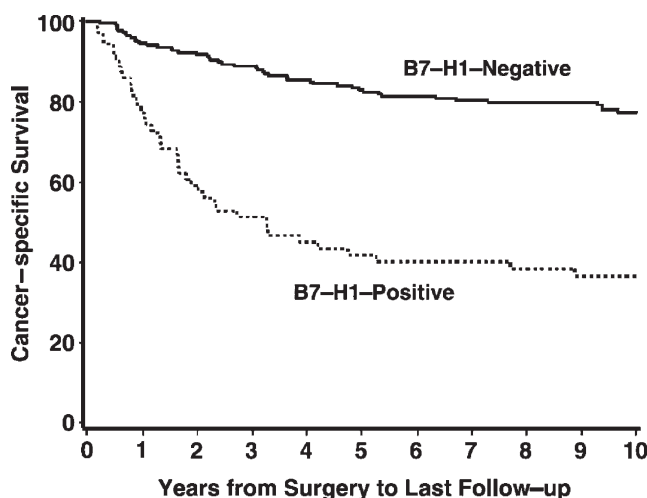


Fig. 5 Tumor PD-L1 expression is associated with worse cancer-specific survival in patients with renal cell carcinoma treated by nephrectomy ($p < 0.001$). This effect was independent of tumor stage, grade, size, and necrosis [from ref. (273)]. Reproduced with permission from the American Association of Cancer Research

PD-L1 increases the risk of metastatic dissemination and cancer-specific death for patients with RCC (Fig. 5). It was also demonstrated RCC-associated PD-L1 represents an independent predictive factor, even after adjusting for all other important clinical predictors such as ECOG performance status, tumor size, tumor stage, tumor grade, and the presence of tumor necrosis (270–273). One in vitro experimental study has also shown that blocking PD-L1 facilitates immune responses against human RCC cells (274). Similar results have been observed in other tumor systems as well (269, 275–277). The implications of this research are important. First, PD-L1 may prove very useful as a prognostic marker to select high-risk patients that might benefit from adjuvant therapy. Second, PD-L1 may represent an important target for immunotherapeutic blockade, particularly when used in combination with other immunotherapeutic modalities (Fig. 3b) (271). Abrogating the inhibition of antitumoral T-cell-mediated immunity through the use of PD-L1 blockade could provide a key strategy to permit the adaptive immune system to more easily detect and destroy malignant cancer cells, for RCC and many other forms of human malignancy. Clinical trials testing blockade of this coinhibitory molecule will certainly be of interest and are anticipated to commence in the next years. A human antibody to the PD-L1, PD-1, is soon to enter phase I clinical trials.

A study evaluating B7-H4 has also recently been published (278). This study showed that like PD-L1, B7-H4 is an important independent predictor of RCC prognosis. Additionally, the prognostic value of B7-H4 is additive to that of PD-L1, suggesting that multiple routes of coinhibition may be active and important in RCC. We are unaware of any published research evaluating ICOS, ICOSL, or B7-H3 in RCC.

3.3 *Novel Pathways: The TNF/TNFR Family*

Members of the TNF/TNFR family of costimulatory molecules have also been evaluated in RCC. For instance, CD70 has been shown to be aberrantly expressed by the majority of clear cell RCCs, and by a lesser proportion of other RCC subtypes (279–281). Expression of the CD70 receptor, CD27, was also noted to be increased on NK cells of RCC patients undergoing IL-2 immunotherapy (282). A strategy to target RCC with anti-CD70 antibody-drug conjugates has been proposed on the basis of this evidence (281). The expression of another TNF/TNFR member, CD40, is increased in both the vasculature and tumor cells of RCC patients (283, 284). Stimulating these CD40 receptors with their ligand, CD40L, results in the increased tumor cell Fas expression and death (283). Similarly, in a murine RCC model, the combination of CD40 stimulation and IL-2 was found to be superior to either agent alone (285). These data suggest that CD40 may play an important role in modulating immunotherapeutic antitumoral responses, though, to date, no clinical trials have tested this hypothesis. We are unaware of any published research evaluating 4-1BB/4-1BBL, OX40/OX40L, or HVEM/LIGHT in RCC.

4 A Model for Immune Dysfunction in RCC

The previous sections of this chapter were intended to impress upon the reader what we believe to be three basic truths. First, the immune system is an important defense mechanism against cancer and is therefore a worthy target for cancer therapy. Second, activating the central effector cell of adaptive immune function—the T cell—is not a serendipitous process. Rather, T-cell activation requires a highly orchestrated sequence of specific steps that can be preempted and/or disrupted by any number of critical events. Finally, multiple limbs of the immune system may be impaired in RCC, and each limb is interrelated with the others. In this section, we propose a pathophysiological paradigm that unites these three aspects into one model of RCC tumor immunology.

The presence of distinct costimulatory and coinhibitory molecules provides a mechanism for the three prongs of immunoediting as described earlier. If sufficient costimulation is provided in the presence of adequate tumor-associated antigenic stimulation, the immune system will act against tumor antigen and, thus, destroy early tumors before they become fully established. Contrarily, if coinhibitory signaling dominates, the immune system will be tolerized to tumor antigens, and the tumor will be permitted to grow unfettered and unmolested by the host immune system. If neither costimulatory nor coinhibitory signals dominate, the adaptive immune system may remain in a tenuous state of equilibrium, militating against tumor outgrowth with varying degrees of success.

If a malignant cells escape early immunoediting and a tumor becomes established, the immune system may still respond. Changes in the local environment of the tumor will result in the recruitment of naive T cells to the tumor. These

T cells once again face three potential outcomes. If the tumor contains a favorable cytokine balance and costimulatory molecules are abundant, the T cells will be activated and the tumor may be eliminated. On the other hand, if the tumor cells or tumor-infiltrating immune cells express abundant coinhibitory molecules, the naive T cell will become tolerized and the antitumor immune response will be crippled. If neither signal dominates, antitumoral T cells may mount a low-level response that only slows tumor progression but does not culminate in tumor elimination.

In the case of RCC, the accumulated evidence suggests that a major costimulatory pathway for the adaptive immune response is CD28-B7-1/2 and that a major coinhibitory pathway is PD-1/PD-L1. Other cosignaling molecules may also play a role in modulating the behavior of RCC tumors, but their importance remains yet to be elucidate. Nevertheless, it is clear that a T cell can be coaxed either into an activated or into a dormant state through manipulations of the intratumoral cytokine environment, by fostering costimulatory signaling and abrogating coinhibitory signaling and by removing inhibitory immune cells that act to impair regional antitumoral immunity. For patients with high-risk RCC, we envision a future in which combination immunotherapeutic approach will be required to improve treatment outcome. Such combination immunotherapies will likely be composed of cytokine treatment with IFN- α and/or IL-2, upregulation of B7-1/B7-2 costimulation, abrogation of coinhibition through blockade of PD-L1 or CTLA-4, and depletion of inhibitory regulatory T cells. In addition, it is likely that such immunotherapies will be given in combination with other modalities of treatment such as surgery or chemotherapy in order to optimize outcome. Thus, it is only through these multiple avenues of attack that the elimination of cancer will become a reality for patients suffering from advanced RCC.

One last word of caution merits mention, given the disastrous consequences of a recent clinical trial that used a CD28-stimulating antibody (286). Targeting costimulatory and coinhibitory molecules can lead to very powerful immune responses and should not be taken lightly. The cytokine release syndrome may results if too much costimulation is provided placing patients' lives at risk (287). Likewise, autoimmunity may develop if coinhibition is too strong and organ damage may ensue (267, 288). The future art of immunotherapy will probably require tailoring the immunotherapy regimen to each patient so that each individual immune system is pushed just far enough in the right direction for the desired effect to occur.

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The Role of Gangliosides in Renal Cell Carcinoma

Philip E. Shaheen, Ronald M. Bukowski, and James H. Finke

Abstract Gangliosides are cell membrane-associated glycolipids with various and unique functions in tumors and normal tissues. Preclinical data suggest that they have a role in modulating the immune response to tumors, modification of biological effects of several trophic and growth factors in vivo and in vitro as well as identifying the phenotype of tumors and their behavior. Their specific role in modulating tumor angiogenesis in renal cell carcinoma (RCC) is an interesting and promising area of research which could potentially have a tremendous benefit in the era of targeted therapy. Although specific expression pattern of certain gangliosides (GM2 and GM1) can be seen in RCC, and “pro-angiogenic” versus “anti-angiogenic” gangliosides have been identified in various tumors and cell lines, more mature data in RCC are needed to integrate these antigens into the diagnostic, prognostic, and therapeutic approaches to this tumor. Their role in the downregulation of the type 1 T-cell response as evident by the inhibition of interferon- γ production from T cells cultured in the presence of renal cell carcinoma-derived gangliosides is another area of interest that would alter the immunotherapy approach to this tumor. Developing antiganglioside antibodies or inhibitors after further understanding of their role in tumor growth and behavior is the ultimate goal in integrating the use of these antigens into antitumor therapy of RCC.

Keywords Gangliosides in RCC • Glycosphingolipids • Gangliosides and VEGF • Gangliosides as therapeutic targets

J.H. Finke (✉)

Department of Immunology, Lerner Research Institute,
The Cleveland Clinic Foundation, Cleveland, OH
e-mail: finkej@ccf.org

1 Gangliosides As Membrane Glycosphingolipids

1.1 Structure and Function

Glycosphingolipids are well-organized membranous ceramide structures of various glycosylation patterns that can be neutral, basic, or acidic. They are classified according to the core structure into ganglio-series, globo-series, lacto-series type 1, and lacto-series type 2. Gangliosides are acidic glycosphingolipids that contain sialic acid and occur as any of the above series, where each can have a tissue specificity and different functional role (1). [Figure 1](#) illustrates the structure of a ganglioside within the cellular membrane. Glycosphingolipids in general are believed to represent cell type-specific markers in normal tissues as well as tumor-associated antigens in malignant cells, and the expression pattern of some glycosphingolipids and gangliosides may decline or change during cell development (2, 3). They can also be reexpressed in various malignancies and the expression pattern might be an indication of the tumor aggressive potential (4, 5).

Gangliosides are found in almost all tissues of the body; however, they are particularly abundant in brain and nervous tissues. Factors that play a role in regulating their synthesis and expression are the specificity and the levels of glycosyltransferases (gangliosides synthases), the location and organization of these enzymes in the cell, and the intracellular pH (6). Gangliosides appear to be involved in various cellular functions including signal transduction, adhesion and cell–matrix interactions, cell proliferation, suppression or downregulation of the antitumor immune response, and can serve as cell-attachment receptors for some microorganisms (7–11). They also have a unique role in modulating angiogenesis and certain growth factors; this is evident by numerous *in vitro* and *in vivo* experiments and will be discussed in more details in Sect. 2.

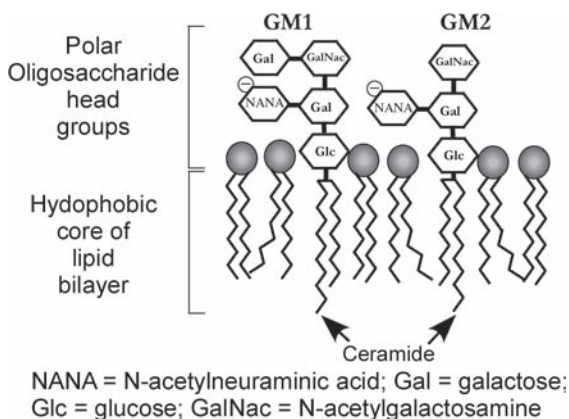


Fig. 1 Structure of a ganglioside within a cell membrane is illustrated

Gangliosides as tumor-associated antigens have gained attention in the field of Oncology, and have been used to develop antitumor therapies such as in vaccine preparation and in the development of antigangliosides monoclonal antibodies. Some of these investigations showed promising results such as the GM2/BCG vaccine in patients with stage III malignant melanoma; this vaccine demonstrated a significant advantage on disease-free survival and overall survival compared to BCG alone in a randomized phase III adjuvant trial (12). Therefore, studying gangliosides and their role in tumor biology, progression, antitumor immunity and the interaction with their environment may facilitate the development of novel therapies to treat various malignancies.

1.2 Gangliosides in Renal Cell Carcinoma

Various gangliosides were found to be expressed in renal cell carcinoma (RCC) tumor biopsy tissues and cell lines, and a higher level of expression of certain gangliosides was found compared to normal kidney tissues. In particular, GM2, GM1, and GD1a were found to have a higher mean expression in tumor tissues compared with normal kidney tissue, and GM2, GM1, and GD2 were found to have a higher mean expression in RCC cell lines compared with tumor biopsy tissues (13). GM3 was the most significantly expressed ganglioside in the RCC and normal kidney biopsy, whereas GM2 was the most significantly expressed ganglioside in the RCC lines. The amount of ganglioside expressed in the RCC biopsy in this study was comparable to that observed in melanoma and neural tissues. In another study, RCC lines derived from metastatic deposits were found to have increased expression of monosialosyl galactosylgloboside (MSGG) and disialosyl galactosylgloboside (DSGG); therefore, they are believed to have a correlation with the metastatic potential of this tumor (14).

In reviewing gangliosides expression in clear cell carcinoma tumor tissues, GM3 was found to be slightly decreased in the majority of specimens (10/14), and slightly increased in few specimens (4/14) compared to that in normal tissues obtained from the same kidneys bearing the tumors (15). In the same study that included tissues from 23 patients, high-performance thin-layer chromatography (HPTLC) was used, and expression corresponding to GM2, GM1, and between GD1a and GD1b was observed in tissues obtained from metastatic clear cell carcinoma and in those obtained from the primary tumors of patients with clear cell RCC who developed relatively early metastases. These observations suggest that RCC may have a specific expression pattern of gangliosides, some of which (e.g., GM2 and GM1) can have a role in tumor behavior.

Studying gangliosides in RCC tissue and patient sera has led to the identification of some of their functions such as their potential role in the immune dysfunction seen in patients with RCC. GM2 in particular appears to contribute to the T-cell apoptosis induced by RCC and by gangliosides isolated from RCC tissues and cell lines. GM2 is also believed to constitute the majority of the apoptogenic activity seen in the supernatants of RCC (Finke et al., unpublished data). This is supported by the findings that gangliosides derived from RCC have the capacity to suppress the activation of NF- κ B,

an antiapoptotic transcription factor in T lymphocytes (16). Gangliosides have also demonstrated the ability to modulate the host immune response *in vivo* and *in vitro* favoring either Th-1 type (cellular and antitumor) or Th-2 type (humoral) cytokine response (11, 17, 18). In RCC, most studies have indicated that the cytokine profile of the tumor infiltrating lymphocytes (TILs) is more consistent with a Th-2 type bias (17); indeed gangliosides isolated from the tumor supernatants suppressed the Th-1 response without inhibiting a Th-2 response *in vitro* (19). Interestingly, other studies have demonstrated that Th-1 response could be the primary immune response to RCC as reflected by the cytokine profile of the TILs (i.e., increased secretion of IFN- γ and IL-2) within the tumor microenvironment (20). This bias is believed to take place at the lymphocytes differentiation level; however, it is unknown whether gangliosides play any role in this setting. Finally, other gangliosides from RCC tissues and supernatants may suppress both Th-1 and Th-2 cytokine response (Richmond et al., unpublished data).

These observations suggest that ganglioside composition in different tumors is a key in determining how Th-1 and Th-2 will be affected.

2 Gangliosides and Growth Factors Modulation

RCC is a heterogeneous disease and its tumorigenesis is believed to be complex and dependent on various growth factors and signaling pathways. *Von Hippel-Lindau* (*VHL*) gene abnormalities play a central role in the development of the most common subtype of RCC (clear cell carcinoma). These abnormalities result in accumulation of hypoxia-inducible factor-1 α (HIF-1 α) and subsequent transcription of multiple genes encoding for various proangiogenic factors. This process appears to have a role in driving the tumor proliferation, differentiation, its vascularity, and possibly its progression and metastatic phenotype. These factors include among others vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), fibroblast growth factor (FGF), and platelet-derived growth factor (PDGF) (21–26).

Treatment of RCC is currently entering a new era where therapy using targeted agents and small molecules are showing promising results. Further understanding of the mechanism of action of these factors and their interaction with the tumor environment can improve the development of targeted therapy, and optimize its use in the appropriate setting. Cumulative evidence indicates that gangliosides modify the biological effects of several trophic factors *in vivo* and *in vitro*, as well as the mitogenic signaling cascades that these factors generate. These observations have not yet been demonstrated clearly in RCC models; however, they remain a promising and worth studying in various malignancies.

2.1 Modulation of Angiogenesis and VEGF Production

VEGF is a glycoprotein that plays a central role in tumor-associated angiogenesis and endothelial cell proliferation, migration, and survival. By binding to the extracellular domain of its receptor (VEGFR), this factor induces intracellular tyrosine kinase

autophosphorylation and other downstream proteins activation (27). Several isoforms of VEGF and VEGFR have been identified; VEGF-A, VEGFR-1, and VEGFR-2 were found to be overexpressed in RCC compared to normal renal tissue, and VEGFR-2 is believed to be the major mediator of the angiogenic effects of VEGF (28, 29).

Various gangliosides have demonstrated the ability to promote or modulate neovascularization and VEGF effects in both cultured cells and xenograft models. Exogenous administration of GD3, GD1b, GM1, and GT1b to human anaplastic astrocytoma cell lines enhanced VEGF production, with the most potent stimulator being GD3 that increased VEGF production by up to 140% when used in high doses. Similar results were found using glioblastoma multiforme cell lines, except that GD1b showed no effect and GM1, which showed only a moderate effect (30). GD3 seems to have a particular role since it was found to be more abundant in high grade and poorly differentiated human glioma tumors, which are dependent on neovascularization for progression. While grade I or II tumors demonstrated no expression, 70–100% of high grade gliomas showed GD3 expression (31). The suppression of this ganglioside by using cells transfected with antisense vectors containing GD3-synthase cDNA resulted in decreased VEGF gene expression in the xenograft (32). However, this did not change the transcription of VEGF gene in the cultured cells despite decreased VEGF protein secretion. The role of this ganglioside in driving tumor vascularization might explain the phenomenon of smaller size and increased necrosis of tumors with suppressed GD3 expression seen in this study.

Promoting angiogenesis by gangliosides was also demonstrated using mouse brain tumor cells in xenograft models. Tumor cells transfected with cDNA for N-acetylgalactosaminyl transferase to synthesize and express GM3, GM2, GM1, and GD1a were more vascularized, had less necrosis, underwent a greater mitosis, and grew more rapidly than tumor expressing GM3 alone (33). Furthermore, tumor cells expressing GM3, GM2, GM1, and GD1a had significantly greater VEGF gene expression measured by VEGF mRNA when grown *in vivo*, as compared to those cells grown in culture.

Gangliosides were also found to promote the angiogenic response to various angiogenesis-inducing factors such as prostaglandin E1 (PGE1) and basic fibroblast growth factor (bFGF) (34, 35). This was demonstrated in animal cornea models where implanting pellets that contain GM1 or GT1b showed a dose-dependent angiogenic response to angiogenic inducers even in the presence of suboptimal dose of the inducer. GM1 or GT1b alone resulted in inefficient angiogenesis. These two gangliosides also improved the growth rate of the newly formed capillaries, but failed to do so when the asialo GM1 (GM1 deprived of its sialic acid) was used. Removing GM1 or GT1b resulted in the complete disappearance of the newly formed capillaries.

There is also some evidence that the role of gangliosides in tumor angiogenesis may be mediated by their effects on endothelial cells; these cells are essential in the process of neovascularization during tumor growth and progression. GM3 ganglioside, for example, was found to block the proliferation of endothelium, which is induced by the potent angiogenic neuroblastoma cells as well as to reduce their binding capacity to fibronectin and to collagens I and IV (36). This was demonstrated when these endothelial cells were cultured or incubated in a GM3-rich medium.

GT1b added to this environment was effective in restoring the proliferation and the motility of microvascular endothelium previously treated with GM3, as well as restoring its binding capacity.

Gangliosides seem to influence cell migration as well; GD1a preincubation significantly increased the number of cells and their migration induced by VEGF compared to the effect of VEGF alone (37).

Other *in vivo* experiments showed that GM3:GD3 ratio in the local tissue environment affected the formation of vessels, as growth and motility of microvascular endothelium in rabbit cornea were reduced by GM3 addition, and returned to normal levels by the addition of GD3. Additionally, GM3 suppression of angiogenic response *in vivo* was associated with decreased rapidity of penetration of the newly formed vessels (38).

In summary, gangliosides appear to have a role in tumor angiogenesis on multiple levels; VEGF production, endothelial cells proliferation, their motility and migration as well as the penetration of the vessels. Although they might not be angiogenic factors, some gangliosides definitely improve the response to angiogenic factors, and this may be in part because they act as modulators of signaling. Some of their effects appear to be dependent on their structure and the distribution of “pro-angiogenic” and “anti-angiogenic” gangliosides in the tumor environment.

Nevertheless, in order to obtain universal conclusions from these studies, it is recommended that investigating gangliosides be done using similar methods. The investigation of the effects of gangliosides on angiogenesis and tumor neovascularization has been done in different settings: (1) exogenous administration or incubation of tumor cells with gangliosides, (2) assessing the endogenous (native) gangliosides expressed by the tumor, (3) transfection of cDNA in cells in order to change their gangliosides expression, and (4) occasionally by adding gangliosides to normal tissue environment. Accordingly, by using universal methods any potential difference in the biologic role and effects on angiogenesis between endogenous and added gangliosides and between endogenous gangliosides and those expressed by transfectant cells can be avoided. Additionally, tumor specificity in response to the particular ganglioside may be unique and the role of gangliosides in modulating angiogenesis may be different in normal cells compared to malignant cells and need to be considered.

2.2 Modulation of EGFR

The human epidermal receptor (HER) family consists of multiple transmembrane receptors. HER1 or epidermal growth factor receptor (EGFR) has an extracellular domain (ECD) for ligand binding, and internal domain with tyrosine kinase activity (39). Ligand binding to EGFR induces the autophosphorylation of its tyrosine kinase resulting in downstream pathways involved in cell proliferation and survival.

Cultured keratinocyte-derived cell lines treated with the gangliosides by adding GM3, GT1b, and GD3 demonstrated a significantly decreased number of EGF

receptor (EGFR) per cell compared to untreated cells or cells treated with GM1 and GM2 (40). Additionally, adding GM3 resulted in suppression of EGFR tyrosine kinase phosphorylation, and the depletion of this ganglioside increased phosphorylation of EGFR, tyrosine kinase activity, and the activation of downstream MAP kinase and PI3 kinase. Other observations were made *in vitro* by using human dermal fibroblasts; preincubation of these cells with GD1a resulted in enhanced EGF-induced receptor autophosphorylation by 70%, as well as enhanced EGF-induced phosphorylation of MAP kinase (41). These effects were also seen in isolated cell membranes after GD1a preexposure, where 90% increase in EGFR autophosphorylation and 60% increase in the tyrosine kinase activity were observed.

Changes in cell membrane composition of gangliosides appear to play an important role in the regulation of the EGF signal transduction. The activity of ERK2 was comparable in cells expressing GM3, and in those transfected with GalNAc-T and expressing GM3, GM2, GM1 and GD1a. However, cells transfected with Sial-T2 and overexpressing GD3 showed decreased EGFR phosphorylation and decreased ERK2 activation by 50% when stimulated with EGF. This decreased activity does not appear to be related to changes in the affinity of EGFR for EGF but to the changes in the activity of the tyrosine kinase itself (42).

The site of potential action of gangliosides on EGFR was also studied, and the ECD was found to have a stronger binding preference for GM3 compared to binding with GM2, GM4, and GD3 as demonstrated by using purified human recombinant ECD from insect cells. No significant binding was detected with GM1, GD1a, GD1b, GT1b, GD2, or GQ1b. The ligand-binding site in the ECD appears to be distinct from the site involved in interaction with gangliosides (43). Neither GM3 nor GM1 appears to have a significant effect on EGF binding to the ECD. In this model that used highly purified recombinant ECD of EGFR, GM3 was also shown to inhibit EGF-stimulated autophosphorylation, while GM2 showed minimal inhibition and GD3 showed no inhibition.

Dimerization of EGFR is an essential part of its activation since the dimer form is the high affinity form of this receptor that binds EGF more effectively. Dimerization appears to be modulated by various gangliosides; pretreatment of human dermal fibroblasts with GD1a resulted in 60% increase of EGFR dimerization over that induced by EGF alone, and this was seen even without the exposure to EGF (38). GM1 enrichment was also shown to increase the ligand-dependent and ligand-independent dimerization, whereas GM3 enrichment did not appear to affect this process. Another observation about this ganglioside is that phosphorylation of EGFR and the downstream ERK1/2 appear to be sustained longer in cells transfected with GM1 synthase gene and overexpressing GM1 compared to control cells lacking GM1 expression (44).

Human keratinocyte-derived cell line treated with antisense oligodeoxynucleotides and overexpressing GM3 (the predominant ganglioside of epithelial cells) demonstrated disruption and blockage of ligand-independent association of the integrin- β 1 subunit with EGFR resulting in the inhibition of cell proliferation. Endogenous depletion of this ganglioside by stable gene transfection on the other hand resulted in increased stimulation of EGFR and MAP kinase signaling (45).

Some experiments suggest that gangliosides may also affect some of the functions of growth factors by changing the distribution of their receptors in the microdomains of cell membrane (raft, glycolipid-enriched microdomain [GEM]/raft), although this was not consistent in all experiments and was dependent on the cells and the particular growth factor (44).

In summary, various gangliosides appear to modulate EGF activity in a ligand-dependent or -independent fashion. This may be secondary to their binding to the receptor, increasing its dimerization, enhancing or suppressing its phosphorylation, or affecting the activity of other downstream kinases.

2.3 Modulating bFGF and PDGF Activity

bFGF is involved in various biologic processes in normal as well as in malignant tissues. It possess angiogenic properties and is believed to play a role in tumor metastasis (46).

The interaction of gangliosides with bFGF, and the resulting effects and modulation of capillary endothelial cell growth, motility, and survival was observed in vitro (47). The addition of GM1, GD1b, or GT1b individually to the culture medium of bovine capillary endothelium was shown to increase the increment of cell number after 72 h of incubation compared to control cells. Individual combinations of bFGF with GM1, GD1b, or with GT1b substantially increased the cell count compared to bFGF alone or any of the gangliosides when used alone. GT1b appears to be more potent in increasing cell number than GM1 or GD1b when all added individually to bFGF at lower dose. The concomitant presence of GT1b and bFGF improved the cell motility as well. The combinations of bFGF with GM1, GD1b, GT1b, or AGM1 increased the percentage of cells able to survive in the medium. Sialic acid alone or the asialo GM1 did not have any impact on survival or motility when added to bFGF.

The effects of different gangliosides on endothelial cells and their proliferation appear to be dependent on the type of the particular ganglioside used in vitro. Slevin et al. (48) reported that GM1 and GM2, for example, inhibited bFGF-induced growth of bovine aortic endothelial cells, whereas GM3 acted synergistically with this factor to enhance the proliferation. By using ^{125}I -bFGF and measuring the radioactivity of cultured cells, GM1 and GM2 resulted in decreased bFGF binding to its receptor, but GM3 had only a marginal effect. GM1 reduced the bFGF receptor phosphorylation, whereas GM2 or GM3 did not. GM1 and GM2 inhibited the receptor tyrosine phosphorylation, while GM3 increased the phosphorylation more than bFGF alone. These results were demonstrated by preincubating or adding gangliosides to the cultured cells.

PDGF is another factor involved in cell growth and various signaling pathways. It binds to PDGF receptor (PDGFR) and activates its receptor tyrosine kinase and other downstream effects involved in cell growth and prevention of apoptosis (49).

In human glioma cells preincubated with GM1, this ganglioside was found to interfere with the interaction between PDGFR- β and specific proteins involved in

PDGF-mediated signaling by attenuating the association of specific kinases with the receptor (50). Therefore, GM1 may modulate the various downstream effects of activating this receptor.

In neural tumor cells transfected with GM2/GD2 synthase and GM1 synthase cDNAs in order to express GM1, PDGF-stimulated cell growth was reduced and PDGFR distribution inside or outside glycolipid-enriched microdomain (GEM) was changed. GM1 also binds with PDGFR in the GEM fractions and regulates its signaling pathway by decreasing the receptor phosphorylation and the activation of MAP kinases (51).

In other studies, GM1 inhibition of PDGFR- β was shown to be variable and dependent on whether the ganglioside is exogenous or expressed by cells stably transfected with the gene for PDGFR- β . Fibroblasts highly expressing PDGFR- β have demonstrated receptor tyrosine phosphorylation inhibition with pretreatment with GM1, whereas cell line transfected for PDGFR- β did not (52). By using chimeric receptor, the inhibition of PDGFR- β by GM1 was found to involve either the extracellular or the transmembrane domain, as the cytoplasmic domain was not sufficient for the GM1-mediated inhibition. Similar effect on receptor tyrosine autophosphorylation with GM1 and GM2 was found when added to cultured vascular smooth muscle cells, in addition to their ability to effectively inhibit the PDGF-BB binding on these cells as well (53).

In summary, gangliosides interfere with bFGF and PDGF activity on multiple levels; these include binding of gangliosides with the receptor, inhibiting the ligand binding, or directly modulating the activity of the kinase. This results in modulation of subsequent downstream effects of these growth factors such as cell growth, motility, and survival.

2.4 Variability in Gangliosides-Induced Modulation

The above observations indicate that gangliosides have a definite role in modulating cell response to various growth factors such as VEGF, bFGF, EGF, and PDGF. Interestingly, the altered cellular response to these factors, which is induced or modulated by gangliosides appear to be dependent on (1) the type of cellular response (proliferation, migration, or phosphotyrosine levels), (2) the growth factor and its receptor (bFGF or EGF), (3) the dose, and (4) the particular ganglioside used in vitro. The exogenous exposure of cultured glial cells to gangliosides GM1, GM3, GT1b, and asialo GM1, for example, had an inhibitory effect on DNA synthesis and cell proliferation when bFGF was used in nonsaturating doses; however, the opposite was observed using higher doses of bFGF as evident by the increase in thymidine uptake (54). In another experiment on the same cells, GT1b accelerated EGFR dephosphorylation, whereas it stimulated the tyrosine phosphorylation of FGFR1 even in the absence of FGF. Furthermore, while increased proliferation was observed with GM3, GM1, asialo GM1, and GT1b, these gangliosides prevented bFGF-induced migration but not EGF-induced migration. Preincubation with GT1b altered EGFR phosphorylation and accelerated its de-phosphorylation, whereas GM1 did not.

Table 1 summarizes the various gangliosides with positive or negative modulatory effects on growth factors with the used cells and methods. **Table 2** illustrates the effects various gangliosides have on growth factor modulation.

3 Using Gangliosides As Therapeutic Targets in RCC

Antitumor therapy by targeting molecular markers requires the identification of highly specific targets that are expressed in the majority of tumors and not in normal tissue. Such targets also need to have a well-defined biologic role in the tumor such as angiogenesis, or be involved in other pathways controlling the tumorigenesis or its progression. These targets would be even more useful if they also had a prognostic value, in order to identify any unfavorable ones and develop specific agents that can be used in tumors that demonstrate aggressive phenotype. In this setting, gangliosides started to receive attention as potential targets in the treatment of RCC.

3.1 Functional Role

Gangliosides have been implicated in the immune dysfunction seen in patients with RCC. They are believed to be involved in the downregulation of the type 1 T-cell response as evident by the inhibition IFN- γ production from T cells cultured in the presence of RCC-derived gangliosides (19). They are also involved in inducing apoptosis of activated T cells as evident by the proapoptotic events seen in these cells isolated from patients with RCC, and the inhibition of these events partially or completely when tumor cells were treated with ganglioside synthesis inhibitor, which reduced tumor-associated ganglioside level by 70–80% (55, 56). Therefore, targeting gangliosides in this setting may theoretically result in the prevention of the apoptosis of T cells and improvement of the host antitumor cytotoxicity. Additionally, there is some preclinical evidence that gangliosides might have a role in RCC motility and invasiveness; this was demonstrated by using transfectant renal carcinoma cells to reduce GM2 and increase GM3 expression. These cells had reduced motility and invasiveness without alteration of growth rate (5). Targeting these gangliosides in this setting may have a positive role in controlling renal carcinoma cell motility and reducing its metastatic tendency.

3.2 Prognostic Value

The relationship of various gangliosides to the metastatic behavior of RCC was also studied. RCC lines expressing DSGG were found to bind strongly to lung tissue and this binding correlated with their DSGG expression, whereas cells expressing no DSGG showed much weaker adhesion (14). Sialic acid binding proteins (siglecs)

Table 1 Ganglioside modulation of growth factor function in different cell types.

Growth factor	Positive modulation	Cells	Method	Negative modulation	Cells	Method
1. VEGF	GD3, GD1b, GM1, GT1b	Human astrocytoma cells	Exogenous administration	GM3	Human saphenous endothelial cells	Exogenous administration to culture
	GM1, GM2, GD1a	Mouse brain cells	Transfectant cells in xenograft			
	GM1, GT1b	Rabbit cornea (in vivo)	Implantation			
	GD1a	Human umbilical vein endothelial cells	Incubation			
2. EGF	GD1a	Human dermal fibroblasts	Incubation	GM3, GT1b, GD3	Keratinocyte-derived cells	Exogenous administration
	GD3	Chinese hamster ovary cells	Transfectant cells	GD3	Chinese hamster ovary cells	Transfectant cells
	GM1	Rat pheochromocytoma cells	Transfectant cells	GM3	Keratinocyte-derived cells	Transfectant cells
				GT1b	Retinal Müller glial cells	Exogenous administration
3. bFGF	GM1, GD1b, GT1b	Bovine capillary endothelium	Incubation	GM1, GM2	Bovine aortic endothelial cells	Incubation
				GM1, GM3, GT1b, asialo GM1	Retinal Müller glial cells	Exogenous administration
4. PDGF	GM1	Mouse fibroblasts	Transfectant cells	GM1	Mouse fibroblasts	Transfectant cells
				GM1, GM2, GM3	Rat aortic smooth muscle cells	Incubation

Table 2 Gangliosides modulate expression and function of different growth factors and/or their receptors.

Ganglioside with positive modulation	Effect(s)
1. VEGF	
GD3	Enhancing VEGF production
GD1b	Enhancing VEGF production
GM1	Enhancing VEGF production Increasing cell mitosis Increasing VEGF gene expression Stimulating cell growth in vivo Enhancing angiogenic response to PG E1 and bFGF
GT1b	Enhancing VEGF production Enhancing angiogenic response to PG E1 and bFGF
GM2	Increasing cell mitosis Increasing VEGF gene expression Stimulating cell growth in vivo
GD1a	Increasing cell mitosis Increasing VEGF gene expression Stimulating cell growth in vivo Stimulating VEGF-induced endothelium proliferation Increasing VEGF-induced endothelial cells migration
2. EGF	
GD1a	Enhancing EGFR autophosphorylation Increasing EGFR dimerization Enhancing EGFR downstream MAP kinase phosphorylation
GM1	Enhancing EGFR phosphorylation Enhancing downstream ERK1/2 phosphorylation Increasing EGFR dimerization Enhancing EGF-induced cell proliferation
3. bFGF	
GM1	Increasing bFGF-induced cell count Increasing cell survival Inhibiting bFGF-induced DNA synthesis in high bFGF concentration
GD1b	Increasing bFGF-induced cell count Increasing cell survival
GT1b	Increasing bFGF-induced cell count Increasing cell survival Improving cell motility Inhibiting bFGF-induced DNA synthesis in high bFGF concentration
Asialo GM1	Increasing cell survival Inhibiting bFGF-induced DNA synthesis in high bFGF concentration
GM3	Inhibiting bFGF-induced DNA synthesis in high bFGF concentration
4. PDGF	
GM1	Increasing PDGFR kinase activity in low doses

(continued)

Table 2 (continued)

Ganglioside with positive modulation	Effect(s)
1. VEGF	
GM3	Blocking endothelial cells proliferation Reducing endothelial cells binding to extracellular matrix
2. EGF	
GT1b	Accelerating EGFR dephosphorylation Decreasing the number of EGFR per cell
GM3	Decreasing the number of EGFR per cell Suppression of EGFR tyrosine kinase phosphorylation Blocking of EGFR association with integrin β 1 Inhibiting cell proliferation
GD3	Decreasing the number of EGFR per cell Decreasing EGFR phosphorylation Decreasing ERK2 activation
3. bFGF	
GM1	Inhibiting bFGF-induced growth Decreasing bFGF binding to receptor Decreasing bFGF receptor phosphorylation Decreasing bFGF receptor tyrosine phosphorylation Inhibiting bFGF-induced DNA synthesis in low bFGF concentration Preventing bFGF-induced cell migration
GM2	Inhibiting bFGF-induced growth Decreasing bFGF binding to receptor Decreasing bFGF receptor phosphorylation Decreasing bFGF receptor tyrosine phosphorylation
GM3	Inhibiting bFGF-induced DNA synthesis in low bFGF concentration Preventing bFGF-induced cell migration
GT1b	Inhibiting bFGF-induced DNA synthesis in low bFGF concentration Preventing bFGF-induced cell migration
4. PDGF	
GM1	Decreasing PDGF-stimulated cell growth Decreasing PDGFR kinase activity in high doses Inhibiting PDGFR- β tyrosine phosphorylation Inhibiting PDGFR- β autophosphorylation Suppressing PDGF-BB-induced DNA synthesis Inhibiting PRGF-BB binding to receptor Changing the distribution of PDGFR between GEM and non-GEM domains favoring non-GEM fraction
GM2	Suppressing PDGF-BB-induced DNA synthesis Decreasing PDGF-induced cell count Inhibiting PDGFR- β autophosphorylation Inhibiting PDGF-BB binding to receptor
GM3	Suppressing PDGF-BB-induced DNA synthesis Decreasing PDGF-induced cell count

are believed to mediate binding of sialoglyco conjugates on tumor cells to target cells expressing siglecs. Siglec7 is expressed on natural killer cells as well as in the lung, and this protein was shown to strongly bind with DSGG, DSLc4, and GalNAcDSLc4 as well as various disialogangliosides with ganglio-series structure such as GD3, GT1b, and GD2 found on RCC cell lines. No binding was found with GM2, GD1a, GD1b, and GM1 (57). These observations reemphasize that gangliosides may have a role in the tumor preferential metastasis, especially to the lung where RCC metastases are frequently encountered. In this setting, identifying renal carcinoma tumors bearing “high-risk gangliosides” for pulmonary metastases may direct the care toward early identification of metastatic disease in patient follow-up. Nevertheless, direct correlation of RCC to tumor stage, grade, treatment outcome, or patient survival according to its ganglioside composition is lacking on the clinical level, and is needed to achieve a better prognostic value of these antigens.

3.3 Antganglioside Antibodies

Targeting gangliosides in vitro and in vivo has been investigated in an attempt to integrate this approach into immunotherapy and enhance the antitumor cytotoxicity. Antganglioside GD2 monoclonal antibody 14.G2a, for example, was utilized in combination with interleukin-2 (IL-2) in pediatric neuroblastoma patients, and resulted in augmentation of the peripheral blood mononuclear cells cytotoxic ability (58). This was even demonstrated when these cells were obtained from healthy donors. The anti-GD2 monoclonal antibody 14.G2a and its mouse-human chimera (ch 14.18) were also found to mediate antibody-dependent cellular cytotoxicity and potentiate lysis of animal malignant melanoma cell lines by IL-2-activated peripheral blood lymphocytes (59). Another monoclonal antibody (DMF10.167.4) was used in vitro and in animal models and demonstrated some therapeutic effects on various cell lines. This anti-GM2 antibody induced apoptosis in lymphoma, melanoma, and small cell lung carcinoma lines expressing GM2 after overnight incubation. In melanoma and small cell lung carcinoma xenograft models, DMF10.167.4 demonstrated the ability to prevent the establishment of these tumors in mice receiving the antibody compared to those receiving no treatment. When melanoma tumors were established in these animals, DMF10.167.4 was able to block the tumor progression and resulted in 50–75% reduction in tumor size especially when higher doses were used (60). Although targeting various molecular markers and their receptors have been investigated and showed benefits in patients with RCC, antganglioside antibodies have not yet been developed or used in this disease.

3.4 Immunization with Gangliosides

Vaccines using gangliosides have been developed and used in animal models as well as in humans. Immunization to develop anti-GD3 antibody response in

patients with melanoma was attempted using vaccines that contain GD3-lactone-keyhole limpet hemocyanin (GD3-L-KLH). This vaccine appeared well tolerated and resulted in immune response with IgM and IgG antibodies that cross-reacted with GD3 in the majority of patients (61, 62). Anti-idiotypic monoclonal antibody that mimics GD3 ganglioside (BEC2) was also used in patients with melanoma and demonstrated the capacity to induce detectable IgG antibody against BEC2 as well as detectable antibody response to GD3 in some patients (63). Similar studies in patients with small-cell lung cancer were performed and resulted in some immune response, and induction of complement-mediated cytotoxicity of tumor cells was demonstrated in patients' sera (64). Although various vaccines have been investigated in patients with RCC, using gangliosides in this setting has not yet been performed. Prior to doing so, a proven benefit from targeting these glycolipids (e.g., meaningful improvement of host immune response or objective tumor response and shrinkage) may be necessary. The tumor specificity of gangliosides may interfere with obtaining similar results in RCC.

3.5 Difficulties in Targeting Gangliosides in RCC

Although gangliosides appear to have a role in RCC and treatment against these antigens is appealing, several difficulties could be encountered when developing effective therapy in patients with RCC using gangliosides as potential targets. More mature preclinical data is needed to identify the mechanisms of gangliosides-induced immune dysfunction and correlate that with any other immune response that may be seen in patients with RCC receiving other types of immunotherapy such as cytokine treatment or targeted therapy. Additionally, no preclinical data is available on ganglioside modulation of various pathways and growth factors involved in RCC pathogenesis and angiogenesis such as VEGF or PDGF as seen in cells derived from neural crest origin. Identifying "pro-angiogenic gangliosides" on RCC tumor, for example, might further indicate the need to use an antiangiogenic agent in treatment as opposed to interferon or chemotherapy. In a different situation using anti- $\alpha v \beta 1$ integrin antibody may be synergistic with using anti-GM3 antibody if this ganglioside showed a functional support of $\alpha v \beta 1$ integrin receptor in RCC as was demonstrated in animal mammary carcinoma lines (8). Since RCC is heterogeneous tumor with various histological subtypes, the prevalence and the role of gangliosides in each subtype is a key and needs further study. Integrating gangliosides expression in the stratification after correlating and validating their role could be a promising goal in various subtypes of RCC. Furthermore, it is unclear whether targeting specific gangliosides in gangliosides-bearing tumors is as beneficial in local and early stages of the tumor as it is in advanced and late stages. This is of a particular importance in RCC as treatment is often deferred when the disease is indolent. Finally, targeting these tumor-associated glycolipids would require prior assessment of these antigens in tumor tissue, in order to identify the gangliosides with prognostic value and those that can be used in vaccine preparation. This approach may not be optimal in advance and metastatic RCC and needs to be considered in early stages or adjuvant setting.

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Tumour Necrosis Factor – Misnomer and Therapeutic Target

Marina Parton, Tanya Das, Gaurisankar Sa, James Finke, Tim Eisen, and Charles Tannenbaum

Abstract Tumour necrosis factor (TNF) is an important immune modulatory signalling molecule. It is synthesized within renal cell carcinomas both by macrophages and tumour cells. The roles of TNF are complex and multiple: TNF has a role in cell killing as well as in evasion of the host immune response, tumour cell growth stimulation and regulation of neo-angiogenesis. These multiple roles have led to TNF being investigated both at supra-physiological doses as an anti-cancer agent and as a therapeutic target. Thalidomide effectively down-regulates TNF mRNA and antibodies to TNF are in clinical use in a number of disease settings. To date the results of targeting TNF in renal cell cancer have been modest.

Keywords Tumour necrosis factor • Cytokine • Immunomodulation • Angiogenesis • Growth factor

1 Introduction

Though tumour necrosis factor (TNF) was originally characterized as a molecule capable of mediating anti-tumour activity, based on its ability to induce tumour necrosis and apoptosis of neoplastic cells (1, 2), it has more recently been recognized as a potent regulator of the innate and adaptive immune responses (2). The rapid release of TNF from mast cells following complement activation (3) and its early synthesis by macrophages following stimulation through Toll receptors (4), results in the induction of many downstream cytokines and chemokines (5). It is these effects, in conjunction with the ability of TNF to stimulate vascular permeability, vascular dilation, acute phase protein production by the liver, and recruitment of

T. Eisen (✉)
Addenbrooke's Hospital, Cambridge CB2, UK
e-mail: tgqe2@cam.ac.uk

C. Tannenbaum
Cleveland Clinic Foundation, Cleveland, OH 44195
e-mail: tannenc@ccf.org

inflammatory cells, complement, and other serum proteins into sites of infection, that render it a very linchpin of the innate immune response (6–9). TNF also modulates adaptive immunity, in large part via the secondary mediators it can induce: IL-1, IL-2, IL-4, IL-6, IL-10, IL-12, IL-18, IFN gamma and prostaglandins all contribute to enhancing or downregulating cellular and/or humoral immune responses (2). TNF itself is additionally reported to serve as an important soluble and membrane-bound effector molecule, through which it plays a prominent role in mediating activation-induced cell death during both normal cell growth and in the course of re-establishing homeostasis following an immune response (10). Thus, based on the pivotal role of TNF in orchestrating the immune response, and on the initial observation that TNF could in some instances mediate the necrosis of neoplastic cells, it was not surprising that histologically distinct tumour cells either treated with recombinant TNF or engineered to overexpress the molecule were growth inhibited or rejected (11–14). It is therefore a paradox that a growing body of evidence now supports the notion that TNF can also promote the growth and metastasis of a variety of tumours, including renal cell carcinoma (RCC). It is this association of TNF with tumour progression, metastasis and immune escape, as well as the mechanisms by which the molecule might be mediating these effects, that will be reviewed here.

2 TNF As a Prognostic Indicator in RCC

While several early studies suggest a positive correlation between TNF serum levels and tumour regression in RCC patients (15), other reports indicate that the molecule is at greater concentrations in the plasma of patients with disseminated disease (16–19). In fact, while some studies indicate that normal plasma TNF levels are highly predictive of a favourable prognosis (18), it is now believed that TNF levels begin to increase at an early stage in RCC patients and are maximal in patients with progressive disease. This thought is supported by Hameo's earlier data indicating that the number of RCC patients with elevated TNF serum levels increased with stage, and that because TNF was already appreciably higher in Stage I patients than in control subjects, it can be used as a prognostic factor useful for early diagnosis and post operative follow-up (19).

TNF was known to be an inducer of IL-6 (20), and was specifically shown by Koo et al. to induce IL-6 (20) in RCC lines (21). Subsequent studies by Ikemoto et al. (16) indicated that even at the extremely low concentrations of 10 and 100pg/ml, TNF stimulated IL-6 production by a number of short term, established RCC cell lines. Given the multiple citations claiming that RCC cells produce IL-6 (22–24), and the report of Miki et al. (25) indicating that anti-IL-6 antiserum specifically inhibits the *in vivo* growth of RCC, it may be that IL-6 is a TNF-inducible, autocrine growth factor for that tumour. In fact, it is likely that the correlation between elevated TNF levels and disseminated disease may be in part related to the positive effect TNF has on IL-6 production. Thus, like elevated TNF levels, IL-6 may be a marker of

tumour aggressiveness. This thought is supported by the work of Blay et al. (24), who reported that 48% of the 138 RCC patients examined had detectable serum levels of IL-6, and that the time frame between the initial diagnosis of primary RCC and the onset of metastatic disease was shorter in patients that had a serum IL-6 level of $>50\text{mg/L}$. Stadler et al. (26) and Dosquet et al. (18) also published that elevated serum IL-6 levels were prognostic for metastatic progression of RCC, and given their finding that IL-6 levels were significantly higher in Stage IV RCC than in stages I, II and II, Yoshida et al. (17) similarly proposed that IL-6 may be a marker of tumour aggressiveness.

3 Intratumoural TNF: Synthesis by Tumour-Associated Macrophages

The observation that RCC occasionally regresses spontaneously, in conjunction with the finding that the tumour demonstrates at least some responsiveness to several immunotherapeutic protocols, strongly supports the notion that RCC is an immunogenic tumour (27, 28). Indeed, immunohistological analysis of multiple, excised tumours indicates that RCC are typically infiltrated by macrophages, dendritic cells and lymphocytes, suggesting that the host immune system has recognized components of the tumour as foreign, and at least has initiated defensive responses (29). Though the extent of macrophage content in RCC can vary widely (30), macrophages are nonetheless invariably a major component of the inflammatory infiltrate of these solid tumours (31); yet the actual significance of tumour-associated macrophages (TAMs) in RCC is not clear. On the basis of previous studies demonstrating the ability of activated macrophages to lyse and destroy tumour cell targets *in vitro* (1), one would expect that the large numbers of tumour-associated macrophages would reflect ongoing anti-tumour responses and hence be prognostic for a favourable outcome. However, the consequence of TAMs is much more complex, since they appear as likely to enhance tumour growth as to be involved in control of tumour progression (31). Banner et al. (32), for instance, correlated inflammatory cell infiltration into RCC with advanced disease and less favourable outcome. Studying stage T1 RCC patients, Waase et al. found a close correlation between TAM, TNF production, and tumour size, and determined using *in situ* hybridization that the TNF mRNA was expressed by infiltrating monocytes and macrophages (33). Interestingly, when TAM and monocytes from the same patients were compared for cytokine production, the TAM synthesized TNF and other cytokines constitutively, while the monocytes required stimulation with LPS to produce any at all (31). In fact, in a study of 83 patients, TNF production was greatest in tumours that exceeded 5 cm in size (31). Such results suggest the possibility that TNF from TAM continuously stimulates the RCC, resulting, as discussed above, in the induction of tumour enhancing growth factors such as IL-6 that can lead to tumour progression and spread.

4 Intratumoural TNF: Synthesis by the RCC Tumour Cells Themselves

Though a variety of laboratories have determined that intratumoural TNF is produced primarily by the macrophages which infiltrate neoplastic masses, it has also been reported that in melanoma (34), head and neck cancer (35) and ovarian cancer (36), the cytokine can be synthesized by the individual tumour cells. Results reported for RCC, however, have been much more equivocal and controversial. When Waase et al. determined that TNF mRNA was expressed by the monocytes and macrophages infiltrating RCC, they simultaneously found, using *in situ* hybridization, that the tumour cells were negative for the molecule (33). This is in contrast to the studies performed by Gogusev et al., who using immunohistochemical staining and northern blot analysis, accumulated data indicating that RCC tumour cells themselves can indeed synthesize TNF protein and mRNA, respectively (22).

One possible explanation for this enigma is the functional inactivation of the von Hippel–Lindau (VHL) tumour suppressor protein in most but not all RCCs (37). The native pVHL protein associates with elongin C, elongin B, Cullin 2 and RBX1, resulting in the formation of a functional E3 ubiquitin ligase that is likely involved in the polyubiquitination and subsequent degradation of specific cellular proteins (38). Detailed analyses of cells expressing functional and mutant forms of pVHL indicate that a subset of mRNAs preferentially associate with polysomes in cells that lack the functional protein, but not with polysomes in cells which express native pVHL. Thus intact pVHL appears to mediate transcriptional inhibition of specific proteins, which is relieved when pVHL is absent or functionally altered (38). It is interesting that cells expressing defective pVHL are characterized by constitutively elevated NF κ B activation, resulting in the enhanced expression of NF κ B-inducible anti-apoptotic molecules such as c-IAP-1, c-IAP-2, c-FLIP and survivin (37); elevated levels of these caspase inhibitors perhaps explains why RCC cells carrying mutant pVHL are highly resistant to TNF-induced toxicity. Indeed, TNF itself was identified as one of the proteins subject to pVHL repression, whose synthesis, therefore, was upregulated in RCC carrying a mutant form of this molecule (38). This might explain the TNF expression characterizing some RCC tumour cells and derived cell lines (38), as well as the elevated levels of anti-apoptotic genes that seem to typify many of those tumours, given the fact that TNF can induce NF κ B.

5 Biological Effects of TNF in the Tumour Microenvironment

As alluded to above, notwithstanding the more than 100-year-old finding that under some circumstances, TNF is capable of killing or suppressing the growth of some tumours, the molecule has paradoxically also been shown to promote tumour growth (16, 38). Whether the TNF is made by the tumour cells themselves, or by the macrophages which so commonly infiltrate a variety of tumour types, the local,

chronic production of TNF in the tumour microenvironment leads to inflammatory conditions that appear to favour tumour development (38). Included among the specific effects induced by TNF that likely lead to tumour progression are stimulation of growth factors, chemokines, metalloproteinases and angiogenic factors. We also find that TNF stimulates the expression of tumour gangliosides such as GM2, which seems to mediate tumour-induced T cell killing and hence the capacity of the tumour to evade host immune responses.

It was already indicated above that TNF production within the tumour environment has a profound effect on tumour growth. When the effects of TNF on the proliferation of long- and short-term RCC cell lines were examined using a ^3H -thymidine incorporation assay, the authors found that DNA synthesis was induced in both cell types even at the low TNF concentrations of 10 and 100 pg/ml¹⁶. This effect appeared to be mediated via the well characterized TNF-induced synthesis of IL-6, as antisera to IL-6 blocked RCC growth in vivo (25).

While metalloproteinases play important roles in normal physiological processes, their activities also contribute to the exacerbation of various pathological conditions, including tumour metastasis (39). MMP-9, for example, is a metalloproteinase regulated by TNF, and its ability to degrade basement membranes is a significant step leading to tumour progression and spread (40). Zhang et al. (41) found that there was a close correlation between MMP-9 expression levels and the pathological stage/histological grade of RCC. Cho et al. (42) additionally reported that in a similar group of patients, high MMP-9 transcriptional levels and the associated elevated-gelatinolytic activities correlated with a high frequency of metastasis and poor survival. Because TNF has an integral role in regulating MMP-9 expression, Lee et al. (40) sought small compounds that could inhibit TNF-mediated upregulation of that proteinase. One compound termed SM-7368 was found to inhibit TNF-induced MMP-9 mRNA and protein expression in a concentration-dependent manner, which correlated with the capacity of the synthetic molecule to inhibit TNF-induced invasion of HT1080 fibrosarcoma cells (40). These results suggest that inhibitors such as SM-7368 might be useful for targeting TNF-induced MMP-9 expression in RCC as well, providing a mechanism for limiting RCC tumour expansion.

Many studies have demonstrated the significant stimulatory effect that angiogenesis has on tumour development, progression and metastasis (43–45). This process is driven by an increased expression of angiogenic factors within the tumour microenvironment, though by ELISA, bFGF and VEGF were also determined to be markedly higher even in the serum of RCC patients with disseminated disease (18). Several reports now suggest that TNF is capable of inducing a number of these angiogenic factors. In a series of correlative studies that included RNA knockdown experiments, Kulbe et al. (46) demonstrated that a network of cytokines, angiogenic factors and chemokines were generated by the autocrine activity of TNF in ovarian cancer cells, which promoted both the neovascularization and spread of developing tumours. TNF-induced VEGF (46), while angiogenic on its own, is even more so in synergy with the TNF-inducible molecule CXCL12 (47). Others reported that IL-8 mRNA expression correlated positively with tumour angiogenesis and negatively with patient survival (48), and that this positive autocrine regulation of tumour

angiogenesis could be partially blocked with anti-TNF antibodies. By inducing angiogenic factors such as VEGF and IL-8, it is likely that the TNF synthesized by tumour infiltrating macrophages or the RCC tumour cells themselves enhance tumour neovascularization and metastatic spread.

6 TNF-Induced Tumour Gangliosides and Immune Suppression

A number of studies suggest that tumour products present within and secreted from the tumour microenvironment can induce T-cell apoptosis, partially explaining the immune dysfunction characterizing cancer patients (49). When RCC tumours are excised and assessed by TUNEL analysis, many are found to contain apoptotic T cells: flow cytometry indicates that >30% of the tumour infiltrating T cells, and sometimes as many as 100%, are annexin V/7AAD positive, a finding that typifies other histological types of tumours as well (50–52). Previous studies from our group established that soluble tumour products play a role in mediating this apoptosis (53). Supernatants collected from *in vitro*, explanted RCC tumours induced T-cell apoptosis within 72 h of coincubation, and experimental evidence suggested that tumour-derived gangliosides were an active, apoptogenic component of the tumour-conditioned medium: neuraminidase abrogated the effect (54), gangliosides extracted from the supernatants demonstrated equivalent apoptogenicity (54, 55), and the ganglioside inhibitor 1-phenyl-2-hexadecanoylamino-3-pyrrolidino-1-propanol (PPPP) reduced the ability of an RCC tumour cell line to kill both Jurkat cells and activated T cells by at least 50% (55). Such glycosphingolipids are overexpressed by a variety of tumour types, and appear to mediate their apoptotic effects by both inhibiting NF κ B (56) and by acting directly on target cell mitochondria to induce cytochrome c release and subsequent caspase activation (57).

We recently demonstrated that RCC tumour cells are rendered highly apoptogenic by a mechanism involving the TNF-enhanced production of the ganglioside GM2 (58). Multiple lines of evidence link RCC-derived GM2 to tumour apoptogenicity and GM2 accumulation by tumours to the paracrine or autocrine TNF-stimulation of renal carcinoma cells. Whether administered exogenously or via a transgene, TNF-stimulated RCC cells overexpressed GM2 and induced cocultured, resting T cells to TUNEL positivity and death. RT-PCR analysis provided insight into the mechanism by which TNF modulated GM2 levels: there was little GM2 synthase mRNA amplified from the parental SK-RC-45 RNA, and only slightly more when RNA from a low TNF expressing clone was used as a template, although the band intensities were dramatically increased when the RNA amplified was derived from a TNF-transfected clone expressing high levels of the cytokine (58). It should be noted that TNF was also found to induce elevated levels of tumour-associated FasL, which while not relevant to our studies with resting T cells, is likely to have an additional apoptogenic effect on activated T cells.

There is precedence to the notion that TNF induces ganglioside expression: incubation of normal melanocytes with TNF increased their production of both

GM3 and GD3 (59), and pancreatic islet cells similarly showed increased ganglioside expression following TNF treatment (60). Additionally, studies with TNFR1-deficient mice revealed the importance of TNF signalling for maintaining expression of select gangliosides in normal tissues, such as lung, muscle, thymus and spleen (61). When compared with wild type mice, TNFR1-deficient animals demonstrated decreased expression of GM3-Neu5Ac, GM3-Neu5GC and GM1b, with a corresponding increase in the neolacto series of gangliosides (61).

Although our studies demonstrating the apoptogenicity of TNF-induced GM2 were initially performed using the SK-RC-45 RCC cell line, TNF was found to have an equivalent effect on cancer lines derived from other RCC tumours. The SK-RC-26b line constitutively synthesized GM2 and killed cocultured resting T cells without the need for exogenous TNF stimulation, but both traits could be abrogated if the tumour cells were pretreated with siRNAs to TNF (58). Indeed, SK-RC-26b was determined by RT-PCR analysis to be constitutively elaborating TNF, thus explaining the constitutive GM2 production and apoptogenic phenotype of the line (58).

Collectively, these findings suggests that TNF contributes to the inhibition of T-cell survival in renal cancer tissue by inducing apoptogenic gangliosides such as GM2, providing tumours a means for inhibiting the development of effective anti-tumour immune responses. It may be that antitumour vaccines could be rendered more efficacious if administered in conjunction with therapies that neutralize gangliosides, which, by ablating tumour-induced T-cell death, could possibly enhance anti-tumour immunity.

6.1 Clinical Applications of TNF α in Cancer

Due to the multifunctional role that endogenous TNF α has in mammalian systems, TNF α can be targeted differentially in various therapeutic roles. Supra-physiological levels of TNF α has been used as the therapeutic agent in isolated limb perfusion (ILP), while physiological levels of TNF α is the therapeutic target in treatments involving TNF α inhibition (Fig. 1).

6.2 TNF α As a Therapeutic Agent

As a result of the availability of recombinant TNF α and promising in vitro data (2, 62), Phase I clinical studies of supra-physiological doses of TNF α were carried out. Intravenous treatment was found to have severe systemic toxicities, most notably hypotension, dyspnoea, hepatic dysfunction and even organ failure as a result of a “septic shock like syndrome” (63). A Phase I study showed some response in renal cell patients (64), and therefore the National Cancer Institute of Canada carried out a Phase II study of 22 patients with renal cell cancer and TNF α given intravenously for 5 days every other week (65). Two patients achieved a response for over 200 days; however, there was considerable toxicity and the programme was abandoned. Other Phase II studies failed to show response in any tumour group (66).

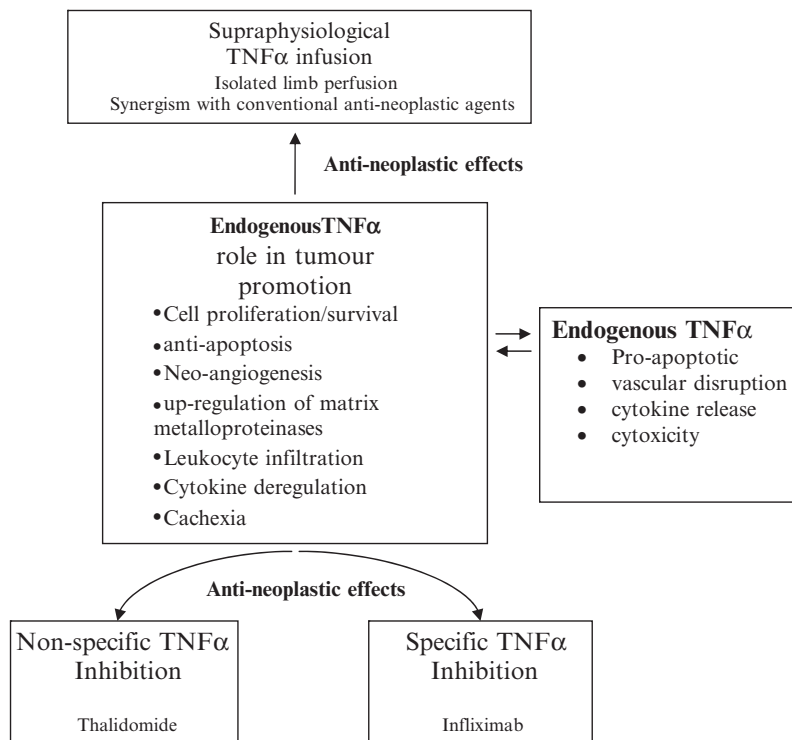


Fig. 1 Role of TNF α in cancer

Methods were developed to deliver local high levels of TNF α in humans without systemic exposure and the associated toxicity. Isolated limb perfusion (ILP) involves the surgical isolation of the vascular inflow and outflow of an extremity, while connected to an extra-corporeal pump to maintain perfusion and oxygenation. The circulation of the affected limb is separated from that of the remainder of the body, allowing chemotherapy doses reaching up to 30-fold the levels possible by systemic administration. Treatment has largely been for in-transit melanomas and sarcomas, which can extensively involve a limb without evidence of disease outside that limb. Without ILP amputation might have to be considered. TNF α alone appeared to have little effect (67), however, in combination with melphalan good outcomes were reported with response rates of over 80% in melanoma and soft tissue sarcomas (68–71). This treatment allows limb sparing, particularly in bulky disease, where response rates appear to be greater (70). Two mechanisms of action of TNF α are likely. There appears to be the immediate effect at the time of perfusion on the integrity of the endothelial cells (72), increasing microendothelial permeability (73, 74) and resulting in higher concentrations of chemotherapy in the tissues (75, 76). This is followed by a later effect, with disruption of the vascular supply of the tumour triggered by a number of mechanisms including selective inhibition of integrin $\alpha v \beta 3$ (77), disruption of endothelial cells causing thrombo-aggregation and

endothelial cell apoptosis, as well as systemic effects with release of interleukin-6 (IL-6) and IL-8 not associated with systemic TNF α leak (63).

6.3 Inhibition of Endogenous TNF α

In contrast with the effect seen with high dose administered TNF α , pathophysiological doses of endogenous TNF α can promote tumourgenesis and growth (78). TNF α is known to contribute to cachexia, hence its early name of cachectin (79), alongside IL-1, IL-6 and interferon γ (80). TNF α derived from tumour appears to be more significant in this role, and also mediates other paraneoplastic features such as hypercalcaemia and leucocytosis in human xenografts (81). Cancer-related cachexia is typically characterized by muscle bulk loss, increased gluconeogenesis, glycolysis and increased fat mobilization and oxidation. It has been postulated that inhibition of TNF α with pentoxifylline or thalidomide could improve cancer-related cachexia, fatigue and depression but this has met with limited success to date (82). Thalidomide, although acting by multiple mechanisms, has had the most success, particularly in tuberculosis (TB) and AIDs-related cachexia (82).

6.4 Thalidomide, a Non-Specific Inhibitor of TNF α

Thalidomide was synthesized in 1954 by the CIBA pharmaceutical company. It was prescribed as a sedative, tranquilizer and anti-emetic for morning sickness. It became a popular sedative marketed under several names throughout the world (83). Unfortunately, thalidomide resulted in limb abnormalities and other congenital defects when children were exposed in utero, and as a result it was widely withdrawn (84). Thalidomide has multiple mechanisms of action, including anti-angiogenic effects, anti-TNF α , immunomodulatory and anti-inflammatory. In 1994, D'Amato and colleagues (85) hypothesized that malformations associated with thalidomide were the result of inhibition of angiogenesis, as demonstrated in a rabbit cornea micro-pocket assay, by inhibition of basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) induced neo-vascularization (86). The anti-angiogenesis effect of thalidomide appears to be separate from the immunomodulatory effects (87).

Thalidomide appears to inhibit production of TNF α in lipopolysaccharide-induced human monocytes and mouse macrophages by enhancing degradation of its mRNA (88–90). Levels of other cytokines, IL-1 β , IL-6, and granulocyte macrophage-colony stimulating factor (GM-CSF), are also reduced by thalidomide, whereas IL-10 is increased (91). However mechanisms may be more elaborate as the thalidomide suppressive action on TNF α production in monocytes and macrophages is contrasted to the IL-2-dependent superinduction of TNF α that takes place in CD4 $^{+}$ and CD8 $^{+}$ T lymphocytes cells with thalidomide (92). Thalidomide has also been shown to

decrease the density of TNF α -induced cell surface adhesion molecules ICAM-1, VCAM-1, and E-selectin on human umbilical vein endothelial cells (93).

Other immunomodulatory properties of thalidomide include stimulation of the Th-1 immune response in healthy humans and scleroderma patients (94, 95), and also the induction of natural killer (NK) cell cytotoxic activity and number (96), factors which appear to contribute significantly to the mechanism of action of thalidomide in multiple myeloma.

Apart from anti-angiogenesis, thalidomide has other non-immunomodulatory effects, such as anti-proliferative effects as a result of IL-2 inhibition (97), pro-apoptotic signalling with activation of caspase-8, enhanced multiple myeloma cell sensitivity to Fas-induced apoptosis, and downregulation of nuclear factor- κ B (NF κ B) activity as well as the expression of apoptosis inhibitory protein (IAP) (98, 99).

Anti-inflammatory clinical uses of thalidomide emerged in the late sixties in erythema nodosum leprosum, a vasculitic complication of leprosy (100). Interestingly, levels of TNF α mRNA were significantly increased in reactional skin and nerve in such patients, particularly in borderline tuberculoid subtypes (101).

Although thalidomide has been used in cutaneous conditions such as sarcoidosis, lupus and Graft versus Host Disease (102–104), it was significantly detrimental in toxic epidermal necrosis (TEN), which is in part mediated by TNF α (105). In fact, there have been case reports of TEN occurring de novo in patients treated with thalidomide (106).

6.5 *Thalidomide As an Anti-Cancer Agent*

Established uses of thalidomide include the treatment of refractory multiple myeloma (107), with response rates of 32%. The mode of action includes inhibition of expression of IL-6 and TNF α by the bone marrow stromal cells that in turn inhibit the growth of multiple myeloma cells, enhancement of T-cell stimulation and proliferation of the activated cells then releasing IL-2 and interferon γ , resulting in activation of NK cells causing lysis of multiple myeloma cells (97, 98) thalidomide and dexamethasone act synergistically on TNF α suppression (88), and this may contribute to the superior results seen with this combination in myeloma (108). Thalidomide is now considered as the new standard of care in myeloma, as well as increasingly used in myelodysplasia (109, 110). Ongoing Phase II trials of thalidomide have also shown potential activity against some solid tumours such as melanoma (111), neuroendocrine tumours (112), prostate cancer (113, 114) and gliomas (115).

6.6 *Thalidomide and Renal Cell Cancer*

Single agent continuous low dose thalidomide was used in a study in advanced renal cell cancer, melanoma, ovarian and breast cancer in 1999 by Eisen et al. (116).

In this Phase II study, 66 patients with advanced metastatic disease (18 renal cell cancers) received 100mg of thalidomide orally every night until disease progression or unacceptable toxicity.

Three partial responses (PRs) were seen in 18 patients treated with RCC. One response lasted 5 months and the other two PRs were continuing at 5 and 11 months of follow-up. Of the other tumour types, none showed any objective responses to thalidomide. None of the biological markers showed any relationship to response or tumour type. Thalidomide at 100mg was very well tolerated with no grade 3 or 4 toxicities, although the majority of patients experienced grade 1 lethargy and two patients developed grade 2 peripheral neuropathy.

Encouraged by these results higher dose oral thalidomide was assessed in 25 RCC patients (117). Doses were escalated to a target dose of 600mg daily over a 5-week period. The combined data from the two Royal Marsden studies ($N = 40$) demonstrated a median survival of 9 months from the start of thalidomide treatment with five patients achieving a PR (23%) and a further nine patients (41%) having disease stabilization. However, a significant number of patients experienced grade ≥ 2 lethargy, constipation and neuropathy at doses above 400mg/day. Decrease in serum TNF α was observed in patients who had an objective response or disease stabilization ($p = 0.05$), but all these patients had higher levels of pre-treatment serum TNF.

Table 1 outlines a summary of all the available trials studying thalidomide as a single agent in metastatic RCC. Overall, there appears to be no dose-dependent relationship between thalidomide and response. In fact, some studies (116, 128) have shown that lower dose thalidomide appeared not only to be better tolerated with the fewer side effects, but also achieved similar results to higher dose treatments. Adverse effects associated with thalidomide use, some of which may be dose related, includes somnolence, constipation, rash, peripheral neuropathy and deep venous thrombosis (129). Other high dose thalidomide studies have shown disappointing results with single agent thalidomide with significant toxicity, with response rates between 4 and 11% (118, 120–124).

In both low and high dose thalidomide trials prolonged periods (over 11 months) of disease stabilization after PRs or stable disease have been observed (116, 117, 122, 125, 126). Although disease stabilization has clinical benefit, many metastatic RCC patients will have a varied course of disease progression over time (130) and it is not always clear if these patients would have progressed without treatment or on hormonal treatment alone. To answer that question in part, Lee et al. (131) published a randomized Phase II/III study to investigate the activity of thalidomide 400mg/day compared with medroxyprogesterone, 300mg/day, as the standard arm in patients with metastatic RCC who had progressed on or were not suitable for immunotherapy. Sixty patients were entered, with 22 patients assessable in the thalidomide arm and 26 patients in the medroxyprogesterone arm. In the thalidomide arm, no responders were seen. Three patients experienced stable disease which lasted 5, 6 and 12 months, respectively, before progression. Most patients had evidence of progressive disease on imaging before study entry. In the medroxyprogesterone arm all assessable patients progressed on treatment. On the basis of intention to treat analysis, there was no difference in time to treatment failure between the two groups.

Table 1 Studies in single agent thalidomide in metastatic renal cell cancer

References	Eisen (118) ^a (119)	Stebbing (119)	Minor (120) (121)	Srinivas (121)	Srinivas (121)	Escudier (122)	Novik (123) (124)	Li (125)	Daliani (126)	Lee (127)
No. of patients	18	25	29	↔ 14		40	27	34	20	60
Prior nephrectomy	NR	24 (96%)	19 (66%)	↔ 11 (79%)		32 (80%)	21 (78%)	31 (91%)	19 (95%)	48 (80%)
Performance status (ECOG or Karnofsky)	≤2	≤2	52% 2 or 3	↔ ≤1		83% ≤1	≤2	KPS ≥70% ≤1	80% ≤1	≤2
Target dose/day	100mg	600mg	1,200mg	200mg	1,200mg	1,200mg	1,000mg	800mg	1,200mg	400mg
No. assessable for response	18	22	24	7	7	39	27	25	19	48
PR 95% CI	3 (17%) (4–41%)	2 (9%) (1–29%)	1 (4%)	0	0	0	0	0 (0–14%)	2 (11%) (1–33%)	0
SD	3 (17%)	12 (55%)	3 (13%)	4 (57%)	2 (29%)	11 (28%)	7 (26%)	16 (64%)	9 (31%)	3 (10%)
Median PFS (months)	NR	NR	2.3	NR	NR	NR	NR	TTP 4	4.7	TTF 2.5
Median survival (months)	↔ 9		3.5	9	9	10	NR	57% alive at 1 year	18.3	8.2

^aAs updated in Stebbing et al. (119)

NR not reported, PR partial response, SD stable disease, CI confidence interval, PFS progression free survival, TTP time to progression, TTF time to treatment failure

Overall, the risk:benefit ratio did not favour the use of thalidomide in metastatic renal cell cancer. However, there is indication that treatment for prolonged periods of time results in a higher number of patients achieving PR (122). A significant percentage of patients (38.5%) in the Lee et al. (127) study received less than 8 weeks of thalidomide, and this may not be sufficient to assess a cytostatic response. There are also intrinsic difficulties in assessing efficacy of such cytostatic drugs such as thalidomide when using conventional response criteria such as tumour shrinkage (132).

Thalidomide has been combined with other known biological and chemotherapy agents. Gordon et al. (133) conducted a large Phase II Eastern Cooperative Oncology Group (ECOG) study of low dose interferon $\alpha 2b$ (IFN) and thalidomide as first line treatment in metastatic RCC. Treatment consisted of 1 MIU SC IFN twice a day alone or in combination with thalidomide. Thalidomide doses were escalated from 200mg/day to 1,000mg/day. Overall, 353 patients were enrolled, of whom 342 were eligible. It was found that although there was no difference in response rates or overall survival, there was significantly longer progression-free survival (PFS) in the IFN + thalidomide arm (3.8 months vs. 2.8 months, $p = 0.04$). However, there was impairment of quality of life and fatigue scores in the thalidomide arm for this modest increase in PFS. Likewise, in other non-randomized studies combining IFN α and thalidomide, there has been little activity above that normally expected for IFN α alone (140, 141). Increased toxicity with thalidomide was observed, requiring treatment discontinuation (134, 135). Thalidomide has also been combined with IL-2 (136, 137), gemcitabine and 5 fluorouracil (138) and bevacizumab (139). These Phase I/II studies have not clearly shown benefit with thalidomide, although the toxicity associated with thalidomide has again limited treatment.

Various analogues of thalidomide have been synthesized and screened for anti-cancer and anti-inflammatory activities. These orally administered immunomodulatory drug (IMiD) analogues include lenalidomide (Revlimid; CC-5013) and CC-4047 (Actimid). These second generation thalidomide analogues have potent immunomodulatory activities (91) and are both currently in trials. Using TNF α inhibition as the basis for comparison, Lenalidomide and CC-4047 are 2,000 and 20,000 times more potent than thalidomide, respectively (140). Combination of these new products with drugs acting by a different mechanism may pave the way for more effective treatments in all solid tumours.

6.7 *TNF α As a Specific Target Anti-TNF α Monoclonal Antibodies Infliximab*

Infliximab (cA2, Remicade, Centocor incorporated) is a chimeric anti-TNF α monoclonal antibody containing a murine TNF α binding region and human IgG1 backbone. It selectively binds TNF α in the cellular microenvironment, thereby preventing TNF α from interacting with membrane-bound TNF receptors (TNFRs) on target cells. It forms very stable complexes with both soluble and transmembrane

TNF α . Infliximab has a half life of about 9 days and is therefore currently given as an intravenous infusion on days 1 and 15 of treatment, followed by maintenance infusions every 6–8 weeks (141). The pivotal placebo-controlled Anti-Tumour Necrosis Factor Trial in Rheumatoid Arthritis with Concomitant Therapy (ATTRACT) in patients with active, refractory rheumatoid arthritis showed that infliximab every 8 weeks plus methotrexate resulted in rapid and sustainable improvements in clinical response, delayed radiographic progression, and/or improved functional status and health-related QOL compared with placebo plus methotrexate (142). This was again confirmed in the larger randomized Phase III ASPIRE trial (143). Subsequent studies have also extended use of infliximab to Crohn's disease, psoriatic arthritis and ankylosing spondylitis (144).

Significant adverse reactions include headache, rash, nausea, diarrhoea, urinary tract infection, upper respiratory tract infection and sinusitis. Infusion reactions (urticaria, pruritus and chills) have been reported in 20% of patients (144). Over 50% of patients will develop anti-nuclear antibodies and some 17% will have double-stranded DNA antibodies, although development of lupus (145) syndrome is unusual. Opportunistic infection such as reactivated TB has been observed. The onset of TB is usually apparent in 15% of cases by the third infusion and 97% of cases by the sixth infusion, which is ~7 months into treatment. Fifty six per cent present with extra-pulmonary disease including cases of atypical mycobacterial infection (146). Other opportunistic infections include histoplasmosis, aspergillosis, listeria and candidiasis. As a result, the drugs information now recommends evaluation for latent TB infection with a tuberculin skin test prior to infliximab therapy. Advice is given particularly with concomitant immunosuppressive therapy regarding opportunistic infection surveillance and risk benefit ratio.

Post-marketing surveillance has also revealed new cases of multiple sclerosis and optic neuropathy of non-demyelinating causes while receiving infliximab, although any direct association is yet to be established (144).

Because of the proposed mechanisms of tumour promotion by TNF α (Fig. 1), infliximab was investigated for the treatment of metastatic RCC in a single arm Phase II study using a 2-stage Gehan design (147). Nineteen patients with advanced renal cell cancer progressing after immunotherapy (interferon α , interferon α and/or IL-2) received infliximab (5 mg/kg) on weeks 0, 2, 6, 14, 22 and 30. Treatment was continued until disease progression and response assessed before each dose from week 6. This was an investigator-initiated and -led study. The primary objective was response rate and duration, with secondary objectives of evaluation of toxicity, survival and biomarkers including plasma TNF α , CCL2 (monocyte chemo-attractant protein 1 MCP1) and IL-6. All evaluated patients were ECOG performance status 0–2 and all had measurable lesions. Patients with active or latent TB or previously known HIV, hepatitis B or C or autoimmune disorder were excluded from the study. Patients with cerebral metastases and prior TNF α -targeted therapy were also excluded.

The first 15 patients are evaluable for toxicity and response at the time of original presentation. Median follow-up was 172 days (range 29–269). Median age was 57 (35–76) years. Two patients achieved PR (12%) at 14 and 30 weeks. One late

response (15 weeks) was observed following initial PD at 6 weeks. Five patients had ongoing disease control (PR + stable disease) at 1, 7, 7, 8 and 8 months. Median progression free survival was 56 days (95% CI 37–75).

Since then, 19 patients have become evaluable for response: three achieved a PR (16%), three stable disease and 13 developed progressive disease (68%). Overall, six patients (32%) showed some clinical benefit. The median duration of response for the partial responders varied from 4.7 to 7 months. One patient experienced grade 3 allergic reaction, and one patient died on holiday of reported septicaemia secondary to pneumonia. This could not be confirmed by the investigators or by post-mortem. Biomarker analysis showed a significant rise in TNF α following the first infusion of infliximab, which was thought to be due to ELISA detection of infliximab–TNF complexes. Although a significant fall in CCL2 and changes in IL-2 were observed between patients during treatment, no correlation with response could be determined. This preliminary study leads to a further high dose infliximab trial involving 20 patients which has completed recruitment.

Other anti-TNF α monoclonal antibodies include adalimumab, a fully human recombinant IgG1 anti-TNF α (D2E7, Humira®, Abbott) and etanercept (Enbrel®, Amgen Wyeth, PA, USA.) which is a soluble p75 TNF α receptor fusion protein that binds TNF α and has a longer half life than the native soluble receptor. As these drugs are established in some auto-immune diseases, their anti-tumour activity is also being explored. A recombinant TNFR IgG chimera (rhuTNFR:Fc) was given with IL-2 with regard to reduction of IL-2 toxicity in a group of 19 melanoma and renal cancer patients. No reduction in the toxicity was observed with the TNFR antibody in this randomized placebo-controlled trial (148), and the direct anti-tumour activity of the anti-TNF monoclonal was not examined. Similarly, etanercept has been used to block some of the TNF-mediated effects of dose-intense chemotherapy (149). Patients who received etanercept with docetaxel experienced significantly less fatigue without apparent compromise of response. Studies have observed biological changes during the treatment of ovarian and breast cancer with etanercept, although no clinical responses were seen (150, 151).

6.8 The Future for TNF α -Related Treatment

The apparently paradoxical effects of endogenous and tumour-derived TNF α belies the complex role played by this cytokine in carcinogenesis. This in turn leads to the seemingly opposite clinical applications of high dose TNF and TNF inhibition in cancer treatment. There have been preliminary studies of TNF-based gene therapy (152), where TNF producing adenovectors have been inserted into tumours and then triggered by radiation. Although renal cell cancer was not examined in this Phase I study, 70% of patients (21/30) experienced a tumour response and there was no dose-limiting toxicity. The interaction of TNF/TNFRs with apoptotic pathways at multiple sites also provides another approach, and this provides the basis of many lines of investigations in various tumour types (153).

It is possible that the overall therapeutic strategy will be tumour dependent, as well as TNF dose dependent. High dose TNF in conjunction with conventional neoplastic agents could be used to achieve a cytotoxic effect followed by maintenance anti-TNF treatments to prevent long-term tumour promotion. Conversely, the anti-TNF treatment could be used in conjunction with conventional cytokine treatment for renal cell cancer to reduce the significant, and often treatment limiting side effects of interleukins and interferon. Certainly, the arena for exploration in this area remains broad.

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Molecular Markers for Predicting Prognosis of Renal Cell Carcinoma

Mark Nogueira and Hyung L. Kim

Abstract Metastatic or recurrent renal cell carcinoma (RCC) carries a poor prognosis and long-term survival is rare. However, many small RCCs that are incidentally discovered have an indolent course even without treatment. The variability in clinical outcome is a reflection of the underlying tumor biology. Currently, clinical variables such as tumor stage and histological grade are widely accepted surrogates for tumor-specific cellular and molecular processes. Ongoing advances in genomic and proteomic technologies have produced an expanding list of molecular markers for predicting prognosis. Expression array studies have produced large numbers of candidate prognostic markers. Many of these markers have been validated in large groups of RCC patients.

Keywords Renal cell carcinoma • Molecular markers • Prognosis

1 Introduction

In the United States, it is estimated that in 2007, approximately 51,190 new cases of renal malignancies will be diagnosed and more than 12,890 individuals will die of the disease (1). As many as 30% of patients have metastatic disease at the time of initial diagnosis, and approximately 30% of patients diagnosed with organ-confined disease develop recurrence following nephrectomy (2, 3). The prognosis associated with RCC can vary widely. Metastatic or recurrent RCC carries a poor prognosis and long-term survival is rare. Historical 3-year survival for patients with metastatic disease is less than 5% (4). However, many small RCCs that are incidentally discovered have an indolent course even without treatment. For patients diagnosed with RCC, accurately determining prognosis is useful for patient counseling, selecting treatment, and considering enrollment for clinical trials.

H.L. Kim (✉)

Roswell Park Cancer Institute, Elm and Carlton Streets, Buffalo, NY 14263

e-mail: Hyung.kim@roswellpark.org

The variability in clinical outcome is a reflection of the underlying tumor biology. Currently, clinical variables such as tumor stage and histological grade serve as widely accepted surrogates for tumor-specific cellular and molecular processes. With advances in genomic technology, molecular markers are being identified that may more directly reflect tumor biology and allow development of effective tools for predicting prognosis. This chapter summarizes expression array studies of prognostic molecular signatures in RCC and describes specific markers that have been validated in studies with sample size of at least 50 RCC patients.

2 Expression Arrays

The emergence of molecular medicine has been fostered by the rapid advances in genomic technology and the completion of the Human Genome Project in 2003. High-throughput tools have been developed for interrogation of tens of thousands of cellular macromolecules such as DNA and RNA in a massively parallel manner. Gene arrays are used to simultaneously monitor the expression of thousands of genes. The first step involves extraction of mRNA from tumor tissue and control tissue (e.g., adjacent normal kidney). The RNA is converted to cDNA, and the cDNA from tumor and control tissue are labeled with two different fluorescent tags. The cDNA is applied to an array of target DNA, representing thousands of genes. The target DNA that binds cDNA is identified by the fluorescent signal. The expression level of a particular gene in the tumor relative to the control tissue can be estimated by examining the intensity of the two fluorescent tags bound to each probe. Gene arrays have been successfully utilized to identify immunomarkers for the various histological subtypes of RCC (e.g., clear cell, papillary, and chromophobe RCC) (5–7). Gene arrays studies have also identified expression profiles associated with prognosis ([Table 1](#)).

The first two gene expression studies reported in the literature suggested that there are two molecularly distinct forms of clear cell RCC, which carry different prognoses. Takahashi et al. performed the first gene expression study identifying prognostic markers (8). They evaluated 29 clear cell RCCs and 29 matching normal kidneys using a custom cDNA microarray of 21,632 genes and expressed sequence tags (ESTs). An unsupervised hierarchical cluster analysis was performed, which groups samples based on similarities in the pattern of gene expression, without considering clinical information. Therefore, following the statistical analysis, clinical characteristics unique to each cluster represent the phenotype resulting from genotype that defines the cluster.

In the study by Takahashi, 3,184 genes were identified, which had at least twofold difference in expression between tumor and normal kidney. A cluster analysis using the 3,184 genes produced two primary groups with significantly different 5-year survivals. Approximately 40 genes were necessary to accurately identify the two clusters. The study was internally validated by simulating the use of expression profiling as a clinical tool for predicting survival. In 96% of the cases, expression profiling accurately predicted the actual clinical outcome.

Table 1 Gene expression studies to predict prognosis

	Sample (RCC subtypes) ^a	Platform	Comparison	Results	Validation
Takahashi (8)	29 clear cell	cDNA array (21,632 clones)	Good vs poor prognosis based on 5-year survival	51 genes were able to cluster tumors into two prognostic groups	Simulation of use as prognostic tool
Vasselli (9)	29 matched normal kidney 51 clear cell	cDNA array (6,400 clones)	Long-term vs short-term survival	45 genes used in cluster analysis identified short-term and long-term survival groups	Simulation of use as prognostic tool
Sütlmann (10)	6 papillary 1 undifferentiated (all from patients with metastatic RCC) 65 clear cell	cDNA array (4,207 clones)	Genes differentially expressed based on metastasis status and survival	45 genes correlated with survival	RT-PCR for 6 genes
Kosari (11)	13 papillary 9 chromophobe 25 normal kidney 10 clear cell (nonaggressive)	HG-U133 Plus2 (38,500 genes)	Nonaggressive primary tumor vs aggressive tumor	85 genes correlated with metastasis status at the time of surgery 35 candidate genes for predicting aggressiveness	RT-PCR using an independent cohort of 55 samples validated 34 of 35 candidate genes
Jones (12)	9 clear cell (aggressive) 9 metastatic deposits 12 matched normal kidney 23 clear cell	HG-U133A (22,283 genes)	Genes differentially expressed based on metastasis status and disease progression status	31 genes correlated with progression	155-gene signature tested on 9 independent T1 clear cell RCC

(continued)

Table 1 (continued)

	Sample (RCC subtypes) ^a	Platform	Comparison	Results	Validation
Zhao (13)	13 papillary			155 genes differentially expressed comparing non-metastatic and metastatic clear cell RCC	
	7 chromophobe 10 TCC 12 oncocytomas 10 metastatic deposits 24 normal kidney				
	177 clear cell	cDNA array (27,290 clone)	Genes differentially expressed based on survival	259 genes used to assign a continuous risk score	Dataset randomly split into a training set and a test set

Abbreviations: *RCC* renal cell carcinoma, *TCC* transitional cell carcinoma, *RT-PCR* reverse transcription-polymerase chain reaction (quantitative)

^aAnalysis was performed on the primary renal tumor unless sample is identified as a metastatic deposit

Vasselli et al. performed gene expression analysis using the primary tumor from patients diagnosed with metastatic RCC (9). The study examined 51 clear cell and 7 nonclear cell tumors using a cDNA microarray of 6,400 genes and ESTs. They used the Cox proportional hazards model to identify 45 genes that most significantly correlated with survival. These 45 genes were used in a cluster analysis, and this resulted in two major clusters representing short-term and long-term survivors. Vascular cell adhesion molecule-1 (VCAM-1) was the most significant predictor of survival. VCAM-1 is a cell surface glycoprotein implicated in the interaction between RCC and epithelial cells. VCAM-1 was uniformly upregulated in long-term survivors, and downregulated in shorter-term survivors.

Others have also utilized expression microarrays to identify candidate genes associated with prognosis. Several studies suggest that molecular features of the primary tumor determine the risk for metastasis. Sultmann et al. examined 65 clear cell and 22 nonclear cell RCCs using a cDNA array of 4,207 clones (10). The array was designed utilizing genes believed to be differentially expressed by RCC and normal kidney based on proposed function of the gene product. The study identified an 85-gene signature in the primary tumor that could predict metastasis status and identified 45 genes that correlated with survival.

The concept that metastasis risk can be predicted by examining the primary tumor is also supported by a study by Kosari et al. (11). Using the Affymetrix HG-U133 Plus2 chip, they compared nine metastatic deposits of clear cell RCC and nine primary tumors from patients with aggressive clear cell tumors. Patients were considered to have aggressive disease if they recurred or died of their disease within 4 years of treatment or diagnosis. The expression profiles for the metastatic deposits and the primary tumors were similar; therefore, these two groups were combined. The resulting group was compared to ten primary tumors from patients with nonaggressive clear cell RCC. Nonaggressive RCC was defined by a predicted 5-year recurrence free survival >84% based on a clinical nomogram. The authors identified 35 candidate genes capable of differentiating aggressive and nonaggressive clear cell RCC and successfully validated 34 of these genes by real-time PCR using a separate cohort of 55 RCCs.

Jones et al. also concluded that the primary tumor contains molecular information to determine metastasis status (12). They used the Affymetrix HG-U133A chip to initially compare eight primary clear cell T1 tumors from disease-free patients and nine metastatic deposits of clear cell RCC. They identified 155 genes that were differentially expressed and tested the 155-gene signature on nine independent T1 primary clear cell tumors. The 155-gene pattern predicted metastasis status with 88.9% accuracy. They also tested their metastasis signature on the dataset generated by Sultmann et al. to demonstrate that gene signatures can be applied across different microarray platforms. For the 155 genes identified, 41 were present on the array used by Sultmann. These 41 genes were used to cluster the patients studied by Sultmann, producing two significantly distinct clusters with regards to metastasis status.

The gene expression associated with metastasis may apply to malignancies in general may not be unique to RCC. In the study by Jones et al., they evaluated an expression signature derived by another group using various non-RCC tumors.

Ramaswamy et al. compared 12 metastatic and 64 primary adenocarcinomas from lung, breast, colon, uterus, and ovary (14). They found a 128-gene signature that correlated with poor outcome. This “global” metastatic signature was applied to RCC. An unsupervised hierarchical cluster analysis produced clusters that correctly identified the status of RCC metastasis.

The largest gene expression study conducted to date has been performed by Zhao et al. (13). They evaluated 177 clear cell RCCs representing all stages of disease. A 27,290 clone cDNA microarray was utilized. The data was randomly split into a training set and a test set. In the training set, a Cox-proportional hazard model was utilized to identify 259 genes that correlated with survival. The Cox score was calculated for each gene, and these genes were used with the test set to derive a supervised principle components (SPC) risk score, which is a continuous variable. In the test set, the SPC risk score was a significant predictor for survival and was independent of clinical stage grouping, histological grade, and performance status.

These gene array studies evaluated thousands of genes to generate a limited list of signature genes (31–259 genes) that predict prognosis. One challenge in interpreting the data is that there is often little overlap between lists of signature genes generated by the various groups. For example, we can use the study by Zhao (13), which had the largest sample size, as a reference; of the 259 prognostic genes identified there is overlap with one of 45 genes from Vasselli (9), 4 of 35 genes from Kosari (11), and 2 of 155 genes from Jones (12). The greatest overlap exists with the 51-gene signature identified by Takahashi (8), with 15 genes overlapping. This lack of overlap can be partially explained by differences in array platform, specimens utilized, statistical analysis, and clinical endpoints evaluated. However, it also underscores the need for large-scale, prospective validation of array-based prognostic models. Candidate genes identified by this technology need validation with larger sample sizes using more robust techniques such as real-time PCR and immunostaining.

2.1 Studies of Individual Prognostic Markers for RCC

Ongoing efforts to elucidate the genetic defects leading to the various histological subtypes of RCC have identified import targets for drug development as well as for predicting prognosis. It is interesting to note that earlier marker studies focused on well-characterized genes of general relevance to oncology and included markers of cell proliferation and growth regulation, such as Ki-67 and p53 (Table 2). However, more recent studies have investigated novel markers identified by expression arrays and genes involved in RCC-specific molecular pathways. Studies of individual prognostic markers for RCC are reviewed in this section. Prognostic markers that are independent of clinical variables on multivariate analysis are emphasized. Commonly used alias are noted; however, markers are also identified (*italics*) in this review using the standard symbols approved by the Human Genome Organization nomenclature committee (81).

Table 2 Molecular markers predictive of survival in patients with renal cell carcinoma

	Year	n	H	A	Year	n	H	A
Apoptosis								
BAX	2004 (15)	138	Mi	U	2004 (16)	67	c	U
BCL2	2004 (17)	101	mi	M	2003 (18)	114	mi	M
	2004 (15)	138	mi	U	2004 (19)	129	c	M
BIRC5 (survivin)	2006 (20)	312	c	M	2002 (21)	67	c	M
CLU (clusterin)	2005 (22)	131	mi	U	2001 (23)	104	c	M
CTSD (cathepsin D)	2005 (24)	150	c	U	2005 (25)	149	c	M
DIABLO	2005 (26)	78	mi	U	2005 (27)	176	mi	U
IGF1R	2003 (28)	280	c	U	2005 (27)	176	mi	U
VEGFA	2004 (29)	48	c	M	2005 (27)	176	mi	M
	2004 (30)	179	c	U	2004 (31)	224	c	M
Degradation of extracellular matrix								
MMP2	2001 (33)	153	c	U	73	M		
	2003 (34)	131	mi	U	2004 (15)	138	mi	U
MMP7	2006 (35)	156	c	M	2003 (34)	131	mi	U
MMP9	2001 (33)	153	c	U	1999 (36)	118	un	M
PLAU (uPA)	2005 (38)	106	mi	U	1997 (37)	50	c	U
PLAUR	2005 (38)	106	mi	U	1997 (39)	87	mi	M
SERPINE1 (PAI)	2005 (38)	106	mi	U	1998 (40)	95	mi	M
Hypoxia-inducible factors					1998 (41)	109	mi	M
CA9	2004 (31)	224	c	M	87	mi	U	
	2004 (42)	318	c	M	2001 (23)	104	c	M
	2003 (44)	321	c	M	2006 (43)	138	mi	U
ENG (endoglin)	2006 (45)	168	c	U	2004 (32)	73	c	M
HIF1A	2006 (46)	176	c	U	2004 (19)	129	c	M
	2005 (47)	66	c	M	2001 (33)	153	c	M
	2005 (48)	56	c	M	2001 (33)	153	c	U
					2005 (49)	193	c	M
Immune regulation					134	c	M	
CCCL4 (MIP-1β)	2006 (51)	67	ns	U	2004 (42)	318	c	M
CCCL5 (RANTES)	2006 (52)	67	ns	U	2001 (23)	104	c	U

(continued)

Table 2 (continued)

	Year		n	H	A	Year		n	H	A
Apoptosis						Cell cycle regulation				
CD274 (B7-H1)	2006 (53)	196	c	M			2000 (54)	73	c	U
CXCL9 (MIG)	2006 (51)	67	c	M			1997 (37)	50	c	M
CXCL11 (I-TAC)	2006 (51)	67	c	U			1997 (55)	72	mi	M
CXCR3(IP10)	2006 (51)	67	c	U		Miscellaneous				
SPP1(osteopontin)	2006 (56)	171	c	U		AQP1	1998 (57)	58	c	M
VTGN1 (B7-H4)	2006 (58)	259	c	U		ADFP	2005 (59)	103	c	M
Cell adhesion						DPYD (DPD)	2003 (60)	65	mi	U
CDH6 (cadherin-6)	2004 (61)	216	mi	M		EBAG9	2005 (62)	78	c	M
CD44	2004 (63)	173	c	U		ECGF1(TP)	2003 (60)	65	mi	M
	2001 (64)	73	c	M		FHIT	2002 (65)	149	c	M
	2001 (66)	92	c	U		IMP3	2006 (67)	371	mi	M
	2000 (68)	52	c	U		KLK6 (HK6)	2006 (69)	70	mi	U
CTNNA1	2004 (70)	124	mi	U		MME (CD10)	2004 (71)	131	c	U
	1997 (72)	90	mi	M		NUDT6 (bFGF)	2005 (73)	259	ns	M
CTNNB1	2004 (70)	124	mi	M		PTGS2 (COX2)	2003 (34)	131	mi	U
	1997 (72)	90	mi	U		VHL	2002 (74)	187	c	M
CTNNG	1997 (72)	90	mi	U			2005 (48)	56	c	U
MUC1 (EMA)	2004 (71)	131	c	U		VIM (vimentin)	2004 (42)	318	c	M
	2003 (75)	92	ns	M						
	2002 (76)	60	c	U						
MUC16 (CA 125)	2003 (75)	92	ns	M						
L1CAM	2005 (77)	72	c	M						
TACSTD1(Ep-Cam)	2005 (78)	96	c	M						
	2004 (79)	417	mi	M						
VCAM1	2006 (80)	485	mi	M						

Abbreviations: *n* number of patients, *H* tumor histology, *mi* mixed, *c* clear cell, *ns* not specified, *A* analysis, *U* univariate, *M* multivariate

Several important research tools have facilitated investigations of prognostic markers. While expression arrays allow for simultaneous analysis of a large number of genes, they are less useful for examining markers across a number of diverse tissues. Tissue microarray (TMA) and quantitative PCR allow hundreds of samples to be rapidly and simultaneously assessed for protein expression and mRNA production, respectively. Both assays are highly sensitive and specific, and have a larger dynamic range when compared to gene expression arrays, making them suitable for validation of candidate prognostic markers. For construction of a TMA, cores of tissue as small as 0.6 mm in diameter are utilized, allowing tissue resources to be conserved. As many as 500 cores of tissue can be placed on a single slide and uniformly immunostained. TMA allows protein expression to be assessed in the context of morphology.

Quantitative reverse transcription-PCR can be performed with frozen as well as archival tissue. RNA is extracted from tumor and control tissue, and converted to cDNA. During PCR amplification of cDNA, fluorescence signal is produced. The original level of mRNA is quantified from a plot of PCR cycle number and log of the fluorescence intensity. Hundreds of reactions can be performed and analyzed simultaneously on multiwell plates. Several representative prognostic markers are discussed below.

3 Prognostic Molecular Markers

3.1 Cell Proliferation/Cell Cycle Regulation

A large number of proteins involved in cell proliferation have prognostic value for patients with RCC (Table 2). Ki-67 (*MKI67*) is a nuclear antigen expressed in the G1, G2, G3, and M phases of the cell cycle and absent in quiescent cells, making it a useful marker for cell proliferation (82, 83). Ki-67 expression has been associated with advanced clinical stage and poor histological grade (84). Increased expression of Ki-67 has been associated with decreased survival in both univariate (15, 29, 37) and multivariate analysis (27, 31, 36, 39, 42, 54). Two studies limited to patients with low stage (T1) tumors found no evidence linking Ki-67 expression and survival (85, 86). Therefore, it is possible that Ki-67 is prognostic only for more advance tumors.

Although Ki-67 has been intensively scrutinized as a prognostic marker, evaluation of Ki-67 in the clinical setting is not practical. RCC has a low growth fraction, making semiquantitative analysis impossible. Differentiation of Ki-67 levels requires more quantitative analyses, which are labor-intensive and not practical for routine use. Therefore, Dudderidge et al. (27) investigated geminin (*GMNN*) and minichromosome maintenance 2 (*MCM2*) to identify prognostic biomarkers with a broader range of expression.

MCM2 is part of a prereplicative macromolecular complex necessary for initiation of DNA replication during S phase (87–89). Geminin blocks reloading of MCM onto chromatin after one round of replication thereby preventing rereplication

and genomic instability (90). Proteins involved in initiating replication have been shown to be useful for determining diagnosis and prognosis in a variety of tumors, and dysregulation of the MCM proteins has been shown to be an early event in tumorigenesis (52, 91–96).

Duddridge et al. found that increased levels of geminin and MCM2 were associated with reduced disease-free survival in univariate analyses of RCC. They also noted that MCM2 expression in RCC is significantly higher than expression of Ki-67 or Geminin, making MCM2 amenable to semiquantitative analysis and routine use in the clinical setting. However, on multivariate analysis, MCM2 expression was not independent of clinical variables as a predictor of outcome.

Spermine (*SAT1*) is a polyamine whose biosynthesis is closely associated with physiological and neoplastic cell growth (97–99). Polyamine levels have been shown to be higher in tumor cells compared to cells of normal adjacent tissue (100, 101). Rioux-Leclercq et al. examined spermine as a prognostic marker in RCC tumors (32). They found increased levels of spermine and Ki-67 to be independent prognostic markers for cancer-specific survival in patients with and without metastasis. Furthermore, spermine exhibited higher specificity and sensitivity as a marker for survival compared to Ki-67 or any clinicopathological variable.

Interruption of cell cycle regulation is a key aspect of tumor growth. Multiple proteins involved in the cell cycle regulation have prognostic significance for RCC. The tumor suppressor gene p53 (*TP53*) is located on chromosome 17p13.1 and encodes a nuclear phosphoprotein of 53 kDa (102). It is believed to regulate cell cycle by serving as a G₁ checkpoint, inducing cell cycle arrest and apoptosis in the presence of damaged DNA (103, 104). There is conflicting evidence regarding the association between p53 expression and RCC prognosis. Several studies have shown p53 to be an independent prognostic factor associated with poor survival (37, 49, 50, 55, 105–107), and others have found no significant association with patient outcomes (54, 86, 108–110).

This discrepancy may have resulted from studies with small sample sizes containing multiple histological subtypes of RCC. In order to overcome these confounding factors, Zigeuner et al. (50) evaluated p53 using a TMA of 246 RCC specimens, of which 134 were clear cell RCC. Patients with clear cell RCC and elevated p53 expression had a significantly worse prognosis in multivariate analysis. There was no association between survival and p53 expression in nonclear cell RCC. Kim et al. examined a TMA of 318 clear cell RCC and reported that p53 status is an independent predictor of survival (42). Subsequent evaluation of subset of patients with localized (49) RCC and subset with metastatic (111) RCC also confirmed that p53 is an independent predictor of survival.

Another regulator of the cell cycle, p27 (*CDKN1B*), is capable of inducing G₁ arrest by inhibition of cyclin E-CDK2 (112). Levels of p27 are increased in quiescent cells and decrease after stimulation with mitogens. It is possible that loss of p27 expression may result in tumor development and/or progression (112). Several studies have found an association between low p27 levels and high-stage, high-grade clear cell RCC (19, 113, 114). Low p27 expression has also been shown to be an independent predictor of poor prognosis in clear cell RCC (19, 21, 23).

3.2 Cell Adhesion

A variety of human malignancies shows changes in expression of cellular adhesion molecules (CAM) (115). L1 cell adhesion molecule (*L1CAM*) is a membrane glycoprotein expressed in neural, hematopoietic, and epithelial cells (116–119). It functions by interacting with other CAMs, extracellular matrix molecules, and signal receptors (120, 121). *L1CAM* has been reported to be overexpressed in several cancers, including malignancies of the uterus, ovary, skin, and lung (122–126). In RCC, Allory et al. (77) reported that increased *L1CAM* expression was associated with a higher risk of metastasis in patients with clear cell RCC, but not in patients with papillary RCC. In multivariate analysis, the concurrent status of two markers, that is high expression of *L1CAM* and absence of cyclin D, was predictive of metastasis.

In addition, in clear cell RCC, there was a strong correlation between expression of *L1CAM* and epidermal growth factor receptor (*EGFR*) (77), which has also been reported to be an independent predictor of survival in RCC (24). *L1CAM* has been shown to activate the *EGFR* receptor in vitro and in vivo (127). Therefore, it has been suggested that the extracellular domain of *L1CAM* may serve as a ligand for receptors such as *EGFR*, and promote cell survival and migration.

An important cell adhesion protein Ep-CAM (*TACSTD1*) mediates calcium-independent cell–cell adhesions (128). Increased Ep-CAM expression is seen in most epithelial tumors, including cancers of the colon, lung, stomach, pancreas, thyroid, breast, ovary, cervix, bladder, and prostate (129–138). Due to its location on the cell membrane, it is a potential target for anti-EpCAM antibody therapy (118, 139–143). Seligson et al. investigated the expression of Ep-CAM in 417 patients treated with nephrectomy for RCC of various histological subtypes (79). They found that clear cell RCC expressed lower levels of Ep-CAM compared to chromophobe and collecting duct RCCs. In a subset of 318 cases of clear cell RCC, Ep-CAM expression was found to be a prognostic factor for improved disease-specific survival in a multivariate analysis. Ep-CAM was also found to be expressed in the distal nephron and in normal renal epithelial cells. Therefore, the utility of Ep-CAM-targeted therapy may be limited by both its presence in normal tissue and relatively low expression in clear cell RCC.

VCAM-1 is a cell surface glycoprotein with an implicated role in binding vascular endothelium and facilitating progression to metastatic disease (144). In a microarray-based study, Vasselli et al. found *VCAM-1* to be predictive of survival in patients with metastatic RCC (9). Shioi et al. investigated the predictive value of *VCAM-1* expression in 429 cases of RCC representing all clinical stages and histological subtypes (80). Clear cell and papillary RCC expressed high levels of *VCAM-1*, whereas chromophobe RCC and oncocytoma expressed low levels. In a multivariate analysis, high *VCAM-1* expression was associated with improved outcome in a subset of patients with low stage, clear cell RCC. In contrast to the report by Vasselli et al. (9), *VCAM-1* expression did not predict survival in univariate or multivariate analysis.

3.3 Apoptosis

Tumorigenesis requires the abnormal inhibition of apoptosis. Survivin (*BIRC5*) is an antiapoptotic protein that belongs to the inhibitor of apoptosis protein family (145, 146). A recent study evaluated 312 clear cell RCC specimens using immunohistochemistry (20). Survivin expression was an independent predictor of death from RCC. Survivin is a promising therapeutic target because it is not found in normal human tissue. Interestingly, survivin is expressed at high levels in almost every human cancer studied to date (145, 147).

DIABLO, the human orthologue of murine Smac, is a proapoptotic molecule. It exists as an inactive precursor protein that is activated and released from the mitochondria in response to apoptotic stimulus. *DIABLO* contributes to caspase activation by sequestering endogenous inhibitors of apoptosis. Mizutan et al. reported that *DIABLO* was downregulated in RCC, with lowest expression in high-stage or high-grade RCC (26). Absence of *DIABLO* expression predicted worse prognosis. These observations suggested a possible treatment strategy. Transfection of RCC with *DIABLO* cDNA sensitized RCC to apoptosis induced by cisplatin and tumor necrosis factor-related apoptosis-inducing ligand.

3.4 Degradation of the Extracellular Matrix

Proteolytic degradation of the extracellular matrix and basement membrane is necessary for tumor growth, invasion, and metastasis. Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases that degrade the extracellular matrix and basement membrane (148). Tissue inhibitor of metalloproteinases (TIMPs) inhibit enzymes of the MMP family (149). Increased MMP levels have been associated with poor prognosis in colon, breast, lung, pancreas, prostate, and kidney cancer (33, 35, 150–154). *TIMP1* and 2 selectively inhibit *MMP9* and 2, respectively (155).

Kallakury et al. investigated the expression of *MMP2*, *MMP9*, *TIMP1*, and *TIMP2* in RCC (33). They found, as did Kugler et al. (156), that increased expression of *MMP2*, *MPP9*, *TIMP1*, and *TIM2* is associated with poor prognosis in RCC. Increased expression of *TIMP1* independently predicted shorter survival. This was an unexpected finding given one of the known functions of TIMPs as inhibitors of MMPs. TIMP expression may contribute to a worse prognosis because of its ability to promote tumor growth, as documented with several cancer cell lines (157–160).

Urokinase-type plasminogen activator (uPA, *PLAU*) is also involved in the degradation of the extracellular matrix. It functions as a serine protease catalyzing the conversion of plasminogen to plasmin, which directly degrades the extracellular matrix (161). It is also involved in cancer invasion and metastasis through the activation of zymogen proteases (161). The function of uPA is modulated through its receptor, uPAR (*PLAUR*) (162). Both uPA and uPAR are regulated by plasminogen activator inhibitor-1 (PAI-1, *SERPINE1*) and PAI-2 (*SERPINE B2*). It was initially proposed that PAIs inhibit tumor invasion and metastasis (162). However, two

studies of RCC found elevated PAI-1 levels to be independent prognostic factors for poor prognosis (38, 163). A potential explanation is that PAI-1 actually protects tumor tissue against uPA-mediated destruction (164). In univariate analysis, uPA and uPAR were found to be significant predictors of survival while there was no association between PAI-2 and survival (38).

3.5 Immune Regulation

There is evidence that RCC promotes tumorigenesis by undermining host antitumor immunity (165–167). B7-H1 (*CD274*) functions as a T-cell costimulatory molecule that has been implicated in antitumor immunity (168–170). Tumor cell expression of B7-H1 has been shown to inhibit apoptosis, impair cytokine production, and diminish the cytotoxicity of activated T cells (169, 171–173). A large, recent study investigated the expression of B7-H1 in 306 patients with ccRCC and over 10 years of follow-up (53). They found increased expression of B7-H1 to be an independent predictor of cancer progression, cancer-specific death, and overall mortality. Interestingly, even in patients with localized disease, median time to progression was less than 1 year in the presence of elevated B7-H1 expression.

B7-H4 (*VTCN1*) is another member of the B7 family of coregulatory ligands, which has been implicated as an inhibitor of T-cell-mediated immunity (58). B7-H4 is a lymphoid marker that can be expressed by human tumors as well as the tumor vasculature. Expression of B7-H4 by tumor cells has been correlated with adverse clinical and pathological features. Patients with B7-H4 expressing tumors were three times more likely to die from RCC when compared to patients with B7-H4 negative tumors. However, the association between B7-H4 expression and survival did not reach statistical significance when controlled for prognostic clinical variables. Patients with tumors expressing both B7-H4 and B7-H1 were significantly more likely to die of RCC than patients expressing only one of the two markers. Both proteins inhibit immune response, therefore, therapeutic strategies to block these molecules may promote immune mediated tumor destruction.

3.6 Hypoxia-Inducible Factors

Under normoxic conditions, the transcriptional regulatory protein, hypoxia-inducible factor 1 α (HIF-1 α) is rapidly degraded after it is tagged by von Hippel–Lindau (VHL) (174–176). Under hypoxic conditions, HIF-1 α is stabilized and translocated to the nucleus (177). Once in the nucleus, it binds a complex that leads to the transcription of genes regulated by the hypoxia response element, including erythropoietin, vascular endothelial growth factor (VEGF), carbonic anhydrase IX (CA9), glycolytic enzymes, and glucose transporters. This adaptive response allows cells to increase the delivery of oxygen and nutrients under stressed conditions.

In clear cell RCC, overexpression of CA9 is the direct consequence of a mutation in the VHL gene, which normally functions to degrade and suppress HIF-1 α . CA9 is not present in normal renal tissue (74, 178), but is present in 80% of primary and metastatic RCC, and in 95–100% of the clear cell variant. VHL mutations and deletions are found in over 50% of sporadic clear cell RCC (74, 178). Hypermethylation represents an additional mechanism for VHL inactivation (74). In patients undergoing treatment for clear cell RCC, VHL mutations and hypermethylation have been shown to independently determine favorable prognosis, suggesting that these tumors are more likely to be treatment responsive (31, 42, 44, 111). Consistent with this observation increased expression of CA9 (46–48) and HIF-1 α (47, 48) have been associated with favorable outcomes.

Although several studies identify HIF-1 α as a prognostic marker for RCC, it appears that HIF-2 α may be more important for clear cell renal carcinogenesis. HIF-2 α and HIF-1 α appear to have contrasting biological activity. HIF-2 α promotes transcription of tumorigenic genes including cyclin D1, transforming growth factor α , and VEGF; while HIF-1 α promotes transcription of proapoptotic genes such as BNip3 (179). Transcription of hypoxia-regulated genes such as VEGF is regulated primarily by HIF-1 α in response to hypoxia; however, in RCC cell lines with dysfunctional VHL, hypoxia-regulated genes are primarily dependent on HIF-2 α (180). Furthermore, in the presence of mutated VHL, inhibition of HIF-2 α , but not HIF-1 α , is necessary and sufficient to suppress tumor formation (181–183).

3.7 Multimarker Prognostic Model

Efforts have been made to combine multiple prognostic markers and develop a clinical tool for predicting individual patient survival. Kim et al. utilized a TMA constructed using clear cell RCC from 318 patients, representing all stages of localized and metastatic RCC, and immunohistochemically stained for molecular markers: MKI67 (alias Ki-67), TP53 (alias p53), GSN (gelsolin), CA9, CA12, PTEN, TACSTD1 (alias Ep-CAM), and VIM (vimentin) (42). In a multivariate analysis of all eight markers, TP53, CA9, GSN, and VIM were independent predictors of survival. These four markers along with metastasis status were used to create a prognostic nomogram, which had a statistically validated C-index of 0.75. A prognostic model based on a combination of clinical and molecular predictors included metastasis status, T-stage, ECOG-PS, p53, CA9, and vimentin as predictors and had a C-index of 0.79, which was significantly higher than that of prognostic models based on Grade alone, TNM stage alone, or the UCLA integrated staging system.

Kim et al. constructed a second prognostic nomogram using results from a subset of 150 patients with metastatic RCC who underwent nephrectomy prior to immunotherapy (184). In a multivariate analysis of all markers, CA9, TP53, VIM, and PTEN were independent predictors of disease-specific survival. These markers were used to develop a nomogram for predicting survival. The statistically validated C-index for a clinical model (stage, grade, performance status), marker

model (CA9, TP53, VIM, PTEN), and the combined clinical/marker model (CA9, TP53, VIM, PTEN, stage, and ECOG-PS) were 0.62, 0.64, and 0.68, respectively. These studies show that a prognostic model based on a small number of markers can perform better than a model based on a set of well-established clinical prognostic variables. However, the best survival predictions were achieved by using a combination of clinical and molecular predictors.

4 Conclusion

Accurate risk stratification is useful for patient counseling, recommending appropriate treatment, selecting patients for clinical trials, and tailoring surveillance strategies. With advances in molecular technology, and greater understanding of subcellular mechanisms characterizing RCC, markers are being identified to predict prognosis. High-throughput techniques such as gene expression arrays allow screening of thousands of genes. TMA and quantitative PCR allow for validation of candidate markers on a large number of tumors. As the list of prognostic markers continue to grow, it will be important to validate these markers on external, multicenter tissue banks, and ultimately develop multimarker tools for predicting outcome.

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Adjuvant Therapy for Renal Cell Carcinoma: Targeted Approaches

A. Karim Kadar and Christopher G. Wood

Abstract Renal cell carcinoma (RCC) remains a significant global health problem. Despite inroads in early detection, ~50% of patients either present with or develop metastatic disease during the natural history of their illness. There remains no consistent curative therapy for metastatic RCC. For patients with locally advanced disease, surgery remains the mainstay of curative therapy, but a significant number of patients still experience relapse, the risk of which is directly related to the presence of high-risk features at diagnosis. The search for effective adjuvant therapy to decrease the risk of relapse following surgery remains elusive. Therapeutic benefit in the setting of metastatic disease has not translated to success in the adjuvant therapy setting. Numerous clinical trials have examined the role of radiation therapy, immunotherapy, chemotherapy, vaccine therapy, and antiangiogenic therapies in the adjuvant setting. To date, none of these trials have demonstrated a significant benefit. Thus, the standard of care remains surveillance following surgical resection of locally advanced disease. Currently, clinical trials with the new targeted therapies developed for RCC are ongoing in the adjuvant setting. We anxiously await the results of these trials to determine whether they can significantly impact recurrence free and overall survival in the locally advanced setting following curative surgical resection.

Keywords Adjuvant therapy • renal cell carcinoma • locally advanced disease

1 Introduction

In those aged less than 85 years, cancer has eclipsed heart disease as the leading cause of death in the USA. Kidney cancer represents the most lethal urological malignancy. Forty percent of patients presenting with this disease eventually die of

C.G. Wood (✉)
Department of Urology, MD Anderson Cancer Center,
University of Texas, Houston, TX, 77030
e-mail: cgwood@mdanderson.org

the disease accounting for an estimated 12,600 deaths this year alone (1). The incidence of kidney cancers has risen gradually since the 1950s (1, 2). Part of this rise is felt to be due to increased detection by imaging performed for unrelated reasons; however, it is believed to also reflect a true increase in incidence (3). Despite this increase in early detection, ~20% of patients present with locally advanced disease and 30% have metastases at presentation (4, 5).

Kidney cancer cannot be viewed as a single disease. The American National Tumor Registries have combined renal parenchymal tumors with those of the renal pelvis. However, the biology of these diseases is very different. The parenchymal tumors arise predominantly from the tubular epithelium (renal cell carcinoma, RCC), whereas the renal pelvic tumors are derived from the urothelial epithelium. For the purposes of clarity, this chapter will focus on the current treatment options and future prospects for patients with advanced RCC.

Further classification of RCC is provided by Linehan et al. from the National Institutes of Health who have made great strides into the molecular, hereditary, and pathological aspects of RCC. This has provided the opportunity for the development of targeted therapies. There are now felt to be five major classifications of RCC including clear cell, papillary, chromophobe, medullary, and collecting duct (6). Sarcomatoid features can be seen with any of these subtypes and portends a poor outcome.

Clear cell RCC is the most common form of the diseases making up ~75% of cases. Seminal to our understanding of the molecular mechanisms of clear cell RCC began initially with the recognition of a familial clustering of clinical manifestations including RCC, hemangioblastoma, pheochromocytoma, and islet cell tumors to name a few. These observations were initially made by Treacher in the late 1800s but were not publicly accepted until noted by Von Hippel, a German ophthalmologist and subsequently Lindau, a Swedish pathologist in the early 1900s (7). This syndrome was later coined Von Hippel–Lindau (VHL) disease. In 1993, researchers at the National Cancer Institute cloned the tumor suppressor gene responsible for the disease at 3p25 which is known as VHL (8). Subsequent work suggested that VHL mutations were seen in 50% of sporadic forms of clear cell RCC with hypermethylation noted in an additional 10–20% (7).

Hypoxia-induced factors 1α and 1β work together to promote transcription of genes whose products are felt to play an important role during hypoxia. These proteins include but are not limited to erythropoietin to stimulate red blood cell formation, vascular endothelial growth factor (VEGF), and platelet-derived growth factor (PDGF), which are involved in angiogenesis and transforming growth factor- α , a powerful ligand for the epidermal growth factor receptor (EGFR), and survival signal as well as carbonic anhydrase IX (CA IX), a tumor-associated antigen, which may be involved in oncogenesis. Studies have shown that CA IX may undergo EGF-mediated phosphorylation leading to interaction with PI-3 kinase, Akt activation, and further downstream signaling (9).

During times of normal oxygen levels, HIF- α is hydroxylated and thus susceptible to ubiquitination by a protein complex including the protein product of VHL (pVHL) Elongin B, Elongin C, Cul2, and Rbx1. Under hypoxic conditions, a lack of HIF- α

hydroxylation results in decreased binding of the pVHL complex and allows it to complex with HIF- β and translocate to the nucleus so that it may act as a transcription factor for hypoxia inducible genes. Inactivated VHL results in a lack of VHL complex binding allowing for constitutive activation of the HIF responsive genes (7).

Because of our understanding of VHL-mediated carcinogenesis, most if not all targeted therapies have focused on treatment of clear cell RCC. The effect of these treatments on tumors of nonclear histology is unclear. It is, however, apparent that these tumors are refractory to immunotherapy.

Papillary RCC is currently thought to exist as two types. Type 1 is associated with a mutation of the c-met oncogene which results in constitutive activation of the tyrosine kinase receptor of the hepatocyte growth factor. This understanding may soon lead to targeted therapies for this disease. Type 2 is significantly more rare and results from the mutation of the fumarate hydratase gene, which encodes an important Krebs's cycle enzyme. Yet another familial cancer syndrome is the Birt-Hogg-Dubé (BHD) syndrome where affected individuals develop cutaneous fibrofolliculomas, lung cysts, and chromophobe RCCs as well as oncocytomas. The gene for BHD has been identified and its function is being elucidated (6, 10).

To date, curative treatment options for patients with RCC have been limited to surgery. Despite the excellent survival with surgery seen in lower stages, many patients with more advanced disease do not fare as well. Unfortunately, no effective adjuvant therapies have emerged for this disease.

Optimally, an effective adjuvant therapy, would only be administered to those patients who need it, would first demonstrate efficacy in the metastatic setting and its efficacy further substantiated by phase II and phase III trials. It would have good oral availability, be relatively nontoxic, and may be administered in the outpatient setting.

Intensive investigation is currently being performed to achieve these goals and great strides have been made. In the future, clinical, pathological, and molecular markers will be used together to more accurately define high-risk patients. Modest response rates with several agents have already been achieved in patients with metastatic disease and many trials on adjuvant therapy are ongoing. With our increasing understanding of the biology of the disease, new potential targets are emerging. There is cause for guarded optimism as much is on the horizon for patients with high-risk disease. In this chapter, we will briefly review the past adjuvant therapy trials and focus on the exciting state of the art and future for targeted therapies for the treatment of locally advanced RCC.

2 Determination of the High-Risk Patient

Despite an increase in early detection, 30% of patients with localized disease will go on to develop metastases following definitive therapy (5). In a recent review of the MDACC experience with a median 23-month follow-up, 24% of 286 patients

developed metastases postnephrectomy for localized disease. Of these, 0.9% had pT1, 25% had pT2, and 39% had pT3 disease (11).

The most widely used staging system in North America is that proposed by the American Joint Committee on Cancer (AJCC) in 2002 (Table 1). Using this system, a recent report by Frank et al. reviewed the Mayo clinic experience with radical nephrectomy in 2,746 patients with localized (N0, M0) RCC. The 5-year cancer-specific survival rates were 97%, 87%, 71%, 53%, 44%, 37%, and 20% in patients with T1a, T1b, T2, T3a, T3b, T3c, and T4 RCC, respectively (12).

Table 1 AJCC 2002 staging system for RCC

T – Primary tumor
TX: Primary tumor cannot be assessed
T0: No evidence of primary tumor
T1a: Tumor 4 cm or less in greatest dimension, limited to the kidney
T1b: Tumor greater than 4 cm, but not more than 7 cm, in greatest dimension, limited to the kidney
T2: Tumor greater than 7 cm, limited to kidney
T3: Tumor extends into major veins/adrenal/perinephric tissue; not beyond Gerota’s fascia
T3a: Tumor invades adrenal/perinephric fat
T3b: Tumor extends into renal vein(s) or vena cava below diaphragm
T3c: Tumor extends into vena cava above diaphragm
T4: Tumor invades beyond Gerota’s fascia
N – Regional lymph nodes
NX: Regional nodes cannot be assessed
N0: No regional lymph node metastasis
N1: Metastasis in a single regional lymph node
N2: Metastasis in more than one regional lymph node
M – Distant metastasis
MX: Distant metastasis cannot be assessed
M0: No distant metastasis
M1: Distant metastasis
Stage groupings
Stage I
T1 N0 M0
Stage II
T2 N0 M0
Stage III
T1 N1 M0
T2 N1 M0
T3 N0 M0
T3 N1 M0
Stage IV
T4 N0 M0
T4 N1 M0
Any T N2 M0
Any T Any N M1

In addition to stage, several factors have been felt to affect survival including clinical presentation, Eastern Cooperative Oncology Group performance status (ECOG PS), grade, and the presence of sarcomatoid differentiation or necrosis in the pathological specimen. Histological subtype was felt in the past to be an important determinant of outcome; however, when corrected for stage and grade, it is now believed to be less important (13). To date, several potential molecular markers of RCC have been identified including Ki-67, p53, and CA IX. It is hoped that these and other markers will one day be incorporated into integrated staging systems, which will allow for better prognostication and prediction of treatment response (14).

In order to define the high-risk patient who may benefit from adjuvant therapy and to prognosticate patients, several predictive tools have emerged to help clinicians and patients decide when to add further, often experimental, therapies.

These include the University of California at Los Angeles Integrated Staging System (UCLA ISS) (15); the Mayo Clinic stage, size, grade, and necrosis score (SSIGN score) (16); the Memorial Sloan Kettering nomogram (17); the European prognostic model (18); and the Hopkins prognostic assessment (19).

Despite our increasing accuracy at detecting those patients who have a greater risk of local and/or systemic failure, to date, no treatment modalities have been identified with proven efficacy in an adjuvant setting. The next sections will describe the past, present, and future of the research in this area.

3 Adjuvant Local Therapy

3.1 Radiation Therapy

Current therapy for local control of RCC is dominated by radical nephrectomy, which has been the mainstay of treatment since first popularized by Robson in the late 1960s. As shown before, 5-year survival using this treatment modality alone in lower stage disease is ~97% (12). It is clear now that partial nephrectomy is as effective as radical nephrectomy for low-stage disease (20). Because of the success of nephrectomy, other minimally invasive treatments for local therapy have emerged, including laparoscopy, radiofrequency ablation, and cryotherapy (21–23). The latter two therapies are newer, experimental procedures for local control in the setting of poor operative candidates with small masses. In the future, they may emerge as the primary treatment modalities for small renal masses. Observation does now appear to be an option for highly selected individuals with nonthreatening masses with other more significant comorbidities (24).

Local recurrence rates with nephrectomy, in the absence of distance metastases, are ~2% (25). Radiation therapy has been utilized in both a neoadjuvant and adjuvant fashion with no appreciable survival advantage (26–28). On the basis of the lack of benefit in these randomized, prospective trials, the use of radiation therapy for this disease has been negligible over the last few decades. There have been anecdotal

reports of the use of intraoperative radiation, as an adjunct to surgery for the treatment of locally recurrent disease (29, 30). However, its use in such a setting is too rare to determine if there is any benefit. The argument for adjuvant therapy for local recurrence is hampered by the lack of effect of radiation therapy and the low incidence of local recurrence in the absence of metastases. If newer, effective, and nontoxic modalities should arise then one could consider adjuvant therapy for local control in selected patients. However, we are far from such treatments at the current time. Therefore, its role has been relegated to the treatment of symptomatic bone and brain metastases.

3.2 Energy Ablation Therapy and Embolization

Technologies are emerging for the minimally invasive management of localized renal tumors. These techniques may also be of some benefit in advanced disease. There is the theoretical ability for preoperative ablation or embolization to release tumor antigens which may vaccinate patients and prevent subsequent recurrence (31, 32). To our knowledge, there are no current trials testing this hypothesis.

4 Adjuvant Systemic Therapy

4.1 Hormone Therapy

The interplay between hormones and cancer was first appreciated in the late nineteenth century when it was noted that one-third of breast cancers in premenopausal women receded after oophorectomy (33). Huggins, the only Urologist to win the Nobel prize, furthered the connection with his landmark work on the androgen dependence of prostate cancer (34). Progestins have been extensively used to treat metastatic endometrial and breast cancer as well as other malignancies (33). The presence of the estrogen receptor on the surface of RCC cells prompted the use of estrogens in its treatment. Pizzocaro et al. performed a phase III multicenter randomized adjuvant therapy trial looking at 500mg medroxyprogesterone acetate (three times per week for 1 year vs no treatment) in 59 T1-T2, 62 T3+ (N+ included) patients. The median follow-up was 3 years. The relapse rate was 25.8% in the adjuvant group as opposed to 23.8% in the control group. Furthermore, 56.9% of patients had toxicity with the adjuvant therapy. Interestingly, recurrence was more common in patients without sex steroid receptors regardless of treatment. This treatment modality was thus abandoned.

Other potential hormonal targets are emerging including somatostatin, leutinizing hormone releasing hormone, and parathyroid hormone-related protein; however, these potential therapies are at the very early stages of investigation (35–37).

4.2 Immunological Therapy

Paul Ehrlich in the early 1900s suggested that without host defenses that cancer would occur with great frequency. Furthermore, innate and adaptive defense systems were at play (38). These ideas would later contribute to him winning the Nobel Prize. This seminal contribution led to the theory of tumor-specific antigens, which was developed by murine tumor transplantation studies in the 1950s and 1960s. Later, there was recognition that T cells recognized intracellular antigens in the context of the major histocompatibility complex (MHC).

The role of immune surveillance in RCC was furthered by the observation of spontaneous regression of metastases and late relapses following radical nephrectomy (25, 39, 40). This prompted a whole field of investigation, which has yielded the best responses to systemic therapy to date. Unfortunately, these gains have been made only in the clear cell subtype, as there is a lack of benefit in nonconventional histology.

Current thinking in tumor immunology points to cellular or T-cell-mediated immunity as they key player in antitumor activity (41). Briefly, CD4 and CD8 positive T cells (T helper and cytotoxic T cells, respectively) interact with intracellular peptides 8–10 amino acids long, presented on cellular surfaces in context with MHC class II and I, respectively. This leads to the release of several cytokines as well as direct cell death. These cytokines are key to the clonal expansion of B cells which produce antibodies specific to recognized antigens. Once bound to antigens, these antibodies are recognized by neutrophils, macrophages, and natural killer (NK) cells which are the mediators of antibody-dependent cytotoxicity (ADCC) system.

Most current strategies attempt to exploit and boost the patient's immune system. This may be done by way of nonspecific or specific immunotherapy (41).

4.2.1 Cytokine Therapy

Cytokines are protein mediators of immunological activity. They are formed by cells which then elicit a response in the same (autocrine) or other (paracrine) cells via cell surface receptors. The use of cytokines in the treatment of metastatic RCC has resulted in the best response rates to systemic therapy. However, these benefits are at the cost of significant toxicity. The predominant cytokines used include interferon (IFN)- α and interleukin (IL)-2. [Table 2](#) outlines adjuvant therapy trials using these cytokines.

IFN- α is a member of a highly conserved family of proteins including IFN- β and γ , which was discovered in the mid 1950s due to their effects on “interfering viral infection”. The entire family has been used for treatment of RCC but efficacy has been limited to IFN- α (39, 51). The first use of IFN- α in the setting of a solid tumor was with osteosarcoma in the early 1970s (52). Ten years later, it was available in recombinant form and its first use in metastatic RCC was described (53). Although response rates as high as 30% have been described, randomized trials show a 15% response rate with only a few complete and durable responses (54).

Table 2 Summary of adjuvant cytokine trials

Author	Agent	Result	References
Interferon- α alone			
Delta-P study group	IFN- α	No benefit	(42)
Pizzocaro et al.	IFN- α	No benefit	(43)
ECOG	IFN- α	No benefit	(44)
Basting et al.	IFN- α	No benefit	(45)
Interleukin-2 alone			
Clark et al.	IL-2	No benefit	(46)
Combination			
Jeon et al.	IFN- α /vinblastine	No benefit	(47)
Migliari et al.	IFN- α /vinblastine	No benefit	(48)
Hong et al.	IFN- α /IL-2/5-FU	No benefit	(49)
Atzpodien	IFN- α /IL-2/5-FU	Decreased survival in treatment group	(50)

ECOG Eastern Cooperative Oncology Group; *CWG* Cytokine Working Group

In the setting of metastatic disease, an additive survival benefit has been seen in patients getting radical nephrectomy in addition to their IFN- α immunotherapy (55, 56). Attempts have been made to use IFN- α in an adjuvant setting in advanced disease with no effect (Table 2).

IL-2 is a cytokine predominantly produced by the activated T helper cell. It results in autocrine and paracrine cellular activation, which lies at the heart of the cellular immune response. Rosenberg et al. showed its effectiveness as an antitumor agent in a murine metastases model in the mid 1980s (57). Studies by Fyfe et al. revealed a 15% objective response rate leading to FDA approval for this indication (58). Significant side effects were encountered including hypotension, pulmonary edema, renal dysfunction, and death (59). Despite the initial excitement, a randomized study looking at a single adjuvant bolus dose of IL-2 postsurgery did not result in a survival benefit (46).

Several randomized trials have been performed to date looking at a combination of these cytokines in the setting of metastatic disease and increased toxicity with no differences with respect to survival have been seen (60, 61).

Other combinations have been tried in the metastatic setting including IFN- α with Vinblastin, IL-2 and 5-fluorouracil, cis-retinoic acid, and IFN- γ (52, 62, 63). These combinations have been met with disappointing or conflicting results and severe toxicities. A recent randomized trial from the German Cooperative Renal Carcinoma Chemo-Immunotherapy Trials Group using the adjuvant IFN- α /IL-2/5-FU combination versus observation in 203 patients with high-risk RCC demonstrated that there was no benefit with respect to progression-free survival and that overall survival was lower in the treatment arm (50).

Overall, the best response rates seen in systemic therapy for metastatic RCC are about 15% seen with either IL-2 or IFN- α as monotherapy. To date, no effect has been seen using this strategy in an adjuvant setting.

4.2.2 Adoptive Immunotherapy

In an attempt to achieve a more targeted response, researchers have attempted to exploit the lymphocytes of the RCC patients as a means of treating them. These lymphocytes are harvested from peripheral blood, tumor draining lymph nodes or the tumors themselves, and activated *ex vivo* in the presence of supraphysiological doses of IL-2 before being reinfused into the patients (64). In the early 1980s, a group out of the NIH described a population of large granular lymphocytes derived from the peripheral blood with antitumor activity which they named lymphokine-activated killer cells (LAK) (65). They were further able to demonstrate regression of metastases in a murine model (66). These initial results prompted great excitement and phase I trials were initiated (67).

Autolymphocyte therapy is a twist on the LAK method which attempts to expand the memory T-cell population *ex vivo* prior to reinfusion. This is done by a combination of T-cell receptor antibodies and chemotherapies. Early on, good results were achieved in the metastatic patient setting (68). This prompted further investigation into the potential benefit of this strategy in an adjuvant setting; however, no benefit was seen (69). Because of these disappointing results, this technique has been largely abandoned.

4.2.3 Tumor Vaccines

Given the immunogenic nature of RCC, great interest has been focused on vaccine-mediated enhancement of the antitumor response. These have been with crude tumor lysates, lysates with immune boosting agents, genetically modified tumor vaccines, and dendritic vaccines.

The first generation vaccines involved crude tumor lysates. These studies showed that an enduring response was possible and even showed some interesting outcomes in small numbers of patients (70). Further study by Repmann et al. showed good tolerability and an impressive 5-year progression free survival in patients with T3N0M0 disease (68.2% in the vaccine group vs 19.4% in historical controls) (71, 72). This prompted further investigation and a recent phase III German trial randomizing 558 advanced RCC patients to adjuvant vaccine therapy showed that the vaccine was well tolerated and that there was a significant 5-year progression free survival of 77.4% in the vaccine group versus 67.8% in the control group (73). Critics of this report, however, argue that upon analysis in an intent to treat analysis, this significance is lost.

Irradiated tumor lysates with BCG has been tried in a randomized trial of 120 patients in an adjuvant setting. Despite a good toxicity profile, no significant difference was seen in disease-specific or overall survival (74).

Second-generation vaccines flourished during the early 1990s when gene transfer technologies matured. The strategy involved the purification of tumor cells and the subsequent transfection of these cells with immunostimulatory cytokine genes including IL-2, and granulocyte, colony stimulating factor (GM-CSF), as well as

the costimulatory molecule B7-H1. Despite initial excitement, trials with this approach have been hard to perform and in the limited number of patients treated, mixed results have been obtained (75).

Dendritic cells are felt to play an important role in antigen presentation and thus tumor rejection. These cells can be derived from monocytes harvested from the bone marrow or peripheral blood of patients or allogeneic donors. They are then cocultured with the patients tumor cell lysates or even transfected with tumor-specific mRNA before being reintroduced to the patient as a vaccine. To date, their use has been limited to the metastatic RCC patient population. A phase I/II trial was performed investing 15 patients treated with autologous dendritic cells pulsed with autologous tumor cells, good tolerability was seen, 1 patient had a partial response, and 7 patients had stabilization of disease (76).

Heat-shock proteins (HSPs) are a family of ubiquitous proteins known to assist in protein folding and localization during the posttranslational period. They bind to intracellular peptides and are highly immunogenic. The experimental use of HSPs in a vaccine strategy began with work by Srivastava in a murine hepatoma model dating back to the mid 1980s (77). The idea is that HSPs and their associated peptide antigens are purified from tumors following nephrectomy. This purified mixture is then used as the basis for a vaccine. Encouraging results have been obtained with Oncophage®, an HSP 96-based peptide complex vaccine (Antigenic, Inc., New York, NY) that has received the generic name vitespen. This vaccine has been studied in clinical trials with pancreatic cancer, melanoma, colorectal carcinoma, glioma, as well as RCC. An initial phase I/II study in metastatic RCC suggests a good safety profile and a 32% objective response rate or disease stabilization. A subsequent phase II trial demonstrated a 15% rate of objective response or stable disease, with a median survival of 1.3 years (Antigenics, Inc., data on file). On the basis of these encouraging preclinical and early clinical results, further study was felt to be warranted. As a consequence, a randomized trial examining the role of vitespen in the adjuvant setting for high-risk clear cell RCC has been completed. This was a phase III open label multicenter trial involving 132 centers worldwide. Patients with high-grade stage IB/II or stage III/IV(nonmetastatic) were randomized to either observation postnephrectomy or 25- μ g intradermally weekly for 4 weeks started within 8 weeks of surgery and then every 2 weeks thereafter until the supply was exhausted. Initial analyses of the data from this trial suggest that there may be benefit derived from vaccine in the intermediate-risk patient (T1–2 high grade, T3a low grade), with significant improvements in recurrence-free survival noted, as well as a trend toward improved survival, in a subset analysis. We await further data from this trial with maturation, as well as a confirmatory study to determine the exact role of vitespen as an adjuvant agent.

4.2.4 Monoclonal Antibody Therapy

Kohler and Milstein's Nobel winning work done at the Ontario Cancer Institute in the mid 1970s fusing antibody producing murine B-cells with myeloma cells

resulted in the development of the first monoclonal antibody (mAb). There was great initial excitement at what many considered to be the “magic bullet” of cancer therapy. After 20 years, they finally became available in the armamentarium of targeted therapies against cancer. Their initial foray was with Rituxan® (Rituximab) (Genentech, San Francisco, CA), approved by the FDA in 1997 for B cell lymphoma. Subsequent mAbs include Herceptin® (Trastuzumab) (Genentech), a humanized mAb used to treat Her2 positive breast cancer, and Zevalin (Ibritumomab Tiuxetan) (Biogen Idec, Cambridge, MA) another non-Hodgkins lymphoma therapeutic antibody. Monoclonal antibodies are now a mainstay in oncological therapy.

There are four mAbs, which have been used to treat RCC. Table 3 summarizes selected studies in which monoclonal antibodies have been incorporated as a therapy against RCC. Each of these targets the hypoxia-induced products synthesized during hypoxia or with VHL inactivation. These include Erbitux® (Cetuximab) (ImClone, NY, NY) and Panitumumab (ABX-EGF, Abgenics, Freemont, CA) which target the EGFR, Avastin® (bevacizumab) (Genentech), which is directed against VEGF and Rencarex® (G250) (Wilex AG, Munich, Germany) whose antigen is CA-IX. Currently, only G250 is being evaluated as a possible adjuvant to surgery in patients with advanced disease. However, bevacizumab holds great promise and may be worthy of testing in an adjuvant setting in the future.

Table 3 Selected monoclonal antibody studies in metastatic patients with agents under current or potential study for adjuvant therapy in RCC

Author	Target	Agent	Phase (no. of patients)	Best response	Reference
G-250 (Rencarex®)	CA-IX				
Steffens		I ¹³¹ G-250	I (12 RCC)	1 S, 1 PR	(78)
Bleumer		Ch G-250	II (35 RCC)	10 S, 1CR	(59)
Bleumer		Ch G250 + IL-2	II (35 RCC)	5 S, 3 PR	(79)
Bevacizumab (Avastin®)	EGF				
Gordon		Bevacizumab	I (25, 7 with RCC)	N/A	(80)
Yang		Bevacizumab	II (116 RCC)	64% PFS at 4 months	(81)
Elaraj		Bevacizumab + Thalidomide	I/II (22 RCC)	TTP 3.0 months	(82)
Spigel		Bevacizumab + Erlotinib	II (59 RCC)	Median OS 23 months, median TTP 11 months	(83)
Hainsworth		Bevacizumab + Erlotinib + Imatinib	I/II (63 RCC)	25% OR, 61% S, median PFS 11 months	(84)

Ch human/murine chimeric antibody, S stable, PR partial response, CR complete response, OR overall response, PFS progression free survival, OS overall survival, TTP time to progression, EGF epidermal growth factor, CA IX-carbonic anhydrase IX

G250 is a mAb raised against the tumor-associated antigen CA-IX. It has been associated with 95% of clear cell RCCs and is not seen at all in normal renal tissue (85). The humanized version has been used alone in an attempt to induce ADCC in the tumor cells recognized by the antibody. Bleumer et al. performed a prospective multicenter single-arm phase II study looking at 50 mg of the G250 antibody injected intravenously, weekly for 12 weeks in patients with metastatic RCC (59). It was well tolerated with only 3 of 36 patients suffering grade 2 toxicity. One patient achieved a complete response, one patient a partial response, and eight patients had stable disease after 24 weeks of follow-up. The median survival was 15 months from the initiation of therapy. Further in vitro work suggested that cytokines may potentiate G250-mediated ADCC-prompted attempts at combining it with IL2 or IFN (86).

Another means to initiate an antitumor effect is to use the tumor-specific antibody to deliver a cytotoxic therapy. This has been attempted with radiolabeled G250. A phase I study looking at ^{131}I -labeled G250 was performed on 15 patients with metastatic RCC which showed good tolerability overall with the dose-limiting toxicity being hematopoietic (87). A phase II trial was then initiated on 29 patients with progressive metastatic RCC. The maximum tolerated dose was 1,665 MBq/m² because of dose-limiting hematological toxicity. Disease stabilization was noted in five patients; however, eight patients only received one out of the intended two high-dose courses due to hematological toxicity, the formation of antichimeric antibodies, or disease progression (88).

These results with G250 were encouraging enough to prompt the first adjuvant trial with a mAb. A large multicenter phase III adjuvant therapy trial randomizing 612 patients to nonradiolabeled G250 versus placebo was started by Willex called ARISER (Adjuvant Rencarex Immunotherapy trial to Study Efficacy in nonmetastasized RCC). Accrual for this trial has been poor, however. The enrollment criteria are currently under revision to help improve patient accrual.

Bevacizumab has demonstrated significant activity in metastatic colorectal cancer increasing survival from 15 to over 20 months when added to patients undergoing combination chemotherapy (89). This prompted study in patients with metastatic RCC who failed prior immunotherapy (81). In this trial, 116 patients were randomized to placebo, 3 mg/kg or 10 mg/kg of bevacizumab. The most significant toxicities included hypertension and asymptomatic proteinuria. The 10 mg/kg dose had a significant benefit with respect to progression free survival (hazard ratio 2.55, $p < 0.001$), an effect which was borderline with the 3 mg/kg dose (hazard ratio 1.26, $p = 0.053$). A follow-up to this study has shown that four patients undergoing long-term bevacizumab treatment have been without progression after 3–5 years of therapy (90). This has prompted some investigators to look at combination therapy with the small-molecule tyrosine kinase inhibitor of epidermal growth factor signaling erlotinib (Tarceva®, OSI Pharmaceuticals, Genentech and Roche). A phase II study in 59 patients with metastatic RCC at the 10 mg/kg dose every 2 weeks for bevacizumab and the 150 mg daily dose of erlotinib demonstrated a very impressive median overall survival of 23 months and median time to progression of 11 months (83). This has prompted further study with bevacizumab with thalidomide,

erlotinib, as well as the c-Kit inhibitor imatinib (Gleevec®, Novartis Oncology, East Hanover, NJ) and IFN. Although these studies are in patients with known metastases, it is felt that it will be tried in the adjuvant setting in the near future.

4.3 Chemotherapy

To date, RCC has proven to be a chemotherapy-resistant tumor. In a review looking at 72 different chemotherapeutic agents, the cumulative objective response rates were a disappointing 2–6% (91). A small Japanese trial investigated a combination of vinblastin/doxorubicin, oral tegafur, and uracil for 2–3 years of adjuvant therapy postnephrectomy for advanced localized disease (92). The 1-, 3-, and 5-year survival rates were 100%, 96%, and 96%, respectively, and were all significantly higher ($p < 0.01$) than the 81%, 72%, and 60% of the historical controls used in this trial. Critics, however, have argued that the control group that was treated from 1974 to 1983 is not comparable to the test group treated between 1984 and 1988. A more recent prospective randomized trial of adjuvant UFT with a 9.4-year median follow-up did not show any benefit in the 5-year recurrence rate in treated subjects versus controls (93). New agents are emerging and are being tested in patients with metastatic RCC; however, these are not yet ready for the consideration of adjuvant therapy.

4.4 Antiangiogenic Therapy

Thalidomide was first shown to have antiangiogenic properties by D'Amato et al. in 1994 (94). Its antiangiogenic properties are believed to be mediated through the inhibition of COX-2 translation as well as inhibition of VEGF-mediated activation of endothelial cells. Eisen was the first to try it in patients with RCC at the 100 mg/day dose (95). Side effects included sedation, constipation, rash, and peripheral neuropathy. Subsequent phase II trials at MDACC investigated elevated doses of 100–1,200 mg/day in patients with metastatic disease. Objective response rates were a disappointing 10%; however, progression free survival at 6 months was ~30% in these trials (96–99).

These results have prompted further study of the agent alone or in combination with chemotherapeutics (5-fluorouracil and gemcitabine) or cytokines (IL2 or IFN- α). In these trials, conflicting results and significant toxicities were seen (100). Because of the lack of benefit of IFN + thalidomide over IFN alone in the metastatic setting, it has been largely abandoned.

Given the plausible biological mechanism and the good progression free survival seen in some of the trials, thalidomide is currently being investigated as a potential adjuvant agent in patients with high-risk (Grade 3–4 stage II, stage III, M0 stage IV, or bilateral tumors) RCC at MDACC. Patients are randomized to 300 mg/day thalidomide for 2 years versus observation. This trial has an accrual goal of 220

patients (110 in each arm) and to date has enrolled 46 patients. Toxicity in the treatment arm as well as competing trials have affected accrual despite this, preliminary analysis on this small number of patients is suggestive of a benefit in disease specific and disease free survival (101).

4.5 Small Molecule Kinase Inhibitors

There has been an explosive increase in kinase inhibitors for the treatment of cancer since the original proof of principle with the bcr/abl, c-Kit inhibitor imatinib (102).

A host of these inhibitors are in clinical trials for the treatment of metastatic RCC, three of which deserve mention in a discussion of adjuvant therapy (Table 4). Two are currently being developed for use in adjuvant therapy, sorafenib (Bay 43-9006, Nexavar®, Bayer Pharmaceuticals, West Haven, CT and Onyx Pharmaceuticals, Richmond, CA), and sunitinib (SU-11248, Sutent®, Pfizer, Inc., La Jolla, CA). Another temsirolimus (CCI-779, Wyeth, Madison, NJ) holds promise and may undergo testing in an adjuvant setting in the future.

Sorafenib is a novel biaryl urea small-molecule Raf kinase inhibitor. Raf is serine/threonine kinase, which is a principal downstream mediator of the Ras GTPase in the mitogen-activated protein kinase (MAPK) pathway, which transmits receptor

Table 4 Selected small-molecule kinase inhibitor studies with agents under current or potential study for adjuvant therapy in RCC

Author	Target(s)	Phase (no. of patients)	Best response	Reference
Sorafenib (Bay-43-9006)	Raf-kinase, VEGFR			
Awada		I (44, 7 RCC)	N/A	(103)
Ahmad		II (41 RCC)	30% S, 40% OR	(104)
Sunitinib (SU-11248)	VEGFR, PDGFR, c-Kit, Flt-3			
Faivre		I (28, 4 RCC)	N/A	(105)
Motzer		II (63 RCC)	27% S, 40% PR, median TTP 8.7 months	(106)
Temsirolimus (CCI-779)	mTOR			
Raymond		I (24, 6 RCC)	1 PR	(107)
Atkins		II (111 RCC)	7% OR (1 CR, 7 PR), 26% MR, median TTP 5.8 months, median survival 15 months	(108)

S stable, *PR* partial response, *CR* complete response, *OR* overall response, *MR* minor response, *TTP* time to progression, *VEGFR* vascular endothelia growth factor receptor, *PDGFR* platelet-derived growth factor receptor, *mTOR* mammalian target of rapamycin

tyrosine kinase activation signals to the nucleus. Mutations resulting in constitutive activation of any component of this system are often associated with malignancy including RCC (109). Strategies used to block activated Raf kinase activity include the use of antisense oligonucleotides (ASON) as well as small-molecule inhibitors. Anti-Raf ASON therapy has completed phase II studies in metastatic, nonrenal, malignancies with the best responses being disease stabilization.

Determination of the crystalline structure of Raf has allowed for the rational design of several potential small-molecule inhibitors. Sorafenib is the furthest along with respect to development. Sorafenib is the first molecule of its class to be used in the clinic. Originally selected for its antikinase activity on Raf, it has subsequently been shown to inhibit other kinases including, VEGFR, PDGFR, Flt-3, c-Kit, and FGFR-1. It has good antitumor activity in *in vitro* and *in vivo* models and has recently undergone FDA approval.

Several phase I studies have been performed looking at toxicity and the pharmacodynamics of this molecule in doses ranging from 50 to 800 mg bid. In general, the drug was well tolerated with dose-limiting toxicities predominantly being fatigue, the maximum tolerated dose was in the 400–600 mg bid range (103, 110, 111). Most common side effects included fatigue, rash, and gastrointestinal (anorexia, nausea, and diarrhea). Disease stabilization was achieved in 25–50% of patients with the rare partial response.

These responses prompted a randomized phase II multicenter trial looking at the 400 mg po bid dose of sorafenib in a variety of different advanced tumors (104). Preliminary results with RCC patients suggested that at 12 weeks, 30% of tumors stabilized, 40% responded (>25% reduction in tumor size), and 30% progressed. Some long-term responses were seen lasting up to 1 year. Several cystic lesions were seen to grow while at the same time losing attenuation at their core a finding, which by some is felt to be an indication of response. A phase III trial is being conducted looking at 884 patients with progressive disease despite immunotherapy. Results should be available soon.

Sorafenib is being investigated in the adjuvant setting in several trials. The British Medical Research Council is conducting a large phase III randomized double-blind controlled study comparing the 400 mg bid dose of sorafenib with placebo in patients with advanced, nonmetastatic disease (the SORCE trial). This multicenter trial is expected to accrue over 1,650 patients randomized to one of three arms; placebo for 3 years; sorafenib for 1 year, followed by placebo for 2 years; and sorafenib for 3 years. Another adjuvant trial comparing sorafenib to sunitinib will be discussed later.

Sunitinib is a small molecule tyrosine kinase inhibitor with demonstrated antiangiogenic and antitumor activity. It is known to inhibit several tyrosine kinases including the VEGF and PDGF receptors as well as Kit and Flt-3. It has completed phase II/III testing in several tumors including gastrointestinal stromal tumors, breast cancer, RCC, and is now being looked at as a possible therapy for acute myeloid leukemia (112). Dose-limiting toxicities have been encountered at the 75 mg po daily dose or greater include grade 3 fatigue and hypertension as well as grade 2 bullous skin toxicity (105).

Motzer et al. have recently published a phase II study looking at sunitinib in 63 patients with cytokine refractory metastatic RCC (106). They investigated the effect of repeated 6 week cycles of 4 weeks of sunitinib at the 50 mg per day dose followed by a 2-week rest period. A partial response was achieved in 40% of patients, stable disease lasting greater than 3 months was achieved in a further 27% of patients, and the median time to progression was 8.3 months.

Given these encouraging results, further testing is being conducted in the adjuvant setting. ECOG is performing a randomized double blind adjuvant trial involving over 1,330 patients (ASSURE, ECOG 2805). This is a 3-arm study comparing nine cycles of sorafenib at 400 mg po bid to 50 mg of sunitinib for 4 weeks followed by a 2 week rest and placebo.

In January 2006, sunitinib gained FDA approval for the treatment of advanced RCC.

Temsirolimus is an inhibitor of mammalian target of rapamycin (mTOR) kinase activity, which ultimately leads to G1 cell cycle arrest. mTOR has been associated with a familial form of RCC known as tuberous sclerosis. In addition, it is known to increase HIF-1 α expression and protein stabilization. For all of these reasons, mTOR felt to be a potential target for RCC treatment. Temsirolimus has completed phase II testing in other cancers including glioblastoma multiforme, breast cancer, and mantle cell lymphoma (113–115). In a recent phase II trial of patients with metastatic RCC, 111 patients were randomized to weekly intravenous infusions of the 25, 75, or 250 mg dose of temsirolimus (108). An objective response rate of 7% and minor response rate of 26% was seen. The median time to progression was 5.8 months and median survival was 15 months. Side effects included rash (76%), mucositis (70%), asthenia (50%), and nausea (43%). Interestingly, neither efficacy nor toxicity was affected by dose level. Although encouraging results were seen in this trial, no randomization was seen against placebo. If these results are validated in future trials, this agent may be amenable to the treatment of RCC patients in an adjuvant setting.

Because of the dramatic increase in novel therapies for this relatively uncommon malignancy accrual to the various trials has become a real concern. In addition to single-agent trials, combination trials of these agents with established therapies or other small-molecule kinase inhibitors are being performed on patients in the metastatic disease setting. The future of adjuvant therapy for RCC will likely include this exciting new class of antineoplastic agents.

5 Conclusion

Despite enormous effort and hundreds of clinical trials, only a handful of targeted therapies are left in the final phase of testing for the adjuvant treatment of RCC. Excitement still exists with many of the tyrosine inhibitors and work is ongoing with mAb therapy.

Patients will soon see the benefits of adjuvant therapy for advanced RCC. The challenge for the clinician is and will continue to be determining which patients

require adjuvant therapy and which adjuvant therapy gives the best therapeutic response with acceptable tolerability for that patient. In light of the major strides in our understanding of the biology of this disease over the last 10 years, these difficult clinical decisions may be easier to make in the next 10 years.

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