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Renal Cell Carcinoma

Molecular Targets and Clinical Applications

Third Edition

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1st Edition Preface

Renal cell carcinoma represents a heterogeneous group of tumors, the most common of which is clear cell adenocarcinoma. The annual incidence of this tumor appears to be rising and approximately 12,000 individuals die from this cancer annually in the United States.

One third of patients who present have metastatic disease at the time of diagnosis, and another 40 % who undergo nephrectomy will ultimately develop this complication. Over the past 10 years, a significant amount of new information concerning the epidemiology, molecular and immunologic characteristics, and therapy for patients with these tumors has appeared.

The recognition that inherited forms of renal cancer exist, and that chromosomal abnormalities can be identified in these tumors, suggested a genetic basis for renal cell carcinoma. The familial cancer syndrome, Von Hippel Lindau disease, provided the setting in which the genetic abnormalites associated with the development of renal cancer were first described. Abnormalities of the *VHL* gene have also been detected in sporadic clear cell carcinoma, and it has now been recognized that approximately 80 % of these tumors will demonstrate characteristic alterations. Currently the functions of the VHL protein are being investigated, and the biology of clear cell carcinoma of the kidney is under study. Additionally, papillary carcinomas of the kidney appear to express different molecular defects, and these are now being unraveled.

Interest in the immunologic characteristics of renal cancer was based on some of the early observations suggesting spontaneous regression of this tumor and responses to immunologicbased therapy. Recently, it has been recognized that tumor-associated antigens may be present in selected renal cell carcinomas and that recognition of these antigenic structures by the immune system may occur. Additionally, abnormal immune regulation or immune dysfunction has also been described, with the molecular basis of these findings now being studied. The interaction between these two areas may have relevance for the effects of immune-based therapy. The treatment of renal cell carcinoma has also evolved, with improvements in surgical therapy for locally advanced tumors, the introduction of partial nephrectomy, and the recent description of laproscopic techniques for tumor removal. The understanding of the role of these modalities and their use in this patient population is now emerging.

For the majority of patients who have metastatic or advanced renal cell carcinoma that is not surgically curable, therapy remains of limited value. Continued investigation of cytokinebased therapy, adoptive immune strategies, and such newer strategies as the inhibition of angiogenesis is being conducted. Management of these patients often involves surgical removal of metastases and/or residual disease following therapy. Finally, the role of symptom palliation for this patient group is an important issue for individuals with this illness.

Renal Cell Carcinoma: Molecular Biology, Immunology, and Clinical Management was designed to assist physicians and researchers who treat and/or investigate patients with kidney cancer. This volume should assist urologists, medical oncologists, and radiation oncologists in their diagnosis and treatment of renal cell carcinoma. The review is designed to assess the pertinent clinical, biologic, and pathologic characteristics of this illness. New developments in the areas of molecular genetics and immune dysfunction have also been included, focusing on therapy for patients with renal malignancies. The roles of partial nephrectomy, radical nephrectomy, and laparoscopy are covered. Treatment of patients with metastatic disease remains a problematic area, and the modalities that have been used or are being developed are discussed.

The last decade has been a time of innovation in the management of renal cell carcinoma, and we believe that *Renal Cell Carcinoma: Molecular Biology, Immunology, and Clinical Management* will provide an overview of the field, as well as demonstrate the progress that has occurred in this area.

Ronald M. Bukowski
Andrew C. Novick

2nd Edition Preface

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 clearcell renal carcinomas [5].

The VHL protein is the product of the VHL gene, functions as a tumor-suppressor gene, and is responsible for ubiquination of hypoxia-inducible factor-α (HIF-α) and its subsequent degradation by the proteosome [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 erythryopoietin (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 or this malignancy, replacing immunotherapy with cytokines.

Systemic Therapy: Metastatic Disease

Immunotherapy consisting of interleukin-2 (IL-2) and/or interferon alpha (IFNα) 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 IFNα (9.9%) and again, no improvement in time to progression (TTP) or survival [7] were seen.

IFNα 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 medial 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 IFNα 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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Chapter 1
Targeted Therapy for Metastatic Renal Cell Carcinoma: Introduction

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

Background

Renal cancer accounts for 3 % of all malignant tumors and is the sixth leading cause of death in the United States. In 2014, an estimated 63,920 new cases with 13,860 deaths secondary to RCC are predicted [1]. At diagnosis, patient ages range from 40 to 70 years, with a male to female predominance persists (1.6–1.0) [1]. Renal cell carcinoma generally arises from the renal epithelium and accounts for approximately 85 % of all renal malignancies [2]. Fifteen to 20 % of patients present with locally advanced or metastatic disease [3], and approximately 20–40 % of those who undergo surgical resection of the primary tumor will develop metastatic disease [4].

Histologically, renal cell cancers represent a group of subtypes with unique morphologic and genetic characteristics. Clear-cell renal carcinoma (ccRCC) is the most common type and accounts for approximately 80–90 % of renal epithelial malignancies [5]. It arises from the proximal convoluted tubule and histologically is characterized by clear cytoplasm with occasional areas of eosinophilia. The majority of sporadic clear-cell renal tumors are associated with defects in the VHL gene [6]. In the past 5 years, efforts to characterize other genetic abnormalities

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in ccRCC have been an important and crucial focus of research in this area. The recognition of the various histologic subtypes and, in some instances, associated genetic alterations now provides the opportunity to develop specific and potentially personalized approaches to therapy.

Molecular Genetics: RCC

Renal cell carcinoma represents a collection of distinct diseases that can be distinguished histologically and genetically. Recent work has focused primarily on clear-cell RCC, the most common subtype. This is a vascular neoplasm, with recent studies revealing the molecular basis of this phenotype. In a recently published dataset from 417 patients, 19 genes were identified as significantly mutated ($q < 0.05$) in clear-cell tumors [7]. Mutations in eight genes emerged as significant, including *VHL*, *PBRM1*, *SETD2*, *KDMRC* (lysine (K)-specific demethylase 5C), *PTEN*, *BAP1*, *mTOR*, and *TP53*. The VHL gene and its product function as a tumor suppressor. Functions of the VHL protein include ubiquitination and proteasome degradation [6] of hypoxia-inducible factor (HIF). HIF-α is a key regulator of the hypoxic response and is the primary target of this protein. Under hypoxic conditions or the presence of 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 vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), transforming growth factor-α (TGF-α), and erythropoietin (EPO). Morphologically, renal cancers are very vascular tumors and have a rich tumor-associated vasculature. The VHL gene controls this process and suppresses angiogenesis; however, loss of VHL gene function in clear-cell tumors results in the increased production of VEGF, PDGF, and TGF-α producing the vascular phenotype characteristic of these tumors.

Recent studies using newer sequencing technologies have reported mutations in *VHL* wild-type tumors in *TCEB1*, which encodes elongin C, a protein that binds to VHL and is required for its function [8]. As noted previously, mutations in additional tumor suppressor genes in the region of the *VHL* on chromosome 3p have also been identified, including *SETD2*, *BAP1*, and *PBRM1*. Mutations of *BAP1* and *PBRM1* appear mutually exclusive and may be associated with different patient outcomes [9].

Finally, mTOR pathway mutations are uncommon, with reported mutation frequency of *mTOR*, *TSC1*, *PIK3CA*, and *PTEN* of less than 10 % in RCC patients [7, 10]. Expression of activated mTOR however has been reported in 60 % of tumors [11]. These initial observations will hopefully provide the rationale for the future to development of molecularly oriented approaches to therapy of advanced RCC.

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 prognostic groups. For previously untreated patients, an initial prognostic model, which included five clinical characteristics, was developed at Memorial Sloan Kettering Cancer Center [12] and later validated and expanded by Cleveland Clinic investigators [13]. These criteria were then utilized in the pivotal phase III clinical trials of the oral tyrosine kinase inhibitors (TKIs), bevacizumab, and temsirolimus. This scheme was updated and modified by Heng and colleagues [14] utilizing patients receiving targeted therapy. These prognostic schemes have clinical utility, but in the future incorporation of tissue-based markers and genomic characteristics of tumors will be required.

Management of Patients with Advanced RCC

The management of patients with metastatic RCC has undergone dramatic changes, and a new treatment paradigm is in place. Blockade of the VEGF pathway and the functions of HIF are now utilized as primary therapeutic strategies. In the past, cytokine administration utilizing either interleukin IL-2 and/or IFNα was the standard approach. Clinical trials [15, 16] demonstrated responses were best with high-dose, intravenous IL-2 (21 %) compared to low-dose intravenous (11 %) or subcutaneous IL-2 (10 %). No progression-free survival (PFS) or overall survival (OS) advantages were observed however. A comparison of overall response rates (ORR) with high-dose IL-2 (23.2 %) to subcutaneous IL-2 plus IFNα (9.9 %) suggested some differences; however, no significant improvement in PFS or OS was found [17]. In contrast, administration of IFNα produced a survival advantage compared to methoxyprogesterone in a prospective randomized trial [18]. Additionally, a median OS of 13.1 months and median PFS of 4.7 months for IFNα-treated patients were reported in a retrospective review [19]. Ultimately, IFNα monotherapy became the standard of care for patients with advanced RCC, in view of these results and the toxicity associated with high-dose IL-2.

Bevacizumab

Bevacizumab (Avastin®) is a fully humanized monoclonal antibody that binds all the isoforms of VEGF. The antitumor activity of the bevacizumab and IFNα combination in patients with advanced clear-cell carcinoma was demonstrated in a sequence of phase III randomized trials [19, 20]. In these studies, the combination

of bevacizumab and IFNα proved superior to monotherapy with IFNα, with increased overall response rates and progression-free survival; however, no survival advantage was detected in either trial. These reports demonstrated that an agent inhibiting VEGF could change the natural history and biologic behavior of RCC when administered with IFNα.

Sorafenib

Sorafenib (Nexavar®) is an orally bioavailable inhibitor of Raf-1, a member of the RAF/MEK/ERK signaling pathway, as well as of multiple growth factor receptors including VEGFR-1, VEGFR-2, VEGFR-3, PDGFR, Flt-3, and c-KIT [21]. In preclinical models, this multitargeted TKI blocked the RAF/MEK/ERK signaling pathway and inhibited tumor angiogenesis. The pivotal study with this agent was a randomized phase III placebo-controlled study [22] in cytokine-refractory patients. Results demonstrated prolongation of PFS in sorafenib-treated patients. Subsequently, a phase II randomized trial in treatment-naïve patients with advanced clear-cell carcinoma [23] compared sorafenib to IFNα. The final analysis demonstrated no differences in PFS for the sorafenib patients and IFNα-treated individuals (5.7 vs. 5.6 months, respectively, HR 0.88, $p=0.504$). Subsequently, sorafenib has been utilized as a comparator treatment in including comparisons to axitinib, temsirolimus, and tivozanib [24–26]. The results from these studies suggest the overall response rates and PFS in earlier trials may have been underestimated.

Sunitinib

Sunitinib (Sutent®) is a multitargeted oral TKI of VEGFR-2, PDGFR with less potent activity against fibroblast growth factor receptor-1 tyrosine. In preclinical studies, direct antitumor activity was reported in cells dependent on signaling through PDGFR, KIT, and FLT3, as well as presence of significant anti-angiogenic effects [27]. A sequence of phase II clinical trials demonstrated the activity of this agent in patients with cytokine-refractory metastatic RCC [28]. These trials accrued 168 patients, and the ORR was 40 % (investigator assessment) and 25.5 % (independent review). The majority of responses were partial. 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), respectively, in the two trials, and the median OS 16.4 months in trial 1. Based on these results, and the ability of this agent to induce objective and meaningful responses, the FDA granted accelerated approval to sunitinib for the treatment of advanced RCC in January 2006.

A large randomized trial in 750 untreated patients with metastatic clear-cell carcinoma was then conducted, which compared sunitinib to IFNα [29].

Patients receiving sunitinib had superior outcomes including improved median PFS (11.0 vs. 5.0 months) and median OS (26.0 vs. 21.0 months) and a response rate over 40 % (investigator assessment). These results established sunitinib as a reference standard for first-line treatment of advanced RCC patients with clear-cell carcinoma.

Pazopanib

The third oral TKI agent investigated in advanced RCC patients was pazopanib (Votrient®), an oral multitargeted TKI, with potent inhibitory activity against the VEGF and PDGF receptors [30]. A pivotal double-blind, phase III study in 435 patients with advanced RCC [31] was conducted. Patients were randomized in 2:1 fashion to pazopanib or a placebo. Pazopanib was found to significantly improve PFS (9.2 vs. 4.2 months), in both untreated and cytokine-refractory patients regardless of performance status, prognostic score, or age. This agent was approved for the treatment of advanced RCC patients in October 2009. The toxicity profile of pazopanib was acceptable, and the data have supported its use as first-line therapy as an alternative to sunitinib.

Axitinib

The most recent TKI studied in RCC patients is axitinib (Inlyta®). It is a potent oral indazole derivative that inhibits all the VEGFR, PDGFR-β, and c-kit [32]. It was evaluated in the AXIS trial [24], a two-arm, randomized, open-label, multicenter phase III study comparing the PFS of 723 patients with advanced clear-cell RCC receiving either axitinib or sorafenib following failure of prior systemic first-line therapy (sunitinib, bevacizumab ± IFNα, temsirolimus, or cytokines). The secondary end points included OS, ORR, safety, and tolerability of axitinib. The results demonstrated a superior PFS for the axitinib-treated patients (6.7 vs. 4.7 months), improved ORR (19.4 % vs. 9.4 %), and similar survival. The side profile of axitinib was moderate and acceptable. The results supported the FDA approval of axitinib as second-line therapy in January 2012 for patients with refractory RCC.

mTOR Inhibitors: Temsirolimus

A third group of medications investigated in advanced RCC patients are the mTOR kinase inhibitors. mTOR is an intracellular kinase that has a central role in controlling cellular functions, including cell division and metabolism [33]. 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 [34]. mTOR has also been identified as an upstream activator of HIF, stabilizing the molecule, preventing its degradation, and thereby increasing HIF activity [35]. Rapamycin was the first mTOR inhibitor developed. It was derived from *Streptomyces hygroscopicus* and was initially developed as an antifungal agent [36]. Subsequently, the mTOR inhibitors were developed to delay and/or prevent graft rejection of solid organ transplant patients. Despite early evidence from in vitro and in vivo studies demonstrating rapamycin possessed cytostatic activity against cancer cells, it was not extensively tested in this area until the late 1990s [36]. These studies suggested this group of drugs may have utility in cancer treatment, with direct anticancer effects as well as producing inhibition of angiogenesis. Recent studies suggest mTOR is phosphorylated and activated in over 60 % of RCC metastatic lesions [11], providing additional evidence that this kinase is a logical therapeutic target in RCC.

Based on the results of a phase 2 randomized trial of intravenous temsirolimus (Torisel®) in treatment-refractory patients [37], a phase III trial investigating temsirolimus was designed. This randomized trial was conducted in patients with poor risk features. Six hundred and twenty-six patients with a poor prognosis and any histology received either intravenous temsirolimus, IFNα monotherapy, or the combination as first-line treatment [38]. The definition of poor risk required ≥3 risk factors, including metastases to multiple organs. Patients receiving temsirolimus monotherapy demonstrated improved median OS and PFS compared to IFNα. This was not seen in the group treated with the combination of temsirolimus + IFNα. A subset analysis suggested OS and PFS were increased in temsirolimus-treated patients regardless of histologic subtype, and the effect was the most pronounced in patients with non-clear RCC. This drug was approved by the FDA for treatment of advanced RCC in May 2007. In Europe, temsirolimus was approved for therapy of advanced RCC patients with a poor prognosis.

Everolimus

A second mTOR inhibitor investigated in advanced RCC was oral agent everolimus (Afinitor®). A pivotal clinical trial was conducted in patients who had failed previous TKI therapy including sunitinib and/or sorafenib [39]. At the time this trial was conducted, patients progressing on TKI therapy were been seen frequently by medical oncologists. And a need to define the effects of therapy in this treatment-refractory group was recognized. A randomized, blinded, placebo-controlled phase III trial (RECORD 1) was conducted in 421 patients with progressive disease following sunitinib and/or sorafenib therapy. The results demonstrated everolimus increased the median PFS compared to placebo, 4.9 (95 % CI, 0.25–0.48) vs. 1.9 months (95 % CI, 1.8–1.9) (HR 0.33, $p < 0.0001$). Everolimus was approved by the FDA for treatment of patients failing sunitinib and/or sorafenib in March 2009.

The Evolving Treatment Landscape for RCC

The TKIs studied in clear-cell RCC were first shown to have activity in cytokine-refractory patients as second-line therapies, with follow-up trials demonstrating their efficacy in the frontline setting. Both VEGF and mTOR pathway inhibitors have received FDA approval based on their ability to prolong either median PFS or OS in large randomized trials. VEGF pathway inhibitors which have shown a high level of clinical evidence supporting their use in metastatic RCC include sunitinib [29], sorafenib [22], pazopanib [31], axitinib [24], and bevacizumab [19]. mTOR pathway inhibitors which have a high level of evidence include temsirolimus [38] and everolimus [39]. These drugs have all been approved as single agents with the exception of bevacizumab, which has been approved in combination with interferon. The TKI tivozanib was investigated in a randomized phase 3 trial and compared to sorafenib [26]. Despite the significant PFS improvement seen, tivozanib was not approved by the FDA secondary to concerns regarding the overall survival data. Although these targeted therapies appear to have better toxicity profiles and improve either PFS or OS compared to cytokine therapy, complete responses uncommon, and survival improvements are measured in months. The need to refine and improve the current paradigm and provide information on the comparative efficacy and toxicity of the available agents is clear.

Comparative Clinical Trials

In the treatment-naïve RCC population, therapy with these agents has improved patient outcomes. The efficacy of the various agents in terms of PFS prolongation, response rates, survival appears similar; however, additional clinical studies were needed to provide this evidence. Therefore, differences in patient tolerability and outcomes have been explored in several studies. In treatment-naïve individuals, clinical trials of sunitinib vs. pazopanib [40, 41] and tivozanib vs. sorafenib [26] suggest similar levels of efficacy with variable toxicity and patient acceptance.

The COMPARZ study which represents an important trial comparing sunitinib and pazopanib as first-line treatment for patients with clear-cell, metastatic RCC has recently been completed [40]. One thousand one hundred and ten patients with clear-cell, metastatic RCC were randomized to receive continuous daily pazopanib or intermittent sunitinib. The primary end point was PFS as assessed by independent review. The study was powered to show the non-inferiority of pazopanib vs. sunitinib. Secondary end points included overall survival, safety, and quality of life. Pazopanib was reported as non-inferior to sunitinib in median PFS (8.4 months, 95 % CI, 8.3–10.9 vs. 9.5 months, 95 % CI, 8.3–11.1, respectively). The hazard ratio for progression of disease or death from any cause was 1.05 (95 % CI, 0.90–1.22), which met the predefined non-inferiority margin (upper bound of the 95 % confidence interval, <1.25). ORRs were 31 % for pazopanib and 24 % for sunitinib.

Quality of life analysis favored pazopanib during the first 6 months of treatment, particularly with regard to fatigue, mouth soreness, and hand-foot syndrome. Final OS data are similar (hazard ratio 0.92, 95 % CI 0.79–1.06; p, 0.24) in both patient groups, 28.3 months for the pazopanib cohort (95 % CI, 26.0–35.5), and 29.1 months for patients randomized to sunitinib (95 % CI, 25.4–33.1).

Another second innovative clinical trial comparing these two TKIs in treatment-naïve RCC patients has also been reported [41]. In the PISCES phase II trial, 169 patients were randomized to either sunitinib or pazopanib. The primary end point was patient preference for a specific treatment, which was assessed by questionnaire at the end of the two treatment periods. In 114 patients meeting prespecified intent to treat criteria, significantly more patients preferred pazopanib (70 %) over sunitinib (22 %) ($p<0.001$); 8 % expressed no preference. These two trials comparing pazopanib and sunitinib have addressed the issues of comparability and patient acceptance; however, the design, analysis, and interpretation of the results have been criticized. Nevertheless, they represent an important milestone in the field and provide comparative data on the administration of TKIs to similar patient populations.

In treatment-refractory patients, results from trials comparing axitinib vs. sorafenib [24] and sorafenib vs. temsirolimus [25] have demonstrated differences in efficacy end points such as median PFS and OS, respectively. In the AXIS trial [24] discussed previously, axitinib therapy was associated with prolongation of the median PFS by 2.0 months and an improved ORR. No survival differences were noted. In the INTORSECT trial [25], 512 patients who had progressed on first-line sunitinib treatment were randomly assigned to receive intravenous temsirolimus once weekly or oral sorafenib. The analysis revealed no significant difference between the primary end point, PFS (hazard ratio, 0.87; 95 % CI, 0.71–1.07; $p=0.19$) or ORR. Median PFS in the temsirolimus and sorafenib arms was 4.3 and 3.9 months, respectively. Interestingly, a significant difference in OS in favor of the sorafenib group (hazard ratio, 1.31; 95 % CI, 1.05–1.63; two-sided $p=0.01$) was found. The median OS in the temsirolimus and sorafenib arms was 12.3 and 16.6 months, respectively. The authors speculated that the longer OS observed with sorafenib therapy suggested sequential VEGF inhibition may be important factor in determining patient outcomes.

Combination Approaches

Overall, the recent results suggest improved efficacy compared to the previous decade, but treatment remains palliative for the vast majority of patients. Therefore, refinement of patient selection for therapy and continued attempts to combine agents are relevant investigative approaches. Initial attempts to combine sunitinib with bevacizumab [42] or sorafenib with IFNα [43] in RCC patients demonstrated enhanced toxicity. Preclinical studies suggested the combination of an mTOR inhibitor such as temsirolimus and bevacizumab was associated with increased efficacy,

and the results from a small phase I/II study [44] in previously treated patients demonstrated an acceptable safety profile for combination at full doses, as well as promising activity (7/12 partial responses). Based on these findings, a randomized, open-label, multicenter, phase 3 study (INTORACT) was initiated in patients with untreated clear-cell RCC [45]. 791 patients received either temsirolimus or IFNα with bevacizumab. The primary end point was independently assessed PFS. In patients receiving temsirolimus + bevacizumab vs. IFN + bevacizumab, the median PFS was 9.1 and 9.3 months, respectively (hazard ratio, 1.1; 95 % CI, 0.9–1.3; $p = 0.8$). Likewise, there were no significant differences in overall survival (25.8 vs. 25.5 months) or ORR (27.0 % vs. 27.4 %). The toxicity associated with temsirolimus and bevacizumab combination was more severe. Similar results were reported in a phase 2 randomized trial (TORAVA) in 171 previously untreated patients [46]. In this study the temsirolimus + bevacizumab combination resulted in higher toxicity than anticipated, which limited the duration of treatment. A median PFS of 8.2 months and ORR of 27 % with temsirolimus + bevacizumab were lower than with IFNα + bevacizumab (16.8 months and 43 %, respectively). These data demonstrate the difficulty encountered in developing combinations of targeted agents, and importantly the lack of evidence demonstrating improved efficacy. These approaches remain investigational.

Sequential Therapy RCC

The results reported in the AXIS [24], RECORD 1 [39], and INTORSECT [25] trials address some of the issues encountered in evaluating sequential therapy in RCC. The clinical effects of this approach are of interest, since data have suggested patients receiving previous therapy with various targeted agents may respond to a second VEGFR TKI [47]. These preliminary observations suggested sequential TKI therapy may be possible, and cross-resistance may not develop. The issue of whether second-line therapy in RCC should involve a TKI inhibiting the VEGF pathway or an agent with a different mechanism of action has attracted interest. The INTORSECT trial [25] results are therefore of interest and suggest sequential administration of agents inhibiting VEGFR may produce superior OS. Another important study investigating sequential therapy is the RECORD 3 trial [48]. This study involved comparison of sunitinib and everolimus. Patients with mRCC (clear or non-clear cell) with no prior systemic therapy were randomized to receive either everolimus or sunitinib. At disease progression, patients then crossed over and continued on the alternate drug until subsequent progressive disease developed. The primary objective was to assess PFS non-inferiority of everolimus compared to sunitinib. The median PFS for the everolimus group was 7.9 months (95 % CI, 5.6–8.2) compared to 10.7 months (95 % CI, 8.2–11.5) for the sunitinib-treated cohort. Importantly, a trend in favoring OS for the sunitinib group was noted and awaits confirmation. In the setting of TKI-refractory disease, a study comparing of mTOR inhibitor such as everolimus with a TKI such as axitinib would be appropriate.

Future Approaches

Continuing investigations of the molecular genetics of RCC and the development of novel drugs recognizing new biologically relevant targets are needed. The development of a rationale molecular-oriented approach to therapy utilizing the genetic background of RCC represents the next step in the evolution of the current treatment paradigm. This will involve discovery of relevant biomarkers, understanding at a molecular level the factors producing resistance to VEGF-targeted therapy, and incorporation of genetic and molecular factors in treatment and prognostic factor schemes.

The previous experience with cytokines in the therapy of RCC and recognition that tumors can overwhelm the immune system utilizing strategies such as altering antigen expression and interfering with T cell activation have resulted in a renewed interest in the role of immunotherapy for this neoplasm [49]. The normal immune response requires two signals for T cell activation and proliferation. The first signal consists of antigen presentation by an antigen-presenting cell and interaction with T cells. The second signal required for immune system activation can involve several costimulatory molecules such as CTLA-4 and PD-1 [50]. These inhibitors are dysregulated in various malignancies such as RCC and can therefore impair immune recognition of tumor cells. Targeting the immune system with monoclonal antibodies producing checkpoint inhibition may impact tumor growth and proliferation. This approach has been utilized successfully in patients with metastatic melanoma and is now being explored in advanced RCC. The area of Immunoncology is now focused on therapy that may improve the body's ability to generate an immune response against cancer. The use of PD1/PDL1 blocking monoclonal antibodies represents a novel investigational approach to immune checkpoint inhibition. Currently several PD1/PDL1 inhibitors are being investigated and clinical trials in advanced RCC are underway. The results of a phase 1 trial [51] utilizing nivolumab, a fully human IgG4 monoclonal antibody directed against PD-1, which included 34 patients with RCC, have been reported. Ten of 34 (29 %) patients had major clinical responses.

These data resulted in initiation of a phase III trial comparing nivolumab to everolimus in TKI-refractory patients with clear-cell RCC [52]. Checkpoint inhibition with agents such as nivolumab will provide data on a new and novel target in RCC, and in view of its favorable toxicity profile, combination therapy with other targeted agents may be possible.

The renewed interest in the immunology of RCC has been accompanied by attempts to develop tumor vaccines for patients with advanced RCC. Ongoing phase III trials involve either an RNA-based autologous tumor vaccine [53] or a peptide-based vaccine [54] administered with sunitinib. The control arm in each study is sunitinib alone, with the primary end point being OS. It is unclear whether these approaches which are based on the reported immunoregulatory functions of sunitinib [55] will succeed; however, the availability of the immune checkpoint inhibitor class of agents should provide a biologically rationale approach and stimulate continued interest in vaccine therapy for advanced RCC.

The need to extend ongoing studies in clear-cell RCC to non-clear-cell RCC variants is recognized. Additional information on therapy, prognosis, and classification of this uncommon group of tumors is required. The limited numbers of patients with these neoplasms make this a challenge, perhaps best met in a cooperative setting.

Finally, postoperative adjuvant therapy has not been demonstrated as useful in preventing relapse following nephrectomy for completely resected, localized RCC. Multiple 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 [56], and a second National Cancer Institute-sponsored phase III trial compares sorafenib and sunitinib to a placebo [57]. Data from these trials should be available in the near future.

Summary

Significant progress has been made in understanding the biology and molecular characteristics of RCC, as well as development of a new treatment paradigm for patients with advanced disease. The chapters in this book were designed to present in detail the clinical, biologic, and genetic features of renal cancer, the molecular targets identified in the various histologic subtypes, and the rationale for the use of the targeted agents. The clinical applications of these agents, as well as novel targeted strategies, are reviewed. The advances in this field have been significant both at the basic and clinical levels and clearly demonstrate that renal cancer continues to represent a model for application of targeted therapeutic approaches.

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Chapter 2
Renal Cell Carcinoma: Pathologic and Molecular Assessment of Targets

Ferran Algaba

Evolution of the Classification of Renal Cell Carcinomas

Knowledge cannot be elaborated and transmitted in the form of isolated observations. For this reason, observations are grouped according to similar features, producing a classification. Classifications can differ depending on their objective and on changes in ways of thinking over time. In cancer, the evolution of knowledge reflects the pattern observed in the general evolution of human understanding, and accordingly cancer classifications are no more than a tool that require revision and refinement from time to time based on the gradual increase in knowledge. The microscopic characterization of renal cell carcinoma (RCC) started in the mid-nineteenth century (1) with the controversy aroused by Grawitz's hypothesis—in 1883, Grawitz stated that *alveolar* (clear cell) tumors, previously considered lipomas, originated in the neoplastic transformation of adrenal cortical residues into renal cortical. One year later, he confirmed his theory when he found ectopic adrenal cortex in the renal cortex. This theory was readily opposed by Sudek, who favored a renal tubular origin. The controversy between supporters and detractors of the Grawitz theory went on for decades. The term *hypernephroma* was introduced in 1909 and made reference to the adrenal origin. Support for the supposed adrenal origin started to grow weaker. Oberling et al.'s ultrastructural studies (2) finally brought the argument to a conclusion by demonstrating the tubular origin of RCC, in the proximal nephron.

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Initial Histological Classifications

For a long time, the mechanical model of disease (according to which man is a complex "machine" and disease is a fault in the machinery) and the limited therapeutic modalities (practically only surgery) resulted in a classification with few histological subtypes. The first international classifications unified all the historical histological types under the common denomination of renal adenocarcinoma; this could be a clear cell or a granular cell carcinoma, its architecture could be tubular, papillary, or cystic, and its appearance was rarely sarcomatoid [3]. Nevertheless, quite soon attempts began to be made to distinguish histological subtypes on the basis of their origins from different parts of the nephron, with efforts to correlate them with different clinical evolutions. Thus, Thoenes et al. described the *chromophobe renal cell carcinoma* [4] morphologically different from the clear cell carcinoma and regarded as probably originating in the intercalated cells of the distal nephron [5]. Subsequently many possible histological variants were described, and attempts made to identify their origin from different areas of the nephron by means of immunohistochemistry. Many of these histological subtypes failed to show a correlation with the clinical evolution, however, bringing into doubt the utility of such morphological classifications. In the wake of these failures, chromosomal studies and developing knowledge of familial RCC syndromes helped to chance the scenario.

Chromosomal Findings in Familial Renal Cell Carcinomas: Impact on the Pathology and Therapy of Sporadic Cases

Approximately 2–3 % of RCCs occur within the context of a familial syndrome. These syndromes are characterized by early onset and/or multifocal/bilateral disease. Some are due to mutated or inactivated tumor suppressor genes and others to activated oncogenes. The recognition that each of these syndromes is associated with specific tumor phenotype, chromosomal changes, and gene alterations had a major impact on knowledge of RCC, and during recent years, various renal cancer syndromes have been characterized (Table 2.1).

Von Hippel–Lindau (VHL) Disease: This is the most frequent familial renal cancer syndrome, estimated to occur at rates of 1:36,000 to 1:45,500 population. It is associated with secondary *VHL* gene (3p25–26) changes. Missense mutations are the most common, but nonsense mutations, microdeletions/insertions, splice mutations, and large deletions also occur. The spectrum of clinical manifestations of VHL reflects the type of germline mutation [6].

Table 2.1 Hereditary renal cell carcinomas

Syndrome	Chr.	Gene	Protein	Tumor type	Extrarenal manifestations	
					Dermis	Other organs
von Hippel–Lindau	3p25	*VHL*	pVHL	Multiple, bilateral clear cell RCC, renal cysts	–	Hemangioblastoma of retina/cns, pheochromocytoma, pancreatic/renal cysts, neuroendocrine tumors, epididymal/parametrial cysts, tumors of the inner ear
Hereditary papillary RCC	7p31	*c-MET*	HGF-R	Multiple, bilateral papillary RCC (type 1)	–	–
HLRC	1q42	*FH*	FH	Papillary RCC (non-type 1)	Leiomyoma	Uterine leiomyoma/leiomyosarcoma
Hyperparathyroidism–jaw tumor (HP-JT)	1q25	*HRPT2*		Epithelial-stromal mixed tumors, papillary RCC	–	Tumors of the parathyroidea, fibro-osseous jaw tumors
Birt–Hogg–Dubé	17p11	*FLCN*	Folliculin	Multiple chromophobe RCC, oncocytic adenoma, papillary RCC	Facial fibrofolliculoma	Pulmonal cysts, spontaneous pneumothorax
Tuberous sclerosis	9q34 16p13	*TSC 1* *TSC 2*	Hamartin Tuberin	Multiple, bilateral angiomyolipomas, lymphangioleiomyomatosis, rare clear cell RCC	Angiofibroma, peau chagrin, subungual fibroma	Cardiac rhabdomyoma, adenomatous small intestine polyps, pulmonal/renal cysts, cortical tuber, subependymal giant cell astrocytomas
Constitutional translocation chr. 3	3p13–14	*FHIT*	FHIT	Multiple, bilateral clear cell RCC	–	–
Succinate dehydrogenase germline mutation	1p36	*SDHB*	SHDB	Renal tumors with unique morphology	–	Paraganglioma Papillary thyroid carcinoma

The typical renal manifestations of VHL disease are kidney cysts and clear cell renal cell carcinoma (ccRCC). Histological examination of macroscopically normal renal tissue may reveal several hundred independent tumors and cysts.

Hereditary Papillary Renal Carcinoma (HPRC): Trisomy or tetrasomy 7, trisomy 17, and loss of chromosome Y are the most common chromosomal changes, with a germline-activating mutation in the MET proto-oncogene (7q31–34) which can cause papillary renal cell carcinoma type 1 (type 1 pRCC), with cuboidal cells with scanty basophilic cytoplasm and low-grade nuclei [7].

Hereditary Leiomyomatosis and Renal Cell Cancer Syndrome (HLRCC): Some families have a linkage to 1q42.3–q43 [8]. At the genetic level, a germline loss-of-function mutation in the fumarate hydratase (FH) gene is present, and the typical kidney pathology is a papillary renal cell carcinoma type 2 (type 2 pRCC) with eosinophilic cells and high-grade nuclei. Recently, however, tubular and solid patterns and the presence of large nucleoli with perinucleolar halos have been described [9].

The Birt–Hogg–Dubé Syndrome (BHD): The BHD (FLCN) gene is located on 17q12-q11.2. It is associated with multiple cutaneous lesions (fibrofolliculomas, trichodiscomas, and acrochordons) and with an increased risk of renal cancers of various histological types, especially chromophobe renal cell carcinoma (chRCC), oncocytoma, and hybrid oncocytoma–chromophobe renal cell carcinoma, although ccRCC and pRCC can also be present [10].

Tuberous Sclerosis: The disease is associated with mutations in the *TSC1* (9q34) and *TSC2* (16p13) genes, leading to hyperactivation of the mTOR pathway. Although angiomyolipoma is the most characteristic kidney tumor in this syndrome, ccRCC and chRCC are also described [11].

Other familial syndromes are much more infrequent (Table 2.1).

Identification of the specific chromosomal and genetic alterations of the familial and hereditary syndromes as characteristics of the distinct histological subtypes of RCC has made it possible to confirm that a high percentage of the sporadic forms of these subtypes display the same genetic changes. The described morphological subtypes can be interpreted as an expression of specific genetic changes; accordingly, based on the morphology, distinct genetic pathways can be recognized. In view of the above considerations, additional entities were included in WHO's 2004 classification [12] (Table **2.2**), which combined morphological and genetic characteristics and began to recognize some variations with evidence of different immunophenotypes or molecular changes with clinical implications. Thus, when developing target therapies against different genetic pathways, the histological subtype can help in selection of the drug.

Table 2.2 Renal cell carcinoma classification

WHO histological subtypes
Clear cell renal cell carcinoma
Multilocular 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 carcinomas
Carcinoma associated with neuroblastoma
Mucinous tubular and spindle-cell carcinoma
Renal cell carcinoma unclassified
Other entities
Tubulocystic carcinoma
Acquired cystic disease-associated carcinoma
Clear cell tubule-papillary carcinoma
Thyroid-like follicular carcinoma
Leiomyomatous renal cell carcinoma
Succinate dehydrogenase (SDHB) germline mutation-associated carcinoma
Anaplastic lymphoma kinase (ALK) translocation-associated carcinoma
Biphasic alveolosquamoid renal carcinoma cell carcinoma

Molecular Pathways in Renal Cell Carcinomas

Study of familial RCCs has identified the involvement of diverse molecular pathways, the main ones being those that mimic a hypoxic status [13], activating angiogenesis, and the mTOR pathway [14].

Pseudo-hypoxic Pathways in Renal Cell Carcinoma

VHL Pathway

The *VHL* gene (3p25.3) encodes the pVHL protein, which regulates HIF-α, a transcription factor involved in the response to oxygen changes. In the hypoxic situation, HIF is not degraded and activates several genes, including platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF) that stimulate angiogenesis and inhibit tumor cell apoptosis [15]. In addition, it upregulates other growth factors (TGFα, EGFR, IGF) that stimulate autocrine cell growth or activate energy supply factors such as glucose transporter protein-1 (GLUT1) and erythropoietin.

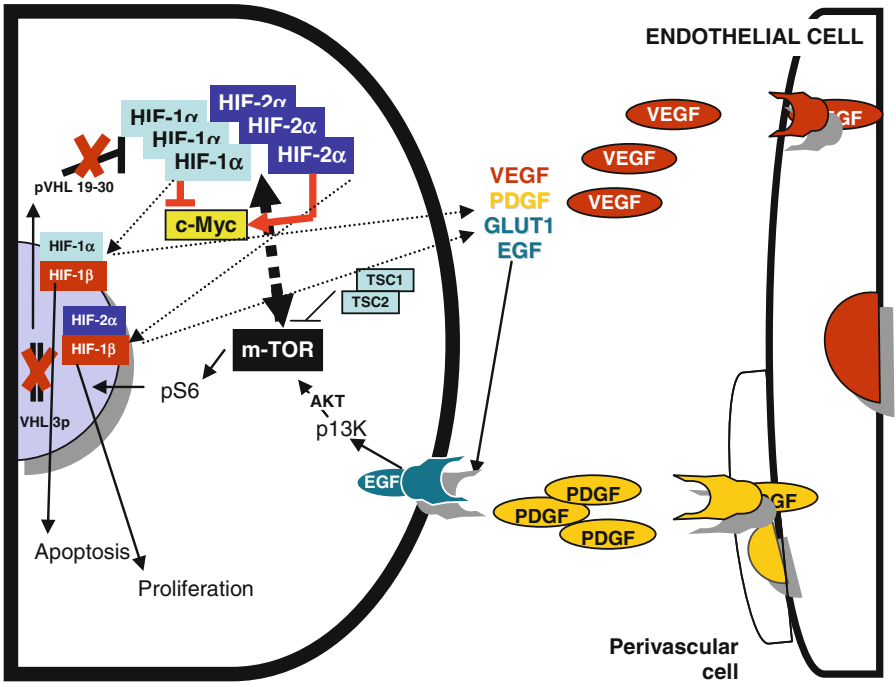

Fig. 2.1 Pseudo-hypoxic pathway. VHL loss with HIF-1α and HIF-2α accumulation and angiogenesis and proliferation increase

The mutation in 3p, present in 70–90 % of sporadic ccRCCs and less commonly in other subtypes, with inactivation of the *VHL* gene results in failure of the pVHL-E3 ubiquitin ligase complex that mediates HIF degradation. This leads to accumulation of HIF-α and binding to HIF-1β, mimicking a hypoxia situation, and transcriptional activation of genes such as VEGF [16].

There are multiple forms of HIF-α, HIF-1α, and HIF-2α being those most commonly involved in RCC. Apoptosis is mediated by HIF-1α, and proliferation is mediated preferentially by HIF-2α, which displays elevated c-Myc activity, resulting in enhanced proliferation and resistance to replication stress [14, 17] (Fig. 2.1). Likewise, HIF-2α can inhibit p53 through a growth factor receptor AKT-MDM2 pathway, contributing to the survival of RCCs during standard treatments such as ionizing radiation or chemotherapy [18].

Krebs Cycle and Pseudo-hypoxic Pathway

Fumarate Hydratase Pathway. The *fumarate hydratase* gene (FH) (1q42.3–q43) encodes the FH protein involved in the conversion of fumarate to malate in the Krebs cycle.

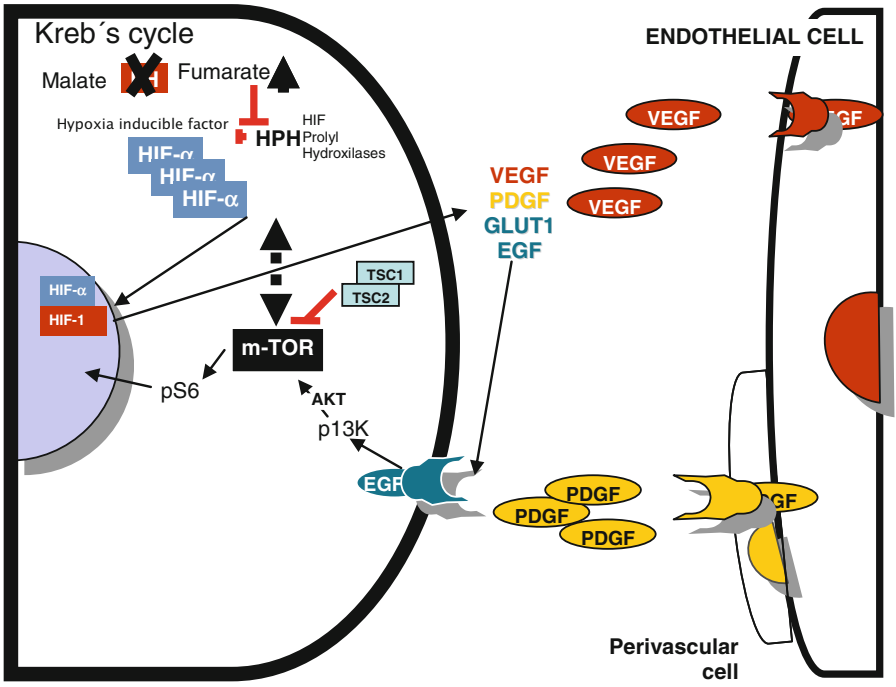

Fig. 2.2 Pseudo-hypoxic pathway. The fumarate hydratase or succinate dehydrogenase mutation can produce a pseudo-hypoxic status similar to VHL gene loss

The mutation in 1q present in the HLRCC syndrome, featuring a pRCC similar to type 2 but with characteristic large nuclei with very prominent eosinophilic nucleoli like a "viral inclusion" [9], results in the accumulation of fumarate. The latter acts as a competitive inhibitor of the activity of HIF prolyl hydroxylases (HPH), which may result in HIF accumulation and pseudo-hypoxic status with all the deregulations characteristic of this situation [19] (Fig. 2.2).

Succinate Dehydrogenase Pathway. Succinate dehydrogenase consists of four different subunits. Their genes (*SDHAF2*-*SD5*-11q13.1, *SDHB*-1q23–25, *SHDC*-1q21–23, *SDHD*-11q23) are encoded in the nuclear DNA, and their proteins are assembled at the inner mitochondrial membrane to form mitochondrial complex 2 and catalyze the conversion of succinate to fumarate [20].

The autosomal germline mutations are the cause of the familial pheochromocytoma/paraganglioma syndromes (PGL1–4), some of which present with gastrointestinal stromal tumors, and the genes *SDHD* and especially *SDHB* are associated with renal neoplasms of different histological features with eosinophilic cells [21].

The mechanism postulated to be responsible for these syndromes involves aberrant apoptosis, oxidative stress, and a pseudo-hypoxic pathway, similar to that observed with increased levels of fumarate [20].

mTOR Pathway

Any pathway with HIF-α accumulation can upregulate the mammalian target of rapamycin or mTOR pathway.

mTOR is an intracellular serine/threonine protein kinase of 289 kDa belonging to the phosphatidylinositol kinase-related kinases coded in 1p36.2. It is involved in the monitoring of cellular nutrition, with effects on protein translation, angiogenesis, cell growth, and apoptosis [22]. mTOR exists in two multiprotein complexes: mTORC1 and mTORC2.

mTORC1 includes the regulatory associated protein of mTOR (RAPTOR). It can be activated by growth factors in the cellular membrane through Ras and PI3K and plays a role in the regulation of cell growth, proliferation, survival, and motility via the phosphorylation of S6K1 and 4E-BP1, which promote mRNA translations and ribosome biogenesis (Fig. 2.1) [23]. On the other hand, HIF-1α represses mTORC1, thereby promoting the release of mTORC2 [18].

mTORC2 is a rapamycin-insensitive companion of mTOR (RICTOR). Knowledge of its functions and control is more limited. Recently the finding that it can directly phosphorylate Akt indicates that mTORC2 may modulate cell survival [24].

HIF-1α seems to be regulated by mTORC1 and mTORC2, whereas HIF-2α expression is mTORC2 dependent but mTORC1 independent [25].

TSC1/TSC2 Pathway

The complex TSC1 (9q34) and TSC2 (16p13.3) is a negative-regulating Rheb/mTOR/p70S6K cascade [26] (Fig. 2.1). The TSC2 loss results in HIF-1α accumulation and pseudo-hypoxic pathway activation [27], which can explain the occasional association of angiomyolipoma with RCC in sporadic cases or in tuberous sclerosis [28].

c-MET Pathway in Renal Cell Carcinoma

The *MET* gene (7q31–34) is amplified in some RCCs. c-MET is a member of the receptor tyrosine kinase family; its ligand is the hepatocyte growth factor (HGF). Both are upregulated after renal injury and tissue repair via PI3-AKT and PI3-RAS-Erk. RCCs with a c-MET mutation presumably overactivate protein products of the *MET* gene, potentially driving uncontrolled growth [29] (Fig. 2.3).

The phosphate and tensin homologue deleted on chromosome 10 (PTEN) is involved in negatively regulating the Rheb/mTOR/p70S6K cascade via PI3K inhi-

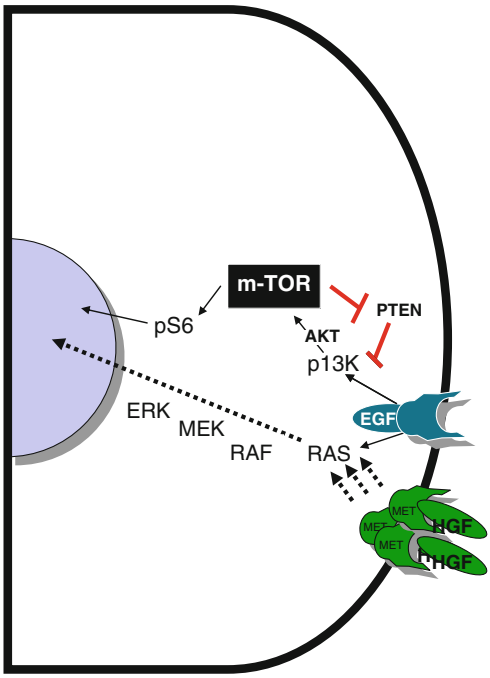

Fig. 2.3 c-MET pathway. Mutation of c-MET overactivate protein products of the *MET* gene, potentially driving uncontrolled growth

bition [22]. Individuals with a germline mutation of the *PTEN* gene (Cowden syndrome) have a risk of tumors in the breast, thyroid, endometrium, and kidney [30].

FLCN Pathway in Renal Cell Carcinoma

FLCN forms a complex with folliculin-interacting proteins (FNIP1 and FNIP2). These components bind to AMP-activated protein kinase (AMPK). AMPK acts to sense cellular energy and assists in the regulation of the mTOR activity level. In tumors that are noted to have FLCN alterations in both alleles, mTOR activation (mTORC1 and mTORC2) and also increased TFE3 transcriptional activity has been observed [31] (Fig. 2.4).

TFE3 is a member of the MiT family of transcription factors (TFE3, TFEB, MITF, and TFEC), which are overexpressed in RCC for translocations in chromosomes 1 and X, t(X:1)(p11.2;p34), and chromosomes 6 and 11, t(6;11)(p21;q13). These translocations create active fusion proteins with MiT transcription factor activity but without their normal regulation [32], conditioning mTOR pathway activation and increase in HIF-1α [33].

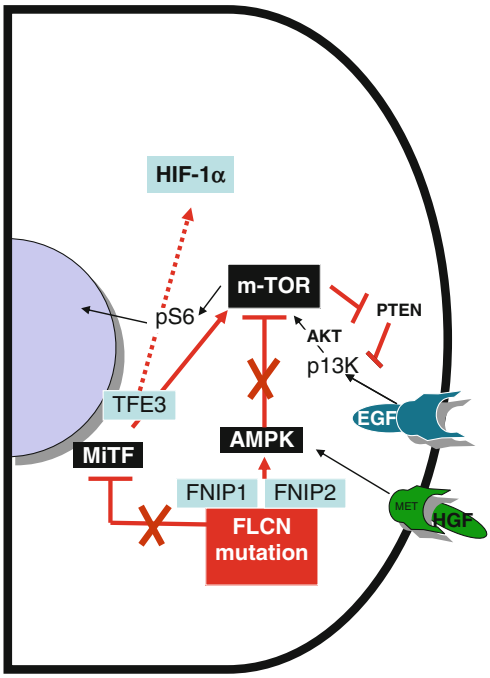

Fig. 2.4 FLCN pathway. Folliculin gene mutations deregulate mTOR activity and increased TFE3 transcriptional activity

Renal Cell Carcinoma Pathology According to Molecular Pathway

Pseudo-hypoxic Pathway

Association with VHL Gene Changes and TSC1/TSC2 Loss

Like the majority of patients with TSC1/TSC2 loss, the familial and sporadic cases with *VHL* gene changes can develop ccRCC [34]. This neoplasm consists of clear cytoplasm (empty) cells (Fig. 2.5). Cells of high nuclear grade can acquire an eosinophilic aspect due to the higher mitochondrial content. The most frequent arrangement is a solid pattern, though tubular and occasionally cystic patterns can also be present. Papillary areas are very rarely observed. Sarcomatoid transformation is observed in 5 % of cases [35]. A prominent vascular stroma is typical. Expression of CAIX and CD10 occurs in the majority of cases [36].

In multilocular ccRCC, 3p deletion is present in 74 % of cases and *VHL* gene mutation in 25 % [37]; for this reason it can be considered a variant of classical ccRCC of low aggressivity.

In addition to the *VHL* gene, other parts of chromosome 3 can be lost, such as 3p12, 3p14, and 3p21, which contain the *PBRM1* gene, with truncating mutations in 41 % of cases of ccRCC [38]. Other chromosomes affected are 5q, 9p, and 14q.

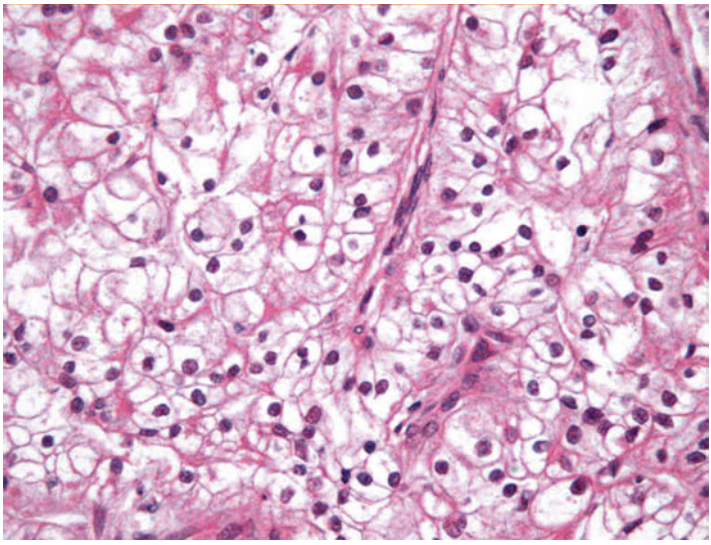

Fig. 2.5 Clear cell RCC. The cells have an empty cytoplasm for lipids and glycogen dissolution during the technical handing of the tumor

Around 10 % of cases of sporadic ccRCC do not have the *VHL* gene mutation, and some of them have somatic *NF2* gene (22q12.2) mutations [39].

Association with Krebs Cycle Mutations

These carcinomas fulfill the Warburg model of cancer because they depend on anaerobic glycolysis instead of oxidative phosphorylation [40].

HLRCC patients (with *FH* gene mutation) and 42 % of those with sporadic papillary RCC have a similar histological subtype to type 2 pRCC, with eosinophilic cells of high nuclear grade and pseudostratified nucleus in papillary cores [41] (Fig. 2.6).

Lack of expression of cytokeratin 7 and positive alpha-methylacyl-CoA racemase (AMACR) are typical. The sporadic type 2 pRCC has a higher frequency of allelic imbalance on 9p21 and a lower frequency of trisomy 17q, which is typical for the other pRCCs [42]. Other changes are in 1p, 3p, and 5q. At present there are doubts over whether HLRCC and sporadic type 2 pRCC are in fact the same entity or not and whether they follow the same pathway as the familial forms. The International Society of Urogenital Pathology (ISUP) has considered HLRCC to be a separate entity from the other histological subtypes, and the majority of authors regard sporadic type 2 pRCC as a heterogeneous variant [43].

Another mutation in the Krebs cycle is at the level of *SDHB* genes. This mutation is associated with the risk of development of succinate dehydrogenase germline mutation-associated carcinoma (SDHB RCC), a variant also characterized by eosinophilic cells with vacuoles and entrapped normal tubules in the periphery [21] (Fig. 2.7).

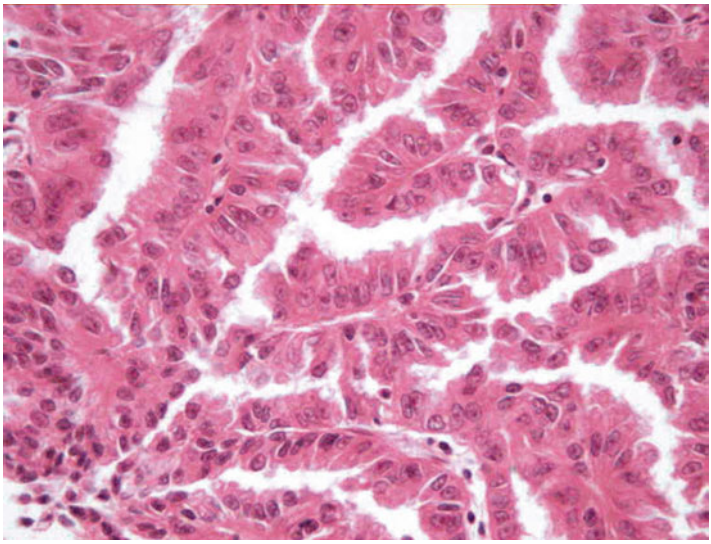

Fig. 2.6 Papillary type 2 RCC. Eosinophilic cells with nucleolus with a pseudostratified papillary arrangements

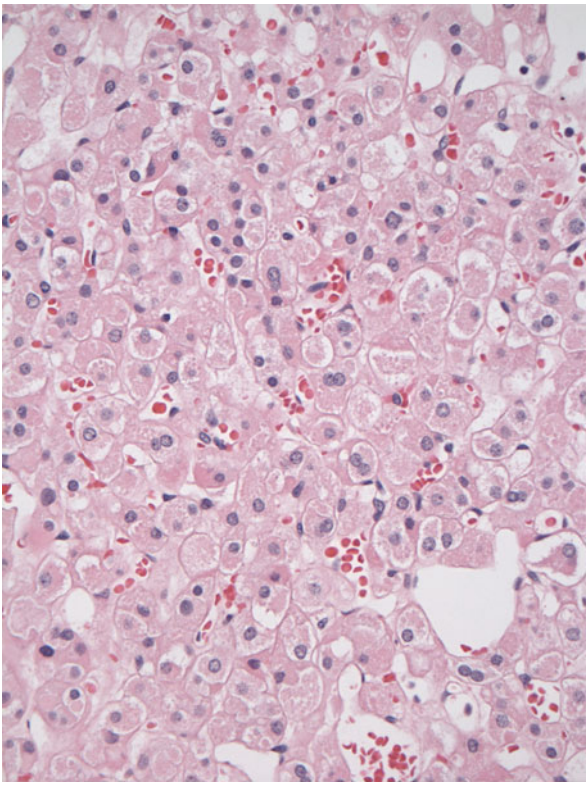

Fig. 2.7 Succinate dehydrogenase (SDHB) germline mutation-associated RCC. The cells have an eosinophilic cytoplasm with occasional vacuolization and bland nucleus (courtesy Dr. K. Trpkov—Calgary)

c-MET Pathway

HPRC and the sporadic cases are characterized by a type 1 pRCC defined by a monolayer of basophilic-cuboidal cells with scant cytoplasm, regular nuclei, and small nucleoli around capillary cores in 50–70 % of the entire tumor (Fig. 2.8). Expression of AMACR is also present [44]. Approximately 75 % of the sporadic forms have trisomy 7q31, which contains genes for c-*MET* and ligand *HGF*, but an activating *MET* mutation is seen in only 13 % of these sporadic cases [45]. In addition, gains in chromosome 17q (full trisomy, isochromosome 17q, or duplication of 17q21-qter) are typical.

Mucinous tubular and spindle-cell RCC is composed of small basophilic-cuboidal cells with round and elongated tubules and spindle cells with mucinous stroma (Fig. 2.9). It has some similarities with type 1 pRCC, with gains in 12q, 16q, 17, and 20q and losses in 1, 4, 6, 8, 9, 13, 14, 15, and 22, but no gains in 7 or 17 [46].

The RCC with PTEN mutation in Cowden syndrome is, in the majority of cases, similar to type 1 pRCC [47].

Tubulocystic RCC is composed of packed tubules and cysts lined by cuboidal or hobnail cells with eosinophilic cytoplasm and large nuclei showing prominent nucleoli (Fig. 2.10). The expression of AMACR and the gains in chromosomes 7 and 17 [48] are considered by some authors to suggest that it is closely related to type 1 pRCC [49].

FLCN Pathway

Mutation or loss of the wild-type allele of the *FLCN* gene has been identified in 70 % of BHD families, with associated risk of development of various RCC subtypes, especially chRCC, oncocytomas, and hybrid oncocytoma–chromophobe renal cell carcinoma. However, this mutation is present in only 10.9 % of the sporadic cases [50].

The cells of chRCC are larger than those of ccRCC. They display polyhedral outlines with good delimitation of the cellular membrane (giving them a vegetal cell appearance) and abundant pale reticular cytoplasm. Numerous, sometimes invaginated vesicles of 150–300 nm in diameter are present, resembling those of type B intercalated cells in the cortical collecting duct. The cytoplasm can be clear or eosinophilic according to the quantity of mitochondria [51] (Fig. 2.11). The architecture is solid, in sheets, and with a trabecular distribution. Losses in chromosomes Y, 1, 2, 6, 10, 3, 17, and 21 are typical of this RCC. The massive chromosomal losses lead to a hypodiploid DNA index. In spite of losses in chromosomes 10 and 17, there are no alterations in PTEN [52], and mutation of the *TP53* tumor suppressor gene is present in only 27 % of cases.

Overexpression of c-kit mRNA is found in not only chRCC but also oncocytomas, and differential expression of c-kit in renal tumors makes it an excellent immunohistochemical marker for diagnosis [53].

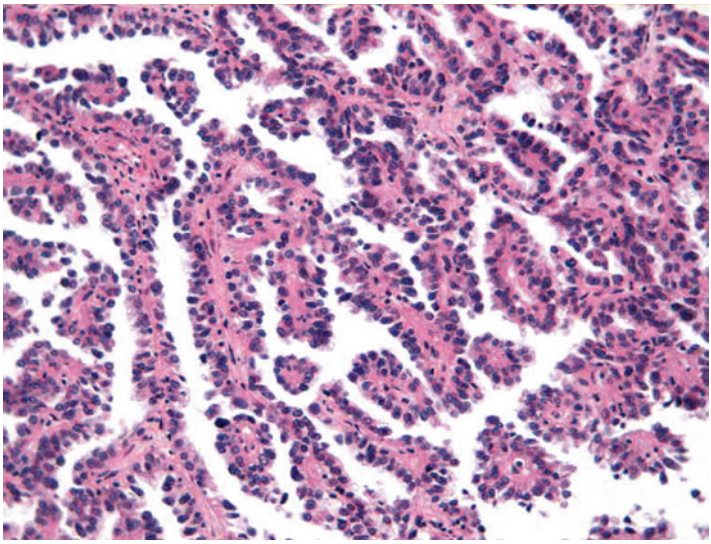

Fig. 2.8 Papillary type 1 RCC. Cuboidal cells with small nucleus and no evident nucleoli with scant cytoplasm (basophilic cells) arranged in a papillary way

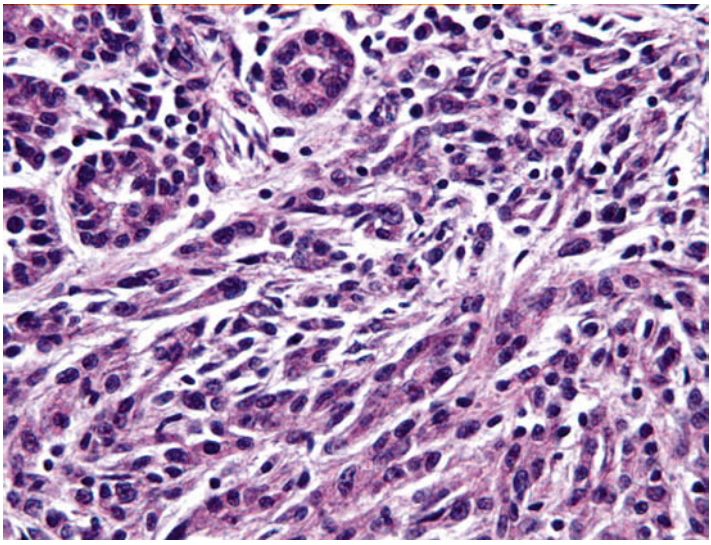

Fig. 2.9 Mucinous tubular and spindle-cell RCC. A neoplasm with bland nucleus cuboidal aspect (basophilic cells) arranged in a tubular way and with areas of spindle appearance for compression and mucinous stroma

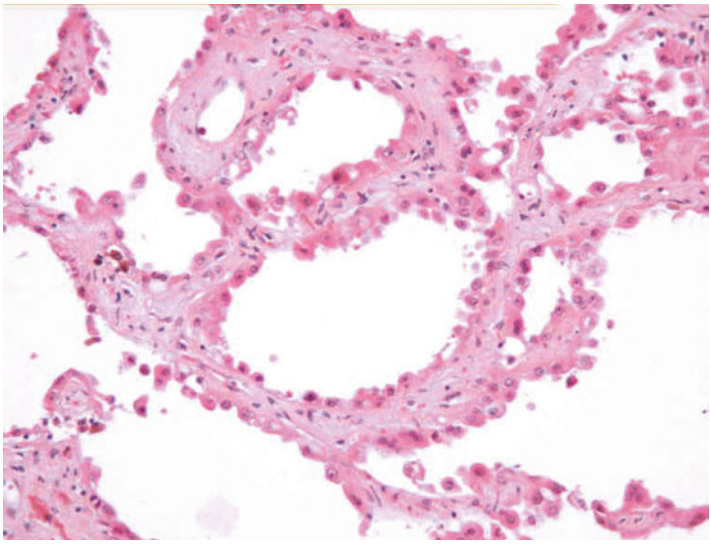

Fig. 2.10 Tubulocystic RCC. Neoplasm with cystic arrangement lined by cuboidal or hobnail cells with scant eosinophilic cytoplasm and occasionally large nuclei with evident nucleoli

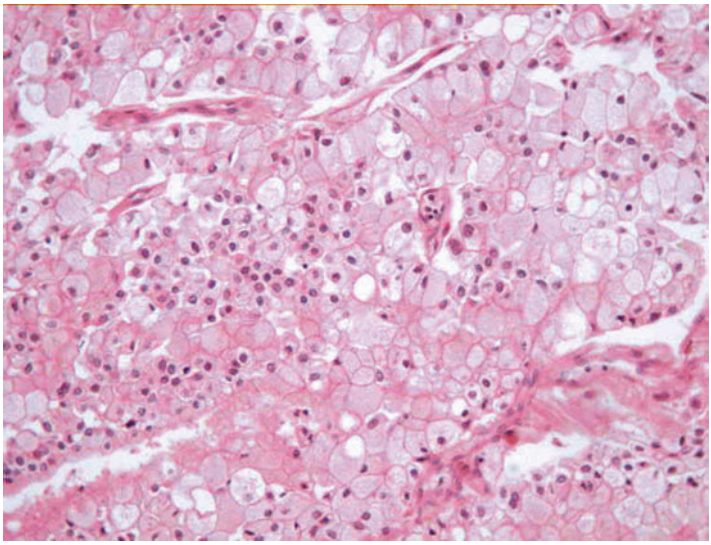

Fig. 2.11 Chromophobe RCC. Large cells with evident cellular outline with granular (clear-like) cytoplasm

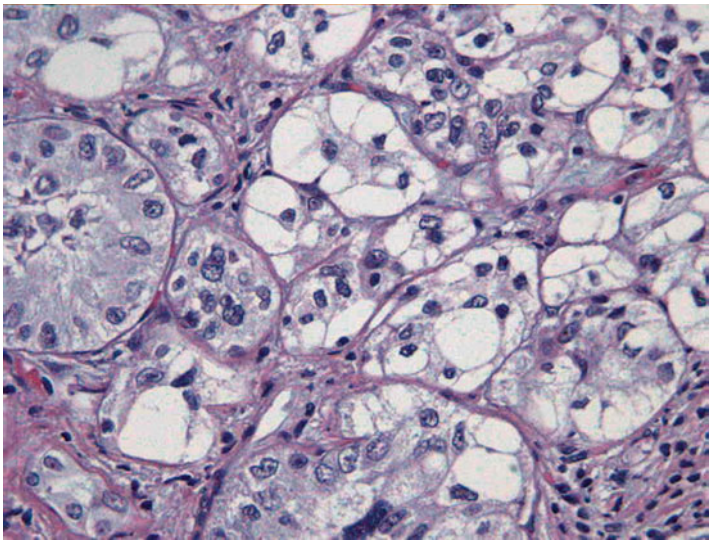

Fig. 2.12 MiT germline mutation RCC. Large cells with clear and eosinophilic cytoplasm, large nucleus, and solid or tubular arrangement

In folliculin-deficient RCC, increased TFE3 transcriptional activity has been found, and this represents a connection with the *MiT germline mutation RCC* [31]. RCCs with TFE3 accumulation display an increase in pS6, activation of the mTOR pathway, and HIF-1α expression [33], but transactivation of the *MET* promoter by ASPL-TFE3 fusion protein has also been reported [54]. TFEB-associated RCCs express HMB45 and melanocytic markers. The morphology of the MiT germline mutation RCC is characterized by large and bizarre clear and eosinophilic cells, some papillary areas, calcifications, and a biphasic pattern in some cases [43] (Fig. 2.12).

Undefined Pathway

Collecting duct (Bellini) RCC (cdRCC) and medullary RCC (mRCC) are infrequent neoplasms characterized by very atypical cells and an overlapping appearance. Eosinophilic cells are present in a solid, papillary, or cribriform arrangement with desmoplasia in cdRCC (Fig. 2.13) while marked inflammatory cells are observed in the stroma in mRCC [43] (Fig. 2.14).

The molecular genetic abnormalities in these tumors are heterogeneous, and there have been few studies on the topic. Recently immunohistochemical loss of INI1 was found in 15 % of cdRCC [55], and many alterations suggestive of mRCC have been observed in the *INI1* gene (hSNF5/BAF47), a remodeling gene of cell differentiation [43].

Some of these tumors have high expression of c-MET and HIF-1α, and for this reason, some authors also relate them to the pseudo-hypoxic pathway [54].

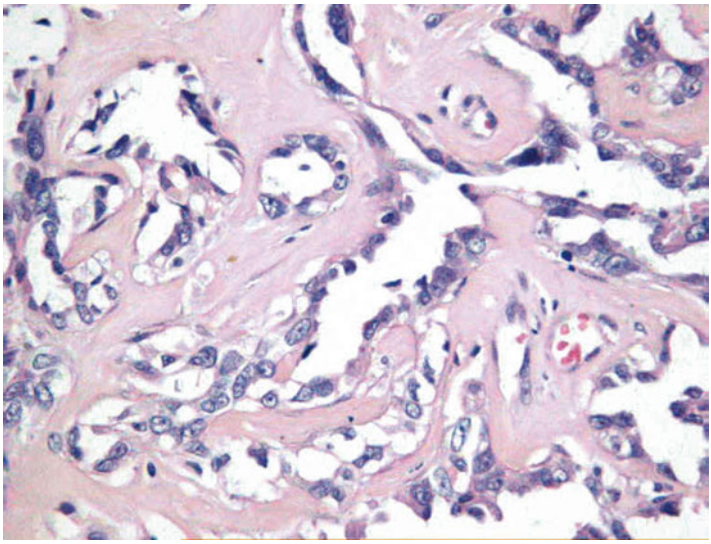

Fig. 2.13 Collecting duct RCC. High-grade carcinoma with tubular pattern in a desmoplastic stroma

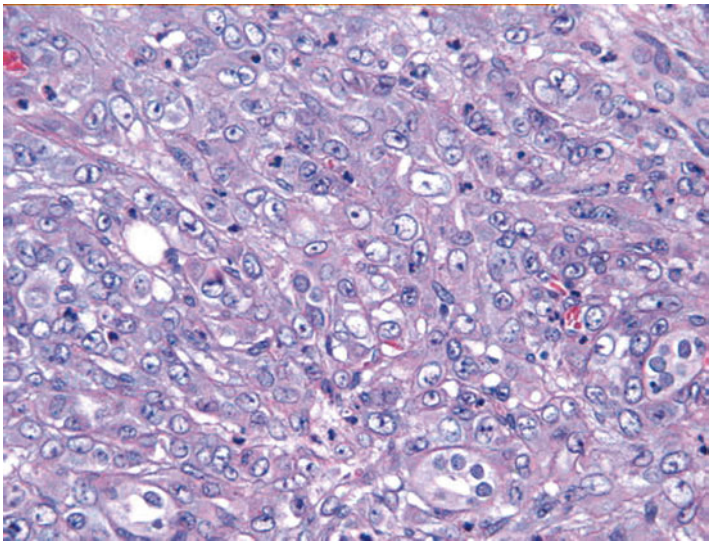

Fig. 2.14 Medullary RCC. Undifferentiated high-grade carcinoma in a solid pattern with some inflammatory cells. Notice sickle-cell erythrocytes

Other Pathological Renal Cell Carcinomas Entities

Other morphological subtypes of RCC with different chromosomal and molecular features have been reported, but the series of these other types are few in number and small; accordingly, conclusive data have not yet been obtained. The entities most frequently cited in the literature are discussed below:

Acquired Renal Cystic Disease-Associated Carcinoma: Patients with end-stage renal disease can have different RCC subtypes [56], but in those with acquired cystic disease of the kidney, the typical composition is large eosinophilic cells with a rounded nucleus and large nucleoli arranged in variety of architectural patterns; in addition, calcium oxalate crystals are observed within the tumors [57] (Fig. 2.15). These carcinomas express AMACR. At the molecular genetic level, gains in chromosomes 1, 2, 6, and 10 and monosomies 3, 9, and 16 are reported, suggesting a distinction from the other RCCs [58].

Clear Cell Tubulopapillary Renal Cell Carcinoma: This tumor was initially described in end-stage kidneys but has recently also been detected in nonterminal kidney disease. In 50 % of cases, a pronounced cystic component is observed; solid, tubular, and microcystic areas are also present. The tumor cells show a clear cytoplasm and low-grade nuclear atypia, with the nucleus situated toward the surface of the papillary tufts [59] (Fig. 2.16). They show neither deletion of 3p nor trisomies of chromosomes 7 and 17 [59].

Thyroid-Like Follicular Renal Cell Carcinoma: Very few cases of this entity have been reported. It has a follicular architecture resembling that of follicular carcinoma of the thyroid and is composed of cells showing low-grade pleomorphism with

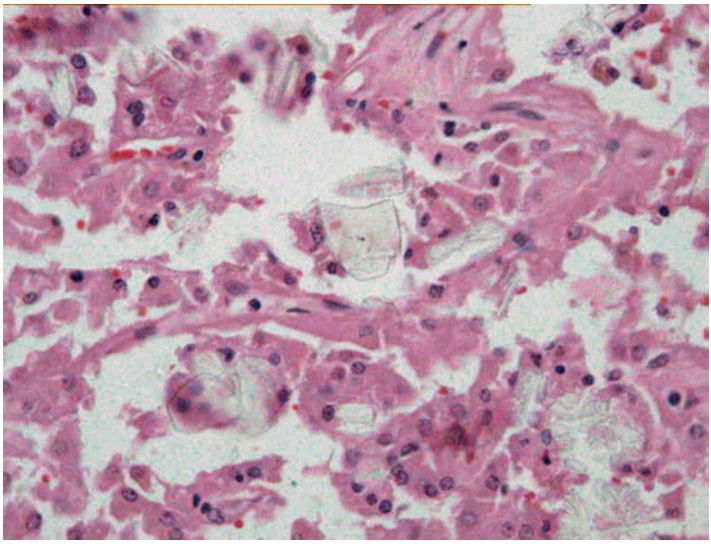

Fig. 2.15 Acquired cystic disease-associated RCC. Eosinophilic cells with calcium oxalate crystals

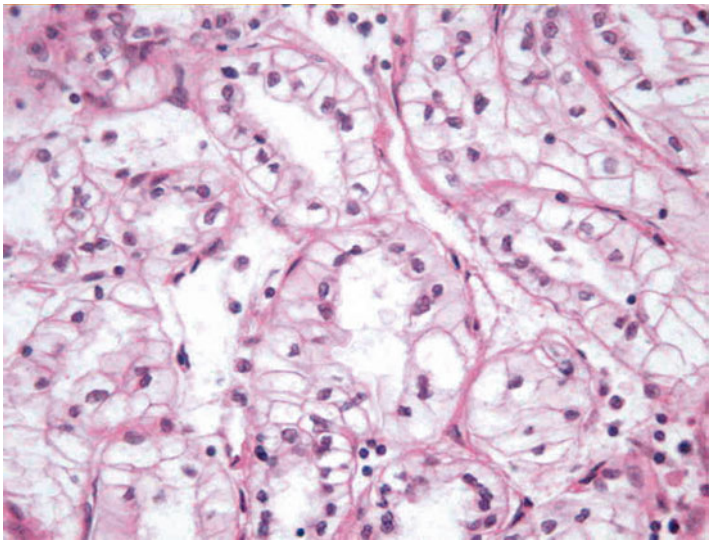

Fig. 2.16 Clear cell tubulo(papillary) RCC. Cuboidal clear cells with tubular arrangement (in some areas can be papillary) with apical nucleus localization

amphophilic to eosinophilic cytoplasm. Gene expression profiling has revealed widespread underexpression or overexpression involving chromosomes 1, 2, 3, 5, 6, 10, 11, 16, and 17 [60].

Leiomyomatous Renal Cell Carcinoma: This entity is composed of tubular aggregates of neoplastic clear cells intermixed in a prominent leiomyomatous proliferation. There is controversy over the chromosome 3 status [61].

Anaplastic Lymphoma Kinase (ALK) Translocation-Associated Renal Cell Carcinoma: This entity displays structural karyotypic abnormalities involving the ALK locus on chromosomal band 2p23 [62]; two cases of VCL (*vinculin*)-ALK fusion have been detected, and two each of TPM3-ALK and EML4-ALK fusions.

Biphasic Alveolosquamoid Renal Cell Carcinoma: There is a dual cell population, and the larger tumor cells with squamous features are arranged in well-demarcated islands, with the smaller cells surrounding them. Partial or complete losses of chromosomes 2, 5, 6, 9, 12, 15, 16, 17, 18, and 22 and partial gains of chromosomes 1, 5, 11, 12, and 13 have been reported [63].

Unclassified Renal Cell Carcinoma

This diagnostic category is for renal cell carcinomas that are impossible to classify as any of the other histological subtypes. It includes pure sarcomatoid RCCs without any evidence of the cellular origin, oncocytic RCCs without sufficient features for a precise diagnosis, any mixture of histological subtypes except oncocytoma–chromophobe varieties, and all RCCs with an unidentifiable morphology.

This diagnostic category can include different biologic entities, and for this reason in each individual case, the prognosis correlates only with the stage and grade [64].

Conclusions

After the identification of the chromosomal and molecular bases of the RCC familial syndromes and the discovery that these correspond with concrete morphological variants, it appeared that these same molecular alterations were present in the sporadic forms, with similar alterations being found in 90 % of sporadic ccRCCs and between 5 and 15 % of the other variants. In recent years, investigations have centered on the development of new therapies based on the consequences of HIF accumulation, the c-MET and FLCN mutations, and the pseudo-hypoxic status that they induce. On this basis it has been possible to reclassify RCCs according to the molecular pathway (Table 2.3) in order to help in therapeutic decision making.

However, not all the sporadic RCCs follow these pathways, and research continues. Recently some deletions in histone-modifying genes immediately next to the *VHL* gene have been detected in ccRCC. This observation has shifted biological interest away from hypoxia-induced epigenetic regulation and specifically toward the methylation of histone 3 and chromatin structure [65], opening potential avenues for new therapeutic approaches [66].

Table 2.3 Proposed renal cell carcinoma classification according to molecular pathway

Pseudo-hypoxic pathway
VHL pathway—*clear (empty) cells*
Clear cell renal cell carcinoma
Multilocular clear cell renal cell carcinoma
Krebs cycle mutations—*granular (eosinophilic) cells*
Papillary type 2 renal cell carcinoma
SDHB germline mutation-associated carcinoma
C-MET pathway—*basophilic-cuboidal cells*
Papillary type 1 renal cell carcinoma
Mucinous tubular and spindle renal cell carcinoma
Tubulocystic carcinoma
FLCN pathway—*large cells*
Chromophobe renal cell carcinoma
Hybrid oncocytoma–chromophobe renal cell carcinoma
MiT family renal cell carcinomas
Undefined pathway
Collecting duct renal cell carcinoma
Medullary renal cell carcinoma
Tubulopapillary clear cell renal cell carcinoma
Unclassified renal cell carcinomas
Pure sarcomatoid renal cell carcinoma
Mixed cellular types no chromophobe and oncocytoma
Oncocytic tumors without characteristics of typical subtype

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Chapter 3
Genomic Assessment of Renal Cancer

Stephen M. Keefe, W. Kimryn Rathmell, and Katherine L. Nathanson

Introduction

Perhaps as much or more than any other category of disease, cancer is fundamentally a genomic disorder. With the advent of a variety of high-throughput techniques for investigating the cancer genome, most notably massively parallel sequencing (MPS) or next-generation sequencing (NGS), the genomics era in cancer medicine would appear finally to have arrived at the threshold of enormous, real possibility. One can trace the arc of clinical observation and the discovery of disease from the identification of families with multiple cases of renal cancer along with the abnormalities at the chromosomal level within the renal cancers themselves to the discovery of pivotal genetic lesions. The exemplar of such success started with the finding that retinal neoplasia occurred in families reported by investigators at the turn of the nineteenth century, which were later found to also have multiple cases of renal cancer [1, 2]. Eventually, the families with von Hippel-Lindau (VHL) disease led to the discovery of 3p loss and, ultimately, to the characterization of the *VHL* gene, its function, and its role in the pathophysiology of clear cell renal cell carcinoma (ccRCC) [3–5].

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Renal cell carcinoma represents a collection of distinct diseases that can be distinguished histologically and genetically. Discovery-oriented work has focused primarily on clear cell renal cell carcinoma, the most common subtype of RCC, characterized by inactivating mutations in *VHL* which led to the initiation of the hypoxia response elements through HIF-mediated transcription resulting in alteration to the intracellular metabolic program. Recently, a number of discoveries also have implicated epigenetic changes as central to the molecular pathophysiology of disease, with mutations in genes responsible for chromatin remodeling and histone methylation. While distinct genetically, less is known regarding papillary renal cell carcinoma subtypes and chromophobe renal cell carcinoma. In this chapter, we will focus primarily on describing that which is now known regarding the ccRCC tumor genome. After a section describing the methods of genomic analysis, consideration of the RCC tumor genome will be organized into sections pertaining to cytogenetic changes, somatic mutations, epigenetic alterations, and the RCC tumor transcriptome.

Methods for Genomic Characterization

Methods for characterizing the genomics of renal cell carcinoma have focused predominantly on the tumor genome of clear cell renal cell carcinoma (ccRCC). Techniques have evolved, rapidly so, over the last 10 years, to allow for analysis of cytogenetics and copy number changes, DNA sequence and somatic mutations, epigenetic alterations, and expression profiling. Technologic advances have made it possible to perform these techniques readily in a massively parallel fashion and at comparatively low cost. Moreover, it has become possible in the context of a single unified project to use multiple analytical techniques together, called integrative genomic analysis, to expand the possibilities for discovery immeasurably. In this section, we will briefly review select methods available in each analytical category.

Cytogenetics and Copy Number Change

The term "genomics" in the context of human cancer research refers to an evaluation of the entire set of genetic information contained across all 23 chromosome pairs of a human cell, including genes, gene-modifying regions, and all other areas in between. Cytogenetics, the oldest genomic method of analysis, emerged early in the twentieth century as a way of detecting changes at the chromosome level of detail using the karyotype. The earliest genomic discovery in RCC pertained to loss of the short arm of chromosome 3 [3, 4]. Clinical cytogenetics analyses still are performed and reported routinely on RCC nephrectomy specimens.

More commonly used in integrative studies today, however, are array-based comparative genomic hybridization (aCGH) or SNP (single-nucleotide polymorphism)

arrays, which are high-throughput techniques for determining the relative copy number of thousands or more genes or specific loci. Two sources of DNA—typically tumor and normal—are isolated, denatured, and labeled with respective fluorophores via nick translation so that their relative frequencies can be compared based on competitive hybridization to known primer sequences. aCGH techniques represent an important way to determine copy number information at the chromosome level, an important first place to look for large-scale and big picture changes in the tumor genome at hand, and this data often cannot be determined using conventional sequencing technologies such as Sanger. The level of resolution of these techniques, however, represents a limitation; although it may be possible to determine that there has been a copy number gain in a chromosomal region, it is not always possible to tell exactly which genes are affected.

DNA Sequence and Somatic Mutations

Massively parallel sequencing (MPS) or next-generation sequencing (NGS) emerged as a commercially available analytic technique in the mid-2000s [6]. Throughput has been optimized to the point that up to 100 million reads of short segments, typically ranging from 50 to up to several hundred bases, can be performed in hours to days at relatively affordable costs. Sensitivity also has been dramatically improved because DNA transcripts can be read many times, with greater than 1,000-fold per given locus, depending on the application, in a given sequencing run which enables the detection of variants that might be present at lower prevalence in a particular sample because of suboptimal tumor-normal admixture or because of tumor genetic heterogeneity. Although the platforms for performing MPS vary and continue to evolve, many share common core processes such as (1) template preparation and library construction, (2) sequencing reactions, and (3) paired-end analysis [7].

MPS represents an important advance for the aforementioned reasons—higher throughput, greater sensitivity, and aligned with continually decreasing costs. However, perhaps its greatest strength lies in its ability to integrate the detection of multiple categories of genomic aberrancy into one methodology. With MPS, it is possible not only to detect mutations or variants but also to detect structural changes, copy number changes, and small insertions or deletions throughout the genome. Although some of these variations require differing and more sophisticated analysis, they are all possible. Limitations of this technology include the fact that massively parallel sequencing generates massive data output which in turn poses real challenges in terms of data storage, manipulation, analysis, and interpretation. Along with this, there can be important implications to consider when potentially thousands of genomic events in a given specimen can be compared with, e.g., clinical outcome. And the discovery of many “new” genomic aberrancies poses a challenge to clinicians in bringing MPS data into the clinic—is the variation in question really important? What does it mean and what should be done about it?

Epigenetic Analysis

A number of recent discoveries in the domain of RCC genomics, described in the sections that follow, pertain to alterations in epigenetic control, and many of these have come from MPS work. Thus, MPS itself represents an important method for elucidating the degree to which epigenetic alteration may contribute to the disease. Numerous methodologies have emerged for detecting DNA methylation, for examining gene silencing, and for identifying other regulatory marks on DNA. Bisulfite sequencing is one strategy, in which DNA is treated with bisulfite (which converts cytosine residues to uracil unless the cytosine has been methylated). DNA can then be sequenced to allow for a determination of methylation status. This method has been used to determine the rate of *VHL* promoter methylation in ccRCC, an important cause of inactivation in cancers, typically about 8 % [8]. Other methods will be described below as they are introduced in the data overview.

Transcript Analysis

Several key papers have used transcript analysis to show that ccRCC can be classified by RNA expression; for example, HIF expression (HIF1α and HIF2α expressing versus HIF2α expressing ccRCCs) influences different transcriptional programs with implications for disease phenotype [9, 10]. Conventional approaches for expression profiling feature high-throughput techniques wherein tens of thousands of probes for specific genes of interest, typically mounted on a glass or on a silicon chip, are allowed to interact with purified mRNA from fresh frozen tissue. Transcript hybridization to probes can be detected and quantified. Data often is analyzed using clustering analysis as a way of identifying, e.g., active signal transduction pathways or the activity of key genes of interest such as *Myc*. MPS techniques also can be used to analyze RNA expression, so-called RNA sequencing, which uses similar end-labeling of cDNA as DNA-based massively parallel sequencing, and sequencing of single or paired-end transcripts using the same read lengths. Strengths of this line of analysis include the abilities to detect altered transcript expression levels, as well as altered allele-specific expression, and differential alternative splicing. With sufficient read depth, sequence alterations can also be detected in expressed genes.

Technique Summary

The technologies for explorations in the human genome, epigenome, and transcript space have become greatly expanded and will continue to evolve at a rapid pace for the foreseeable future. The advantages are increased capability for discovery of events or changes, improvements in speed and cost, and provision of opportunity for integrated analysis. However, the component data have become bioinformatically

enormous and complex to analyze and interpret. It is essential for the consumer of this information to have at least a fundamental knowledge of the data platforms, to recognize limitations and opportunities for errors to exist in the data, as well as to interpret and recognize interesting or novel findings. The role of bioinformatics expertise in this space is increasingly important, and these individuals play a critical role in interfacing between cancer biologists, physician scientists, and emerging genomic data.

Cytogenetics and Copy Number Changes in Renal Cancer

As with all cancers, the initial studies falling into the genetics and genomics realm focused on examining the karyotype of renal cancers using standard cytogenetic techniques. It has been recognized for some time that ccRCC has chromosomal aberrations at significantly fewer sites when compared to other tumor types; [11] however, those that are present are very commonly observed. Copy number alterations in ccRCC also are more likely to involve full chromosome arms than to target them [11]. Hyperploidy (ploidy >2.5) of ccRCC has been associated with a higher rate of metastases and poor prognosis [12]. Multiple types of renal cancer were used together as an important initial model to show that copy number aberrations as defined by array-based comparative genomic hybridization (aCGH) could differentiate among cancer types. Using only 40 renal cancers in total, Waldman and colleagues were able to differentiate between ccRCC, papillary renal cancer, chromophobe renal cancer, and oncocytoma, demonstrating how different these subtypes of renal cancer are in copy number profile [13]. This study not only was able to easily discriminate among types of renal cancer with small numbers but underscored the usefulness of aCGH.

Copy Number Changes in ccRCC

Multiple studies of ccRCC have reported regions of gains on chromosomes 1q, 5q, 7, 8q24, 11q, 12q, and 20q and regions of losses on chromosomes 1p, 3p, 4q, 6q, 8p, 9p, 9q, and 14q [14–17]. Chromosome 3p losses (60–90 %) and 5q gains (33–67 %) are the most prevalent genetic abnormalities in sporadic ccRCC tumors [11, 14, 16, 18, 19]. The four most commonly mutated genes in ccRCC—*VHL* (von Hippel-Lindau), *PBRM1* (polybromo 1), *SETD2* (SET domain containing 2) and *BAP1* (BRCA-associated protein 1)—are located on 3p [20–23]. Biallelic loss of *VHL* has long been known to occur in the vast majority of ccRCC, with loss of heterozygosity accompanied by either *VHL* mutation in most cases or methylation [20]. Although the gain of 5q has been commonly observed for many years, the driver gene(s) has not been elucidated. In the tumors included for the Cancer Genome Atlas (TCGA) effort, focal amplifications were found which narrowed the region to 5q35, encompassing 60 genes [11]. Within the region are several genes of interest, one of which

also was investigated by Dondeti et al. [16] *STC2* (*stanniocalcin 2*), a secreted glycoprotein, is upregulated under hypoxia and is thought to help cells adapt to the stress of the tumor microenvironment. Using siRNA experiments, the authors were able to show that *STC2* promotes tumor growth by inhibiting cell death in ccRCC cells [16]. This gene also has been found to be hypomethylated in ccRCC, supporting a tumorigenic role [15]. The loss of 14q, containing *HIF1A*, also is commonly observed (30–50 %) [14] and associated with prognosis, as discussed below.

Additional common amplifications and deletions are described in ccRCC. Targeted copy number changes include *CDKN2A*/*B* (9p) and the *Myc* oncogene (8q), which are deleted and amplified respectively; [14] however, *Myc* amplification appears to be more important in renal cancer cell lines than in tumors. The region that includes TP53 also has been observed to be recurrently deleted and that encompassing *EPAS1* (HIF2a) is amplified [24]. Additional common regions of amplification and deletion observed in the TCGA, by descending frequency, included deletions of 6q26 (*QKI*, *ARID1B*), 8p11, 10q23 (*PTEN*), 1p36 (*ARID1A*), and 4q35; amplification of 3p26 (*MECOM*; MDC1 and EVI1 complex locus); and deletions of 13q21 (*RB1*), 15q21, and 2q37 (*CUL3*) [11, 12]. The TCGA also identified several additional regions of focal amplification and deletion targeting specific genes including amplifications of *MDM4* (1q32), *PRKCI* (name), and *JAK2* (9p24) and deletions of *NEGRI* (1p31), *CADM2* (3p12), *PTPRD* (9p23), and *NRXN3* (14q24). Many of these findings explain previously described deletions and amplifications identified through less precise methodologies such as karyotyping and aCGH.

Prognostic Associations with Copy Number Changes in ccRCC

Several studies have examined whether karyotypic and copy number changes in ccRCC are associated with prognostic differences in ccRCC. Standard karyotyping has been done in 282 ccRCCs in patients with nephrectomies to examine whether cytogenetic changes were prognostic; this study remains influential in the field [18]. The deletion of 3p was associated with a better prognosis ($p=0.03$), whereas deletions of 4p ($p<0.001$), 9p ($p<0.01$), and 14q ($p<0.01$) were associated with a decrease in disease-specific survival. In multivariate analysis, loss of 9p remained, along with stage and grade as independently associated with survival. *CDKN2A* is located on 9p, loss of which is associated with poor prognosis in other tumor types as well, such as melanoma [25]. Additionally, 1p, 9q, and 13q loss and 12q gain have been associated with stage and grade [17]. Some of these findings may come down to single gene changes, which have now been better delineated by the TCGA effort, such as *RB1* on 13q.

Of particular interest in regard to worsened prognosis is the deletion of chromosome 14q, which contains the *HIF1A* locus [18, 26]. When tumors expressing HIF1α and HIF2α (H1H2) are compared to those expressing HIF2α (H2) alone, losses in 9p and 14q are more significant in the H2 group compared to the H1H2 group [16]. In patient samples, frequent targeted deletion of *HIF1A* has been observed, particularly in those renal cancers associated with more aggressive disease [27].

Taken together, these findings by multiple investigators provide evidence that the loss of *HIF1A* is a poor prognostic marker in ccRCC and support multiple other avenues of evidence suggesting that HIF2α is the major HIF driver in this cancer type. Of note, copy number analyses of ccRCCs, sporadic and associated with VHL disease in several studies, have been compared and show generally a similar profile between groups, although the sporadic tumors are more heterogeneous and consistently demonstrate more copy number aberrations per tumor, significantly so in one study [14, 17, 24].

Familial Renal Cancer Due to Chromosome 3 Translocations

Multiple families with inherited susceptibility due to balanced translocations involving chromosome 3 have been described [28–33]. The mechanism behind the increased risk of multifocal clear cell renal cancer is thought to be the loss of the rearranged chromosome during mitosis, which requires a quadrivalent (four chromosomes coming together), leading to greater errors during chromosomal segregation. As multiple genes involved in the pathogenesis of clear cell renal cancer are located on chromosome 3p, including *VHL*, *PBRM1*, *BAP1*, and *SETD2* [11], it is not surprising that a mechanism of increased loss of one allele leads to an increased risk of clear cell renal cancer.

Somatic Genetics of Renal Cancer

With the advent of massively parallel sequencing, as with other cancer types, the somatic genetic and genomic profiles of renal cancers have become increasing well detailed. Multiple studies focusing on ccRCC using whole exome sequencing (usually in fact covering 85–90 % of the genome) have been published [21, 23], and more comprehensive studies also including copy number analysis, methylation, RNA sequencing, and some whole-genome sequencing have been done [11, 12]. These studies have greatly contributed to our understanding of ccRCC, which had been poorly characterized compared to various other cancer types. The mutational profile of renal cancer is characterized by an enrichment of T>C/A>G transitions, followed by C>T/G>A transitions [12]. In the ccRCC TCGA effort, 1.1 ± 0.5 nonsilent mutations per megabase were identified [11].

Mutated Genes in ccRCC

In the TCGA dataset of 417 patients, 19 genes were identified as significantly mutated ($q < 0.05$) [11]. Among those, 8 emerged at the highest level ($q < 0.00001$), whereas the remaining 11 remained significant but several orders of magnitude less so ($q < 0.01$–0.05). The eight genes included *VHL*, *PBRM1*, *SETD2*, *KDM5C*

(lysine (K)-specific demethylase 5C), *PTEN*, *BAP1*, *MTOR*, and *TP53*. Mutations in the histone-modifying genes *SETD2*, *KDM5C*, and *KDM6A* (*lysine* (*K*)*-specific demethylase 6A*) and the tumor suppressor *NF2* (*neurofibromin 2*) had been previously emerged as important in ccRCC in a whole exome-sequencing study from Futreal and colleagues [22]. Varela et al. had identified truncating mutations in *PBRM1* (*polybromo 1*), a SWI/SNF complex member, also using massively parallel sequencing [23]. Of these targets identified as mutated by massively parallel sequencing, only *PBRM1* is involved in a large proportion (30–40 %) of ccRCC tumors. Most recently somatic mutations in *BAP1* (BRCA-associated protein 1) also were identified through whole exome-sequencing studies [21]. Interestingly, in the whole exome sequencing of clear cell renal cancer, which required both tumor and germline samples, mutations of *BAP1* were found to originate from the germline in a few patients. Thus, two recent studies have suggested that *BAP1* mutations predispose to familial clear cell renal cancer, along with uveal and cutaneous melanoma, and mesothelioma, the known tumor types associated with germline *BAP1* mutations [34, 35]. Infrequently, as compared to the other genes, mutations in the known tumor suppressor genes, *TET2*, *KEAP1*, *NRF2*, *CUL3*, and *TP53*, also have been identified [11, 12].

Recurrent mutations in *TCEB1*, which encodes elongin C, part of the pVHL complex that ubiquinates the HIFs [36], have been recently identified [12]. Although these mutations are relatively infrequent (3 % of cases), they are found only in *VHL* mutation negative ccRCC and accompanied by loss of the wild-type allele at 8q21. The missense mutations are found at Tyr79 and Alal100, more frequently at the former. The identified mutations are within the binding domain for pVHL and are predicted to abolish the interaction between elongin C and pVHL, resulting in accumulation of the HIF proteins, similar to VHL inactivation. Tumors containing *TCEB1* mutations demonstrate increased HIF1α staining by immunohistochemistry.

Activation of the PI3K/AKT Signaling Pathway in ccRCC

Mutations and copy number changes affecting multiple genes within the PI3K/AKT signaling pathway are found in ccRCC, totaling ~30 % of cases, and are generally mutually exclusive with each other. Activating mutations in *MTOR* have been identified in 6 % of ccRCC, with recurring mutations at Phe1888 within the FAT domain [11, 12]. Additionally, rare activating mutations are found in *PIK3CA* and *AKT1/2/3* and inactivating mutations in *TSC1*, *TSC2*, and *PTEN*, with the latter being more frequent with homozygous deletions observed as well. Amplifications of *FGFR4*, *GNB2L1* (RACK1), and *SQSTM1* (p62) also have been observed which are associated with activation of PI3K signaling [37, 38]. These data provide insight into the clinical activity of MTOR inhibitors (temsirolimus, everolimus) in ccRCC, perhaps opening the door for molecular stratification of patients and optimization of selection for therapy based on tumor genetic profile.

ccRCC Tumorigenesis and Prognosis in Relation to Genetic Mutation

Mutations in the non-*VHL* 3p target genes, *PBRM1*, *BAP1*, and *SETD2*, all occur within the background of *VHL* mutations [11, 12]. In the 421 and 188 ccRCCs from the TCGA and MSKCC, respectively, mutations were present in *PBRM1* 33.5 % and 30.3 % of the time, *SETD2* 11.6 % and 7.4 %, and BAP1 9.7 % and 6.4 % [39]. The mutation profiles can include all combinations of the genes on 3p, but they tend to be negatively correlated with each other. Recent studies suggest that *SETD2* and *BAP1* mutations may be acquired during progression, whereas PBRM1 mutations may be early or initiating mutations [39]. Independent studies from UTSW and MSKCC, both of which were validated using the TCGA dataset demonstrated that *BAP1* mutations were associated with a higher tumor grade and decreased overall survival, as compared to those with *PBRM1* mutations (hazard ratio 2.8 (95 % CI 1.4–5.9 in the TCGA dataset), which are negatively correlated [11, 39, 40]. The few patients whose ccRCCs had mutations in both *BAP1* and *PBRM1* had the worst survival [40]. *SETD2* mutations also have been associated with decreased overall survival [12, 39].

Epigenetic Regulation in ccRCC

Renal cell carcinoma has recently emerged as a paradigm shaping cancer owing to several recent discoveries linking epigenetic regulation with clear cell (conventional) renal cell carcinoma. These discoveries build on an existing body of evidence documenting that gene regulation in RCC occurs commonly via altered DNA methylation. The specific genes are described in detail elsewhere in this text and recently in a review of renal cell carcinoma [41]. In general, as overviewed in other portions of this chapter, renal cell carcinomas display a low mutation frequency and relatively consistent copy number alterations [14]. These findings are in stark contrast to tumors driven by defects in DNA repair or other hypermutable scenarios. As a result, it should be not surprising that epigenetic regulation should emerge as a major mechanism promoting tumorigenesis.

The landscape of epigenetic regulation in renal cell carcinomas is only now beginning to be understood, and much of this fascinating tumor biology remains to be discovered [42]. A flood of new data is likely to appear in the next several years which will elucidate the mechanisms by which these changes promote cancer growth, along with perhaps greater insights as to why renal cell carcinoma, at this point in time, predominantly the clear cell subtype, favors this strategy of tumorigenesis. In this chapter, we will summarize “what” we currently know about epigenetic features and profiles in kidney cancer. The “how” and “why” will remain to be discovered.

Mutations in Epigenetic Regulatory Genes

Key to this discussion is the recent observation that mutations in quintessential chromatin-modifying genes are among the most frequently altered genes in clear cell renal cell carcinoma [22, 23]. These mutation frequencies, discussed elsewhere in this chapter, suggest a potentially strong causal association with cancer progression. Several genes fall into this category, and their frequency of association with clear cell renal cell carcinoma is second only to mutations in *VHL*. This group of genes were initially identified by a series of deep sequencing studies and have been independently verified by the Cancer Genome Atlas data [11, 21–23]. Collectively these genes, including *PBRM1*, *BAP1*, and a set of histone-modifying genes, are mutated in up to 25 % of tumors. In addition to mutations in these genes, hypermethylation has also been detected specifically to reduce expression [43]. We will examine each group separately, although it is important to note that at the time of this chapter, their specific roles in tumorigenesis have not been well described.

PBRM1

PBRM1 (polybromo-1), also known as *BRG1*-associated factor 180 (*BAF180*), is a component of the SWI/SNF-B (PBAF) chromatin-remodeling complex, which contains at least *SMARCA4*/*BRG1*, *SMARCB1*/*SNF5*/*INI1*/*BAF47*, *ACTL6A*/*BAF53A* or *ACTL6B*/*BAF53B*, *SMARCE1*/*BAF57*, *SMARCD1*/*BAF60A*, *SMARCD2*/*BAF60B*, and actin [44]. The SWI/SNF complex functions as a nucleosome-remodeling complex [45]. In packaged chromatin, nucleosome positioning is key to regulating available DNA sequences for sequence-specific transcription factor, enhancer, or repressor protein binding and for assembling DNA packaging properly for cell function. In simple terms, this complex uses ATP hydrolysis to unwind and rewind DNA around assembled nucleosomes [46]. Although discovered with much excitement and fanfare in the mid-1990s, this essential process of nucleosome repositioning remains relatively poorly understood. In addition, mutations in several members of the SWI/SNF complex have been associated with various cancers [45]. For reasons that remain unclear, within the complex, only *PBRM1* inactivating mutations, with accompanying loss of heterozygosity, are associated with renal cell carcinoma. Although a complex coordinating DNA packaging might intuitively be associated with cancer suppression, the exact mechanism by which disruption of this complex by *PBRM1* or other mutations promotes cancer remains poorly understood.

BAP1

BAP1, also known as the *BRCA1* associated protein-1, is a deubiquitinating enzyme that is a member of the polycomb group proteins that act as transcriptional repressors. *BAP1* is the catalytic subunit of the polycomb repressive deubiquitinase (PR-DUB) complex, which controls gene regulation by titrating the amount of

ubiquitinated histone H2A present in nucleosomes at the promoters of key developmental genes [47]. It also serves as an adapter molecule for a variety of transcription factors that associate with chromatin-modifying complexes. The effect of mutations in *BAP1* to remodel chromatin or affect chromatin-mediated transcriptional processes in tumors is not known. However, among the mutated genes implicated in a chromatin regulatory function, *BAP1* is most closely associated with clinical outcome. As discussed above, *BAP1* mutations have been linked to a new familial form of renal cell carcinoma [34, 35] and also are associated with a class of ccRCCs typified by poor outcome and aggressive disease [21, 40, 48].

Histone-Modifying Genes (HMGs)

Massively parallel sequencing of renal tumors has identified an increased rate of mutation in genes associated with modifying histones. Although individually many of these genes are mutated in a minority of ccRCC tumors (<5 %), collectively, mutations in this set of genes may contribute to nearly 30 % of tumors. The impact of mutations in genes that modify histones has potential to dramatically alter cellular dynamics, as the histone modifications of methylation, acetylation, and other alterations program the chromatin for efficient and proper "reading" by interacting proteins of the "histone code." [49] This code provides an important sequence agnostic level of the regulation of genes for effective transcription control. The most commonly mutated gene in this set is *SETD2* [22, 50]. This factor is well known to have a nonredundant role as a histone methyltransferase. SETD2 trimethylates histone 3 on lysine 36, placing a repressive mark on actively transcribed genes. The loss of SETD2 causes histones to lose this mark. The predicted effect of losing this activity would be to permit RNA polymerase II reentry on already transcribed genes or to miss exon and splicing cues. Human tumors were recently analyzed, demonstrating accumulated alternatively spliced transcripts, intron retention, and alternatively used transcriptional start sites and termination cues. In addition, a massive increase in accessible, non-nucleosome-bound DNA is observed, suggesting a global chromatin reprogramming effect. The net effect in human tumors has not yet been established, although mutations in *SETD2* also are associated with poor outcome [39]. Other genes mutated in this group include *JARID1C* (*KDM5C*, an H3K4 demethylase) and *UTX* (*KMD6A*, an H3K27 demethylase). Mechanistically, the link to advancing the tumor phenotype of these mutations remains to be discovered, but ultimately the high frequency of these events is provocative to consider them as a whole as a key step in the evolution of clear cell renal cell carcinoma.

Genomic Assessments of Chromatin

Ongoing studies to examine gene level changes in histone marks and the resultant alteration in the histone code will be essential to these advances. The future of epigenetic assessment of chromatin in cancer will require increasingly

bioinformatically intensive processes to compile short read maps of the genome, essentially "decorating" the genome with regional information. Several tools are being actively applied to characterize the genomic in this way: Chromatin immunoprecipitation sequencing (ChIP-seq) uses high-specificity antibodies to capture regions of DNA bound by proteins, which are amenable to massive parallel sequencing, and may be a valuable tool going forward for delineating the function of chromatin-interacting proteins, such as BAP1, as well as to map the regions of the genome displaying specific histone marks (such as methylation, acetylation) using epitope-specific antibodies. Widely used to localize transcription factors in the genome, these technologies will create a cancer genome model very unlike from current versions and will hopefully provide insights regarding the derangements of epigenetic marks and programs occurring as a result of these mutations. Genomic studies that have the capacity to map nucleosome placement, such as micrococcal nuclease sequencing (MNase-seq), and regulatory element occupancy, such as formaldehyde-assisted isolation of regulatory elements sequencing (FAIRE-seq), are complementary technologies that capture fragmented regions of the genome to either localize nucleosomes genome-wide or expose open regulatory regions (promoters, enhancers, etc.). These tools have been applied in cell lines but are being developed for use in the complex tumor tissue to examine changes in the epigenome in ways previously impossible.

DNA Methylation Phenotypes

Perhaps the most well-studied epigenetic mark in cancer biology is CpG island DNA methylation. The relationship between mutations in chromatin epigenetic regulators as those discussed above and DNA methylation remains unknown. However, renal tumors consistently demonstrate differences in DNA methylation compared with normal tissue. In the TCGA analysis, hypermethylation was observed using bisulfite sequencing at a variety of tumor suppressor loci [11]. Gene mutation-specific differences in DNA methylation, such as changes associated with *SETD2* mutation, suggest that DNA methylation change may result directly or indirectly from this mutational event. In particular, loss of DNA methylation was found in non-promoter regions in *SETD2*-mutated tumors, potentially suggesting a role in maintaining the heterochromatic state [51]. This "reprogramming" of available promoters for gene expression can provide a powerful mechanism to repress or enhance gene expression. High-throughput bisulfite sequencing has become a standard tool in the armamentarium of cancer genome scientists and can not only complement the marks indicated above by ChIP-seq or other methods but also provide specific information that augments gene expression profile information.

Renal Cancer Transcript Assessments

Transcriptional dysregulation exists at the heart of clear cell type renal cell carcinoma. This is largely owing to the classical association with deregulated hypoxia signaling, covered in detail elsewhere in this text, although many other factors contribute to transcript variance in this cancer. Assessments of mRNA signatures have been examined in a variety of platforms. As indicated above, because it was recognized more than 30 years ago that clear cell renal cell carcinoma was associated with key hypoxia-regulated genes, transcription profiles have been studied from a time when such profiles were only evolving [52–54]. The hypoxia signature consists of massive upregulation of over 100 genes now known to be induced because of activation by the transcription factors HIF1α and HIF2α, each present in a complex with a ubiquitous nuclear transporter HIF1β, also known as ARNT. The induction of these transcripts ranges from subtle increases of twofold or less to genes that are activated more than tenfold in expression. Genes induced transcriptionally as a part of the HIF-driven hypoxia response include genes involved in: angiogenesis, glucose metabolism, cell survival, and cell migration/invasion properties. All tumors that harbor *VHL* mutation or loss display stabilization of one or both of these HIF factors. Although the consensus binding site for these transcription factors is the same, the factors themselves have overlapping but not identical sets of target genes [31, 55, 56]. Notably, gene expression profiling using array platforms identified that HIF1α specifically targets enzymes involved in glycolysis, which was verified by PCR [56]. Subsequent detailed analysis of human tumors demonstrated that tumors could be classified for expression of HIF1α and HIF2α (H1H2), HIF2α alone (H2), or VHL wild type [9]. The transcript profile analysis of these classifications confirmed this distinction, as well as demonstrating evidence of increased MTOR signaling in H1H2 and wild-type VHL tumors.

In addition to the hypoxia response transcription factors, other features may participate in adapting the transcriptome. Many gene expression mRNA factors have been identified by association with outcomes. The power of gene expression profiling is in the massive numbers of genes that can be simultaneously analyzed for level of expression at a single point in time. By examining genes or gene sets associated with poor outcome tumors, several gene sets have been identified that can aid in the classification of tumors according to the risk for disease progression or death [57–61]. Clustering methods have also emerged which use very high level pattern recognition algorithms to find inherent subgroups within groups of tumors. Clear cell renal cell carcinoma conforms to these pattern recognition algorithms by sorting into two dominant groups, designated as ccA and ccB [10, 62], which are also associated with disease outcome. Using a platform of metadata, which combines the majority of available gene expression data, these two dominant classifications were again observed [63]. In addition, this analysis revealed a small group of variant tumors, not readily classifiable as clear cell tumors, and on histologic assessment these tumors were more readily classified as the rare clear cell papillary subtype [64], demonstrating the power of molecular assessment to define groups and variants.

Overall, these transcript analyses provide a powerful and ready tool to measure gene expression by a variety of means, from PCR- and hybridization-based platforms to massively parallel sequencing, which has brought RNA sequencing into the mainstream. This tool, utilized in the TCGA dataset, provides isotype agnostic expression data as well as opportunities to identify gene fusions or mutations that are not detected by arrays or other means. For example, several recurrent key fusions were identified by the Cancer Genome Atlas [11]. These indicate that some translocations more commonly found in pediatric cancers may be relevant in adult forms of ccRCC as well [65]. Future work will discern the biological relevance of these fusions to promote kidney cancer.

Non-clear Cell Disease

As has been the case for ccRCC, genomic insights into non-clear cell disease have been driven by discoveries related to tumors arising in a familial or inherited context. Germline mutations in the *Met* proto-oncogene have been shown to be associated with inherited risk for the development of multiple, synchronous papillary RCC type 1 (pRCC-1). Hereditary leiomyomatosis and renal cell carcinoma (HLRCC), characterized by a solitary papillary RCC type 2 (pRCC-2) tumor, appears to arise from germline mutations in the fumarate hydratase gene (*FH*) [66–69]. It is important to note that HLRCC may only define one subset of pRCC-2. Germline mutations in folliculin (*FLCN*) have been characterized as the key genetic alterations with Birt-Hogg-Dubé syndrome associated with chromophobe RCC as well as, perhaps, oncocytoma and ccRCC [70]. The FLCN protein has no homology to previously identified proteins, and its function has been controversial. Most recently, it has been suggested that it is a ciliopathy that is involved in cell polarity, regulates cell-cell adhesion, and negatively regulates rRNA synthesis [71, 72]. These genes and their respective genetic changes have been implicated in disrupting core metabolic programming. Understanding the relationship between these changes in tumor cell metabolism and tumorigenesis and progression across RCC subtypes remains a critical area of need for future work.

Little of the somatic genetics of the sporadic (non-inherited) non-clear cell RCC has been elucidated. On a genomic level, non-ccRCCs are known to vary considerably from ccRCC. For example, although chromosome 3p deletion is commonly present in ccRCC and often encompasses *VHL*, *PBRM1*, *BAP1*, and *SETD2*, chromophobe RCC tumors often accumulate heterozygous losses of multiple whole chromosomes, including chromosomes 1, 2, 6, 10, 13, 17, and 21 [73, 74]. Although chromosome 1 also is often lost in the oncocytoma variant of renal cancer, this pattern of copy number alterations provides a discrete footprint for assigning the diagnosis of chromophobe RCC on the basis of cytogenetic analysis.

Histologically, papillary RCC takes on two forms, type 1 and type 2, but, genetically, it is not clear that the distinction will remain clear cut. Cytogenetically, patterns of gains and losses include common gains of chromosome 17 in pRCC-1 and

losses of chromosomes 8, 11, and 18 in pRCC-2 [75], but the driving mutations in any of these rarer variants of sporadic renal cancer, however, remain largely unknown. The genetics and genomics of these tumor types are being further elucidated, and the inclusion of both papillary and chromophobe-type RCC in TCGA projects will enable dramatic clarification of these diseases at a genomic level.

Conclusions

The methods of genomic assessment have evolved rapidly over the recent past creating remarkable possibility. Beyond the mutations and hypermethylation involving *VHL* which have been known for some time, the use of these modern methods of genomic analysis in ccRCC has identified a number of important additional changes related to tumor cell genetics, genomics, gene expression, and epigenetic control. Progress in the elucidation of the non-clear cell tumor genomes has lagged behind discoveries in ccRCC; however, new findings are emerging here too. Key challenges that we face now in this work pertain to refining the storyline of how these new findings are interrelated in the pathophysiology of RCC and to determining how best to leverage these findings therapeutically. We have crossed the threshold of enormous possibility in our understanding of these tumors. We hope with some optimism now that these findings and the many others that soon will follow can be translated into real improvements in the care of our patients with renal cancer.

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Chapter 4
VHL and HIF in Clear Cell Renal Cell Carcinoma: Molecular Abnormalities and Potential Clinical Applications

Lucy Gossage

Introduction

Von Hippel-Lindau (VHL) disease (also known as VHL syndrome) is a hereditary, autosomal dominant, neoplastic disease caused by germline mutations in the VHL tumour suppressor gene [1]. Patients inherit a single faulty copy of the gene, but the development of disease depends on spontaneous inactivation or loss of the second, wild-type VHL allele. VHL disease is associated with clear cell renal cell carcinomas (ccRCCs), central nervous system and retinal haemangioblastomas, phaeochromocytomas and pancreatic neuroendocrine tumours, in addition to pancreatic cysts, endolymphatic sac tumours and epididymal papillary cystadenomas.

Two key observations led groups to question whether mutations within the VHL gene are also responsible for the development of sporadic ccRCC. First, the leading cause of death in patients suffering from VHL disease is ccRCC [2]. Second, the reintroduction of wild-type, but not mutant, VHL into RCC cell lines that lack the protein has no demonstrable effect on their growth in vitro but inhibits their ability to form tumours in nude mice [3–5]. Subsequently, it became clear that most sporadic ccRCCs do exhibit VHL alterations [6–9]. Consistent with the two-hit hypothesis of the tumour suppressor gene theory [10], biallelic inactivation of VHL occurs in the majority of sporadic ccRCCs due to a combination of somatic mutations, VHL promoter hypermethylation (which effectively turns off gene expression) and loss of heterozygosity (LOH) by allele deletion. In those ccRCCs which harbour VHL mutations, VHL is mutated ubiquitously in all areas of the tumour, supporting a key driver role for VHL mutations in the pathogenesis of most ccRCCs [11]. This is in contrast to mutations in other proposed driver genes which are often not detectable across every region of the tumour.

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The compelling correlation between VHL inactivation and the development of ccRCC has been the subject of extensive research for almost 20 years, resulting in a heightened appreciation of the intricate relationship between the tumour endothelial vascular network and ccRCC inception and progression. In turn, this has facilitated the development of a variety of targeted therapies for ccRCC which not only reduce tumour burden but also significantly improve the clinical outcome in patients with advanced disease.

In this chapter, we examine in detail the role of the VHL gene and protein in ccRCC, alongside that of its main downstream target, hypoxia-inducible factor (HIF).

VHL Gene and Protein

The VHL gene is located on 3p25, has been evolutionarily conserved and consists of 854 nucleotides in three exons [12] (Fig. 4.1). An alternative splice variant that lacks exon 2 has been described but is thought to lack tumour suppression activity [7]. As a result of two alternative in-frame start codons, two pVHL isoforms exist in the cell: a 213-amino acid, 30 kDa form ($pVHL_{30}$), and a 160-amino acid, 19 kDa form ($pVHL_{19}$) [5, 13, 14]. $pVHL_{19}$ lacks a 53-amino acid N-terminal pentameric acid repeat domain and seems to predominate in many tissues. Since both isoforms behave similarly in biochemical and functional assays and possess tumour

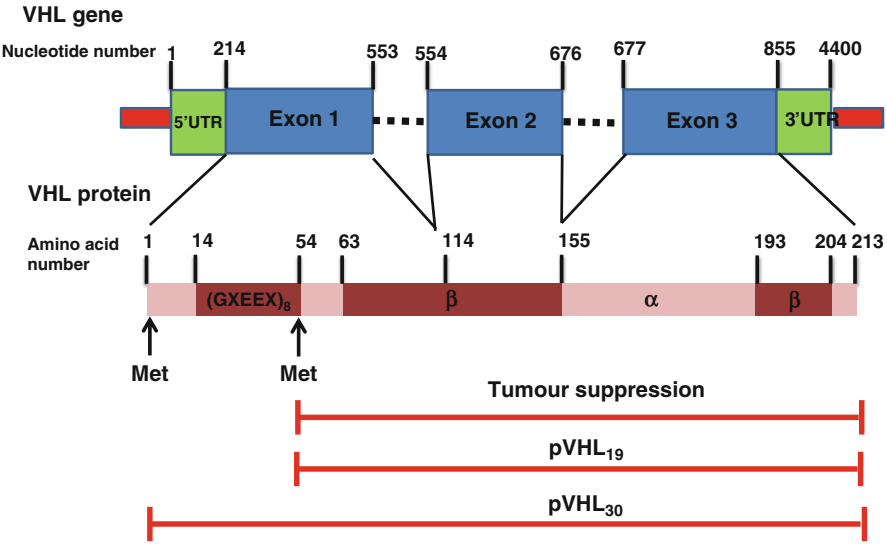

Fig. 4.1 VHL gene and protein structure. The VHL gene consists of 854 nucleotides in three exons. Two pVHL isoforms, $pVHL_{19}$ and $pVHL_{30}$, exist as a result of two alternative in-frame start codons. $pVHL_{19}$ lacks an N-terminal (GXEEX) repeat domain, but both isoforms possess tumour suppressor activity in vivo

suppressor activity in vivo [5], the term pVHL is used to describe both these proteins generically. Though pVHL shuttles between the nucleus and the cytoplasm, under steady-state conditions, most of the protein is located in the cytoplasm [4, 15–22]. Some pVHL is also found in mitochondria and associated with the endoplasmic reticulum [23, 24].

*p*VHL *Structure*

pVHL consists of two tightly coupled domains, α and β (Fig. 4.2). The β domain consists of a 7-stranded β-sandwich (amino acids 63–154) and an α-helix (residues 193–204) and has the properties of a substrate docking site [25]. The α domain, residues 155–192, consists of three α-helices and binds elongin C; an α-helix of elongin C completes a folded leaf four-helix structure instigated by the three pVHL α-domain helices [25]. In turn, this nucleates a complex containing elongin B, cullin 2 (Cul2) and RING finger protein Rbx1 (VCB-CR complex) [17, 25–28]. The elongin B/elongin C complex thus acts as an adaptor that links a substrate-recognition subunit (pVHL β domain) to heterodimers of Cul2 and Rbx1. pVHL is directly stabilised by associating with elongins B and C, and in turn elongins B and C are stabilised through their interactions with each other and pVHL [29]. The entire pVHL-elongin complex is thus resistant to proteasomal degradation. In contrast, VHL proteins harbouring mutations which disrupt elongin binding are unstable and rapidly degraded by the proteasome. Structurally, the VCB-CR complex resembles yeast Skp1-Cdc53-F-Box protein (SCF) ubiquitin ligases, and functionally, both the VCB-CR and SCF complexes have ubiquitin ligase activity and are capable of targeting proteins for proteasomal degradation [30, 31].

*p*VHL *and Hypoxia-Inducible Factors*

The best-documented function of pVHL relates to its role as the substrate-recognition component of the VCB-CR E3 ubiquitin ligase complex. This complex is best known for its ability to target hypoxia-inducible factors (HIFs) for polyubiquitination and proteasomal degradation [32, 33] (Fig. 4.3). The alpha subunit of HIF interacts exclusively with the beta domain of pVHL, binding alongside the β-sandwich [34, 35] (Fig. 4.2). This binding is dependent on the hydroxylation of one of two conserved proline residues within HIFα by prolyl hydroxylases (PHDs) 1–3, which require oxygen as a co-substrate and are thus only active under normoxic conditions [35–39] (Fig. 4.4). Prolyl hydroxylation of HIFα enables its recognition and ubiquitination by the VCB-CR complex, and polyubiquitinated HIFs are recognised and degraded by the cellular proteasome (Fig. 4.3). Under hypoxic physiological conditions (or in the absence of functional pVHL), HIFα accumulates and forms heterodimers with HIF1β. These heterodimers translocate to the nucleus where they

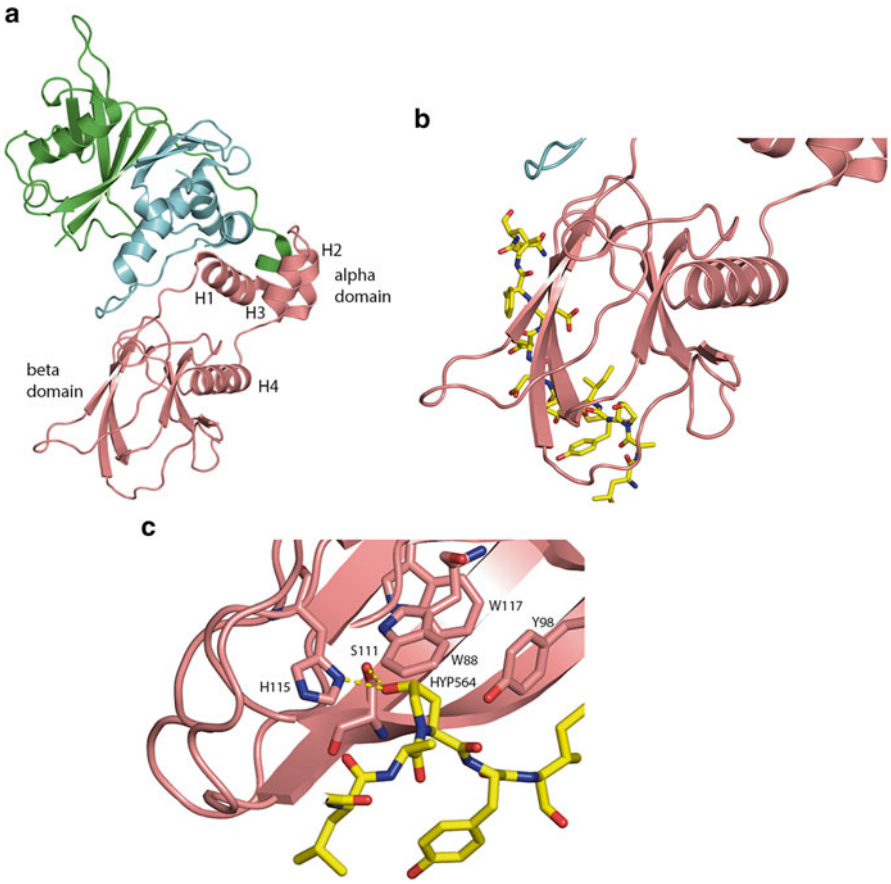

Fig. 4.2 Ribbon diagrams illustrating the secondary structure of the VHL-elongin C-elongin B complex and interactions with HIFα. (**a**) Secondary structure of the VHL-elongin C-elongin B complex. pVHL (*pink*) consists of two tightly coupled domains, α and β. The β domain consists of a 7-stranded β-sandwich and an α-helix and has the properties of a substrate docking site. The α domain, residues 155–192, consists of three α-helices and binds elongin C (*blue*). The H4 helix of elongin C fits into an extended groove formed by the H1, H2 and H3 helices of the VHL α domain. The VHL-elongin C complex nucleates a complex containing elongin B (*green*), cullin 2 (Cul2) (not shown) and RING finger protein Rbx1 (not shown) (VCB-CR complex). (**b**) The HIFα hydroxyproline binding pocket of pVHL. A 15-amino acid portion of HIFα (*yellow*) adopts an extended beta strand-like conformation and interacts exclusively with the beta domain of pVHL (*pink*) binding alongside the beta sandwich. (**b**) Key interactions between pVHL and the HIFα hydroxyproline (HYP). The hydroxyproline of HIF binds in a pocket on pVHL lined by residues W88, Y98, S111, H115 and W117. The hydroxyl group of S111 and the H115 imidazole amino group serve as hydrogen-bonding partners to the HYP564 hydroxyl group. All of the residues that form the pocket are frequently mutated in ccRCC

bind to hypoxia response elements that contain the consensus sequence 5′RCGTG-3′ [40]. Based on genome-wide chromatin immunoprecipitation combined with DNA sequencing or mRNA microarrays, the number of direct HIF target genes is currently greater than 800 [41, 42]; many of these genes promote adaptation to acute or

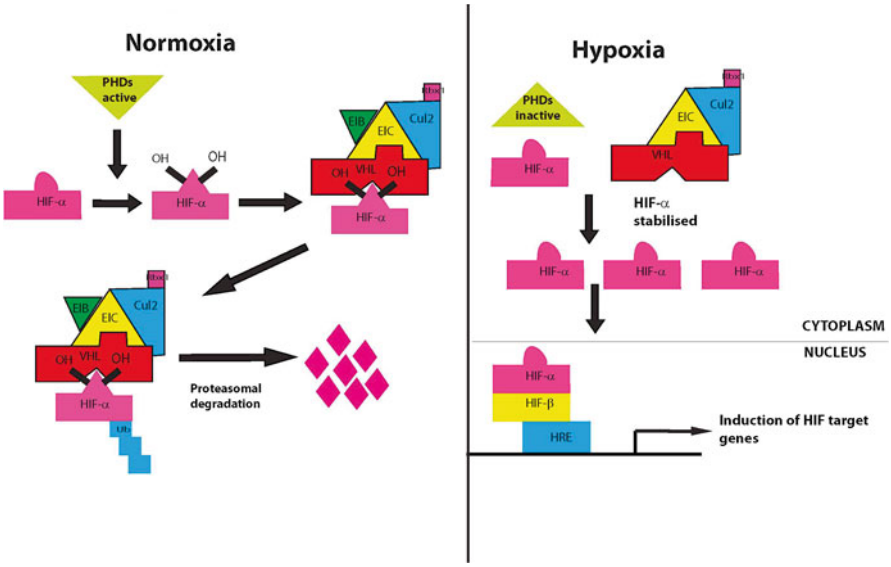

Fig. 4.3 Oxygen-dependent HIF regulation. In normoxic conditions, HIFα is hydroxylated by prolyl hydroxylases (PHDs) 1–3. Prolyl hydroxylated HIFα is recognised by the VHL-elongin C-elongin B-cullin 2-Rbx1 (VCB-CR) E3 ubiquitin ligase complex and targeted for ubiquitination and proteasomal degradation. In hypoxic conditions, prolyl hydroxylases 1–3 are inactive. HIFα therefore accumulates and forms heterodimers with HIF1β which translocate to the nucleus, bind to hypoxia response elements and induce transcription of genes involved in adaptations to hypoxia. Though HIF1α and HIF2α have significant overlap in function, they are not functionally redundant and activate different pathways to different extents

chronic hypoxia [43]. A list of selected HIF-induced genes with their functions is shown in Table 4.1. HIFs also indirectly regulate gene expression by transactivating genes encoding microRNAs [44] and chromatin-modifying enzymes [41, 43, 45].

HIF thus plays a critical role in cellular adaptation to reduced oxygen tension; functional pVHL is necessary to switch off this adaptation under normoxic conditions. The loss of pVHL function, occurring, for example, secondary to biallelic inactivation of the VHL gene, impairs HIFα destabilisation. This promotes inappropriate activation of downstream target genes which would normally only be activated under hypoxic conditions and thereby contributes directly to tumorigenesis. This phenomenon provides an explanation for why pVHL-defective tumours including haemangioblastomas, phaeochromocytomas and ccRCCs are sometimes associated with paraneoplastic erythrocytosis [46]. In keeping with the notion that the regulation of HIFα is the key tumour suppressor function of pVHL, a large proportion of disease-associated VHL mutations are predicted to and have been demonstrated to abolish the interaction between pVHL and HIF [34, 35, 47] (Table 4.4).

Other than HIFα, additional potential pVHL ubiquitination substrates, including atypical protein kinase C [48] and the large subunit of RNA polymerase II [49], have been described (reviewed within [32, 50–52]), though their significance in ccRCC tumorigenesis is uncertain.

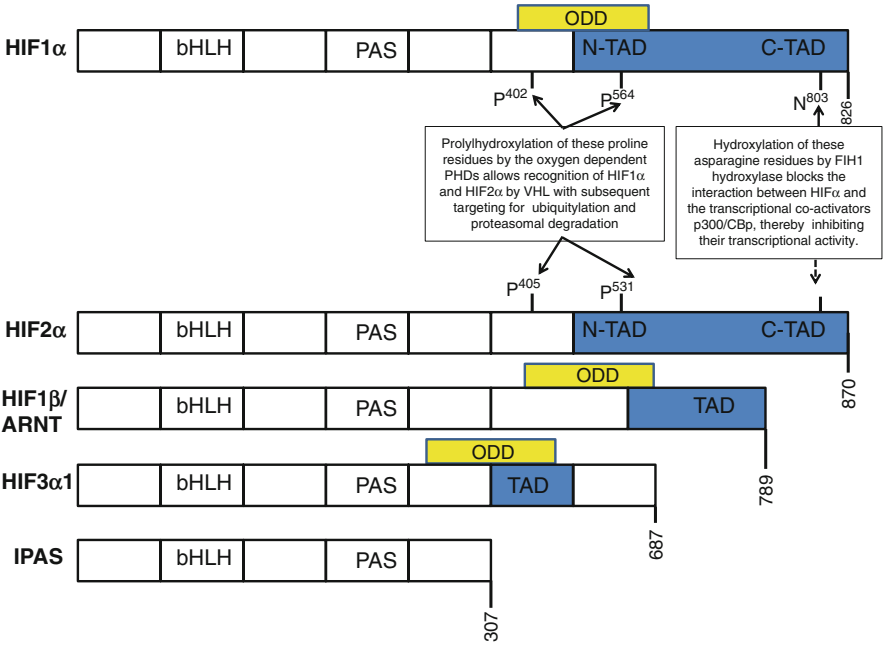

Fig. 4.4 HIF transcription factors. HIF proteins are members of the basic helix-loop-helix (bHLH per-Arnt-SIM (PAS) family of DNA-binding transcription factors. The bHLH and PAS domains are involved in DNA binding and heterodimerisation; the oxygen-dependent degradation (ODD) domain is required for oxygen-dependent hydroxylation and degradation; and the N-terminal and C-terminal transactivation domains (NTAD and CTAD, respectively) are required for transcriptional activation. HIF1α and HIF2α both have two transcriptional activation domains. HIF1β has just one transcriptional activation domain. The hydroxylation of conserved proline residues in the ODD of HIFα proteins by oxygen-dependent prolyl hydroxylase enzymes (PHDs) is required for pVHL to bind and degrade HIFα subunits under normoxic conditions. Hypoxia limits PHD activity. Hypoxia also inhibits hydroxylation of a conserved asparagine in the CTAD by factor-inhibiting HIF1 (FIH1); this blocks the interaction between HIFα and the transcriptional co-activators p300/CBp. FIH1 hydroxylates HIF2α at a lower efficiency (*broken arrow*) than HIF1α (*unbroken arrow*). HIF3α undergoes extensive mRNA splicing; many of the ensuing splice variants (e.g. IPAS) lack a transactivation domain and function as dominant-negative regulators of HIFs

The HIF Transcription Factors

Three HIFα family members (HIF1α, HIF2α, HIF3α) and two HIFβ family members (HIF1β and HIF2β) exist (HIFβ is often referred to as ARNT [aryl hydrocarbon receptor nuclear translocator]) (reviewed in [40]). While HIF1α is ubiquitously expressed, the expression of HIF2α is restricted to endothelial, lung, renal and hepatic cells. HIF proteins are part of the basic helix-loop-helix PER-ARNT-SIM (PAS) family of DNA-binding transcription factors (Fig. 4.4). HIF1α and HIF2α both have two transcriptional activation domains: the N-terminal transactivation domain (NTAD) and the C-terminal transactivation domain (CTAD) [52, 53].

Table 4.1 Selected shared and unique target genes regulated by HIF1**α** and HIF2**α** in ccRCC

Gene	Protein	Function	HIF1α	HIF2α	Reference
GLUT1	Thioredoxin-interacting protein	Glucose transport	+	+	[69]
ADRP	Adipose differentiation-related protein	Lipid metabolism	+	+	[69]
CAIX	Carbonic anhydrase IX	pH homeostasis	+	+	[69]
IL-6	Interleukin-6	Immune cytokine	+	+	[69]
ADM1	Adrenomedullin 1	Angiogenesis	+	+	[69]
VEGF	Vascular endothelial growth factor	Angiogenesis	+	+	[69]
BNIP3	BCL2/adenovirus E1B-interacting protein 3	Autophagy, apoptosis	+	–	[70]
HK2	Hexokinase 2	Glycolysis	+	–	[69]
PFK	Phosphofructokinase	Glycolysis	+	–	[69]
ALDA	Aldehyde dehydrogenase	Glycolysis	+	–	[69]
PGK1	Phosphoglycerate kinase 1	Glycolysis	+	–	[69]
LDHA	Lactate dehydrogenase A	Glycolysis	+	–	[69]
EPO	Erythropoietin	Erythropoiesis	–	+	[83]
TGFα	Transforming growth factor α	Growth factor/ angiogenesis	–	+	[70]
CCND1	Cyclin D1	Cell cycle progression	–	+	[70]
REDD1	DNA-damage-inducible transcript	Cell growth, proliferation and survival	_	–	[75]
TXNIP	Thioredoxin-interacting protein	Cell growth, cell cycle progression	+	–	[75]

+ stimulates – no effect

In contrast, HIF3α undergoes extensive mRNA splicing; many of the ensuing splice variants lack a transactivation domain and can competitively inhibit transcriptional activation by HIF1α and HIF2α [54–57], although little is yet known about the impact of HIF3α on hypoxic tumour progression.

HIF1α, but not HIF2α, can also be recognised by at least two hydroxylation-insensitive ubiquitin ligase complexes that do not contain pVHL [58, 59] (Fig. 4.5). Firstly, HIF-associated factor (HAF) binds and destabilises HIF1α under normoxic and hypoxic conditions in a pVHL-independent, proteasome-dependent manner, but has no effect on HIF2α levels. Instead, HAF binds HIF2α at a distinct C-terminal region and promotes HIF2α transcriptional activity, effectively switching cells from an HIF1α to an HIF2α transcriptional programme. Secondly, heat shock protein 70 (HSP70) and carboxyl terminus of Hsc70-interacting protein (CHIP), a recently identified E3 ubiquitin ligase, bind and degrade HIF1α (but not HIF2α) under conditions of prolonged hypoxia in cultured cells [58, 60]. In addition, the hydroxylation of HIFα can occur on a conserved asparaginyl residue within the CTAD by the asparaginyl hydroxylase factor-inhibiting HIF1 (FIH1). This hydroxylation prevents its interaction with the transcriptional co-activator p300 and thereby impairs CTAD activity [61–63]. Though the asparaginyl hydroxylation reaction also requires

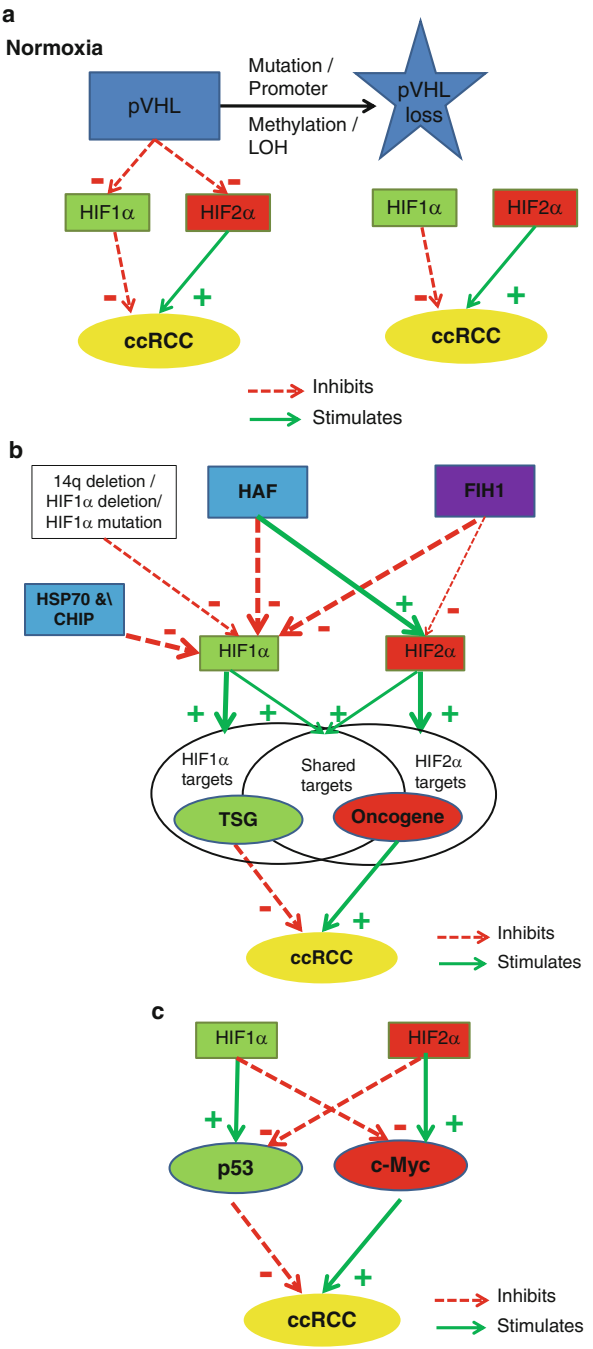

Fig. 4.5 Role of VHL, HIF1α and HIF2α in clear cell renal cell carcinoma. (**a**) In normoxic conditions, pVHL inhibits activity of both HIF1α and HIF2α by targeting them for ubiquitination and degradation. Evidence suggests that on balance, HIF2α is a renal oncoprotein which promotes

molecular oxygen, FIH1 remains active at intermediate levels of hypoxia which would render the prolyl hydroxylases inactive [64]. On the whole, FIH1 seems to play a role in fine-tuning the hypoxic response [65, 66]. The HIF1α CTAD is more sensitive to FIH1 than the HIF2α CTAD [67, 68]. Consequently, different HIF target genes exhibit different sensitivities to FIH1 inhibition, presumably resulting from their relative dependency on HIF1α versus HIF2α, and/or on NTAD versus CTAD activity. Interestingly, some HIF target genes are induced by HIF in a broad variety of cells and tissues, while others are more constrained. For example, the expression of erythropoietin in adults is principally restricted to specialised cells in the kidney.

HIF2α Is More Oncogenic than HIF1α

Both HIF1α and HIF2α are stabilised and activated by hypoxia and dimerise with HIF1β. Likewise, both isoforms activate transcription of target genes by binding to the same hypoxia response element. However, although significant overlap in their function exists, HIF1α and HIF2α are not functionally redundant. Array studies indicate that while HIF1α induces apoptotic pathways not targeted by HIF2α and preferentially drives the expression of genes involved in the glycolytic pathway, HIF2α preferentially promotes growth and angiogenesis [69–71]. Furthermore, the relative contributions of the two paralogs to the control of specific HIF target genes can differ in different cellular contexts. For example, VEGF is primarily regulated by HIF2α in pVHL-defective renal carcinoma cells but by HIF1α in breast cancer cells [71].

Moreover, in vitro and cell line xenograft studies suggest that although HIF2α is both necessary and sufficient for the growth of transformed RCC cell lines, HIF1α is not (reviewed in [72, 73]). There are four lines of evidence for this. First, all VHL null ccRCC cell lines examined to date express HIF2α, while not all express HIF1α [74, 75]. Second, the downregulation of HIF2α expression (using short hairpin

Fig. 4.5 (continued) growth of ccRCC, while HIF1α is a renal tumour suppressor which inhibits growth of ccRCC. Biallelic loss of VHL secondary to a combination of mutation, promoter methylation and/or LOH results in increased HIF1α and HIF2α levels. (**b**) Loss of HIF1α's tumour-suppressive activity relative to HIF2α's oncoprotein activity may result from the following: (1) Reduced HIF1α expression or activity secondary to 14q deletion, deletion of the HIF1α locus or loss-of-function mutations in HIF1α. (2) The greater sensitivity of HIF1α to FIH1 compared to HIF2α. HIF1α would therefore theoretically be silenced by FIH1 in VHL null cells unless those cells were profoundly hypoxic. (3) HIF-associated factor (HAF) binds and destabilises HIF1α but promotes HIF2α activity. (4) Heat shock protein 70 (HSP70) and carboxyl terminus of Hsc70-interacting protein (CHIP) bind and degrade HIF1α but not HIF2α. The gene sets regulated by HIF1α and HIF2α overlap but are not entirely congruent. HIF1α targets may be biased towards ccRCC tumour suppressors, while HIF2α targets might be biased towards ccRCC oncoproteins. (**c**) Alternatively, it is possible that the differences between HIF1α and HIF2α result from differential regulation of HIF target genes. For example, in some systems, while HIF1α suppresses c-Myc activity, HIF2α enhances c-Myc activity. Similarly, HIF1α enhances and HIF2α suppresses p53 function

RNAs delivered by a viral vector) in human VHL null RCC cells is sufficient to prevent tumour formation in nude mice [76, 77]. Conversely, the overproduction of HIF2α but not HIF1α can override pVHL's tumour suppressor activity in such xenograft assays [70, 76, 78]. Third, in animal models, HIF2α variants that lack prolyl hydroxylation sites (and cannot therefore be targeted for ubiquitination by the E3 ubiquitin ligase complex) prevent tumour inhibition by pVHL, whereas analogous HIF1α variants do not [78, 79]. Finally, the levels of HIF2α are highest in cells engineered to produce variants of pVHL associated with type 1 and type 2B VHL disease (associated with a high risk of RCC), intermediate in cells with forms of pVHL linked with type 2A VHL disease (associated with a low risk of RCC) and essentially normal in cells with pVHL mutations seen in type 2C disease (which are not associated with ccRCC) [80, 81]. HIF2α seems to be both necessary and sufficient for much of the pathology that has been described in genetically engineered mouse models in which VHL has been inactivated in specific tissues [82–86]. Interestingly, HIF2α polymorphisms have recently been linked to the risk of developing kidney cancer in the general population [87].

HIF1α Is a Renal Tumour Suppressor Gene

Four further lines of evidence suggest that HIF1α is not merely dispensable in the context of ccRCC but actually functions as a tumour suppressor gene. First, targeted exon sequencing of ccRCC has shown rare inactivating mutations in HIF1α [88], and copy-number analyses of RCC cell lines and primary tumours suggest that the HIF1α locus is frequently lost, either alone or along with the long arm of chromosome 14 (14q) where the HIF1α gene resides [75]. The loss of chromosome 14q in this setting is associated with a poor prognosis [89, 90]. In other cases, alternative mRNA splicing around deleted HIF1α exonic sequences leads to the production of aberrant HIF1α isoforms [75]. Second, though all VHL-defective ccRCCs overexpress HIF2α, approximately one third of these tumours also lack HIF1α expression [74]. Third, functional in vitro and in vivo studies suggest that the overexpression of HIF1α in VHL wild-type cells restrains tumour growth, whereas the suppression of HIF1α in VHL-deficient cells enhances tumour growth [75, 91]. Finally, though uncommon, HIF1α mutations have been described in ccRCC. These include missense mutations which compromise HIF1α's ability to suppress proliferation when reintroduced into ccRCC cell lines that lack endogenous, wild-type, HIF1α [75, 77, 88].

In keeping with these findings, many ccRCCs produce no, or very low, levels of HIF1α, and 14q-deleted tumours exhibit a transcriptional signature indicative of decreased HIF1α activity [74, 75, 92]. However, in contrast to ccRCC cell lines, ccRCC tumours often appear to retain a wild-type HIF1α allele [75], suggesting that HIF1α haploinsufficiency is sufficient to promote tumorigenesis in vivo. Since many ccRCC cell lines are established from metastatic lesions, it is possible that reduction to nullizygosity is a late event in renal carcinoma and thus underrepresented in primary tumours, particularly in patients with early disease who have undergone a nephrectomy with curative intent.

Collectively, these observations suggest that while HIF1α is a tumour suppressor gene in renal cancer development and is one of the relevant targets of the 14q deletions that are typical of ccRCC, HIF2α is the key driver of renal cancer progression.

Differences Between HIF1α and HIF2α

Explanations for the difference in oncogenicity between HIF2α and HIF1α may relate to the relative resistance of the HIF2α CTAD to FIH1 compared with the HIF1α CTAD; HIF2α may be able to escape from proteins such as FIH1 that would otherwise limit HIFα activity in cells lacking VHL (Fig. 4.5). In contrast, the HIF1α CTAD would theoretically be silenced by FIH1 in VHL null ccRCC cells, unless the cells were severely hypoxic. A transcriptionally inactive HIF1α could, in principle, act as a dominant negative, by competitively displacing HIF2α, which is relatively insensitive to FIH1, from specific HIF target genes. In support of this theory, some HIF target genes are paradoxically increased when HIF1α is downregulated in VHL null RCC cells [70], and the HIF2α NTAD and CTAD cooperate to promote renal tumorigenesis in vivo [68]. The differential sensitivities of HIF1α and HIF2α to HAF, HSP70 and CHIP may also play a role.

Alternatively, it is possible that some genes that are preferentially activated by HIF2α relative to HIF1α are particularly oncogenic. One which has attracted significant interest relates to the opposing roles of HIF1α and HIF2α in the regulation of c-Myc activity; while HIF1α suppresses c-Myc activity, HIF2α promotes the transactivation or transrepression of c-Myc-specific target genes [74, 93, 94]. In keeping with this notion, RCC tumours that exclusively express HIF2α have increased proliferation rates [74]. Intriguingly, a subset of ccRCC tumours seem to have copy-number amplification of 8q24 where c-Myc resides [95].

Similarly, HIF target genes that are regulated primarily by HIF1α may suppress ccRCC growth. For example, HIF1α enhances and HIF2α suppresses p53 function [96, 97]. Other potential tumour suppressor genes that are regulated by HIF1α in VHL null ccRCCs include BNIP3, REDD1, TXNIP and ZAC1 [75]. Interestingly, ZAC1 maps to chromosome 6q23, which is often deleted in VHL-associated renal cancers, haemangioblastomas and phaeochromocytomas. Other potential tumour suppressor genes that are regulated by HIF1α in VHL null ccRCCs include BNIP3, REDD1, TXNIP and ZAC1 [75]. Interestingly, ZAC1 maps to chromosome 6q23, which is often deleted in VHL-associated renal cancers, haemangioblastomas and phaeochromocytomas [98, 99].

*HIF-Independent Functions of p*VHL *(Table 4.2)*

Despite pVHL's well-characterised role in targeting HIFs for polyubiquitination and proteasomal degradation, evidence has accrued to indicate that pVHL also has functions independent of HIF1α and HIF2α that may be important for its tumour suppressor action. These include the assembly and regulation of the extracellular

Table 4.2 Mechanisms that involve pVHL

	HIFα dependent	HIFα independent
Angiogenesis	Vascular endothelial growth factor	NA
	Platelet-derived growth factor	
	Adrenomedullin 1	
	And many others	
Glucose uptake and metabolism	Glucose transporters 1 and 3	NA
	Hexokinase 2	
	Aldehyde dehydrogenase	
	Phosphoglycerate kinase 1	
	Lactate dehydrogenase A	
	Phosphofructokinase 1 Pyruvate dehydrogenase	
	And many others	
Chemotaxis	Stromal-cell-derived factor 1/ CXC chemokine receptor 4	NA
Cell proliferation and survival	Transforming growth factor α, epidermal growth factor receptor	NA
Regulation of extracellular pH	Carbonic anhydrase IX	NA
Assembly and regulation of the extracellular matrix	E-cadherin	Interacts directly with fibronectin and collagen IV
		Regulation of adherens and tight junctions and integrins
	Matrix metalloproteinases	Regulation of matrix metalloproteinases
Microtubule stabilisation and maintenance of the primary cilium	NA	Associates with and stabilises microtubules
Regulation of apoptosis	p53	Increase p53 transcriptional activity
	NF-κB	Modulation of NF-κB activity
	BCL2/adenovirus E1B-interacting protein 3	Downregulation of JUNB (which is known to blunt neuronal apoptosis during NGF withdrawal)*
Control of cell senescence	NA	Dependent on the retinoblastoma protein and the SWItch/Sucrose NonFermentable SWI2/SNF2 chromatin remodeller p400
Transcriptional regulation	NA	Mediates the ubiquitination of the large subunit of RNA polymerase II in response to oxidative stress
		Controlling influence on HuR
		Binds specificity protein 1 transcription factor
Erythropoiesis	Erythropoietin	NA
Cell cycle progression	Cyclin D1	
Lipid metabolism	Adipose differentiation-related protein	

NF-κB nuclear factor kappa-light-chain-enhancer of activated B cells
*Dysregulation of this pathway is speculated to be important in the pathogenesis of phaeochromocytomas.

matrix, microtubule stabilisation and maintenance of the primary cilium, regulation of apoptosis, control of cell senescence and transcriptional regulation (Table 4.2).

These roles are less thoroughly characterised than those that involve HIFs, and many have been discovered through biochemical interactions. Nonetheless, gene expression studies also support the notion that there are HIF-independent gene expression changes induced by VHL loss [100, 101], though to what extent the HIF-independent functions of pVHL cooperate with HIF dysregulation in ccRCC tumorigenesis currently remains unknown.

VHL and Regulation of the Extracellular Matrix

One of the better characterised, though still incompletely understood, HIFα-independent roles of pVHL is its effect on the assembly of the extracellular matrix (ecm). The ecm is a complex structural component, composed of proteoglycans, hyaluronic acid and glycoproteins such as fibronectin and collagen [102]. The disruption of its architecture has been associated with tumour growth, angiogenesis and metastasis, and pVHL plays an important role in its regulation. pVHL interacts directly with fibronectin and collagen IV, and all pVHL mutants tested to date fail to bind fibronectin and collagen IV and lose the ability to assemble an ecm [80, 81, 103–108]. The inability of VHL-deficient cells to bind ecm components is not mediated by HIF [103, 107, 109] and does not require binding to the other components of the pVHL complex such as elongins B and C and cullin 2.

The inactivation of the VHL-ecm assembly pathway results in highly vascularised tumours with a remodelled fibronectin and collagen IV matrix and increased invasive capacity [106], and it has been speculated that ecm remodelling may promote angiogenesis by providing a path for blood vessels to infiltrate tumours, thereby supporting tumorigenicity. This is in contrast to inactivation of the VHL-*HIFα* regulation pathway, which results in tumours with high VEGF levels but decreased angiogenesis, a tightly assembled fibronectin and collagen IV matrix and low invasive capacity.

The interaction of pVHL with fibronectin is mediated by pVHL neddylation [108] which acts as a molecular switch in conferring selectivity to fibronectin binding over CUL2 [110]. The interaction of pVHL with collagen IV is dependent on endoplasmic reticulum hydroxylation [105] and is competed by hydroxylated, but not unmodified, HIF1α peptides, implying that this interaction requires the hydroxyproline binding pocket in the β domain of pVHL. pVHL does not affect fibronectin and collagen IV production or secretion and does not result in collagen IV proteasomal degradation [105, 106].

It is well recognised that tumour cell invasion is dependent on the adhesion and proteolytic remodelling of the ecm, and it is now known that pVHL influences both these processes. VHL promotes E-cadherin transcription through HIF-dependent mechanisms [104], and inactivation of pVHL results in downregulation of the adherens junction protein E-cadherin [111]. VHL inactivation also leads to downregulation

of the tight junction proteins occludin and claudin in an E-cadherin-independent manner [112], and pVHL has been reported to downregulate integrins in an HIFα-independent manner [113]. As a consequence of these mechanisms, pVHL inactivation results in the disruption of both adherens and tight junctions. Cells lacking pVHL also fail to form β1 fibrillar adhesions, which may contribute to the increased cell motility and invasiveness seen in the absence of a functional pVHL [114].

VHL also regulates the matrix metalloproteinases, a family of matrix-degrading enzymes involved in ecm turnover. Firstly, HIF2α has been shown to influence RCC cell invasiveness by regulating membrane type 1 MMP expression [115, 116]. Secondly, the loss of VHL-ECM pathway regulation in RCC cells results in increased cell invasiveness and activation of MMP-2 [106]. Compared to VHL WT ccRCC cell lines, VHL null ccRCC cells show increased invasiveness in growth factor-reduced Matrigel, overproduce MMP-2 and MMP-9 and display an extensive branching morphogenesis phenotype in response to hepatocyte growth factor/scatter factor [117]. MMP-induced proteolytic remodelling of the ecm has been shown to expose cryptic sites in collagen IV which are required for in vivo angiogenesis [118].

As yet, the precise mechanisms of the interplay between pVHL, the ecm and suppression of tumorigenesis, angiogenesis and invasiveness are not resolved. In particular, how an intracellular protein such as pVHL can modulate the assembly of the extracellular ecm components remains to be elucidated. pVHL may mediate fibronectin and collagen IV modification to allow their proper assembly into the ecm. Loss of these interactions resulting from loss of VHL would lead to an aberrant ecm, activation of MMPs, release of ecm-sequestered growth factors and stimulation of tumorigenesis, angiogenesis and invasion. The disruption of integrins and cell adhesion molecule regulation would further enhance the invasive RCC phenotype. In principle, a more complete understanding of the mechanisms of ecm regulation by pVHL could lead to novel therapies for patients with ccRCC, though extensive future work in this field is required.

Microtubule Stabilisation and Maintenance of the Primary Cilium

pVHL can also associate with microtubules [119]. This association has been reported to result in microtubule stabilisation [119] and directional growth of microtubules towards the cell periphery [120] and appears to be independent of pVHL's ability to downregulate HIF or its ubiquitin ligase function. In turn, this may explain the loss of primary cilia seen in renal cysts from VHL disease patients and ccRCC cell lines devoid of functional pVHL [120–122]. The primary cilium is a specialised structure on the cell surface that acts as an antenna of the cell and regulates the transduction of both chemical and mechanical signals [123]. The ciliary axoneme is composed of microtubules that are arranged out from the basal body or mother centriole; thus, microtubule dynamics and formation and maintenance of the primary cilium are intimately linked.

An interaction between pVHL and the Par3-Par6-atypical protein kinase C polarity complex has been suggested as a mechanism for linking polarity pathways to microtubule capture and ciliogenesis [120]. The phosphorylation of pVHL by glycogen synthase kinase 3β (GSK3β) has been reported to prevent pVHL from stabilising microtubules, without disrupting their interaction with pVHL [119]. This phosphorylation occurs on Ser68, after a priming phosphorylation at Ser72 by an unidentified kinase. One hypothesis is that GSK3β maintains cilia independently of pVHL. However, when GSK3β is inactivated, pVHL is active and can regulate the microtubules and primary cilia independently of GSK3 [119]. Interestingly, a pVHL variant with phosphomimetic substitutions at Ser68 and Ser72 was also impaired with respect to HIF polyubiquitination, suggesting GSK3β may regulate more than one pVHL function. In keeping with the notion that GSK3β and pVHL redundantly maintain primary cilia, it appears that the PTEN tumour suppressor protein cooperates with pVHL to suppress cyst development in the kidney [124]; the combined loss of VHL and PTEN in a genetically engineered mouse model cooperate to promote renal and genital tract cysts. pVHL's effects on microtubule dynamics appear to be HIF independent, though some studies suggest that HIF dysregulation may play at least a partial role in the loss of microtubule stability imparted by VHL inactivation [121, 122, 125].

Surprisingly, pVHL's ability to stabilise microtubules is lost in VHL mutations that predispose to the development of haemangioblastomas and phaeochromocytomas, but not those associated with the development of ccRCC [119]. This apparent paradox, whereby VHL mutants predisposing to RCC maintain the ability to regulate microtubule dynamics, is perplexing. It has been suggested that renal cysts which develop secondary to the loss of primary cilia on renal tubular cells lack significant malignant potential and that the majority of ccRCCs associated with VHL disease may arise without a preceding cystic phase [126]. This speculation is not proven, though, to some extent, it is in keeping with the observation that patients with polycystic kidney disease, despite having large numbers of renal cysts, are not clearly at a significantly higher risk of developing ccRCC [127].

Regulation of Apoptosis

In comparison to the majority of other tumour types, ccRCCs are insensitive to cytotoxic chemotherapies. Failure of cytotoxic chemotherapy is tightly associated with failure of p53-mediated apoptosis [128]. However, p53 mutations or loss is rare in ccRCC [129–131]. Subsequently, groups have considered whether HIF or pVHL is able to influence p53 function or activate alternative anti-apoptotic pathways in ccRCC.

Indeed, pVHL loss has been demonstrated to result in p53 inactivation by both HIF-dependent and HIF-independent effects. Firstly, HIF can directly bind to and modulate p53 activity [132–134]. Secondly, pVHL has been shown to directly associate with and stabilise p53 by suppressing Mdm2-mediated ubiquitination and

nuclear export of p53 and by subsequently recruiting p53-modifying enzymes, resulting in an increase in its transcriptional activity [135]. VHL-deleted RCC cells show attenuated apoptosis and abnormal cell cycle arrest upon DNA damage, which normalises on restoration of pVHL [136].

Resistance to chemotherapy-induced apoptosis is also mediated through the nuclear factor κB (NF-κB) pathway. pVHL has been shown to facilitate TNFα-induced cytotoxicity in RCC cells, at least in part, through the downregulation of NF-κB activity and subsequent attenuation of anti-apoptotic proteins c-FLIP, survivin, c-IAP-1 and c-IAP-2 [137, 138]. pVHL's effect on NF-κB is at least in part dependent on HIF signalling [139]. In addition, pVHL can modulate NF-κB activity directly by serving as an adaptor that promotes the inhibitory phosphorylation of the NF-κB agonist CARD9 by casein kinase 2 [140].

Interestingly, 11 % of apparently sporadic phaeochromocytomas (defined by a lack of a family history or a spectrum of tumours suggestive of VHL disease) are actually due to occult germline mutations of VHL [141]. However, somatic VHL mutations are uncommon in truly sporadic phaeochromocytomas. Furthermore, type 2C VHL disease mutations (which are associated only with phaeochromocytomas and not with other tumour types) retain their ability to downregulate HIFα [80, 81], suggesting that the development of VHL-associated phaeochromocytomas is related to an HIF-independent function of pVHL. Phaeochromocytomas derive from sympathetic neuronal precursor cells, many of which undergo c-Jun-dependent apoptosis during normal development as nerve growth factor (NGF) becomes limiting. Phaeochromocytoma-associated VHL mutations result in the HIF-independent accumulation of JUNB, which is known to blunt neuronal apoptosis during NGF withdrawal [142]. Failure of developmental apoptosis may thus play a role in the development of phaeochromocytomas in patients inheriting phaeochromocytoma-associated VHL mutations.

Control of Cell Senescence

Cellular senescence is the phenomenon of irreversible growth arrest in response to DNA damage and is an important in vivo tumour suppressor mechanism [143]. Studies have shown that the stabilisation of HIF occurring as a consequence of physiological oxygenation can extend the replicative lifespan of cells in culture [144, 145]. However, acute VHL inactivation, which would also result in the stabilisation of HIFα, has been shown to cause a senescent-like phenotype in vitro and in vivo [146]. Interestingly, this phenotype was independent of p53 and HIF but dependent on the retinoblastoma protein (Rb) and the SWItch/Sucrose NonFermentable SWI2/SNF2 chromatin remodeller p400. This finding is somewhat surprising, since the induction of senescence would be expected to restrict the development of renal carcinoma in vivo. A subsequent study demonstrated that the induction of senescence secondary to VHL loss occurs under atmospheric conditions (21 % O_2), but not under physiological oxygenation (2–5 % O_2), suggesting

that VHL inactivation sensitises cells to oxidative stress [147]. The authors suggest that in vivo oxygenation may promote a tolerance of VHL loss in renal epithelia, which may allow cells to progress further towards a transformed state.

Transcriptional Regulation

VHL has been shown to mediate the ubiquitination of the large subunit of RNA polymerase II, Rpb1, in response to oxidative stress, in a manner dependent on the hydroxylation of a specific proline [49, 148]. VHL has also been suggested to regulate transcription through a controlling influence on the RNA-binding protein HuR [149–151] and has been reported to bind the SP1 transcription factor [152–154].

VHL *Proteostasis*

It has long been known that the proper folding and functionality of pVHL requires its tight association with elongins B and C to form the VCB complex and that failure of correct folding and interaction with elongins B and C results in the proteolytic degradation of pVHL [29].

Following synthesis on ribosomes, nascent VHL is shuttled from the ribosomal machinery with the assistance of heat shock protein 70 (HSP70) [155]. Formation of the VCB complex is then mediated by the chaperonin TCP-1 ring complex (TRiC; also called chaperonin-containing TCP-1 [CCT]) [155], a hetero-oligomeric complex which consists of two stacked rings with a central chamber in which unfolded polypeptides bind and fold [156]. TRiC facilitates VHL folding, thereby enabling its association with elongins B and C to form the VCB complex which develops while VHL is bound to TRiC [155]. Upon the formation of a mature VCB complex, pVHL is released from TRiC.

The binding of pVHL to TRiC occurs at amino acids 114–119 and 148–155 (called Box 1 and Box 2, respectively) [157]. These two motifs are located in adjacent strands of the β domain, and both harbour tumour-associated mutations (e.g. W117A) that disrupt the association of pVHL with TRiC and lead to misfolding of newly translated pVHL and the absence of a mature VCB complex in the cell [158, 159]. Failure to generate a properly folded pVHL or a mature VCB results in the degradation of pVHL through the ubiquitin-proteasome system. pVHL degradation specifically requires another chaperone, Hsp90, which does not participate in pVHL folding [160]. Since distinct chaperone pathways mediate the folding and quality control of pVHL, an enhanced understanding of the mechanisms by which destabilised pVHL mutants are targeted for proteasomal degradation may lead to strategies for refolding and stabilisation of pVHL, to allow its incorporation into the VCB complex and potential restoration of its tumour suppressor activity. Bortezomib and MG132 are both capable of increasing VHL levels, and a cell-based Prestwick

compound screen has identified several compounds that upregulate VHL-W117A in VHL-W117A-infected cell lines [161]. Further work is underway to analyse the functional consequence of pVHL upregulation using these compounds, as well as attempt to identify new compounds which rectify the interaction between point-mutated pVHL and the chaperones and chaperonins, and it is conceivable that such compounds may resuscitate the function of pVHL and thereby alter the disease phenotype and provide clinical benefit for patients with lesions possessing certain missense VHL mutations.

Genotype-Phenotype Correlations in Von Hippel-Lindau Disease

In VHL disease, there is clear evidence for strong genotype-phenotype correlations with specific classes of VHL mutations predisposing to different spectrums of morbidity and mortality (reviewed within [1], Table 4.3). While true null VHL alleles (i.e. large genomic deletions, frameshift mutations or nonsense mutations) are associated with a low risk of phaeochromocytoma (type 1 VHL disease), the majority of VHL mutations identified in families with an increased risk of phaeochromocytoma (type 2 VHL disease) are missense mutations. Type 2 VHL disease is further subdivided into type 2A (low risk of ccRCC), type 2B (high risk of ccRCC) and type 2C (phaeochromocytoma but no other manifestations of VHL disease). Subsequent analysis has suggested that surface amino acid substitutions confer a higher phaeochromocytoma risk than substitution of amino acids buried deep within the protein core [162].

Furthermore, the risk of developing ccRCC in VHL disease appears to be linked to the degree to which HIF activity is compromised ([47, 80, 81, 163, 164]. While type 1 and type 2B mutations (which are associated with a high risk of developing ccRCC) are grossly defective with respect to HIF regulation, type 2A mutations

Table 4.3 Genotype-phenotype correlations in VHL disease

VHL disease subtype	Clinical phenotype	Type of VHL mutation	HIF expression relative to wild type
1	ccRCC	Deletion, nonsense, frameshift (occasionally missense)	↑↑↑
	Haemangioblastoma		
2A	Haemangioblastoma	Missense	↑
	Phaeochromocytoma		
2B	ccRCC	Missense	↑↑
	Haemangioblastoma		
	Phaeochromocytoma		
2C	Phaeochromocytoma	Missense	Normal
Chuvash hereditary polycythaemia	Hereditary polycythaemia	Homozygous for VHL 598C>T (pVHL Arg200Trp) mutation	↑
	No elevated cancer risk		

ccRCC clear cell renal cell carcinoma, ↑ increased relative to wild type

(associated with a low risk of ccRCC) appear to be far less compromised with respect to HIFα regulation. Perhaps the clearest demonstration of this phenomenon is seen by undertaking a detailed biochemical analysis of the two most frequent type 2A mutations, Y98H and Y112H, in comparison to type 2B mutations in the same residues, Y98N and Y112N [47]. While none of these mutations affected the assembly of the VCB complex, the type 2A mutant proteins exhibited higher stabilities at physiological temperature and higher binding affinities for HIF1α compared with the type 2B mutant proteins. Consistent with these results, the type 2A but not type 2B mutant VHL proteins retained significant ubiquitin ligase activity towards HIF1α in vitro [47].

Fascinatingly, individuals with homozygosity for the germline R200W VHL mutation develop Chuvash polycythaemia, a rare benign congenital erythrocytosis with no associated cancer risk [165, 166].

*Therapeutic Implications of p*VHL *in ccRCC*

It has long been known that angiogenesis is a critical component for malignant tumour progression [167]. This observation coupled with the hypervascular nature of ccRCCs led to intense interest in the molecular mechanisms of angiogenesis in ccRCCs. It is in this context that the discovery of the VHL gene and the identification of its critical role in regulating the HIF-mediated response to hypoxia have facilitated the dramatic recent shift in paradigm for the treatment of ccRCC. As described earlier, the inactivation of VHL triggers pro-angiogenic mechanisms through the activation of HIF with subsequent activation of transcription of many pro-angiogenic factors including the vascular endothelial growth factor (VEGF) family of proteins and TGFα. Numerous subtypes of VEGF exist (including VEGF-Agreed, VEGF-B, VEGF-C, VEGF-D, VEGF-E and placenta growth factor-1) (reviewed within [168]). Most of these are regulated by pVHL and HIF and play key roles in angiogenesis by binding to cell membrane-associated tyrosine kinases, the VEGF receptors. The binding of VEGF ligand to its receptor initiates the activation of downstream signalling pathways which ultimately lead to endothelial cell activation, proliferation, migration and survival.

Drugs which modulate the VHL-HIF-VEGF pathway have proven benefit in treating ccRCC and are now the standard of care for patients with metastatic disease, with established superiority over cytokine therapies (reviewed within [72, 169–171]. Such drugs include sunitinib, sorafenib, pazopanib and axitinib (multiple tyrosine kinase inhibitors which inhibit the VEGFRs among others), inhibitors of the mTOR pathway such as temsirolimus and everolimus and the monoclonal anti-VEGF antibody bevacizumab. The mechanisms of action, benefits and toxicities of these drugs are detailed further in chapter x.

In general, these targeted therapies increase progression-free survival (PFS) compared with placebo or immunotherapy [171]. However, very few trials have performed a direct head-to-head comparison of different targeted therapies or

investigated which agents are most suitable for particular patient types, for example, patients at different levels of risk or different ages. The development of validated predictive and prognostic markers would be particularly valuable to optimise clinical management strategies and enhance clinical efficacy and cost-effectiveness. In ccRCC, the most widely used prognostic and predictive tools are based around the Motzer criteria or the Memorial Sloan Kettering Cancer Center risk status [172], which were developed in the immunotherapy era. In 2009, Heng et al. [173] conducted a large multi-centre study of 645 patients to better define the prognostic indicators for overall survival in mccRCC patients treated with VEGF-targeted therapy. They showed that six factors (performance status, haemoglobin levels, serum calcium concentration, time from diagnosis, neutrophil count and platelet count) could segregate patients into three prognostic categories: favourable, intermediate and poor prognosis groups. This model has since been externally validated and performs favourably compared with the MSKCC model [174]. However, even this model performs only moderately well (concordance index for overall survival 0.71), begging the question as to whether it is possible to achieve better discrimination in outcomes.

On this note, it is highly likely that the addition of patient-specific or tumour-specific genetic biomarkers could help. These biomarkers can be broadly classified as prognostic markers (those mainly associated with the course or outcome of a disease) or predictive markers, which can be used to identify subpopulations of patients who are most likely to respond to a given therapy. Increasingly, treatments for other cancers are targeted to patients with alterations in specific molecular pathways known to be important in the pathogenesis of these tumours (reviewed within [175]). For example, a breast cancer with amplification of *HER2* might be treated with the anti-HER2 monoclonal antibody trastuzumab or the HER2 tyrosine kinase inhibitor lapatinib. In contrast, there are currently no validated molecular markers used to guide therapeutic decisions in ccRCC. We now discuss whether VHL mutational status may be a clinically useful genetic biomarker in sporadic ccRCC.

Functional Loss of VHL *in Sporadic ccRCC*

The reported incidence of somatic VHL gene mutations in sporadic ccRCC varies from 18 % to 91 % in various studies [79, 176–191]. The reported frequency on COSMIC at the time of writing is 1,485/3,479 ccRCC samples (46.8 %), though this includes many older studies which used less sensitive sequencing methods [129]. In addition, the methylation of VHL resulting in gene silencing occurs in between 5 and 30 % of sporadic ccRCC cases, and LOH occurs in up to 98 % of sporadic ccRCC cases [7, 188]. The wide variation in the reported prevalence of mutations may be explained by numerous confounding factors including the patient population examined, tumour histopathology, ratio of tumour to normal DNA in a sample and the method and depth of sequencing. As methods for detecting VHL gene alterations improve, the reported frequency of mutations in VHL is increasing.

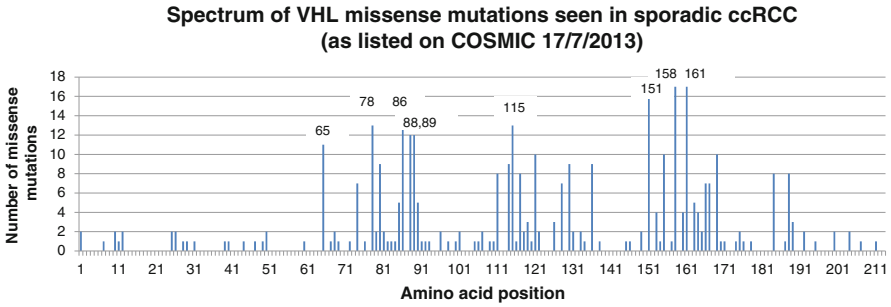

Fig. 4.6 Spectrum of missense mutations in sporadic ccRCC. Distribution of coding mutations in VHL found in sporadic ccRCC samples as listed on COSMIC on 17 July 2013. The amino acid positions at which mutations were most commonly found are *highlighted*

Over 900 different mutations have been identified in sporadic ccRCC and VHL disease [129, 159]. More than half of these are frameshift and nonsense mutations which are likely to cause the loss of pVHL function. However, in total, nearly 200 different missense mutations have been described in sporadic ccRCC. Since both $pVHL_{19}$ and $pVHL_{30}$ possess tumour suppressor activity in vivo, it is unsurprising that mutations are rare in the first 53 amino acids [5]. Otherwise, missense mutations are distributed fairly evenly across the three exons of VHL, with no dramatic hotspots for mutations (Fig. 4.6).

Dissecting the Impact of Missense Mutations

The simplest assumption is that all missense mutations disable pVHL's activity in equal amounts. However, numerous published studies which have examined the functional effects of VHL missense mutations in vitro and in cell culture systems demonstrate that this is not the case (Table 4.4). In fact, experimental data suggest that the impact of missense mutations on the function of pVHL is highly diverse, ranging from imperceptible to complete functional loss, particularly with respect to stability of the VCB complex and effects on HIFα ubiquitination and degradation. Interestingly, some mutations selectively influence HIF1α and HIF2α degradation [192].

To date, the preponderance of evidence can classify most missense mutations into four clear classes: (1) mutations which interfere with the binding of VHL to HIFα (e.g. Y98H, Y112H) [34, 35, 47], (2) mutations which inhibit the interaction between pVHL and elongins B and C (e.g. L158P, C162F and R167W) [17, 29, 80, 81, 193, 194], (3) mutations which inhibit the interaction with TRiC (e.g. G114R and A149P) [157] and (4) mutations which severely destabilise pVHL (e.g. G93D, W117R, L101P) [192]. However, not all mutations fit into this classification system; indeed, there are a significant number of mutations described in sporadic ccRCC which

Table 4.4 Functional effects of missense VHL mutations as determined experimentally

References	Amino acid change	Clinical phenotype (S, sporadic; F, familial)[a]	Summary of findings
[264]	L63P	F-1, type 2C	Defective in HIF2α degradation in 7,860 cells
[81, 106, 142, 264]	R64P	F-1, type 2C	Conflicting results with respect to HIF2α downregulation in 7,860 cells. Reduced binding to fibronectin
[80, 264, 265]	S65W	S-3; F-6, type 1	Unstable. Decreased HIFα binding and downregulation. Incomplete downregulation of HIFα
[264]	S65L	S-9; F-11, type 1	Unstable. Normal elongin binding. Defective HIFα degradation
[264]	S68W	F-2, type 2 (1 with variable penetrance, 1 with no phenotype described)	Defective in HIF2α degradation in 7,860 cells
[125, 192]	S68T	S-1	Phosphorylation site. Normal stability and HIFα ubiquitination
[264]	R69C	S-1; F-1 (compound heterozygote with pL158fs*15)	Normal VCB complex formation. Decreased HIF1α binding
[264, 266]	E70K	F-1, type 2C	Defective in HIF2α degradation in 7,860 but near normal HIF1α degradation. Retained the ability to promote ubiquitin-mediated destruction of pJAK2
[125, 192]	S72P	S-1; F-2, type 1	Phosphorylation site. Defective HIFα downregulation
[192]	V74D	S-4	Defective HIFα downregulation
[192]	N78K	S-3,	Normal HIF1α downregulation. Slightly less than WT for HIF2α
[192]	N78Y	S-1	Normal HIF1α downregulation. Slightly less than WT for HIF2α
[80, 192]	N78S	S-2; F-14	Less stable than WT. Defective HIFα binding, ubiquitination and downregulation. Possibly greater relative effect on HIF2α than HIF1α
[80]	S80Q	S-1	Normal suppression of HIFα subunits in stably transfected RCC4 cells
Personal data, as yet unpublished	N78D	S-1; F-1, type 2A	Moderately stable. Retained HIFα binding. Downregulated glut1, upregulated VEGF
[192]	S80N	S-3; F-5 (2 type 1, 1 type 2, 1 type 2A, 1 no phenotype)	Normal HIFα downregulation

[267]	P81S	S-14 (13 RCC after trichloroethylene exposure); F-7 (type 1 and other variations). Two families P81S and L188V, type 1 and type 2c	May be slightly less stable than WT. Normal HIF1α downregulation
[163]	R82P	S-3; F-2 (type 2B and phenotype not described)	Defective elongin B and C, HIFα and fibronectin binding. Defective HIFα downregulation
[80, 81, 265, 267, 268]	V84L	F-2 (type 2c and phenotype not described)	Possibly very minor effect on stability. Normal suppression of HIFα. Reduced fibronectin binding. Retained ability to bind and downregulate JAK2. Failed to correct the spindle misorientation phenotype of VHL null 7,860 cells
[192]	V84D	S-1	Defective HIFα downregulation
[163]	P86H	S-3	Defective elongin B and C, HIFα and fibronectin binding. Defective HIFα downregulation
[192]	W88S	S-1; F-2 (type 1). Also 1 sporadic cerebellar HB	Defective HIFα downregulation
[163] Personal data, as yet unpublished	N90I	S-3; F-2 (type 1)	Defective elongin B and C, HIFα and fibronectin binding. Unstable. Defective HIFα downregulation
[192, 193]	G93D	F-1 (type 2A)	Less stable than WT but retained elongin binding. Defective HIFα downregulation
[266]	Q96P	S-1; F-2, type 1	Retained the ability to promote ubiquitin-mediated destruction of pJAK2
[29, 47, 265, 268]	Y98H	F-10, type 2A	Minimal effect on stability. Retained ability to bind and downregulate pJAK2
[17, 47, 163, 164, 193, 265]	Y98N	S-1; F-1, type 2B	Normal complex formation. Less stable than WT. Defective HIFα downregulation in all but one study
[192]	L101P	S-2	Unstable. Defective HIFα downregulation
[193]	S111N	S-3; F-6	Normal elongin binding
[157]	S111H		Mild impairment of TRiC binding

(continued)

Table 4.4 (continued)

References	Amino acid change	Clinical phenotype (S, sporadic; F, familial)[a]	Summary of findings
[47, 265, 268, 269]	Y112N	F-2 (1 type 1, 1 type 2B)	Destabilised. Only partial downregulation of HIF2α and failed to downregulate glut1 in 7,860 cells. Retained ability to bind and downregulate JAK2. Failed to correct the spindle misorientation phenotype of VHL null 7,860 cells
[47, 80, 157, 163, 265, 270]	Y112H	F-3, type 2A	Conflicting effects with respect to stability. Partial restoration of HIFα downregulation. Did not bind fibronectin
[192]	Y112D	1 case	Defective HIFα downregulation
[271]	G114S	S-2; F-3 (details unclear)	Unfolded. Hugely decreased HIF1α binding and ubiquitination. TRiC binding site
[157, 192]	G114R	S-1; F-1, type 2	Abnormally folded and failed to interact normally with elongins. Defective HIF downregulation. TRIC binding site
[17, 107, 164, 192, 194, 271]	W117R	S-3	Decreased VCB formation and decreased stability. Defective HIFα binding, ubiquitination and degradation. Defective fibronectin binding
[192]	W117L	S-1	Defective HIFα downregulation
[157, 272]	W117C	S-1; F-7, type 1	Unfolded. Does not bind TRiC
[266]	L118R	F-1, type 2	Retained the ability to promote ubiquitin-mediated destruction of pJAK2
[157]	L118P	S-4; F-6 (4 type 1, 1 type 2B, 1 no phenotype described)	Unfolded. Does not bind TRiC
[81, 142, 157]	F119S	F-1, type 2C	Reduced stability. Normal downregulation of HIF2α in 7,860 cells. Reduced binding to fibronectin
[192, 193]	F119L	S-3; F-5 (4 type 2, 1 no phenotype described)	Retained elongin binding. Defective HIFα downregulation
[192]	D121Y	S-3	Partial HIF1α but not HIF2α downregulation
[271, 273]	D121G	S-3; F-3 (2 no phenotype, 1 type 2A)	Contradictory findings with respect to the formation of VCB complex. Bound HIF1α. Some HIFα ubiquitination (less than WT). Normal glut1 downregulation
[157]	L128F	S-3; F-3 (2 type 2, 1 no phenotype)	Significant impairment of TRiC binding
[192]	V130F	S-1; F-2, type 1	Defective HIFα downregulation, possibly relative greater effect on HIF1α
[192]	V130D	S-1	Defective HIFα downregulation

[264]	Q145H	S-1	Normal formation of VEC complex. Defective HIF2α downregulation
[157]	A149P	S-1	Abnormally folded. Did not bind TRiC
[266]	N150K	F-1 (details unknown)	Retained the ability to promote ubiquitin-mediated destruction of pJAK2
[157]	I151S	S-6	Abnormally folded. Did not bind TRiC
[192]	L153P	S-4	Normal downregulation HIF1α. Slightly reduced for HIF2α
[80, 155, 157, 193, 265, 271]	L158P	S-3; F-7 (2 type 1, 2 type 2, 2 no phenotype, 1 retinal angioma only)	Decreased elongin binding. Remains bound to TRiC. Unstable. Defective HIFα ubiquitination and degradation
[266]	L158S		Retained the ability to promote ubiquitin-mediated destruction of pJAK2
[108]	K159E	S-1; F-1, type 2	Normal downregulation of glut1. Did not bind fibronectin
[192]	K159N	S-1	Neddylation site. Partially defective downregulation of HIFα
[192]	R161Q	S-1; F-15 (mixture of 2A, 2B and 2C)	Normal downregulation of HIF1α. Slightly reduced for HIF2α
[192]	R161P	S-3; F-2 (1 type 1, 1 type 2)	Defective downregulation of HIFα
[47, 81, 107, 194, 271]	C162F	S-2; F-2 (type 1)	Defective elongin, HIFα and fibronectin binding. Defective HIFα downregulation. Retained ability to bind and downregulate JAK2
[194]	V166F	S-3; F-6 (4 type 2, 1 bilateral phaeochromocytoma [sporadic/ germline], 2 type 2A)	Decreased cul2 and elongin B binding
[17, 29, 193, 194, 265, 274]	R167W	S-4; F-34 (4 type 1, 30 type 2 [all subtypes], 3 germline [would fit with type 2])	Decreased elongin and cul2 binding. Unstable. Normal HIF2α and partial HIF1α downregulation
[17, 80, 193, 265, 269, 270, 273, 275]	R167Q	S-4; F-39 (mixture of type 1 and type 2, 4 germline [would fit with 2A])	Decreased elongin C binding but could pull down cul2. Unstable. Retained but diminished HIFα binding. Conflicting results on HIFα downregulation. May have differential effect on HIF1α and HIF2α
[192]	L169P	S-8; F-2, type 2A	Normal HIF1α downregulation. Slightly less than WT for HIF2α
[192]	V170D	S-1; F-4 (2 type 2, 1 type 2B, 1 no phenotype)	Partially defective HIFα downregulation
[192]	I180V	S-1; F-2 (1 type 1, 1 type 2B)	Normal HIFα downregulation

(continued)

Table 4.4 (continued)

References	Amino acid change	Clinical phenotype (S, sporadic; F, familial)[a]	Summary of findings
[80, 81, 121, 142, 192, 194, 265, 267, 270, 273, 276–278]	L188V	S-1 (after trichloroethylene); F-4, type 2C	Conflicting results with respect to stability and VCB complex formation. Relatively normal HIFα binding, downregulation and ubiquitination. Defective fibronectin binding. Elongated cell shape and disrupted organisation of IC junctions. Restored formation of the primary cilium in RCC4 and RCC10 cells. Increased E-cadherin expression in RCC4 and RCC10 cells similarly to WT. Retained ability to bind and degrade JAK2
[80, 265]	L188Q	S-2; F-1 (type 1)	Unstable. Decreased elongin C interaction. Could bind but not ubiquitylate HIFα. Defective HIFα downregulation
[266]	H191D	F-Hereditary polycythaemia	Exhibited normal fibronectin matrix deposition. May increase SOCS binding
[80]	Q195X	S-5; F-2 (types unclear)	Did not restore HIFα downregulation in transfected RCC4 cells
[25, 125, 166, 266, 270] Me	R200W	S-2 cases; F-1 (type 1). Multiple associated with hereditary polycythaemia	Inhibits HIF1 transcriptional activity less effectively than WT VHL. Normal fibronectin matrix deposition. May increase SOCS binding

[a]Numbers refer to number of sporadic ccRCC cases or familial kindreds in which mutation has been described
JAK2 Janus kinase 2, *SOCS* suppressor of cytokine signalling, *TRiC* TCP-1 ring complex, *VCB* VHL-elongin C-elongin B, *WT* wild type

appear to behave similarly to wild-type pVHL with respect to HIFα regulation and formation of the VCB complex [192] (Table 4.4). The most likely explanation is that these mutations are simply passenger mutations which don't influence tumour growth, though the possibility remains that they may somehow interfere with HIFα-independent functions of pVHL.

Mutations in Non-coding Regions of VHL

There is accumulating evidence that somatic mutations in non-coding regions of DNA may be pathogenic in other tumour types [195–208]. Furthermore, recent studies report that changes in the non-coding regions of VHL may have important physiological effects [209], suggesting that mutations in non-coding regions of VHL may be important physiologically. To date, there are 75 confirmed somatic variants (of which 47 are unique) in non-coding regions of VHL in tumour samples listed on COSMIC as of 13 June 2013 [129], and an additional study reports 49 somatic variants in non-coding regions of VHL in 37/128 sporadic ccRCC patients [293].

VHL *as a Biomarker in ccRCC*

For several years, groups have been addressing the question as to whether VHL mutational status, namely, the presence or absence of mutation, the type of mutation/alteration or the effect of the mutation/alteration on the function of pVHL, is a useful biomarker in ccRCC. Are so-called "loss-of-function" mutations, such as nonsense or frameshift mutations, associated with a different prognosis to missense mutations, some of which appear to cause minimal changes in VHL function? Can VHL mutational status be used to predict response to treatment? Finally, do mutations in non-coding regions of VHL have important functional effects?

VHL *Alterations as Potential Prognostic Markers in ccRCC*

Numerous studies have investigated whether or not functional loss of VHL may influence prognosis in ccRCCs (Table 4.5). ccRCCs in VHL disease have an earlier age of onset than cases of sporadic ccRCC. However, they also appear to grow more slowly than cases of sporadic ccRCC and are associated overall with a better prognosis [210, 211]. However, while the results of some studies appear to support this hypothesis in principle [182, 187, 188, 212], others have found no association between the presence or absence of VHL alterations and prognosis or other adverse clinicopathological features ([79, 176, 178, 179, 181, 183–186 189–192, 213]), and one study reported

Table 4.5 VHL alterations as potential prognostic markers in sporadic ccRCC

References	N[a]	Methods used to look for mutations	Mutation frequency (%)	Hypermethylation frequency (%)	LOH	% With alteration[b]	Associations with clinicopathological variables and survival
[189]	609	PCR amplification and sequencing	55	NA	NA	NA	None
[213]	185	PCR amplification and sequencing	49	NA	NA	NA	None
[192]	256	PCR amplification and sequencing	71	NA	NA	NA	None
[188]	177	PCR amplification and sequencing	75	31	89	88	VHL mutations were not associated with survival. Trend towards a worse prognosis for truncations resulting in late termination (termed as after codon 123) compared to truncations resulting in early termination (before codon 123). No VHL involvement may confer a worse prognosis than having either methylation or LOH and mutation
[184]	185	SSCP and direct sequencing	52[c]	11	NA	57	None
[182]	100	PCR amplification and sequencing	58	NA	NA	NA	VHL mutations associated with lower T stage, absence of distant metastases and longer PFS in univariate analysis
[181]	205	Endonuclease scanning and sequencing	82	8	NA	91	Overall prevalence of mutations not associated with clinicopathological characteristics
							Late-stage metastatic lesions had more double mutations, nonsense mutations and mutations located in exon 3 than Mx or M0 cases. Nonsense mutations associated with Fuhrman nuclear grade and lymph node positivity
[178]	62	PCR amplification and automatic sequencing	27	NA	NA	NA	None
[176]	97	DHPLC and sequencing	71	20	78	90	LOH significantly less common in grade 4 tumours

[186]	187	SSCP and sequencing	61	NA	NA	NA	None
[179]	56	SSCP and automated sequencing	20	14	NA	29	P582S was detected in six patients and was correlated with M1 disease. VHL alterations were not associated with survival. Patients with loss-of-function mutations showed decreased PFS and OS
[183]	113	PCR amplification and sequencing	34	NA	NA	NA	Loss-of-function mutations[d] were associated with significantly worse prognosis in univariate analysis. Presence/absence of mutations not associated with clinicopathological features
[187]	187	SSCP and direct sequencing	52	5	NA	57	In pts with stage I–III tumours who underwent a radical nephrectomy, VHL alteration was associated with better cancer-free survival and cancer-specific survival independently of other outcome predictors. VHL alterations were not associated with survival for patients with stage IV disease. Mutational subtype was not associated with survival
[279]	202	SSCP and sequencing	51	5	90	56	VHL alterations not associated with clinicopathological features. Mutations were less common in younger patients
[180]	82	SSCP and sequencing	54	NA	NA	NA	Multiple mutations more common in grade 3 tumours than in grade 1 or 2
[177]	151	SSCP and sequencing	38	7	93	45	VHL mutation or hypermethylations significantly associated with pT3 tumour stage
[185]	49	SSCP and sequencing	53	NA	NA	NA	Mutations not associated with clinicopathological features

[a]Only included ccRCC cases and excluded other histological subtypes
[b]Alteration defined as mutation and/or methylation. Only listed for studies which documented mutation and methylation status
[c]Data only presented for loss-of-function mutations defined as "a mutation that causes a change in the protein versus silent or no mutations"
[d]Loss-of-function mutations defined as "mutations predicted to alter the open reading frame of VHL"
NA not assessed, *DHPLC* denaturing high-performance liquid chromatography, *PCR* polymerase chain reaction, *SSCP* single-strand conformational polymorphism, *PFS* progression-free survival, *OS* overall survival

that VHL mutation/hypermethylation was associated with advanced tumour stage [177] (Table 4.5). Overall, there is certainly no clear evidence that the presence or absence of VHL mutations per se influences the outcome in sporadic ccRCC.

In part, the conflicting results may be explained by different methods used to assess the presence or absence of alterations, with not all studies assessing for the presence or absence of hypermethylation. Furthermore, different studies have included different patient populations with tumours of varying stages and grades. The majority of studies have used scanning techniques such as denaturing high-performance liquid chromatography or single-strand conformational polymorphism, followed by annotation of variants using Sanger sequencing. However, newer scanning techniques such as endonuclease scanning, and the incorporation of next-generation sequencing techniques with greater sensitivity and depth, may be more sensitive, and it is likely that the true prevalence of VHL alterations is higher than previously suspected [181]. Including multiplex-ligation-dependent probe amplification and Southern blotting to detect copy-number changes in the VHL gene in the search for alterations may also increase the detected frequency of alterations, and it is possible that non-coding alterations in VHL may also have physiological significance.

Many groups have done further analyses on subgroups of VHL mutations to try and determine if the type or nature of mutation is associated with prognosis. For example, are mutations which would be predicted to result in complete loss of pVHL function, such as nonsense or frameshift mutations, associated with a worse prognosis than missense mutations, some of which may only mildly affect pVHL's function? Alternatively, could nonsense mutations perhaps result in more aggressive tumours, perhaps due to retained "partial" activity of pVHL? Of course, categorising mutations in this way does not take into account the diverse effects of missense mutations on the function of pVHL (Table 4.4). While some missense mutations, even those distant from specific binding sites such as the HIF binding site, can have serious functional effects by globally destabilising protein folding, others seem to have little or no effect on pVHL's function. The position of a missense mutation within the gene may therefore also be important prognostically. However, at present, there is no reliable way to easily discriminate between passenger mutations which do not influence tumour growth and driver mutations which do. The impact of individual missense mutations on either HIF regulation or other pro-tumorigenic activities thus remains largely uncertain, and the diverse spectrum of mutations presents a significant barrier to understanding the functional effects of missense mutations and other loss-of-function type events. In this respect, the development of validated in silico tools which can accurately predict the functional effects of missense mutations may be of benefit.

Consequently, it has proven difficult to develop a rational way of categorising mutations within VHL, and various groups have elected to use different classification systems. While some have divided mutations into "loss-of-function" mutations predicted to alter the open reading frame of VHL and "non-loss-of-function" mutations not predicted to alter the open reading frame [183], [184], others have classi-

fied mutations either according to their impact on the DNA sequence (frameshift/missense/nonsense) or their location within the gene (e.g. exon number) or protein (e.g. deep/surface amino acids). To date, only one group has attempted to discriminate between those missense mutations with functional consequences and those without [192]; they subgrouped VHL missense mutations into predicted destabilising and neutral missense mutations using in silico prediction tools and in vitro assays but report no differing effects on patient outcome.

In VHL disease, there is an intimation that mean age of onset is earlier and age-related risk of ccRCC is higher in patients with frameshift or nonsense mutations than in those with deletions or missense mutations that disrupt the structural integrity of pVHL [162]. Another study of VHL disease patients reported an increased frequency of renal involvement in cases with a truncating mutation or large rearrangement than in those with missense mutations [214]. Subsequently, some have speculated that truncating mutations may be associated with the expression of a truncated gene product that has retained a β-domain HIF binding site but not a functional α-domain elongin C binding site [162]. Such a mutant protein might compete with wild-type pVHL to bind the HIFα subunits in normal renal cells, thereby protecting it from degradation. Further support for such a "dominant-negative" hypothesis comes from a study of sporadic ccRCC which reported a trend towards a worse prognosis for truncations resulting in late termination (termed as after codon 123) compared to truncations resulting in early termination (before codon 123) [188]. Mutations prior to codon 123 would be expected to disrupt the HIFα binding site, while later truncations may leave an intact HIFα binding site but disrupt the elongin C binding site and thus potentially protect HIFα from degradation.

In sporadic ccRCC, there are a handful of studies which suggest that nonsense mutations may be associated with worse prognosis and adverse pathological features [179, 181, 183]. Analysis of VHL mutations as part of a large kidney cancer case-control study suggested that nonsense mutations were significantly associated with increased grade and lymph node positivity and that these mutations were more prevalent among M1 than M0 cases [181]. Another study of 113 ccRCCs subdivided VHL mutations into "loss-of-function" mutations predicted to alter the open reading frame of VHL and "DNA sequence variants of unknown biological consequence" (in-frame, missense, silent or intronic mutations) [183]. Though "loss-of-function" mutations were not associated with tumour phenotype (grade, stage or metastasis), there was a significantly worse prognosis in tumours with "loss-of-function" mutations leading to truncated pVHL than in tumours with no mutations or mutations with unknown consequences for pVHL structure and function. However, on multivariate analysis, only histological grade and pT stage were independent predictors of adverse outcome, though this may simply reflect the small sample size. It should be noted that the prevalence of VHL mutations reported in this study was only 34 %, which is lower than that described in the majority of studies. Another small study of 56 patients found that loss-of-function mutations were associated with significantly decreased progression-free survival and overall survival [179].

It is not uncommon for a single tumour to have multiple VHL mutations with the reported prevalence of this up to 8 % in two studies [183, 215]. Multiple mutations of VHL within the same tumour have been described in up to 42 % of patients exposed to trichloroethylene [216]. Though one study reported that late-stage metastatic lesions had more double mutations than M0 or Mx cases [181], other studies have not replicated these findings.

VHL *Alterations as Potential Predictive Markers*

As yet, only a few studies have examined a role for VHL as a potential predictive marker in ccRCC, largely because it is only recently that effective treatment options have come into widespread use (Table 4.6). One of the major hurdles relates to the collection of adequate quality tissue for DNA extraction and sequencing; the majority of clinical trials collect formalin-fixed paraffin-embedded (FFPE) tissue rather than fresh frozen tissue, and the quality of DNA extracted from FFPE tissue is generally inferior to that acquired from fresh frozen tissue. As such, the frequency of VHL mutations reported in many of these studies is lower than might be expected, implying a possible skewing of the results.

A recently reported study analysed 78 tumour tissue samples from a cohort of 225 metastatic clear cell RCC patients who received pazopanib, a standard first-line VEGF-targeted agent, as part of a clinical trial [217]. The authors evaluated the association of several components of the VHL-HIF pathway (VHL gene inactivation [mutation and/or methylation], HIF1α and HIF2α immunohistochemistry staining and HIF1α transcriptional signature) with best overall response rate to pazopanib and progression-free survival. 70/78 (90 %) of patients had VHL mutations or methylation. Neither VHL gene status nor HIF1α or HIF2α protein expression or HIF1α gene expression signature was associated with clinical outcome to pazopanib.

The phase III Treatment Approaches in Renal Cancer Global Evaluation Trial (TARGET) randomised 903 patients with advanced ccRCC to sorafenib or placebo. VHL mutational status was available for 134 patients (though only 48 patients had all three coding exons of VHL successfully sequenced), and no correlation between VHL mutational status and sorafenib benefit was found [218].

One of the biggest earlier studies included 123 patients with metastatic ccRCC who had received treatment with sunitinib (51 %), sorafenib (23 %), axitinib (12 %) and bevacizumab (14 %) as part of a clinical trial [219]. The incidence of VHL mutations in this group of patients was 49 %, with 78 % of these classified as "loss-of-function" mutations (i.e. frameshift, nonsense, splice and in-frame deletions/insertions). In addition, 10 % of patients had promoter methylation, though these patients were excluded from the analyses. Though there was no difference in response rate between patients with inactivated (mutated or methylated) VHL and those with wild-type VHL (41 % versus 31 %, $p=0.34$), on subgroup analysis, patients with "loss-of-function" mutations had a significantly higher response rate than those with wild-type VHL (52 % versus 31 %, $p=0.04$). This remained an

Table 4.6 VHL alterations as potential predictive markers (all studies of patients with metastatic ccRCC)

Ref	*N*[a]	Methods used to look for mutations	Treatment	Mutation frequency (%)	Hypermethylation frequency (%)	Predictive findings
[217]	78	PCR amplification and sequencing	Pazopanib	68/78 (87 %)	8/69 (12 %)	VHL mutation and/or methylation status did not correlate with ORR or PFS
[231]	80	Not reported	Sunitinib	Not reported	Not reported	VHL mutation did not correlate with clinical outcome
[218]	48[b]	PCR amplification and sequencing	Sorafenib or placebo (TARGET trial)	35/68 (51 %)	NA	No correlation between VHL mutational status and sorafenib benefit
[219]	123	PCR amplification and sequencing	VEGF-targeted therapy[a]	60/123 (49)	12/123 (10)	Increased RR for patients with loss-of-function mutations compared with patients with wt VHL. No responses in 21 patients with wild-type VHL treated with bevacizumab or sorafenib, compared to 16/30 (53 %) patients treated with sunitinib or axitinib
[280]	78	Not stated	Pazopanib	70/78 (90) (methylation or mutation)		No correlation between VHL status and response
[222]	13	PCR amplification and sequencing	Axitinib	2/13 (15)	NA	Objective response in six patients, two of whom had VHL alterations. No alterations found in remaining patients
[221]	53	PCR amplification and sequencing	Sunitinib, axitinib or interferon-a plus bevacizumab	25/43 (58)	1/43 (2)	No association between the presence of a VHL mutation or methylation and ORR or tumour shrinkage. Median TTP in patients with VHL alteration was 10.8 months versus 5.5 in patients with no VHL alteration. Patients with methylation or mutations that truncated or shifted the VHL reading frame had increased TTP
[179]	56	SSCP and automated sequencing	Immunotherapy	11/56 (20)	8/56 (14)	No significant association between VHL alteration and response to immunotherapy
[187]	53	SSCP and direct sequencing	Immunotherapy or combined chemo-immunotherapy	30/53 (57)	NA	VHL mutational status had no influence on cancer-specific survival rates

[a]Sunitinib, sorafenib, axitinib, bevacizumab

[b]Nine-hundred and three patients in study. 134 patients had at least one of the VHL coding exons sequenced successfully, but only 48 patients had all three exons sequenced successfully

mccRCC metastatic clear cell renal cell carcinoma, *RR* response rate, *VEGF* vascular endothelial growth factor, *wt* wild type, *NA* not assessed, *ORR* objective response rate, *TTP* time to progression

independent predictor of response on multivariate analysis, even after adjusting for the specific anti-VEGF drug used. Further analyses showed that while patients who received sunitinib or axitinib had significant responses independent of VHL status, no responses (0/21) were seen in patients with wild-type VHL treated with bevacizumab or sorafenib. At present, the survival data are immature. A separate small study of ccRCC patients treated with first-line sunitinib found no association between VHL alterations and response to sunitinib [220].

These results at first appear to challenge those of previous studies, suggesting that "loss-of-function" mutations are associated with adverse outcomes. This may be because the advent of VEGF-targeted therapy means that patients who once had the worst outlook no longer do. The authors speculated that the differential effect of VHL mutations on therapeutic response could be explained either if sunitinib and axitinib have additional non-VHL-related inhibitory mechanisms on ccRCC or perhaps have superior inhibition of the VEGF receptor compared with bevacizumab and sorafenib. A subset of the data included in this analysis had previously been analysed and reported retrospectively in a study of 43 patients with metastatic ccRCC who received therapy with sunitinib, axitinib or interferon-α plus bevacizumab [221]. In this report, there was no association between the presence of a VHL mutation or methylation and objective response rate or tumour shrinkage, and VHL alterations did not impact upon overall survival.

A very small study of 13 patients included in a phase II study testing the efficacy of axitinib found no correlation between the somatic mutational status of the VHL gene and the objective response to axitinib [222]. However, this was a tiny study which did not analyse the methylation status of the VHL promoter, and among the 13 patients, only two showed the presence of sequence variants, suggesting the patient cohort within this study may not be entirely representative of the usual ccRCC population. There is very little work examining VHL mutational status as a predictive marker for response to mTOR-directed therapy, though a retrospective analysis on a subset of patients ($n=20$) enrolled within a phase II trial of temsirolimus in advanced ccRCC found no correlation between VHL mutational status and response to temsirolimus [223]. VHL alterations were not associated with response to immunotherapy in a retrospective analysis of 56 patients [179].

Categorising *VHL* Mutations According to Their Functional Effects

The preponderance of evidence suggests that not all VHL mutations are equal and that, in fact, a significant percentage of mutations described in sporadic ccRCC may simply be passenger mutations. Designing a system of categorising VHL mutations, perhaps by using in silico prediction tools to predict the effect of the mutation on the function of pVHL, may allow us to use this information practically to separate high- and low-risk patient groups and select patients appropriately for targeted therapies.

Is VHL *Alone Too Simplistic?*

As our understanding of the finer details of the molecular pathways downstream of VHL expands, various groups are investigating whether a combination of VHL mutational status and other molecular markers may prove more useful as prognostic markers than VHL alone. For example, one study reported that low CAIX expression and absence of VHL gene mutation are associated with aggressive clinicopathological features and poor survival of ccRCC [182], and another study by the same authors reported that the absence of VHL gene alteration, or altered VHL and high VEGF expression, is associated with poor survival [212].

An alternative way of classifying VHL-deficient tumours was described by Gordan et al. [74] who analysed VHL genotype, HIF1α, HIF2α and c-myc expression in 160 primary tumours. Based on immunohistochemical assays and mRNA profiling, they subdivided the tumours into three groups with distinct molecular characteristics: (1) tumours with wild-type VHL alleles and undetectable HIFα protein expression (designated VHL WT), (2) VHL-deficient tumours expressing detectable HIF1α and HIF2α proteins (designated H1H2) and (3) VHL-deficient tumours expressing HIF2α exclusively (designated H2). H2 tumours displayed enhanced c-Myc activity and higher rates of proliferation relative to H1H2 tumours regardless of stage and also displayed increased expression of genes involved in DNA repair, decreased levels of endogenous DNA damage and fewer genomic copy-number changes. In contrast, H1H2 and VHL WT tumours displayed increased activation of Akt/mTOR and ERK/MAPK1 growth factor signalling pathways and increased expression of glycolytic genes relative to H2 tumours. This study argues for the existence of two biologically distinct types of VHL-deficient ccRCCs: those that produce HIF1α and those that don't.

Recent studies have used biologically driven clustering to define two robust subgroups of ccRCC, ccA and ccB, that are highly dichotomous by molecular phenotype and cancer-specific survival [224]. ccA and ccB subtypes do not appear to be divided based on the expression of defined VHL or HIF profiles; however, a smaller, third subtype of tumours had a WT VHL signature and indications of variant histologies [225]. Other studies have identified transcript patterns related to the expression of HIF1α and HIF2α [74, 95] and genetic sequence [88].

VHL *and Chromatin-Remodelling Genes*

Studies of kidney cancers arising in VHL patients suggest that VHL inactivation in human kidneys leads to preneoplastic cysts but is not sufficient for malignant transformation [226, 227]. This explains why a mouse model of ccRCC does not exist today. Malignant transformation seems to require the accumulation of additional genetic and possibly epigenetic changes. Recently, genomic sequence analysis has identified several genes that are frequently mutated in ccRCC. These include polybromo 1 (*PBRM1*), SET domain

containing 2 (*SETD2*), BRCA1-associated protein-1 (*BAP1*) (all of which lie on a relatively small, 43 Mb region of chromosome 3p and are therefore potentially deleted alongside VHL in tumours with 3p loss) and lysine (K)-specific demethylase 5C (*JARID1c*). It is likely that these genes function in pathways which would otherwise limit transformation driven by VHL loss. In this respect, acute VHL loss leads to senescence in many cell types [146, 147]. Interestingly, pVHL inactivation leads to the induction of JARID1c which then acts to block the proliferation in this setting [228].

It has been proposed that ccRCC development may evolve along two different paths [190]. Following a VHL mutation and the loss of 3p which is frequently observed, mutations in the remaining *PBRM1* or *BAP1* allele may lead to tumours with different characteristics. Interestingly, in the mouse, Vhl is on a different chromosome to that of Pbrm1 and Bap1; thus, the loss of heterozygosity of the mouse Vhl region would not simultaneously inactivate one copy of Pbrm1 and Bap1. If this model is correct, simultaneous inactivation of Vhl and either Pbrm1 or Bap1 in the mouse should lead to the development of ccRCC.

It will be interesting to see whether subtypes of VHL alterations are linked in any way to mutations in chromatin-remodelling genes and whether these changes link to therapeutic response. As therapeutic strategies improve and specifically achieve more successful inhibition of VEGF activity and/or replacement of pVHL activity, closer correlations between drug responses and VHL alterations may be detected. A deeper understanding of VHL targets other than HIFα and particularly how VHL cooperates with *BAP1*, *PBRM1* and other genes to cause ccRCC may ultimately lead to the identification of additional biomarkers and potentially novel therapeutic strategies.

HIFα as a Biomarker in ccRCC

Many studies have investigated whether HIF1α or HIF2α is a useful prognostic marker in ccRCC (Table 4.7). Several earlier studies linked the expression of HIF1α to poor prognosis in ccRCC, though more recent studies suggest that the relative expression of HIF1α and HIF2α may be more important [74, 229]. A subset of ccRCC tumours seem to have copy-number amplification of 8q24, where c-Myc resides [95]. Since HIF1α acts to suppress c-Myc activity while HIF2α promotes the transactivation or transrepression of c-Myc-specific target genes [74, 93, 94], it would be interesting for future studies to look at the expression of these genes in combination, particularly in conjunction with the mutational status of VHL, *PBRM1* and *BAP1*.

Only a few studies have evaluated HIF expression as a predictor of response to targeted therapies (Table 4.7). Pretreatment HIF levels were associated with response to sunitinib in a cohort of 43 metastatic ccRCC patients [230], and positive HIF1α and HIF2α protein expression was reported to be an independent predictor of outcome for VEGFR tyrosine kinase inhibitor therapy in 71 patients with metastatic ccRCC [231]. However, another study reported that the loss of chromosome

Table 4.7 HIF1α and HIF2α as potential biomarkers in ccRCC

Reference	Number of patients	HIF1α and/or HIF2α	Method	Associations with clinical and pathological variables
[217]	66	Both	Immunohistochemistry and HIF1α transcriptional signature	HIF1α and HIF2α protein levels did not correlate with overall response rate or progression-free survival to pazopanib. HIF1α gene expression signature was not associated with clinical outcome to pazopanib
[232]	75	HIF1α	Copy-number analysis	14/14q- did not affect PFS or response to pazopanib
[231]	71	Both	Immunohistochemistry	HIF1 and HIF2 positive expression showed a significant correlation with PFS and OS. HIF1α was also predictive for response rate (RR). On multivariate analysis adjusting for other prognostic factors, HIF1α and HIF2α remained the most significant independent predictive factors for survival
[230]	43	Both	Western blot	High pretreatment HIF1α and HIF2α levels were associated with response to sunitinib in 43 metastatic ccRCC patients who received sunitinib
[229]	168	Both	Immunohistochemistry	HIF1α(high)HIF2α(low) tumours had a worse overall survival compared with HIF1α(low)HIF2α(low) ($p = 0.04$)
[281]	135	HIF1α	Immunohistochemistry	High HIF1α expression was associated with poor prognosis in patients treated with cytokine therapies or new targeted drugs
[282]	176	HIF1α	Immunohistochemistry	HIF1α levels were higher in primary and metastatic ccRCCs compared with benign tissues ($p < 0.0001$). High HIF1α expression was an independent predictor of disease-specific survival and tumour progression in primary ccRCC
[283]	82	Both	Immunohistochemistry	HIF1α and HIF2α are not expressed in the sarcomatoid component in about 50 % of 24 ccRCCs with sarcomatoid differentiation, while HIF2α was consistently overexpressed in the epithelial component in a majority of the tumours. HIF1α expression was an independent predictor of cancer-specific survival in ccRCC with sarcomatoid differentiation

(continued)

Table 4.4 (continued)

Reference	Number of patients	HIF1α and/or HIF2α	Method	Associations with clinical and pathological variables
[284]	94	HIF1α	Immunohistochemistry	Overexpression of cytoplasmic HIF1α was associated with higher nuclear grade, larger tumour size, higher stage and shorter survival. Overexpression of nuclear HIF1α was associated with lower nuclear grade, smaller tumour size and longer survival
[285]	249	HIF1α	Immunohistochemistry	Baseline HIF1α expression did not predict response to temsirolimus or interferon-α
[286]	170	HIF1α	Immunohistochemistry	HIF1α expression was not associated with disease-specific survival
[287]	202	HIF2α	Real-time polymerase chain reaction	There was an inverse correlation between HIF2α mRNA levels and TNM stage I and II–IV tumours ($p=0.01$) and nuclear grade ($p=0.006$). No correlation of HIF2α mRNA to survival was observed
[74]	160	Both	Immunohistochemistry	ccRCCs with intact VHL, as well as pVHL-deficient HIF1α-/HIF2α-expressing ccRCCs, exhibited enhanced Akt/mTOR and ERK/MAPK signalling. pVHL-deficient ccRCCs expressing only HIF2α displayed elevated c-Myc activity, resulting in enhanced proliferation and resistance to replication stress
[288]	50	HIF1α	Immunohistochemistry	HIF1α expression correlates with prognosis in high-stage tumours
[289]	357	HIF1α	Immunohistochemistry	HIF1α expression was greater in ccRCC than in benign tissue. In metastatic patients, patients with high HIF1α expression had significantly worse survival than patients with low expression
[290]	136	HIF1α	Immunohistochemistry	HIF1α nuclear positivity correlated with reduced tumour-specific survival
[291]	176	HIF1α	Immunohistochemistry	In patients with conventional RCC, HIF1α levels were significantly lower in locally aggressive tumours versus localised tumours, and patients with high HIF1α levels tended to have a better prognosis
[292]	92	HIF1α	Quantification of Western blot	HIF1α was an independent prognostic factor

14 or 14q- (HIF1α locus) was not correlated with clinical response to pazopanib [232], and a very recent study found no association between HIF1α (65 samples) and HIF2α (66 samples) protein levels and overall response rate or progression-free survival to pazopanib [217].

Targeting HIF2α

Since HIF2α seems to be an oncogene in ccRCC, targeting HIF2α would seem to be a sensible therapeutic strategy for ccRCC. However, with the exception of the steroid hormone receptors, targeting DNA-binding transcription factors with drug-like small organic molecules has historically been relatively unsuccessful. Despite this, several potential strategies to inhibit HIF2α have been identified. Proof-of-concept experiments intimate that, in principle, it may be possible to target HIF2α with DNA-binding polyamides that disrupt the HIFα-DNA interface, though at present the bioavailability of such agents is inadequate [233–235]. Acriflavine is a small molecule that inhibits the ability of HIF1α and HIF2α to dimerise with HIF1β and has been shown to inhibit tumour growth and vascularisation [236]. Alternatively, if reliable methods for systemic delivery of siRNA become available, siRNA targeting of HIF2α may become a future therapeutic option. Two groups have been screening for drugs that, at least indirectly, inhibit HIF2α in VHL null ccRCC cells, though the specificity of these compounds remains to be established [237–240].

Many other compounds are also known to indirectly inhibit HIFα, including mTOR inhibitors, HSP90 inhibitors and HDAC inhibitors [241]. Unfortunately, the two currently used rapamycin-like mTOR inhibitors, everolimus and temsirolimus, primarily inhibit mTOR in the TORC1 complex and have less activity against mTOR present in the TORC2 complex [242]. The inhibition of TORC1 inhibits HIF1α more than HIF2α [243] and can also paradoxically increase upstream receptor tyrosine kinase signalling due to a loss of TORC1-dependent negative feedback pathways [244, 245, 246].

Of course, many of the drugs currently used to treat ccRCC in the clinic indirectly inhibit HIF2α by inhibiting the action of one of its most tumorigenic downstream targets, VEGF. These include bevacizumab, sunitinib, sorafenib, pazopanib and axitinib.

*p*VHL *and Synthetic Lethality*

Two genes are synthetic lethal if mutation of either alone is compatible with viability but mutation of both leads to death [247]. Synthetic lethality thus provides a framework to discover drugs that might preferentially kill cancer cells harbouring a cancer-relevant gene yet leave normal cells unharmed. To date, the results from two synthetic lethality screens attempting to target VHL-deficient cells have been reported. Firstly, a cell-based small-molecule synthetic lethality screen identified a

compound, STF-62247, that selectively induces autophagic cell death in VHL-*deficient* cells but not in those expressing wild-type VHL [248]. From the same screen, a second compound, STF-31, was identified that inhibits glucose uptake by the Glut1 transporter and exhibits enhanced cytotoxicity against VHL-deficient ccRCC [249]. Secondly, an shRNA screen targeting 88 kinases reported that silencing of CDK6, MET and MEK1 preferentially inhibited the growth of VHL null cells compared with their wild-type pVHL-reconstituted counterparts [250]. Interestingly, in both screens, the selective killing of cells lacking VHL was HIF independent, suggesting that therapies targeting these pathways may cooperate with those targeting HIF. Another study showed that the lack of a functional VHL gene product sensitises renal cell carcinoma cells to the apoptotic effects of the protein synthesis inhibitor verrucarin A [251].

VHL, HIFα *and Metastasis*

Since the main tumour-suppressive function of VHL is its role in mediating the degradation of HIF2α, and since at least one HIF2α target gene, chemokine (C-X-C motif) receptor 4 (*CXCR4*), is a direct mediator of metastatic colonisation [252, 253], it has previously been suggested that loss of VHL might directly lead to metastatic tumour phenotypes through HIF activation [253]. However, even though CXCR4 expression correlates with metastasis in ccRCC [253–255], as described above, VHL mutation has not convincingly been shown to correlate with poor disease outcome and metastatic disease.

Therefore, Massagué and colleagues examined whether the increased expression of *CXCR4* and other potential metastatic genes downstream of the VHL-HIF axis occurs as a result of epigenetic changes. They selected highly metastatic subpopulations of the VHL-deficient ccRCC 786-0 cell line (which was originally established from a patient with metastatic disease) through tail vein injection into immunocompromised mice. Using genome-wide transcription profiling, they identified 155 genes associated with the metastatic phenotype of these cell variants. They then refined this gene set to a core set of 50 genes (termed the renal cancer metastasis signature 50 (RMS50)) that are also expressed in ccRCC gene expression profiles that form the GSE2109 data set in the Gene Expression Omnibus.

Additional gene expression profiling studies showed that a subset of these genes responded to VHL inactivation and were transcriptional targets of HIF2α. Focusing on the two most prominent pro-metastatic VHL-HIF target genes, they showed that the loss of polycomb repressive complex 2 (PRC2)-dependent histone H3 Lys27 trimethylation (H3K27me3) activates HIF-driven chemokine (C-X-C motif) receptor 4 (CXCR4) expression in support of chemotactic cell invasion, whereas the loss of DNA methylation enables HIF-driven cytohesin 1-interacting protein (CYTIP) expression to protect cancer cells from death cytokine signals.

Previously, the pathways that drive metastasis have been considered to be separate from tumour-initiating functions [256]. In contrast, this study suggests

that metastasis in ccRCC is based on an epigenetically expanded output of the tumour-initiating pathway, namely, loss of VHL function.

HIF- and Hypoxia-Mediated Epigenetic Regulation

As discussed in high-throughput genetic studies of RCC have identified recurrent mutations in genes encoding several epigenetic regulators, including *PBRM1*, *SETD2*, *JARID1c*, *KDM6A* and *MLL2* (reviewed in [257]). Interestingly, the hypoxia response pathway has been shown to have a direct effect on histone modification. Firstly, HIF has been shown to directly activate several chromatin demethylases, including *KDM3A, KDM4B, KDM4C* and *KDM5B* [41, 258–260]. Secondly, VHL inactivation has been shown to decrease H3K4Me3 levels through an HIF-dependent increase in the expression of *JARID1c* (an H3K4 Me3 demethylase) expression. The re-expression of pVHL in VHL-deficient cell lines reduces HIFα expression, resulting in decreased levels of *JARID1c* with a consequential increase in levels of H3K4me3 [228].

In contrast, hypoxia may also *increase* methylation through HIF-independent mechanisms; histone demethylases are members of the dioxygenase superfamily, which require oxygen for activity, and hypoxia suppresses JARID1A (KDM5A) activity, resulting in increased H3K4me3 levels [261]. The loss of demethylases, and by implication, increased histone methylation, may thus be part of a hypoxia phenotype that is selected for in ccRCC. This hypoxia phenotype, which is mimicked by VHL loss, would also be mimicked by the loss of histone demethylase activity resulting from inactivating mutations.

Chromatin organisation also influences HIF function, and it seems that HIF is preferentially targeted to previously nucleosome-depleted chromatin regions [262]. SWI/SNF has also been shown to regulate the cellular response to hypoxia by regulating HIF1α transcriptional activity [263].

As yet, the extent to which mutations of epigenetic regulators influence chromatin or HIF targeting is unknown. Since hypoxia directly influences demethylase activity, the relationship between epigenetic variation and HIF targeting might well differ depending on the conditions of hypoxia in primary cells and the context of specific epigenetic alterations in tumour cells.

Conclusions

VHL and HIF are undoubtedly key players in ccRCC pathogenesis. The loss of VHL with consequent deregulation of HIFα and its downstream targets is an early event for the majority of patients with ccRCC. In this disease, HIF2α acts as an oncoprotein, while HIF1α acts as a tumour suppressor. This knowledge

underpins the rationale behind the development of drugs that inhibit HIF or selected HIF targets for the treatment of ccRCC. As our understanding of ccRCC biology continues to evolve rapidly, we must amalgamate the wealth of newly acquired information regarding chromatin-remodelling genes and ccRCC metabolics with the existing data regarding VHL and HIF in ccRCC. The challenge now is to establish a road map of tumour ontogeny for precursor lesions and early ccRCC and to clarify the mechanisms of tumour progression for more advanced disease. Studies of genomics, transcriptomics, epigenetic data and molecular biology must be co-ordinated and targeted. International collaboration and communication among experts is vital to ensure this information is used and interpreted as efficiently and effectively as possible.

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Chapter 5
PBRM1: A Critical Subunit of the SWI/SNF Chromatin Remodeling Complex

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Recent Identification of Tumor Suppressors in ccRCC

Emergence of ccRCC has long been associated with loss-of-function mutations of the *von Hippel-Lindau* (*VHL*) tumor suppressor gene. Over the preceding decades, significant efforts have led to the recognition that the VHL protein functions as the E3 ubiquitin ligase that ubiquitinates and targets the transcription factor hypoxia-inducible factor (HIF) for proteasome-mediated degradation. During tumorigenesis, loss of VHL function leads to an accumulation of HIF, which in turn induces changes in cellular metabolism and growth. This is largely mediated through the increased expression of pro-angiogenic factors such as vascular endothelial growth factor (VEGF). Consequently, the targeting of VEGF with molecularly targeted therapies has revolutionized the treatment of ccRCC. Despite these advances, metastatic ccRCC remains a poorly understood disease that is largely incurable. Accumulating clinical evidence from patients and preclinical evidence from mouse models suggest that loss of VHL alone is insufficient for tumorigenesis [1]. Collectively, these results suggest that additional genetic "hits" are at play, and multiple alterations are likely to conspire to promote ccRCC. Using massively parallel sequencing technologies, several clinical correlation studies have identified candidate tumor suppressor genes that may play important roles in restricting the emergence of

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ccRCC. Although no other single gene has been identified to have the same frequency of *VHL* inactivation in ccRCC, these commonly mutated genes show unequivocal and consistent associations with ccRCC. These finds have now been demonstrated by multiple independent studies and hence define these genes as tumor suppressors. These genes include *PBRM1*, *SETD2*, *BAP1*, and *KDM5C* [2].

Mutations in ccRCC Tumor Suppressors as Potential Clinical Biomarkers

ccRCC is commonly characterized by loss of the p arm of chromosome 3(3p) distal to band 3p11.2–p13 [3]. Mutations in tumor suppressors within the remaining 3p arm results in loss of heterozygosity (LOH). Contained within the 3p arm are the most commonly mutated genes in ccRCC, which include *VHL*, *PBRM1*, *SETD2*, and *BAP1*.

PBRM1 *Is Often Mutated but Not Correlated to Survival*

PBRM1 is the second most commonly mutated gene in ccRCC, which is located at band 21(3p21) [4]. Mutations of *PBRM1* in ccRCC were originally identified using an exploratory cohort of seven patient cases, which included both *VHL* mutant and *VHL* wild-type tumors. In that subset, 4 out of the 7 patients were found to have mutations in *PBRM1*. To better understand the broader prevalence of *PBRM1* mutations, a follow-up analysis was expanded to a larger cohort of 257 RCC cases, which included 36 cases of non-clear cell RCC. This analysis revealed truncating *PBRM1* mutations in 34 % (88/257) of cases. In the tumors with available SNP data, mutations in PBRM1 all induced LOH with concurrent loss of chromosome 3p (38/38 of cases) [4]. Immunohistochemical (IHC) analyses of ccRCC confirmed a high correlation between loss of protein expression and truncating mutations. 90 % of samples found negative by IHC also contained mutations in *PBRM1*, while 90 % of samples found positive by IHC contained wild-type *PBRM1* [5].

Since these seminal discoveries, multiple groups, including The Cancer Genome Atlas (TCGA) project and Seishi Ogawa and colleagues from Japan, as well as others have examined large collections of tumor originating from several hundred kidney cancer patients and have confirmed that mutations of *PBRM1* occur at a high frequency, up to 41 % of ccRCC cases [6–9]. For example, the TCGA evaluated tumor samples from 421 ccRCC patients and reported a frequency of genetic *PBRM1* loss of 33 % [6]. The overall rate at which *PBRM1* is mutated, however, appears to be lower than the frequency reported in the literature for *VHL* inactivation, which can range from 50 to 90 % of patients. In the large collections of tumor samples mentioned above, the TCGA and Sato et al. reported that *VHL* alterations occurred in 52 and 82 % of cases, respectively [6, 9]. Interestingly, losses of *PBRM1* and *VHL* are not mutual exclusive, but instead tend co-occur [8]. Similar to the

findings in RCC, *PBRM1* truncation mutations are commonly seen in breast cancer and pancreatic cancer [10, 11]. In breast cancer, chromosome 3p loss is common, and nearly 50 % of tumor samples show LOH at the *PBRM1* loci [12]. In contrast, in lung cancer, chromosome 3p loss is also common; however, *PBRM1* mutation is rare [13, 14]. *PBRM1* was screened in 56 lung cancer cell lines (30 NSCLC and 26 SCLC), and mRNA expression of *PBRM1* was seen in all 56 cell lines along with protein expression in 46/46 assayed cell lines. Ten selected cell lines were sequenced, and no mutations in PBRM1 were seen. Together, this suggests that *PBRM1* loss plays a functional role in the tumorigenesis of specific malignancies rather than simply a passenger mutation that is associated with 3p loss.

Given the significant frequency with which *PBRM1* is inactivated in ccRCC, its mutational status has been the subject of recent biomarker studies. In particular, these studies have examined if lesions in *PBRM1* might predict clinical outcomes such as cancer-specific survival (CSS). It has become increasingly apparent that loss of *PBRM1* function, like that of *VHL* inactivation, may be an early event in cancer initiation. This notion is supported by evidence derived from both animal and clinical studies. In a mouse pancreatic cancer model, a transposon system was used to randomly inactivate genes, and inactivation of *PBRM1* was seen in early dysplastic histologies including pancreatic, intraductal (PanIN), and high-grade dysplasia. In ccRCC patients, loss of *PBRM1* was correlated with lower Fuhrman grade ($p = 0.025$) [5]; however, it was also associated with increased invasiveness, as demonstrated by a sixfold increased risk of small tumors (<4 cm) extending into the renal vein or muscle containing branches or extending into the sinus or perinephric fat [2]. However, with respect to patient survival, mutations of *PBRM1* had no prognostic bearing on mortality risk as assessed by CSS [8]. As such, *PBRM1* loss appears to be like *VHL* inactivation that likewise does not predict CSS. These conclusions were reached based on the analyses of two large, independent ccRCC cohorts. Among 188 patients who underwent resection of primary ccRCC at the Memorial Sloan Kettering Cancer Center (MSKCC) and 421 patients evaluated by the TCGA, neither alteration of *PBRM1* nor *VHL* had an adverse outcome with respect to CSS within either cohort [8]. Collectively, these pieces of evidence imply that loss of either gene may not overtly affect ccRCC disease progression. Rather, they suggest that mutations of *PBRM1* and *VHL* may be pivotal to cancer initiation and/or maintenance.

Mutation of SETD2 *and* BAP1 *Is Correlated with Worse Survival*

In contrast to mutations of *PBRM1*, alterations in *SETD2* and *BAP1* may have negative prognostic significance on patient survival. Mutations of *SETD2* were originally identified by a large-scale exome sequencing of 3544 genes in tumor samples from 101 ccRCC cases [15]. In this set, *VHL* mutations were identified in 55 % of samples. Gene expression profiling classified tumors as hypoxic or non-hypoxic phenotypes, with 82 % of tumors showing an upregulation of genes associated with hypoxia. Hypoxic tumors have increased inactivating point mutations on *VHL* (65 %) and increased loss of 3p, where *VHL* is located (87 %).

Additionally, mutations of *SETD2*, *KDM5C*, or *KDM6A* were observed in approximately 15 % of tumor samples collectively. Regarding adverse clinical outcomes, the TCGA study of 421 patients reported that alterations of *SETD2* were associated with a worse CSS. Curiously, however, in the MSKCC cohort, mutation of *SETD2* showed no such association with CSS [8]. The basis for this discrepancy is unclear.

Regarding *BAP1*, inactivating mutations of this gene were found to correlate with clinical outcome. *BAP1* mutations were first identified by exome sequencing of an exploratory cohort of seven ccRCC tumors and paired normal tissue samples, which included six high-grade tumors [5]. Subsequently, using a cohort of 176 tumors, the *BAP1* mutation rate was determined to be 14 %, and loss of *BAP1* was associated with higher Fuhrman grade ($q = 0.005$) and mTORC1 activation as assayed by phospho-S6 ($q = 3 \times 10^{-4}$) and 4E-BP1 ($q = 0.029$). Loss of *BAP1* function was associated with a worse cancer-specific survival (0.013) and trended toward a worse overall survival ($p = 0.072$) [2]. *BAP1* mutations have also been associated with other malignancies including cutaneous and uveal melanomas and mesotheliomas. Patients with germline mutations in *BAP1* have increased incidence of malignancy, with 69.7 % cancer incidence among 76 mutation carriers [16]. Therefore, *BAP1* loss appears to convey tumor-suppressive function in multiple tissue types. One possible mechanism of BAP1 action may involve the regulation of mitotic growth. Cell lines with *BAP1* loss display an increased rate of proliferation, which could be decreased by the reintroduction of wild-type BAP1 [5, 17].

Interestingly, mutations in *BAP1* and *PBRM1* tend to be mutually exclusive of one another [2, 5]. In the small number of tumors that lose both *BAP1* and *PBRM1* function, a more aggressive behavior was observed. These tumors typically displayed high-grade acidophilic cytoplasm, eccentric nuclei, and prominent nucleoli, which are consistent with a rhabdoid morphology. RCC with rhabdoid features represents ~5 % of RCC yet results in disproportionate amounts of grade 3 and grade 4 tumors, 9 and 35 %, respectively [18]. Such tumors also show a greater propensity for extrarenal extension, 52 % for rhabdoid and 28 % for non-rhabdoid. Consequently, patients with a tumor harboring mutations of both *BAP1* and *PBRM1* displayed a poorer rate of survival [19]. In a UT Southwestern cohort of 145 patients, patients with a *PBRM1* mutation had an overall survival (OS) of 10.6 years as compared to *BAP1* mutation with an OS of 4.6 years. This study also preliminarily evaluated a TCGA cohort of 327 patients. Those patients with a *PBRM1* mutation had an OS of 5.4 years vs. a BAP1 mutation had an OS of 1.9 years. In both sets, simultaneous mutation of *PBRM1* and *BAP1* was rare, with three and four cases in the UT Southwestern group and TCGA, respectively; however, the results suggest a worse survival with OS confidence intervals of 0.3–3.8 years for the UT Southwestern group and 0.0–1.2 years for the TCGA group. Taken together, it may be possible to stratify a patient's prognosis based on their *PBRM1* and *BAP1* mutational status, with *PBRM1* mutation being a marker for good risk disease, *BAP1* mutation being a marker for poor risk disease, and double mutation being a marker for very poor risk disease. However, it is still unclear how and whether *PBRM1* and *BAP1* mutations cooperate to become a more aggressive disease.

Mutation in KDM5C *Is Less Common but May Be Associated with Aggressive Disease*

KDM5C, which is also known as *JARID1C*, was originally identified as an X-linked mental retardation gene. KDM5C functions as the histone demethylase that removes methyl groups from trimethylated histone 3 at lysine position 4 (H3K4me3) to di- or mono-methylated products [20]. KDM5C is a HIF-dependent transcription target, and loss of VHL leads to an upregulation of *KDM5C* mRNA and decreased H3K4me3. Interestingly, in *VHL*-deficient RCC cell lines, experimentally reducing expression of KDM5C by RNA-interference-mediated knockdown leads to an increase in levels of H3K4me3 and increased proliferation [21]. Together, this suggests that KDM5C functions as a tumor suppressor. Analysis of TCGA data is suggestive that mutation of KDM5C is associated with increased tumor grade and stage; however, the numbers of cases were small and did not reach statistical significance [2]. Larger studies with longer follow-up are likely necessary to elucidate the role of KDM5C in RCC tumorigenesis.

Epigenetic Regulation of Gene Expression

Remarkably, aside from *VHL* mutation, the most commonly mutated genes in ccRCC are unified by their potential role in epigenetic modification. Gene expression is a highly regulated process, which allows for complex and diverse phenotypes using the same genetic code. Regulation of gene expression can occur at the chromatin and mRNA level. In eukaryotes, DNA is packaged into chromatin, bound to histone proteins. In the most basic structure, 147 base-pair units of DNA form an individual nucleosome, when it wraps around histones, an octomeric protein complex comprised of two copies each of histone subunits H2A, H2B, H3, and H4. This packaging of DNA is important to maintain the integrity of the genome, yet it is thought to hinder accessibility for vital cellular processes such as transcription, replication, and DNA repair and recombination. Both DNA and protein components of the nucleosome can be modified to alter access to the DNA, thereby restricting or permitting transcription. After transcription, mRNA is subject to further regulation by microRNAs (miRNAs), which are noncoding RNAs ranging from 17 to 25 nucleotides which can downregulate gene expression. Together, these cooperate and build upon the complexity of genome.

Chromatin Remodeling: Covalent Modification vs. ATP Hydrolysis

Chromatin remodeling enzymes can be broadly categorized into two general classes of complexes. Whereas one group mediates covalent modification of histones, the other uses ATP hydrolysis for modification [22]. Covalent modification includes

Table 5.1 Major families of ATP-dependent chromatin remodeling complexes

	SWI/SNF	ISWI	CHD
Domains	Bromodomain, ATPase	SANT domain, ATPase	Chromodomain, ATPase
Intact nucleosome	Independent	Dependent	Dependent
Histone tail	Independent	Dependent	Independent

acetylation, methylation, phosphorylation, SUMOylation, and ubiquitination. For instance, *SETD2*, *KDM5C*, and *BAP1* are examples of a histone-lysine *N*-methyltransferase, a lysine-specific demethylase, and a deubiquitinating enzyme, respectively. On the other hand, PBRM1 is a critical member of an ATP-dependent chromatin remodeling complex. ATP-dependent chromatin remodeling complexes can be subdivided into three separate families, named the SWI/SNF (SWItch/Sucrose NonFermentable), ISWI (imitation SWI), and CHD (chromodomain and helicase-like domain) [22]. All three families have a similar ATPase domain but differ by the presence of unique domains, specifically, bromodomains, SANT domains, or chromodomains, respectively, which are thought to convey separate nucleosome and histone recognition [23]. Furthermore, while the different families show similar rates of ATP utilization and nucleosomal remodeling in vitro, they differ with respect to whether an intact nucleosome is required for their ATPase activity and regarding whether their remodeling function is dependent on the presence of histone tails. These differences are summarized in Table 5.1.

The SWI/SNF Complex and Cancer

Although PBRM1 is a defining member of the PBAF SWI/SNF complex, its role in tumorigenesis must be understood in the context of other SWI/SNF complexes. The SWI/SNF chromatin remodeling complex is a multi-subunit protein complex that is combinatorially assembled and defined by unique subunits of the distinct complexes. Given the tight stoichiometry relationship between subunits, phenotypes from specific mutational losses could be the consequence of a loss of function of that particular complex or a gain of function of alternative complexes. In other words, the phenotype from loss of PBRM1 could be due to either loss of PBAF activity or increased activity of competing SWI/SNF complexes, such as BAF due in increased shared subunit availability.

Organization of the SWI/SNF Complex: ATPase, Core Subunits, and Accessory Subunits

SWI/SNF is evolutionarily conserved across species, and the multi-subunits of the SWI/SNF complexes are highly homologous from yeast to *Homo sapiens* [24]. Genes encoding components of the SWI/SNF complex were originally identified in yeast through screens examining defects in mating-type switching and sucrose fermentation [25–27]. In higher eukaryotes, including *Drosophila* and humans, SWI/

SNF complexes are formed by 9–12 different subunits (Table 5.2) [24], which can be grouped into three categories: the ATPase, the core subunits, and the accessory subunits. The ATPase is the critical catalytic subunit of the SWI/SNF complex, which is capable of nucleosome remodeling in vitro in the absence of any other subunits, although the degree of activity is attenuated compared to that of the full complex [28]. The core subunits are the minimally necessary set of subunits that are required in addition to the ATPase for in vitro nucleosome remodeling activity equivalent to that of the full SWI/SNF complex. The accessory subunits are defining members of specific SWI/SNF complexes and are presumed to contribute to assembly, regulation, and targeting of the complexes. Each SWI/SNF complex consists of a single ATPase (BRM or Brg1), a single core protein SNF5, and two core proteins of either BAF155 or BAF170 or one of each. Considerable evidence now exists and continues to accumulate that indicates that alterations in SWI/SNF function lead to cancer susceptibility (Table 5.3). Notably, lesions in genes encoding various

Table 5.2 Comparison of SWI/SNF complexes from yeast to humans

	Yeast		*Drosophila*		Human	
Complex	SWI/SNF	RSC	BAP	PBAP	BAF	PBAF
ATPase	Swi/Suc2	Sth1	BRM/Brahma		BRM or BRG1	BRG1
Core	Snf5	Sfh1	SNR1/BAP45		hSNF5/BAF47/INI1	
	Swi3	Rsc8/Swh3	MOR/BAP155		BAF155, BAF170	
Accessory	Swi1/Ard6		OSA/eyelid		ARID1A-b	
				Polybromo		PBRM1
				BAP170		ARID2
				CG7154	Brd9	Brd7
	Arp4		BAP55 or BAP47		BAF53a-b	
			Actin		Actin	
			BAP111/dalao		BAF57	
					BAF45a-d	
	SNF12				BAF60a-c	

Table 5.3 SWI/SNF alterations and cancer

SWI/SNF subunit	Associated malignancy (frequency of alteration)	References
Brg1	Prostate cancer	
	3 % heterozygous deletion	TCGA
	Breast cancer	
	20 % heterozygous deletion	TCGA
	Lung cancer	
	4.5 % mutation, 21.5 heterozygous deletion, total 78 squamous tumors	TCGA
	52.7 % heterozygous deletion, total 129 adenocarcinoma tumors	
SNF5	Malignant rhabdoid tumors	
	49 % mutation, 51 % homozygous deletion, total 29 tumors	50
	75 % homozygous deletion, total 16 tumors	53

(continued)

Table 5.3 (continued)

SWI/SNF subunit	Associated malignancy (frequency of alteration)	References
BAF155/ BAF170	Lung cancer	
	97 % 3p loss, total 97 tumors	13
	46 % heterozygous deletion, total 129 tumors	TCGA
	Breast cancer	
	30.8 % heterozygous deletion, total 766 tumors	TCGA
	Ovarian cancer	
	33.1 % heterozygous deletion, total 311 tumors	TCGA
	Cervical cancer	
	50 % heterozygous deletion, total 36 tumors	TCGA
PBRM1	Renal cell carcinoma	
	29 % mutation, total 185 tumors	2
	41 % mutation, total 227 tumors	4
	50 % mutation, total 10 tumors	7
	26 % mutation, total 106 tumors	9
	Pancreatic cancer	
	8 % mutation, total 25 samples	85
ARID1A	Ovarian cancer	
	57 % mutation, total 42 tumors	71
	43 % heterozygous loss, total 316 tumors	TCGA
	Lung adenocarcinoma	
	8 % mutation, total 183 tumors	81
	6 % mutation, total 129 tumors	TCGA
	Esophageal dysplasia	
	15 % mutation, total 20 samples	84
	Gastric cancer	
	78 % mutation, total 23 microsatellite instability tumors	78
	47 % mutation, total 15 EBV-infected tumors	
	10 % mutation, Total 71 microsatellite stable EBV-negative tumors	79
	20 % mutation, total 15 tumors	
	Pancreatic cancer	
	47 % heterozygous deletion, total 70 tumors	85
	32 % heterozygous deletion, total 50 tumors	TCGA
	Hepatocellular cancer	
	13 % mutation, total 13 % HBV-associated tumors	86
	Burkitt's lymphoma	
	17 % mutation, total 29 tumors	82
	Childhood neuroblastoma	
	11 % mutation (ARID1A and ARID1B combined), total 71 tumors	83
	Transitional cell carcinoma	
	13 % mutation, total 97 tumors	80
	Renal cell carcinoma	
	15.8 % heterozygous loss, total 418 tumors	TCGA
ARID1B	Pancreatic cancer	
	74 % heterozygous deletion, 3 % homozygous deletion, total 70 samples	85
	52 % heterozygous deletion, total 50 samples	TCGA
ARID2	Hepatocellular cancer	
	18 % mutation, total 23 HCV-associated tumors	88

components of these complexes have been connected to the emergence of solid tumors of diverse tissues. Each of these individual subunits and their relationship to tumorigenesis will be discussed in detail later in the chapter.

SWI/SNF ATPase: The Catalytic Subunit

The ATPase is the critical catalytic subunit in the SWI/SNF complex, which is capable of nucleosome remodeling in vitro in the absence of any other subunits [28]. In yeast and *Drosophila*, there is only a single ATPase, Swi/Suc2 and Brahma (BRM), respectively [29]. In humans, there are two homologous ATPases, *BRM* and *Brg1* (Brahma-related gene 1) [30]. Other names for *BRM* in humans include *Brahma*, *SNF2α*, and *SMARCA2*. Other names for *Brg1* in humans include *Brahma-related gene 1*, *SNF2β*, and *SMARCA4* (see Table 5.4 for list of SWI/SNF subunits and their alternative names). *BRM* and *Brg1* contain three conserved domains, a C-terminal

Table 5.4 Major SWI/SNF subunits and alternative names

Name in review	Aliases
Brm	SMARCA2 (SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily a member 2); SNF2; SWI2; hBRM; NCBRS; Sth1p; BAF190; SNF2L2; SNF2LA; hSNF2a
Brg1	SMARCA4 (SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily a member 4); SNF2; SWI2; MRD16; RTPS2; BAF190; SNF2L4; SNF2LB; hSNF2b; BAF190A
SNF5	SMARCB1 (SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily b member 1); RDT; INI1; hSNF5; Snr1; BAF47; MRD15; RTPS1; Sfh1p; hSNFS; SNF5L1
BAF155	SMARCC1 (SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily c member 1); Rsc8; SRG3; SWI3; CRACC1
BAF170	SMARCC2 (SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily c member 2); Rsc8; CRACC2
ARID1A	AT-rich interactive domain 1A (SWI-like); ELD; B120; OSA1; P270; hELD; BM029; MRD14; hOSA1; BAF250; C1orf4; BAF250a; SMARCF1
ARID1B	AT-rich interactive domain 1B (SWI1-like); OSA2; 6A3-5; DAN15; MRD12; P250R; BRIGHT; BAF250B; ELD/OSA1
Brd9	Bromodomain containing 9, PRO9856; LAVS3040
Pbrm1	Polybromo-1; PB1; BAF180
ARID2	AT-rich interactive domain 2 (ARID, RFX-like); p200; BAF200
Brd7	Bromodomain containing 7; BP75; NAG4; CELTIX1
BAF53a	ACTL6A (actin-like 6A); Arp4; ACTL6; BAF53A; INO80K; ARPN-BETA
BAF53b	ACTL6B (actin-like 6B); ACTL6
BAF57	SMARCE1 (SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily e member 1)
BAF45a	PHF10 (PHD finger protein 10); XAP135
BAF45b	DPF1(D4, zinc and double PHD fingers family 1); NEUD4; neuro-d4
BAF45c	DPF3 (D4, zinc and double PHD fingers, family 3)
BAF45d	DPF2 (D4, zinc and double PHD fingers family 2); REQ; UBID4; ubi-d4

(continued)

Table 5.4 (continued)

Name in review	Aliases
BAF60a	SMARCD1 (SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily d member 1); Rsc6p; CRACD1
BAF60b	SMARCD2 (SWI/SNF-related matrix-associated, actin-dependent regulator of chromatin subfamily d member 2); Rsc6p; BAF60B; CRACD2; PRO2451
BAF60c	SMARCD3 (SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily d member 3); Rsc6p; CRACD3

proline-rich domain, a DNA-dependent ATPase, and a bromodomain that share considerable sequence identity [30]. These domains are also highly conserved from humans to yeast. Despite significant sequence homology between *BRM* and *Brg1*, knockout mice of *BRM* and *Brg1* demonstrate remarkably disparate phenotypes.

Knockout mice of *BRM* are largely phenotypically normal; but they are 14 % heavier than their wild-type counterparts [31]. This mostly reflects increases in the bone, muscle, and connective tissue mass. In *BRM* knockouts, Brg1 is upregulated and replaces BRM in the SWI/SNF complex. The degree of Brg1 upregulation correlates to the degree of BRM expression in the wild-type tissue. In tissues with high BRM expression such as the brain, Brg1 expression increases five- to sixfold compared to only twofold in liver/spleen.

In contrast, loss of Brg1 is homozygous lethal, and embryos die peri-implantation [32]. However, Brg1 is not necessary for general cell viability. $Brg1^{-/-}$ cells can be isolated and cultured in vitro, which suggests that Brg1 knockout mice die from a developmental defect. Heterozygotes are viable; however, they showed increased rates of exencephaly and apocrine tumors. Together, this suggests that BRM may be functionally redundant, while Brg1 is critical. Biochemically, this may be explained by the exclusion of BRM from the PBAF complex. In other words, both BAF complex and PBAF complex can be formed in the absence of BRM; however, only BAF complex can be formed in the absence of Brg1.

Both Loss of BRM and Brg1 Play an Important Role in Tumorigenesis

Prostate cells are an androgen-sensitive cell type, whose growth rate is often increased by endogenous or exogenous androgens. However, prostate cells are still able to grow in the absence of androgen, albeit at a slower rate. $BRM^{-/-}$ knockout mice show increased prostate cell proliferation during androgen deprivation [33]. Both $BRM^{-/-}$ and wild-type mice have similar prostate growth in response to testosterone stimulation; however, $BRM^{-/-}$ mice have a ninefold increase in BrdU incorporation compared to wild-type animals in response to androgen deprivation. In cells with low BRM, the transcription factor E2F1 is dysregulated and leads to a proliferative advantage. No increase in Brg1 expression is seen in the prostate tissues, implying that $BRM^{-/-}$ loss may have some direct effects in prostate cell proliferation.

BRM Loss Plays a Role in Prostate Cancer

Mechanistically, *BRM* plays an important role in androgen signaling. Using double knockouts for Brg1 and BRM, steroid receptor activation is absent [34]. However, steroid-dependent transcription can be restored by ectopic expression of BRM or Brg1. Ectopic expression of BRM robustly restored androgen receptor (AR)-dependent transcription, while ectopic expression of Brg1 only modestly restored AR-dependent transcription. In contrast, estrogen receptor (ER)-dependent transcription showed equal responses to ectopic BRM or Brg1. Mechanistically, upon androgen stimulation, the BRM containing ATPase complex is recruited to the chromatin [35]. In contrast, untreated cells or treatment with a partial AR agonist did not recruit BRM to the chromatin.

In primary tumor samples, it is unclear whether prostate cell proliferation is due to $BRM^{-/-}$ loss or increased Brg1 [36]. Microarray analysis of both malignant and benign prostate tissue shows a reciprocal relationship between BRM and Brg1 levels, which was not seen in mouse models of BRM loss [33]. In primary tumors, Brg1 is elevated in malignant cells, and higher Brg1 levels are correlated with increased tumor grade, tumor mass, and invasiveness. However, other studies have also reported loss of Brg1 in prostate cancer cell lines [37]. In these cell lines, the reintroduction of Brg1 reverses their transformed phenotype. Upon ectopic expression of Brg1, the cells undergo cell cycle arrest and morphologically flatten. Although this seems to contradict other reports of elevated Brg1 in prostate cancer, these cell lines also show decreased levels of BRM. Taken together, BRM loss may be important for prostate cancer pathogenesis, and upregulation of Brg1 helps compensate for BRM loss. Elevated Brg1 expression levels may be an indirect marker of BRM loss, and redundancy between BRM and Brg1 can help explain the recovery of normal physiology with Brg1 reintroduction. However, more studies are necessary to understand the mechanism for ATPase loss in prostate cancer pathogenesis.

Brg1 Loss Plays a Role in Breast Cancer

$Brg1^{+/-}$ heterozygous mice show a greater propensity for apocrine tumors, which resemble breast epithelium [32]. Breast tumors isolated from $Brg1^{+/-}$ mice likely result from haploinsufficiency and not loss of heterozygosity (LOH) [38]. Sequencing of these tumors reveals no evidence of mutations in the remaining copy of Brg1 or any copy number changes at the *Brg1* locus; however, the tumors express 60 % of WT levels of Brg1. Outside the Brg1 locus, multiple genomic changes are seen involving both the loss and gain of chromosome 2 and gain of chromosomes 4, 15, and 19, implying that breast cancers are genomically unstable; however, maintenance of a single copy of *Brg1* is necessary for survival. Similarly, mutations in *Brg1* are also seen in breast cancer cell lines [37].

Brg1 Loss Plays a Role in Lung Cancer

Loss of BRM and Brg1 has also been identified in both lung cancer cell lines and primary lung tumors [37, 40, 41]. Low or absent expression of BRM and Brg1 is seen in multiple non-small cell lung cancer (NSCLC) cell lines. In this study, 6/60 primary lung tumors (41 adenocarcinoma and 19 squamous) lost both BRM and Brg1. Solitary Brg1 loss was not seen, and for technical reasons, solitary BRM loss could not be assayed. Interestingly, in lung cancer, there was no evidence of discordant BRM and Brg1 expression as seen in prostate cancer. Loss of BRM/Brg1 correlates with worse survival, likely independent of stage. Brg1-positive stage 2A/2B patients had a median survival of 46 months. Brg1-positive stage 3A patients had a median survival of 25 months. Brg1-negative patients had a median survival of 9.5 months ($p = <0.0001$).

Differential Effects of Brg1 Loss in Normal vs. Transformed Cells

Lung-specific conditional Brg1 knockout mice also demonstrated increased lung cancer [42]. Wild-type, lung-specific $Brg1^{+/-}$ mice and lung-specific $Brg1^{-/-}$ mice were treated with a lung-specific carcinogen, ethyl carbamate. In untransformed cells, heterozygous inactivation of *Brg1* increases tumor development fivefold; however, homozygous inactivation of Brg1 in untransformed cells reduced proliferation and enhanced apoptosis. However, if homozygous loss of Brg1 occurs after the development of the adenoma, this results in a tenfold increase in tumors. Together, this suggests that heterozygous loss of Brg1 enhances tumor initiation; however, homozygous Brg1 loss enhances proliferation after transformation has already occurred. This data helps reconcile observations that development of breast tumors in $Brg1^{+/-}$ mice result from haploinsufficiency and not LOH; however, in primary tumors, complete loss of Brg1 is commonly seen.

Loss of BRM and Brg1 has differential effects on genomic stability [43]. Loss of Brg1 had dramatic disruption of peri-centromeric heterochromatin domains, while loss of BRM only had modest effects. Brg1 loss is also associated with changes in histone trimethylation, but this is not seen with BRM or SNF5 loss. Furthermore, Brg1 loss is associated with mitotic defects and polyploidy with many cells having >4 N DNA content. These cells are at a significant proliferative disadvantage with many undergoing cell death. It is likely untransformed cells are unable to tolerate this level of genomic instability; however, after transformation, the physiologic cell cycle checkpoints are disrupted, and the nuclear defects are both tolerable and pathogenic.

Differential Effects of BRM and Brg1 on Growth

Oncogenes like Ras also provide differential regulation of BRM and Brg1 [44]. In untransformed fibroblasts, growth arrest is associated with accumulation of BRM. Interestingly, Ras transformation induces reentry into the cell cycle and downregulation of BRM expression, while Brg1 remains unchanged.

In contrast, both BRM and Brg1 are capable of mediating Rb-induced cell cycle arrest [45]. Concurrent loss of BRM and Brg1 prevents Rb-induced arrest, which can be restored via ectopic expression of BRM or Brg1. Mutants of BRM and Brg1 unable to bind Rb are also unable to induce cell cycle arrest. However, heterozygous loss of Brg1 is insufficient to enhance the $Rb^{+/-}$ phenotype [38]. Mice that are $Brg1^{+/-}$ $Rb^{+/-}$ and $Rb^{+/-}$ mice are similar in phenotype with both mice acquiring pituitary tumors. The single copy loss of Brg1 did not change the latency period or penetrance or Rb-related tumor, nor did it result in early death. It is possible that Rb-mediated interactions with the SWI/SNF complex can utilize either ATPase; therefore, singular loss of an ATPase is insufficient to drive the cell cycle progression.

Brg1 Binds BRCA1

Brg1 can directly bind to BRCA1 (breast cancer type 1 susceptibility protein) [39]. Pulldown of BRCA1 recovers a Brg1-containing SWI/SNF complex including at least BRG1, SNF5, BAF155, BAF170, BAF60B, BAF53, and SNF5; however, direct binding only occurs with BRG1 and not the rest of the members of the complex. Together, this complex mediates BRCA1 transcriptional activity. However, interaction between BRCA1 and BRM is yet to be identified. The interaction between BRCA1 and Brg1 may be unique to this ATPase.

Potential Targeting of Tumors with BRM Loss

In contrast to Brg1, which is often lost through deletion and truncating mutations, BRM loss in malignancies is often due to epigenetic silencing [46]. Given the reversible nature of epigenetic silencing, there is interest in pharmacologic restoration of BRM expression. Sequencing of the BRM promoter identified two insertion polymorphisms, which are associated with loss of BRM in primary NSCLC tumors and cell lines [47]. A case-control study of 484 smokers with lung cancer and 715 smokers without lung cancer demonstrates increased adjusted odds ratio of 2.19 (CI = 1.4–3.4, $p = 0.0006$) of having lung cancer, when patients are homozygous for both polymorphisms. The insertional polymorphisms generate sequences with 92 % homology to myocyte enhancer factor 2 (MEF-2) binding sites, which is known to recruit histone deacetylases (HDAC). Treatment with HDAC inhibitors can restore BRM expression in BRM-negative cell lines [46, 47]. Taken together, it is attractive

to speculate that these polymorphisms induce acetylation at the BRM promoter and silence the gene. Although HDAC inhibitors can restore BRM expression, they also induce BRM acetylation, which inhibits BRM function. Consequently, HDAC inhibitors are not well suited for reactivation of BRM in deficient cells. Through large library screens, other compounds have been identified which can restore BRM expression and function in deficient cell lines [48]. Further studies are necessary to determine the mechanism of action and whether they may yield clinical utility.

SWI/SNF Core Subunits: Activating the SWI/SNF ATPase

Purified ATPases alone are capable of nucleosome remodeling in vitro; however, their activity is below that of the whole SWI/SNF complex [28]. In vitro reconstitution of the SWI/SNF complex identified subunits that are necessary to provide remodeling activity equivalent to the whole SWI/SNF complex. These subunits are defined as the "core" subunits of the SWI/SNF complex and include SNF5, BAF155, and BAF170. The core subunits function with either BRM or Brg1 and can increase ATPase activity in the presence of nucleosomes three- to sevenfold.

SNF5

SNF5, which is also known as INI1, BAF47, or SMARCB1, was originally isolated in yeast as a transcriptional activator in the SWI/SNF complex. The human homolog of SNF5 was identified as an interaction partner with HIV integrase, hence named integrase interactor 1(INI1) [49].

Loss of SNF5 Is Sufficient for Tumorigenesis

In humans, malignant rhabdoid tumors (MRT) are associated with germline loss of one allele of SNF5 and LOH in the tumor [50]. MRT is an aggressive pediatric malignancy that occurs before age 2 and often arises from the kidney and other soft tissues, including the brain, skin, liver, thymus, and orbit. In mice, homozygous loss of SNF5 is lethal at embryonic day 7, while heterozygous loss of SNF5 allows for grossly normal mice at birth. At week 5, the mice begin to develop tumors similar to MRT with 12 % penetrance [51]. Similarly, complete conditional loss of both alleles of SNF5 leads to pancytopenia followed by death, or conditional loss of both alleles leads to development of MRT and rapid death with 100 % penetrance [52]. Interestingly, $BRM^{-/-}$ $Brg^{+/-}$ mice do not recapitulate the phenotype seen with SNF5 loss [38]. In addition to the malignancies seen in $Brg1^{+/-}$ mice, $BRM^{-/-}$ $Brg1^{+/-}$ mice also develop hemangiosarcomas; however, there is no change in $Brg1^{+/-}$ malignancy penetrance nor is there development of MRT [38]. Together, this suggests that SNF5 loss is a unique phenotype.

Unlike loss of Brg1, which induces genomic instability in breast cancer [38], loss of SNF5 induces tumorigenesis without the development of genomic instability [53]. Loss of SNF5 did not show defects in DNA repair nor did it show hypersensitivity to DNA damaging agents, such as etoposide, cisplatin, or UV radiation. High-density SNP array analysis of SNF5-deficient malignancies also did not show genomic alterations such as deletions or amplifications other than at SNF5 locus. Gene array analysis did show overexpression of cyclin D1 and c-Myc. Whole-exome sequencing and SNP array analysis of MRT patients also show relatively simple genomes [54]. Other than mutations in SNF5, 2/32 tumors showed no other mutations by whole-exome sequencing at mean 83× coverage, and 4/32 tumors only had subclonal mutations. This suggests that epigenetic dysregulation is sufficient to drive tumorigenesis. The maintenance of DNA damage checkpoints in these epigenetic-driven malignancies may help explain the resistance of these tumors to traditional chemotherapeutics and radiation.

Cooperation Between SNF5 and the ATPase Subunit in Tumorigenesis

Although SNF5 is a core member of the SWI/SNF complex, SNF5 is dispensable for SWI/SNF function [55]. Only a subset of SWI/SNF-dependent transcripts is also dependent on SNF5. In SNF5 null cell lines, SWI/SNF-dependent functions such as induction of hsp70 in response to arsenite or induction of CIITA to IFNγ remain possible. At a minimum, PBAF SWI/SNF complex (BRG1, BAF155, BAF170, Pbrm1) can still be assembled without SNF5. Despite the dispensability of SNF5 in the SWI/SNF complex and the difference in phenotype between SNF5 loss and ATPase loss, the tumorigenicity of SNF5 loss is dependent on Brg1 activity [56].

Inactivation of Brg1 in SNF5-deficient MEFs (mouse embryonic fibroblasts) leads to rapid cell death. Similarly, knockdown of BRM and Brg1 in MRT cells, which commonly have loss of SNF5, also leads to cell death in vitro, and conditional loss of Brg1 prevents the development of MRT in SNF5 conditional mice. It remains to be explained whether the residual SWI/SNF activity in the absence of SNF5 is responsible for tumor formation or, alternatively, concurrent loss of SNF5 and Brg1 is incompatible with general viability.

Loss of SNF5 Activates Multiple Signaling Pathways

Loss of SNF5 leads the aberrant activation of multiple signaling pathways including the Polycomb, Hedgehog/Gli, and Wnt/β-catenin pathways [57–59]. Polycomb-group (PcG) proteins play an important role in tumorigenesis. PcG member EZH2 is highly expressed in many cancer types and has been associated with poor prognosis. EZH2 helps silence many tumor suppressors via trimethylation of histone 3 lysine 27. Loss of SNF5 induces elevated expression of EZH2 and downregulation of PcG-silenced genes. Furthermore, there is an upregulation of stem cell-associated genes, which could be reversed by knockdown of EZH2. Loss of EZH2 also significantly decreases proliferation of $SNF5^{-/-}$ cells in vitro and tumor formation in vivo.

This suggests that loss of SNF5 induces stemlike features partly through upregulation of EZH2, which contributes to oncogenic transformation.

The Hedgehog (Hh) signaling pathway also plays a critical role in regulating development and tumorigenesis [60]. Gli1 is a transcription factor that mediates Hh signaling and has been implicated in the development of stemlike features and metastasis [61]. SNF5 localizes to Gli1-dependent promoters, and loss of SNF5 enhances Gli1-dependent transcription, which includes Gli1 itself and Patched 1(Ptch1) [59]. Furthermore, the SNF5/Brg1 complex suppresses Gli1 expression, where loss of either SNF5 or Brg1 enhances Gli1 levels. Elevated Gli1 contributes to MRT tumorigenesis, and loss of Gli1 inhibits proliferation and colony formation. Given the availability of Gli1 inhibitors, this may suggest some rationale for their use in SWI/SNF-altered malignancies.

Similarly, β-catenin also regulates development, and loss of SNF5 upregulates β-catenin-dependent genes such as AXIN2, APC, βTRCP, LEF1, and HDAC4 [58]. Loss of SNF5 acts independently of the canonical Wnt pathway, as treatment with Wnt inhibitors and downregulation of β-catenin had no effect on growth and proliferation; however, SNF5 was found to bind to TCF4. In the absence of nuclear β-catenin, TCFs bind to β-catenin-targeted promoters and repress their transcription. However, either the loss of SNF5 or deletion of the SNF5 binding site on TCF4 prevents TCF4 from repressing transcription. It is unclear whether TCF4 recruits the SWI/SNF complex for transcriptional regulation or the converse where the SWI/SNF complex is required for proper localization of TCF4 to the promoter.

These data demonstrate that dysregulation of the chromatin remodeling complex renders tumors independent of their external signaling, such as Hg or Wnt. Furthermore, dysregulation of a single subunit in the SWI/SNF complex leads to simultaneous alteration of multiple pathways critical for proper cell development and tumorigenesis. Given the difficulty of restoring proper function of the SWI/SNF complex, it may be necessary to simultaneously target multiple signaling pathways instead of our current paradigm of single-agent targeted therapy.

BAF155/BAF170

BAF155, which is also known as SWI3, SRG3, or SMARCC1, and BAF170, which is also known as SMARCC2, are the human homologs of yeast SWI3 and *Drosophila* MOIRA. BAF155 or BAF170 can exist as either a homodimer or heterodimer (BAF155/BAF155, BAF155/BAF170, BAF170/BAF170). Binding between the dimers occur through their leucine zipper [62]. BAF155 was originally thought to be highly expressed and localized to the thymus and low in the periphery; however, it is now believed that BAF155/BAF170 is more ubiquitously expressed. At this time, it is still unclear why two homologs for SWI3 occur in humans. It is possible that they may confer some subtle alteration in the formation of the SWI/SNF complex; however, that is yet to be determined.

BAF155/BAF170 Function as a Regulatory Scaffolding Protein

BAF155/BAF170 functions primarily as a scaffolding protein and can directly interact with BAF57 [63]. BAF57 levels are tightly regulated, where exogenous addition of BAF57 leads to a decrease in endogenous BAF57. BAF155/BAF170 levels help dictate BAF57 by preventing its degradation [64]. BAF57 is subject to ubiquitination by the E3 ligase TRIP12 and subsequent degradation. BAF155/BAF170 prevents BAF57 degradation through both ubiquitin-dependent and ubiquitin-independent processes. Binding of BAF155/BAF170 obscures the ubiquitination sites on BAF57; however, mutation of the ubiquitination sites on BAF57 is insufficient to fully protect unbound BAF57 from degradation. Similarly, binding to BAF155/BAF170 also stabilizes BRG1, SNF5, and BAF60a by preventing proteasomal degradation [65]. Together, this suggests that SWI/SNF subunits are maintained in a tight stoichiometric relationship, which may be critical for its proper function. This is further evidenced by compensatory upregulation of homologous subunits when individual subunits are lost, which adds a layer of complexity to understanding SWI/SNF function. It is unclear whether the phenotypes seen reflect a loss of function associated with a particular mutation or a gain of function related to the upregulation of the compensatory subunits.

Regulation of BAF155/BAF170 may have implications in global SWI/SNF activity by capping the total amount of complex formation possible. Downregulation of BAF155/BAF170 also plays a physiological role in T-cell receptor (TCR) signaling and thymocyte maturation [66]. Under physiologic conditions, BAF155/BAF170 transcription is downregulated by TCR activation, and constitutive expression of BAF155/BAF170 prevents positive selection by TCR stimulation.

BAF155/BAF170 and Tumorigenesis

The role of BAF155/BAF170 on tumorigenesis still remains unclear. BAF155 is located on chromosome 3p21.31, and 3p loss is frequently seen in multiple cancers including breast cancer (87 %) and lung cancer (97 %), with specific loss of 3p21.3 at 69 and 77 %, respectively [13]. BAF155 loss is also identified in multiple cell lines including colon and ovarian lines [67]. These cell lines show no changes in Rb-mediated cell cycle arrest; however, expression of the BAF155 decreases colony formation suggesting a tumor-suppressive effect of BAF155. Consistent with this data, elevated mRNA expression of BAF155 in stages I–III of colon cancer is associated with improved OS ($p = 0.0275$) [68].

However, the role of BAF155 as tumor suppressor may be a tumor type-specific phenomenon. Instead of BAF155 loss, BAF155 overexpression can also be seen. Gene expression analysis of cervical intraepithelial neoplasias (CIN) and normal cervical tissue shows elevated BAF155 in CIN III lesions, which are the most dysplastic [69]. Similarly, in prostate cancer, elevated BAF155 by IHC is correlated with increased Gleason score ($p < 0.05$), increased stage ($p < 0.01$), and decreased

time to recurrence ($p = <0.001$). Elevated BAF155 yields an increased odds ratio for recurrent cancer of 16 and metastatic disease of 4.5 [70].

These differences in effects on tumorigenesis by BAF155/BAF170 may reflect its non-selectivity for participation in competing SWI/SNF complexes. Changes in BAF155/BAF170 may simply amplify and depress the effects of the complex. In other words, if the SWI/SNF complex is dysregulated in such a way to promote tumorigenesis, amplification of BAF155/BAF170 will enhance tumorigenesis; however, if the complex is dysregulated such that tumor formation is suppressed, loss of BAF155/BAF170 will also enhance tumorigenesis.

Accessory Subunits and Specific SWI/SNF Complexes

The accessory subunits are thusly named for the role in targeting and regulating the SWI/SNF complex. These subunits have no direct effect on mediating ATPase function; however, they play an important role in scaffolding the ATPase and core subunits with other receptors and transcription factors and also target promoter recognition. The accessory subunits are combinatorially assembled into multiple complexes; therefore, individual subunits can either be shared across multiple specific complexes or define specific SWI/SNF complexes. In general, complexes can only contain one subunit from a particular subset, e.g., ARID1A or ARID1B, but not both.

PBAF vs. BAF: The Best Characterized Eukaryotic SWI/SNF Complexes

Many distinct SWI/SNF complexes have been identified that have a variety of functions. These complexes mediate nuclear receptor signaling and can play key roles in the development and differentiation of progenitor cells. However, dysregulation of these SWI/SNF complexes has profound effects on tumorigenesis. The two best characterized SWI/SNF complexes are PBAF and BAF.

The PBAF SWI/SNF complex exclusively utilizes Brg1 as its ATPase, and it is defined by the accessory components PBRM1, ARID2, BAF45A, and Brd7. BAF, on the other hand, utilizes either BRM or Brg1 as its ATPase, and it is defined by ARID1 and Brd9. It is unclear what determines the ATPase specificity for PBAF. Although these two complexes are functionally distinct, their roles are closely intertwined by the sharing of ATPase, core, and accessory subunits. Other distinct SWI/SNF complexes have also been identified and have been named as per their putative function in development. These include esBAF (embryonic stem cell BAF), cBAF (cardiac BAF), nBAF (neuronal BAF), and npBAF (neural progenitor BAF). It is not yet clear how these other complexes cooperative with or antagonizes PBAF and BAF. As this field rapidly evolves, additional subunits of the SWI/SNF complex are being identified. This chapter will attempt to summarize the evidence that these subunits play in tumorigenesis; however, the ultimate composition of each of these complexes is yet to be defined.

BAF Dysregulation and Tumorigenesis

Alterations in ARID1A are most frequently seen in ovarian malignancies; however, they are broadly found in other malignancies including those of the kidney, upper GI tract, liver, and others. The high frequency of ARID1A mutations in ovarian clear cell carcinomas was initially identified through next-generation sequencing of eight tumors, which covered 18,000 genes at 84× coverage [71]. Mutations in more than one tumor were seen in four genes, which include PIK3CA (phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha), K-RAS, PPP2R1a (protein phosphatase 2, regulatory subunit A), and ARID1A. Targeted sequencing of additional tumors showed mutation rates of 40, 4.7, 7.1, and 47 %, respectively. Other groups have shown targeted sequencing of other ovarian cancers shows mutation of ARID1A in 46 % of ovarian clear cell (55/119) and 30 % of endometrioid (10/33) histologies; however, no mutations were seen in 76 cases of high-grade serous ovarian cancer [72]. Similarly, loss of ARID1A detection by IHC is seen in low-grade endometrioid ovarian cancers (15/58, 26 %), but not in serous or mucinous subtypes [73]. Interestingly, in one patient, loss of ARID1A is seen in the endometriosis adjacent to the tumor; however, it is not seen in the distant endometriosis [72]. This may suggest that loss of ARID1A may be an early lesion in ovarian cancers.

Homozygous deletion of ARID1A is seen in 16 % of RCC tumors (71/436) and cell lines [74, 75]. Decreases in ARID1A at both the protein and mRNA levels are seen in ~65 % of ccRCC tumors ($p<0.001$). Loss of ARID1A is a poor prognostic feature, which is associated with worse disease-free survival ($p=0.01$) and worse overall survival ($p=0.003$). Similarly, decreased ARIDA1 is associated with larger tumors (<4 cm vs. >7 cm, $p=0.03$), higher grade (65 % grade 1 vs. 26 % grade 3/4, $p=0.02$), and higher stage (48 % stage I vs. 24 % stage II/III, $p=0.004$). This can be contrasted to mutations in PBRM1, which is in the PBAF complex and is a positive prognostic feature.

In breast cancer, copy number loss of ARID1A is seen in 13 % of tumors (11/82) [76]. Decreased ARID1A mRNA is correlated with poor prognostic features including increased grade (grade 3 vs. grade 1/2, $p<0.0001$), hormone receptor negativity ($p<0.05$), increased Ki-67 ($p<0.05$), and increased ERBB2 expression ($p<0.05$). The frequency of low nuclear ARID1A also increases with later stages of disease: 37 % normal breast cells, 57 % ductal carcinoma in situ, 64 % invasive breast cancer, and 80 % metastatic lymph nodes. Furthermore, decreased nuclear ARID1A is associated with a twofold increase in breast cancer mortality (HR 1.98, $p=0.088$).

In other cancer types, the data is more conflicted. In gastric cancer, a study has shown that loss of ARID1A expression is a poor prognostic feature like what is seen in RCC and breast cancer, with loss of ARID1A associated with increased stage ($p=0.001$) and grade ($p=0.006$) and decreased survival ($p=0.029$) [77], while another study has shown that alterations in ARID1A were a good prognostic feature in multivariate analysis independent of stage [78]. This difference may reflect association of ARID1A alterations with specific subtypes of tumors and the relationship of that subtype to the relative prognosis within that malignancy. For example, in gastric cancer, ARID1A alterations are associated with associated with microsatellite instability and Epstein-Barr virus infection and PIK3CA mutations [78, 79].

Multiple other malignancy types demonstrate alterations in ARID1A: transitional cell carcinoma 13 % [80], NSCLC 8 % [81], Burkitt's lymphoma 17 % [82], childhood neuroblastoma 11 % [83], and high-grade esophageal dysplasia 15 % [84]. Together, this suggests that dysregulation of the BAF complex plays an important role in tumorigenesis with downregulation of ARID1A associated with increased growth and invasion [84]. Despite the importance of this complex, it remains to be seen whether this is a reasonable therapeutic target as effective targeting of transcription factors or restoration of tumor suppressors remains elusive.

Tumorigenesis Involving PBAF and BAF Complexes

Tumors often show mutations in both BAF- and PBAF-containing complexes. In pancreatic cancer, mutations are seen in ARID1A, ARID1B, and PBRM1 [85]. In hepatocellular carcinoma (HCC), ARID1A and ARID2 mutations are mutually exclusive, and ARID1A mutations are associated with hepatitis B virus infection or alcohol use; however, ARID2 mutations are associated with hepatitis C virus infection [86–88]. Given that ARID1A, ARID1B, and PBRM1 form complexes that are often antagonistic in function, it remains to be determined whether these will ultimately stratify into unique subtypes that are distinct in natural history and response to therapy or if the overlapping functions between SWI/SNF complexes are more critical to tumor pathogenesis.

p53 loss is the most common mutation in cancer. Interestingly, p53 mutation is mutually exclusive with ARID1A and ARID2 loss in multiple malignancies including gastric, ovarian, and hepatocellular [78, 88, 89]. However, mutations in SWI/SNF family members yield tumors that are functionally negative for p53. Loss of ARID1A in gastric cancer decreases the nuclear translocation of p53 ($p=0.028$) [84], and in breast cancer tumors that appeared to be "functionally" p53 negative by gene expression profiling, ARID1A expression was significantly lower ($p<0.001$) [76]. Restoration of these defects not only decreased proliferation but also restores p53-dependent transcription of targets such as p21(CDKN1a) or SMAD3 [89].

Loss of p53 transcriptional activity has also been associated with loss of PBRM1, Brd7, and BAF60A [90–92]. Both Brd7 and BAF60A are thought to directly bind p53. Brd7 binds to p53 independent of the bromodomain [90, 91]. Knockdown of Brd7 increases fibroblast proliferation and delays replicative senescence. This is also associated with decreased p53-dependent transcription of p21 and MDM2; however, loss of Brd7 also decreases p21 transcription via other stimuli including TGFβ and vitamin D. Similarly in breast cancer, downregulation of Brd7 is mutually exclusive with p53 mutation. BAF60A interacts with p53 via the tetramerization domain on p53 [92]. Amino acids 108–150 on BAF60A have a homology to the p53-interacting domain on MDM2; mutations of this region generate a dominant-negative BAF60A, which decreases p53-dependent apoptosis and cell cycle arrest through decreased transcription of BAX and p21.

PBRM1 Is a Defining Member of the PBAF SWI/SNF Complex

The *PBRM1* gene encodes the BAF180 protein that contains six tandem bromodomains (BD1-6), two bromo-adjacent homology regions (BAH), and one high-mobility group (HMG). Each of these domains is thought to confer a different functional activity to the overall BAF180 protein. Bromodomains, found in all members of the superfamily of bromodomain-containing proteins, recognize and bind acetylated lysine residues, often found on the "tails" of histone proteins. It appears that each bromodomain of BAF180 has a different affinity toward acetylated peptide in vitro. This has been deemed to suggest that BAF180 might cooperatively bind to unique patterns of acetylated lysine residues in the histone components of chromatin, thereby directing the PBAF complex to potentially discrete regions where nucleosome remodeling is occurring. HMG domains generally have the ability to bind the minor groove of DNA. Thus, like bromodomains, the HMG domain of the BAF180 protein might also provide specificity to PBAF as to where in chromatin the PBAF complexes are to localize. For example, BAF180 seems to localize to the kinetochores to help mediate chromatin remodeling during mitosis [93]. Furthermore, based on the solved crystal structure of the BAH domain [94], it is believed that the BAH domain of BAF180 mediates protein-protein interaction. Thus, it is probable that BAF180 is recruited into the PBAF complex, which itself is composed of multiple proteins, through its BAH domain. Together, the functional domains of BAF180 might be responsible for directing the PBAF complex to distinct chromatin locations.

PBRM1 as a Mediator of Nuclear Receptor Signaling

Genetic studies in the mouse originally revealed the physiological importance of PBRM1 function. *Pbrm1* is widely expressed throughout the mouse embryo and extraembryonic tissues including the yolk sac and placenta. Loss of *Pbrm1* function through null mutation of *Pbrm1* leads to embryonic lethality due to defects in ventricular development and placental trophoblast [95]. Interestingly, these abnormalities in the Pbrm1-mutant appear to phenocopy the developmental aberrations observed in mice deficient of *RXRα* (retinoic acid receptor) or *PPARγ* mice. Prior in vitro-based studies had implicated BAF180 to be an important regulator of nuclear receptor signaling. For example, the PBAF complex has also been reported to be crucial for vitamin D receptor and PPARγ receptor-mediated transcription. Thus, the similarities between the *Pbrm1* knockout mice and animals deficient in the nuclear receptors mentioned above provide further support to this possibility. In accordance with this view, during retinoic acid exposure, *Pbrm1*-deficient cells show a reduced responsiveness as determined by a decrease in the induction of RXRα-dependent genes.

PBRM1 as a Mediator of Angiogenesis and Development

Despite the association of PBRM1 with the regulation of nuclear receptor signaling, it is unclear whether PBRM1's role in this activity is relevant to tumor suppression. The mechanism(s) by which PBRM1 mediates tumor suppressor activity remains to be determined. Various studies suggest that PBRM1 could be involved in the regulation of cell differentiation, cell cycle control, and replicative senescence. Most dramatically, deletion of *Pbrm1* in the mouse leads to premature severe hypoplastic cardiac ventricular development, improper formation of the coronary vessels, and a failure to undergo epithelial-to-mesenchymal transition (EMT) [95, 96]. In addition, cells lacking *PBRM1* were found to display reduced migration through collagen, and this decrease in cell motility was associated with lower levels of expression of mRNAs involved with angiogenesis, growth regulation, and migration. Specifically, in these PBRM1-deficient cells, transcripts encoding *VEGF*, *ANG-2*, *FGF9*, *PDGFB*, *TGFβ2*, and *TGFR2* were found to be decreased [96]. Therefore, *Pbrm1* has a developmental role and seems to participate in lineage specification and/or cell differentiation.

PBRM1 as a Mediator of Cell Cycle Inhibition

Another way PBRM1 may restrict the emergence of tumor cells is through a control of cell proliferation. Breast cancer cell lines that lose *PBRM1* fail to properly upregulate p21, the cyclin-dependent kinase inhibitor protein, and, as a result, were resistant to G1 growth arrest triggered in response to TGFβ stimulation or γ-irradiation. Upon restoration of PBRM1 expression through transfection studies, these cells subsequently reacquire stimulus-induced growth arrest [10]. BAF180 could be found occupying the promoter region of the p21 gene, suggesting that PBRM1 exerts a direct influence over p21 transcriptional expression. Together, these results indicate that PBRM1 plays a role in regulating stress-induced control of cell proliferation within the model systems that were examined. In consistence, cell- and RNA-interference-based studies using short hairpins to knockdown endogenous PBRM1 levels appear to similarly support this view. Downregulation of PBRM1 expression in multiple renal tumor cell lines, which had normal copies of *PBRM1*, led to a higher rate of mitotic growth and enhanced colony formation [4]. Gene expression profiling indicated that this increase in cellular proliferation correlated with an upregulation of pro-growth genes [4].

Other Components of PBAF (ARID2, Brd7, BAF45a)

ARID2

ARID2 is also known as BAF200 and is a unique and essential subunit of the PBAF SWI/SNF complex [97]. ARID2 is the human homolog to *Drosophila* BAF170. It contains an ARID domain (DNA binding), multiple LXXLL motifs (protein-protein interaction), and two zinc fingers (DNA or protein interaction). ARID2 is capable of

joining the PBAF complex in the absence of PBRM1. Knockdown of ARID2 decreases PBRM1 levels; in contrast knockdown of PBRM1 had no effect on ARID2 levels. Binding of ARID2 to PBRM1 is a DNA-independent interaction. Interestingly, despite both being members of the PBAF complex, loss of PBRM1 and ARID2 is not functionally equivalent. For example, knockdown of ARID2 prevents interferon-induced transcription of IFITM1 (interferon-induced transmembrane protein 1); however, knockout of PBRM1 has no effect.

BAF SWI/SNF Complex Is Functionally Distinct with an Associated Phenotype

In addition to the core subunits, the BAF SWI/SNF complex contains ARID1A/ARID1B, Brd9, and BAF45d. It is capable of utilizing either BRM or Brg1 as its ATPase. Mutations in the BAF complex may be responsible for Coffin-Siris syndrome [98, 99]. Coffin-Siris syndrome (MIM 135900) is a rare congenital autosomal dominant disorder that is characterized by growth and cognitive defects. Typical features also include coarse facial features and hypoplastic digits of the hands and feet. Whole-exome sequencing of patients reveals mutations in BAF complex members in 87 % of cases (20/23). Mutations are spread across the BAF complex including both shared components (BRM, Brg1, SNF5, and BAF57) and BAF-specific components (ARID1A, and ARID1B).

ARID1A Is a Defining Member of BAF SWI/SNF Complex

ARID1A (AT-rich interactive 1A), which is also known as BAF250a, SMARCF1, or OSA, is a defining member of the BAF complex and is not found in PBAF [100]. It can partner with either BRM or Brg1 to mediate transcriptional activity. It was originally identified as a 270 kDa protein sharing similar epitopes as transcription factors such as p300 and CREB-binding protein (CBP) [101]. ARID1A contains an ARID binding region, a Q-rich region, and an LXXLL motif, which are implicated in DNA binding, transactivation, and nuclear receptor binding, respectively. The motifs in ARID1A show similarity to yeast SWI1, which also contains an ARID binding region, Q-rich region, and LXXLL motif; however, there is no direct sequence homology between the two proteins.

ARID1A May Be Necessary for Long-Term Renewal of Stem Cells

ARID1A is critical for maintaining embryonic stem (ES) cells [102]. ARID1A is highly expressed in both the late embryo and ES cells, and its expression begins to drop off during the blastocyst stage. Loss of ARID1A induces the loss of pluripotency, which can be restored by growth on a feeder layer. Whether the developmental defects with ARID1A loss are cell intrinsic or due to the microenvironment remains

to be determined. In fibroblasts, which contain a mutant ARID1A that lacks exon2/3, there is improved capacity to support fetal liver hematopoietic stem cells (HSCs) [103]. This mutant is still capable of binding Brg1; however, the deleted exons include WW and STAT3 interaction motifs. Additionally, this region also includes putative MAPK, GSK3, and PI3K phosphorylation sites. Although these site are yet to be verified, it is attractive to speculate that dysregulation of regulatory signaling alters the capacity to support HSCs. It would be interesting to further test whether these alterations are also capable of supporting tumor-initiating cells.

ARID1A Mediates Glucocorticoid Signaling

ARID1A activity depends on both Brg1 binding and DNA binding [104, 105]. ARID1A enhances glucocorticoid-dependent transcription via direct binding of the ARID1A C-terminus to the glucocorticoid receptor (GR) [100]. Furthermore, GR-dependent transcription is dependent on ARID1A and not PBRM1, the defining member of PBAF [106]. ARID1A binds to the helicase/SANT-associated (HSA) domain on Brg1. Mutation of the HSA domain prevents GR-mediated BRG1-dependent transcription. Similar, mutations within ARID domain induce defects in SWI/SNF function [105]. Mutation of V1068G in ARID1A is also homozygous lethal. Homozygotes appear largely normal until embryonic day 8.5; however, they subsequently show smaller size and developmental defect with death at embryonic day 13.5. Defects include decreased brain size, heart defects, improper vascular branching, and poor vascular integrity. The mutant ARID1A is assembled in the BAF complex and is capable to nucleosome remodeling in vitro; however, DNA binding is attenuated. Consequently, ARID1A V1068G-containing BAF complex has decreased but not absent in nucleosome binding. Together, this suggests that ARID1A plays a part in nucleosome binding, but is not solely response; however, this decrease is sufficient to induce significant developmental defects. Loss of BAF binding not only leads to upregulation of genes such as THBS1 (thrombospondin-1), which is known to be repressed by BAF, but increased binding of PBAF is also seen in its place. It is possible that BAF and PBAF can compete for binding sites and are thereby antagonistic in activity.

ARID1B Is a Homolog of ARID1A

ARID1B was isolated as a protein homologous to ARID1A [107]. These two proteins are mutually exclusive, and not only have a similar tissue distribution, but are also capable of binding BRM or Brg1. They typically occur at a 3.5:1 ratio of ARID1A: ARID1B in differentiated tissues. In embryonic stem cells, predominately ARID1A is incorporated into the BAF complex; however, after stimulation to differentiate with retinoic acid, incorporation of ARID1B increases and ARID1A decreases [108].

In addition on ATPase-dependent nucleosome modification, ARID1B can also participate in histone ubiquitination [109]. Elongin C (Elo C) binds in the BC box motif in ARID1B to form an E3 ligase with Cullin 2 (Cul2) and Roc1 to target histone

H2B on 120. Knockdown of ARID1A and ARID1B decreases monoubiquitination of H2B, which can decrease histone H3 lysine 4 trimethylation and indirectly regulate Hox gene member mRNA levels. It is unclear whether the role of ARID1A and ARID1B in histone ubiquitination is as part of the SWI/SNF complex.

Brd7 vs. Brd9 Analogous Subunits in PBAF and BAF Respectively

Brd7 (bromodomain 7) and Brd9 (bromodomain 9) are both part of a larger family of bromodomain-containing proteins (BCPs) to which >42 family members have been identified and >20 are likely to be transcription factors [110]. Bromodomains are 110-amino-acid domains that form a 4-α-helix bundle that recognizes *N-acetyl-lysines* on histones and thusly are found in many histone and nucleosome remodeling proteins, including SWI/SNF family members and histone acetyltransferases (HATs) [111]. Brd7 and Brd9 are highly conserved in higher eukaryotes from zebrafish to humans, and both contain a bromodomain and a DUF3512 (domain of unknown function). However, Brd7 is found exclusively in PBAF, and Brd9 is found in BAF [108, 112]. Brd9 is less well studied; however, given the significant homology to Brd7, it possibly serves similar roles in BAF as Brd7 serves in PBAF.

Brd7 has been implicated as a mediator of gene transcription when bound to other partner proteins. Brd7 was originally identified as a binding partner for E1B-AP5, a member of the heterogeneous nuclear ribonucleoprotein family (nHRNP) [113]. E1B-AP5 typically functions as a repressor of transcription; however, binding between Brd7 and E1B-AP5 induces transcription of glucocorticoid-dependent genes in the absence of ligand, which could be disrupted by mutating the Brd7 binding site on E1B-AP5. Brd7 is also found to mediate BRCA1-dependent transcription by recruiting BRCA1 to target promoter sequences [114]. In the absence of Brd7, other SWI/SNF family members including Brg1, BAF155, and BAF57 are still localized to the promoter; however, BRCA1 is unable to induce transcription of estrogen receptor α (ERα).

Brd7 may have some non-PBAF-related functions, as it has also been identified as a binding partner with dishevelled-1(Dvl-1) [115]. Dvl-1 functions as part of the canonical Wnt signaling pathway downstream of the transmembrane receptor Frizzled. Upon Wnt stimulation, Dvl-1 in conjunction with Brd7 inactivates GSK3β by mediating dephosphorylation of Tyr216, which leads to the accumulation of β-catenin and subsequent β-catenin-dependent transcription.

BAF57 Is a Stoichiometrically Regulated Component of PBAF and BAF

BAF57 is also known as SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily e member 1 (SMARCE1) and is only found in higher eukaryotes and not yeast [116]. BAF57 is ubiquitously expressed and can be found

in both BRM- and Brg1-containing complexes. It is highly conserved from humans to flies. It contains a HMG (high-mobility group) domain and a kinesin-like coiled-coil domain. Similar to other HMG-containing proteins, it has intrinsic DNA-binding ability; however, the HMG domain is dispensable for the formation of the SWI/SNF complex and DNA binding of the SWI/SNF complex. However, transgenic mice with mutation BAF57 in the HMG domain or deletion of the HMG domain present with a phenotype similar to heterozygous loss of Brg1 [117]. The mice exhibit a reduction in thymic cellularity with reduced CD8 but enhanced CD4 expression. This phenotype is exacerbated by concurrent Brg1 loss. As a result of tight regulation of BAF57 protein levels, in the transgenic mice, WT BAF57 expression is repressed tenfold, and 90 % of BAF complexes are inactivated. The BAF57 HMG mutants retain chromatin targeting and binding ability; however, it loses DNA bending ability.

BAF57 Mediates Nuclear Receptor Binding

BAF57 directly binds to steroid nuclear receptors and is important for receptor-mediated transcription. Nuclear hormone receptors contain both a DNA-binding domain and ligand-binding domain [118]. In estrogen receptor α (ERα), it also contains two transactivator domains, AF-1 and AF-2. AF-1 is in the N-terminal region and is ligand independent. AF-2 is within helix 12 of the ligand-binding domain and is ligand dependent [119]. Upon ligand stimulation, ERα recruits p160 family coactivators, such as SRC1, SRC2, or SRC3. BAF57 is capable of binding SRC1 in vitro and in vivo. Consequently, ligand stimulation recruits BAF57 to ERα. Loss of BAF57 decreases ERα-dependent transcription, which can be restored in a dose-dependent manner with exogenous BAF57. Recruitment of BAF57 depends on AF-2, with some suggestion that AF-1 may suppress the interaction between ER and BAF57 [120].

Similarly, BAF57 also mediates the interaction between BRM and androgen receptor (AR) [121]. In normal prostate tissue, only BRM is expressed but not Brg1 [122]. The SWI/SNF complex mediates androgen-dependent transcription, such as PSA; however, BRM is unable to bind AR directly. Instead, BAF57 binds AR directly in a ligand-dependent manner, with mild binding in the absence of ligand and enhanced binding in the presence of androgen [121]. Like the interaction between ER and BAF57, this also occurs in a p160 coactivator-dependent manner; however, in AR, BAF57 can also bind the DNA-binding domain.

BAF57 also binds the glucocorticoid receptor (GR) in a complex containing BAF60a, SRC1, and SRC3 [123]. Both BAF60a and BAF57 bind to the DNA-binding domain and hinge region of GR; however, BAF60a and BAF57 do not bind to each other. Furthermore, it is not possible to pull down Brg1 and BAF155/BAF170, which may suggest that assembly of the full complex may occur as a later event.

BAF57 as a Signal Transduction Mediator

BAF57 can also directly bind calcium/calmodulin (CaM) via the HMG domain [124]. In macrophages, Toll-like receptor 4(TLR-4) signaling activates SWI/SNF-dependent chromatin modification; however, activity is dependent on CaM binding. In the presence of a CaM inhibitor, SWI/SNF complexes are localized to the chromatin; however, they remain functionally inactive. The mechanism by which this occurs still remains unclear, and it is possible that SWI/SNF complexes at the promoter sites remain inactive until an activating signal such as those mediated by BAF57 reaches the chromatin.

BAF57 also mediates Sonic Hedgehog (SHH) and bone morphogenetic protein-4 (BMP-4) signaling via Teashirt-3 (TSHZ3) [125]. Adult muscle cells are typically in a quiescent state; however, in response to exercise or injury, they can be triggered to reenter the cell cycle and proliferate. This is in part mediated by TSHZ3 which is expressed in adult satellite cells (SC) and increases during activation of SC during muscle regeneration, and it is subsequently downregulated during differentiation. TSHZ3 is a zinc finger transcriptional factor downstream of SHH and BMP-4 signaling [126]. Homozygous loss of TSHZ3 leads to failure of neuronal and smooth muscle differentiation. BAF57 binds to the C-terminus of TSHZ3 and forms a complex that inhibits the transcription of genes related to muscle differentiation, such as myogenin, which is a key regulator of skeletal muscle terminal differentiation.

BAF57 Stabilizes Other SWI/SNF Family Members

In addition to serving as a binding partner for other transcription factors, BAF57 also plays a role in stabilizing other SWI/SNF family members [127]. Knockdown of BAF57 also leads to decreases in PBRM1 and BAF60A protein levels, a 14-fold decrease and a 10-fold decrease, respectively. In comparison, BAF47, BAF53A, BAF60B, BAF155/BAF170, ARID2, BRM, Brg1, ARID1A, and ARID1B show a < fivefold decrease. Despite the dramatic change in PBRM1 protein level, PBRM1 mRNA levels remain unchanged. Knockdown of BAF57 did not change in vitro nucleosome remodeling activity; however, it decreases cell proliferation and growth in soft agar. BAF57 knockdown cells show accumulation in G2/M phase and inhibition of genes required for progression through G2/M (CCNB1, CDC2, CENPE, CENPF, CCNF, CCNG1, and CENPA) and decreased MYC. Although BAF57 is found in multiple SWI/SNF complexes including BAF and PBAF complexes, it is unclear why loss of BAF57 selectively alters the subunits found in specific complexes. It is possible that BAF57 globally stabilizes many more SWI/SNF subunits; however, PBRM1 and BAF60a are intrinsically degraded at a faster rate; hence, the changes in protein level become more dramatic.

BAF57 and Tumorigenesis of Hormone-Dependent Malignancies

BAF57 is important for functionally mediating ER and AR signaling in hormone-dependent malignancies [120, 121]. In breast cancer and prostate cancer cell lines, hormone-dependent proliferation could be inhibited by loss of BAF57 or BRM. BAF57 is also found to be elevated in a subset of prostate cancers [128]. Furthermore, expression of the N-terminal region of BAF57, which can bind AR, exerts a dominant-negative effect and inhibits AR-dependent proliferation in AR positive cell lines. Conversely, this phenomenon is not seen in AR-negative cell lines.

BAF45a-d Helps SWI/SNF Complexes Recognize Histones

BAF45a (PHF10), BAF45b (DPF1), BAF45c (DPF3a/b), and BAF45d (DPF2) belong to the D4 family of proteins [129]. The D4 domain is a double-paired zinc fingerlike motif that is high conserved across species [130]. NMR and structural analysis shows that the two PHD motifs in the D4 domain function as a single unit and is capable of recognizing unmodified histones; however, acetylation on H3K14 increases the binding affinity fourfold, while acetylation or methylation on H3K4 decreases binding 15- or 20-fold, respectively. Consequently, BAF45d and full-length BAF45c are found to bind acetylated histone H3, but not unmodified or methylated histone H3 [131, 132]. Additionally, full-length BAF45c can also bind acetylated histone H4. However, a splice variant of BAF45c, which truncates the D4 domain, is unable to bind histones.

BAF45b, BAF45c, and BAF45d also share a Requiem-N domain (N-terminal domain of DPF/REQ). This is a putative domain that is also conserved across species; however, the function of this domain remains to be determined. BAF45a, on the other hand, contains a SAY (supporter of activation of yellow protein) domain instead of Requiem-N. BAF45a is found exclusively in the PBAF complex [112]. Functions of BAF45a include transcriptional repression of caspase 3, through binding to the caspase 3 promoter. Knockdown of BAF45a leads to apoptosis. BAF45b is a neuron specific and plays a role in neuronal differentiation [133].

BAF45c and BAF45d Mediate NF-κB Signaling

Interestingly, BAF45c and BAF45d interact with the canonical and noncanonical NF-κB signaling pathway, respectively. In the canonical NF-κB pathway, stimulation leads to phosphorylation and subsequent degradation of IκB, which allows the RelA/p50 heterodimer to translocate into the nucleus to stimulate transcription [134]. This is mediated in a SWI/SNF-dependent manner, and knockdown of BRM and Brg1 reduces NF-κB target transcription by 82 and 66 %, respectively [135]. All of the D4 family members have some activity in mediating transactivation of

NF-κB targets; however, BAF45c is most efficient. There is also some suggestion that this complex uses BAF60a. On the other hand, BAF45d mediates noncanonical NF-κB transcription [136]. In the noncanonical NF-κB pathway, RelB/p52 starts as RelB/p100 until stimulated, which induces cleavage of cytoplasmic p100 to p52. After cleavage, p52 translocates into the nucleus as the RelB/p52 dimer [137]. RelB/p52 transactivation requires both BAF45d and BRM; however, it does not require Brg1. In cell lines expressing high levels of RelB/p52, knockdown of BAF45d decreases colony formation in 3D culture without changing the proliferation rate in 2D monolayers. The specificity for the noncanonical NF-κB pathway is in part dictated by the N-terminal region of BAF45d, which is capable of binding p52, but not p50.

BAF45d Suppresses Estrogen-Related Receptor α (ERRα) Signaling

ERRα was isolated based on its sequence homology to estrogen receptor [138]. However, ERRα is unable to bind steroid hormones and their derivatives; instead ERRα belongs to a family of orphan nuclear receptors [139]. ERRα is found to regulate multiple metabolic processes including fatty acid oxidation, mitochondrial biogenesis and oxidative capacity, glucose metabolism, and transcriptional control of metabolism. Consequently, ERRα is ubiquitously expressed; however, it is increased in highly metabolic tissues, such as the CNS, GI tract, and cardiovascular organs. Increases in ERRα are also seen in breast cancer models of brain metastasis, along with its activators proliferator-activated receptor γ coactivator-1α (PGC-1α) and PGC-1β [140]. PGC-1α enhances ERRα transactivation without a ligand binding to ERRα. However, BAF45d acts to suppress ERRα transactivation activity by displacing PGC-1α within the ERRα complex [131]. BAF45d also helps recruit HDAC1 to ERRα-dependent promoters to further suppress ERRα transactivation activity. ERRα is also known to physically interact with hypoxia-inducible factor (HIF), which is elevated with VHL loss and activates hypoxia-dependent transcription [141]. Loss of ERRα or treatment with an ERRα inhibitor prevents the transcription of hypoxia-related genes in response to hypoxia. It still remains to be determined whether alterations in BAF45d may indirectly interact with HIF function.

Less Well-Understood SWI/SNF Complexes

In addition to PBAF and BAF, other distinct SWI/SNF complexes have also been identified. These include neuronal progenitor BAF (npBAF) and neuronal BAF (nBAF), embryonic stem cell BAF (esBAF), and cardiac BAF (cBAF). These SWI/SNF complexes play an important role in cellular differentiation and development and are mechanistically characterized by subunit switching during development.

npBAF (BAF53a/BAF45a)/nBAF (BAF53b/BAF45b)

The substitution of homologous subunits during development can be illustrated in the transition from npBAF (neural progenitor BAF) to nBAF (neural BAF). npBAF, which is necessary for neuronal progenitor cells, is made up of BAF53a and BAF45a; however, after mitosis and differentiation, subunits in npBAF are replaced with BAF53b and BAF45b or BAF45c to form nBAF [133].

BAF53a-b/Actin

BAF53a (also known as actin-like 6A) and BAF53b (also known as actin-like 6b) are part of the family of actin-related proteins (ARP). The ARPs are thusly named for their sequence identity to actin at the ATP-binding cleft. Together, the ARPs participate in a wide range of cellular processes. BAF53a and BAF53b are highly conserved from yeast to humans.

BAF53a is highly expressed in progenitor cells and is downregulated during differentiation. Conditional loss of BAF53a in mice specifically targeting both the bone marrow and skin leads to exhaustion of progenitor cells [142, 143]. In the bone marrow, loss of BAF53 leads to loss of long-term hematopoietic stem cells followed by severe pancytopenia and death [142]. In the skin, regions of BAF53 loss become severely hypoplastic without evidence of increased cell death [143]. Knockdown of BAF53a leads to cell cycle exit of epidermal progenitor cells and terminal differentiation. This process is likely mediated by an ARID1A-containing BAF complex. ARID1A knockout mice exhibit a phenotype similar to BAF53a loss. In contrast, loss of ARID1B is phenotypically dissimilar from BAF53a loss. This may be due to the antagonistic relationship between ARID1A- and ARID1B-containing complexes

BAF53a Helps Stabilize Brg1

Mutations in BAF53a change the preferences of BAF53a from BAF to histone acetyltransferase complexes [144]. BAF53a K226A/E227A mutants lose binding affinity for actin and Brg1 and can direct BAF53a toward histone acetyltransferase complexes with increased binding to TIP60 and KAT2a. Furthermore, loss of BAF53a also increases the Brg1 degradation rate. Mechanistically, BAF53a acts by targeting Kruppel-like factor 4 (KLF-4) [143]. Loss of BAF53 increases the transcription activators KLF4, GRHL3, PRDM1, and HOPX; however, ~50 % of BAF53a-regulated genes are also regulated by KLF4, with the latter three accounting for only 8 % of BAF53a-regulated genes.

BAF53a and Tumorigenesis

Interestingly, in the TCGA, BAF53a is amplified in many different malignancies including 37.4 % lung squamous ca, 20 % head and neck squamous, 22.9 % cervical squamous, and 28.6 % ovarian cystadenocarcinoma. It remains to be determined

whether this represents a reversion to a more stemlike phenotype. Coincidentally, BAF53a amplification also seems to occur more frequently in malignancies with squamous histology. This coupled with the observation that loss of BAF53a predominately leads to defects in epidermal formation may imply that BAF53a plays an interesting biological role in these cell types. Further study will be necessary to identify the underlying mechanisms for these observations.

BAF53b Is a Neuron-Specific Subunit and Defines nBAF

BAF53b, on the other hand, is only found in neurons in the nBAF complex [145]. BAF53b is 84 % identical and 93 % similar to BAF53a. BAF53a is ubiquitously expressed in all tissues; however, BAF53b is only found in the brain. BAF53b exhibits strong binding to both BRM and Brg1 and excludes PBRM1 from the nBAF complex. The nBAF complex contains ARID1A, BRG1, BAF155/BAF170, BAF60b, BAF57, SNF5, and β-actin. During neuronal development, there is a critical SWI/SNF subunit switch that occurs from BAF53a to BAF53b and BAF45A to BAF45b and BAF45c, which is mediated by microRNAs [146, 147]. Defects in subunit switching lead to aberrant neuronal development [148, 149].

esBAF (Brg1, BAF155, BAF60A, no Brm, BAF170) vs. CBAF (BAF60c/BAF45c)

esBAF (embryonic stem cell BAF) and CBAF (cardiac BAF) are other examples of compositionally similar but functionally distinct SWI/SNF complexes. esBAF consists of Brg1, BAF155, and BAF60A but excludes Brm, BAF170, and BAF60C and maintains pluripotency [150]. CBAF, on the other hand, incorporates BAF60C and BAF45C and is essential for heart development and muscle development [151].

BAF60a-c

BAF60A, BAF60B, and BAF60C are also known as SMARCD1, SMARCD2, and SMARCD3, respectively. They are the human homologs to yeast SNF12. BAF60A directly binds to Brg1 via its N-terminal domain and binds BAF155/BAF170 via its C-terminal domain [119]. Furthermore, the N-terminal domain mediates receptor binding by binding to glucocorticoid receptor, progesterone receptor b (PRb), ERα, and PPARγ. However, it did not show any interaction between vitamin D receptor and retinoid X receptor α (RXRα). The C-terminal binding of BAF155/BAF170 is also critical for BAF60A function, as truncation of BAF60A generates a dominant-negative protein [152]. Additionally, BAF60A also interacts with Fos/Jun heterodimers [153] and mediates hepatic lipid metabolism by facilitating the interaction between PPARα and PGC-1α [154, 155].

The role of BAF60B is less well understood. To date there is no clearly defined functional role for BAF60B; however, it is uniquely regulated independent of BAF60A and BAF60C, which likely implies a unique function for BAF60B, which

is yet to be discovered. BAF60B is ubiquitinated by Unkempt (Unk) upon Rac GTPase activation [156]. This ubiquitination is unique to BAF60B as it is not seen in BAF60A and BAF60C; however, the functional significance of BAF60b ubiquitination remains to be determined.

BAF60C is essential for CBAF, a BAF complex critical for chromatin remodeling during heart development and muscle differentiation [151, 152, 157]. Knockdown of BAF60C leads to defects in heart, neural tube, and somite development. BAF60C is expressed in two different splice forms BAF60C1 and BAF60C2, which differ by their first exon [152]. Both splice forms are broadly expressed, but there is some tissue specificity. BAF60C1 is higher in the brain, spleen, and trachea, while BAF60C2 is higher in the adipose, skeletal muscle, lung, heart, and thyroid. The N-terminal domain change between the two isoforms allows the two isoforms to alter their coactivator preferences; BAF60C1 has greater affinity for RXRα, while BAF60C2 has greater affinity for PPARγ.

With the discovery of additional SWI/SNF subunits and functional characterization of distinct SWI/SNF complexes, more insight will be obtained into an already complicated network of regulation. To date the majority of our evidence of SWI/SNF function has been elucidated from genetic perturbation of SWI/SNF subunits. It remains to be seen how major signaling pathways that are critical for ccRCC pathogenesis will integrate into SWI/SNF regulation. In silico analysis of SWI/SNF subunits has identified many putative phosphorylation sites; however, validation and understanding the clinical implication of their regulation remains to be done.

PBRM1: Therapeutic Implications

With the recognition that SWI/SNF is involved in tumorigenesis, focus is being placed on developing anticancer strategies that focus on this class of chromatin remodeling proteins. Significant attention has already been directed toward other broad types of chromatin modifiers, in efforts to intervene against a wide range of diseases where they have been implicated. Such efforts have relied on the optimism that the secondary "downstream" effects of aberrant chromatin modification, induced by the primary mutations in chromatin remodeling genes, are "epigenetic" in nature. Here, unlike mutations of DNA that are essentially irreversible, chromatin changes are potentially reversible. Exploration of this possibility has led to inhibitors against broad forms of histone deacetylases (HDACs) and DNA methyltransferases. These therapies have shown promise to reactivate certain tumor suppressors, reduce proliferation in some cancer cell lines, and inhibit tumorigenesis in various mouse models of cancer. Indeed, some HDAC inhibitors and DNA methylation inhibitors are being evaluated in clinical trials for use against a wide range of cancers, including solid tumors like hepatocellular carcinoma, ovarian cancer, and many hematopoietic malignancies, and have received FDA approval for use in certain cancers. Most notably, Vorinostat, also known as suberoylanilide hydroxamic acid (SAHA), and Vidaza (5-azacytidine) are approved for cutaneous T-cell lymphoma (CTCL) and myelodysplastic syndrome (MDS), respectively [158, 159].

However, development of selective therapeutic targeting of specific chromatin modifiers currently seems to be a formidable challenge. One of the major obstacles in targeting chromatin modifiers as drug targets lies in determining what vulnerabilities exists in the tumor cells. As is evident from the alterations of *PBRM1* and indeed the majority of chromatin modifiers identified thus far, including other proteins of SWI/SNF, mutations in chromatin remodeling genes are largely loss-of-function alterations. As a result, they do not afford obvious opportunities for a direct targeting of an oncoprotein. For the converse case of gain-of-function lesions, at least one prominent example has recently emerged and demonstrated that a successful targeted therapeutic approach against a chromatin-modifying protein can be achieved [160–162]. BRD4 is a member of the BET bromodomain family of proteins. BRD4 was found to be susceptible to the small-molecule inhibitor termed JQ1. Specifically, this class of BRD4 inhibitor appears to competitively target the acetyl-binding pocket of the BRD4 bromodomains. As a result, JQ1 was found to be highly effective at suppressing acute myeloid leukemia in vitro and in xenograft studies. The mechanism by which this compound exerts anticancer effects involves the eviction of the BRD4 protein from active chromatin. BRD4 eviction leads to a significant downregulation of *Myc* transcription. MYC activity is a pivotal cancer driver in the forms of AML that were investigated. Subsequent studies now show that tumor cells from a variety of hematopoietic malignancies, including multiple myeloma as well other cancer subtypes, like lung cancer and glioblastoma, show sensitivity to the anticancer effects of BRD4 inhibition by JQ1. Therefore, a targeting of the chromatin-binding activity of the BRD4 bromodomain protein serves as a proof of principle that in certain cancers, a chromatin regulatory factor can be inhibited for therapeutic benefit. From this perspective, some PBAF complexes might likewise be attractive targets of anticancer therapy.

However, for *PBRM1* that exhibits loss-of-function mutations, especially in ccRCC, the therapeutic targeting is likely to be fundamentally more elusive. Nevertheless, opportunities may still exist. Crucial to these opportunities is to better understand the downstream molecular consequences of PBRM1 loss. Specifically, it is easy to speculate that with PBRM1 inactivation, PBAF complexes would fail to properly regulate the normal expression of target genes. Therefore, it would become valuable to identify how the genes become dysregulated and their contribution to tumorigenesis. Toward this goal, we anticipate that synthetic lethal screens and/or screening of small-molecule libraries are attractive approaches that could be used to achieve this goal. Furthermore, advancement toward targeting of cancer with PBRM1 loss would likewise benefit from developing RCC in mouse models that could serve for evaluation of candidate therapeutics.

Conclusions

The SWI/SNF complexes play a critical role in chromatin remodeling and are tightly regulated in order to coordinate physiological functions such as cell fate determination, cell proliferation, and cell growth. Dysregulation or loss of SWI/

SNF family members has profound consequences and can lead to a variety of pathologic conditions including malignancy and stem cell failure. Given the intricate relationship between SWI/SNF components, it is unclear whether a perturbation in any specific subunit represents a loss-of-function or gain-of-function alteration. Most of the studies available have examined the genetics of SWI/SNF and disease. However, it is becoming apparent that SWI/SNF subunits themselves are subject to posttranslational modification, and as a result, this regulation of SWI/SNF subunits should be taken into account in future analyses of SWI/SNF function. Transcriptional activation is a tightly coordinated process that extends beyond coordinating the shuffling of coactivators and corepressors to the correct promoter elements. Further studies will be needed to elucidate how alterations in chromatin remodeling induce defects in cell signaling and promote tumorigenesis. *PBRM1* mutations comprise the second most frequent genetic lesions and make up the bulk of SWI/SNF mutations in RCC. Currently, there is only circumstantial evidence from correlative studies that suggests that *PBRM1* functions as a tumor suppressor in RCC. Mouse models of PBRM1 loss would be necessary to definitively prove this hypothesis. Given that *PBRM1* mutations in RCC reflect loss of function and that this loss presumably leads to broad alterations in gene expression, major challenges exist to devise targeted therapies for RCC patients harboring genetic aberration of this gene.

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Chapter 6
Sporadic RCC: Abnormalities in Histone-Modifying Genes

Ruhee Dere and Thai H. Ho

Introduction

Renal cell carcinoma is histologically classified into several subtypes, with the clear cell variant alone accounting for 80 % of renal tumors. ccRCC is most commonly associated with biallelic inactivation or loss of heterozygosity (LOH) of the *VHL* gene in 90 % of RCC cases, accompanied by mutations in 50 % of the cases or by *VHL* promoter hypermethylation associated with 10 % of the cases [3–6]. VHL is a multifunctional protein with its best-studied role being the substrate recognition component of an ubiquitin degradation complex. VHL in its role as a substrate recognition component forms a multi-subunit complex with elongin B, elongin C, Cullen 2, and Rbx1 referred to as the VCB complex [7]. Hypoxia-inducible factor—HIFα—is the most notable substrate for VHL, with VHL binding HIFα and promoting degradation under well-oxygenated conditions. Under conditions of hypoxia, HIFα escapes recognition by VHL and forms a heterodimer with HIFβ to promote transcriptional activation of 100–200 genes, some of which are involved in adaptation to low-oxygen conditions [7]. In its non-proteasomal role, VHL localizes to the microtubules and is involved in microtubule stabilization with emerging studies linking loss of VHL to the absence of primary cilia [8–11], an organelle that modulates several critical signaling pathways in response to fluid flow in the kidney. VHL was also reported to be required for the appropriate assembly of the extracellular matrix

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[12–14], and in regulating apoptosis via modulation of apoptotic effectors p53 and nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) [15–18].

VHL disease is characterized by loss of one short arm of chromosome 3 (allele 1) and a mutation in the second arm of chromosome 3p25 (allele 2), where VHL is localized. More than 800 VHL mutations have been identified in hereditary and sporadic cases of RCC [19], with the most common being frameshift and nonsense mutations (classified as Type 1 disease), followed by missense mutations (classified as Type 2 disease). VHL is a tumor suppressor gene, and although VHL is critical in both hereditary and sporadic RCC, inactivation of VHL alone is not sufficient to develop RCC, suggesting the existence of other genes involved in the development of RCC. Recurrent loss of the short arm of chromosome 3 yielded clues to the identification of several additional tumor suppressors involved in the pathology of RCC.

Use of whole genome and/or whole exome and RNA sequencing along with array-based gene expression, copy number, and methylation analysis revealed the most commonly mutated genes in a cohort of 106 ccRCCs. These analyses revealed that in addition to VHL, three additional tumor suppressors—*PBRM1* (polybromo-1), *BAP1* (BRCA1-associated protein-1), and *SETD2* (SET domain-containing protein 2) gene mutations—occurred with high frequencies [20, 21] in the setting of ccRCC. Importantly, all of these genes are found on chromosome 3p in a region most commonly (90 %) deleted in ccRCC. Thus, loss of this chromosomal region in RCC would essentially eliminate all four of the tumor suppressor genes.

In addition to the high frequencies of mutations and loss of *VHL*, *PBRM1*, *BAP1*, and *SETD2*, another gene, *TCEB1*, was also found at a high frequency of mutation [20]. *TCEB1* mutations were accompanied by an obligatory loss of chromosome 8 in 36–42 % of ccRCC with intact *VHL* [20]. *TCEB1* encodes elongin C, a 112 residue protein, that interacts with VHL to form the VCB ubiquitin degradation complex. *VHL* and *TCEB1* mutations were found to be completely mutually exclusive [20]. Integrated pathway analysis revealed that the largest most frequently mutated network was the one with VHL and its interacting partners, followed by genes involved in the SWI/SNF chromatin remodeling complex [21].

Epigenetics of RCC

Genomic DNA is organized into higher-order structures with histone proteins to form protein/DNA complexes called chromatin (Fig. 6.1). Chromatin is composed of nucleosomes of ~146 base pairs (bp) of DNA wrapped around histone proteins. DNA methylation and histone modifications are major epigenetic mechanisms involved in gene regulation and are often perturbed in cancers [22]. DNA methylation involves chemical modifications to the cytosine residues of DNA and regulates gene expression. The N-terminal tails of histones are heavily modified with numerous posttranslational modifications including acetylation, methylation, phosphorylation, ubiquitination, sumoylation, deamination, and ADP ribosylation [23, 24]. The different modifications on specific residues of the histones alter chromatin

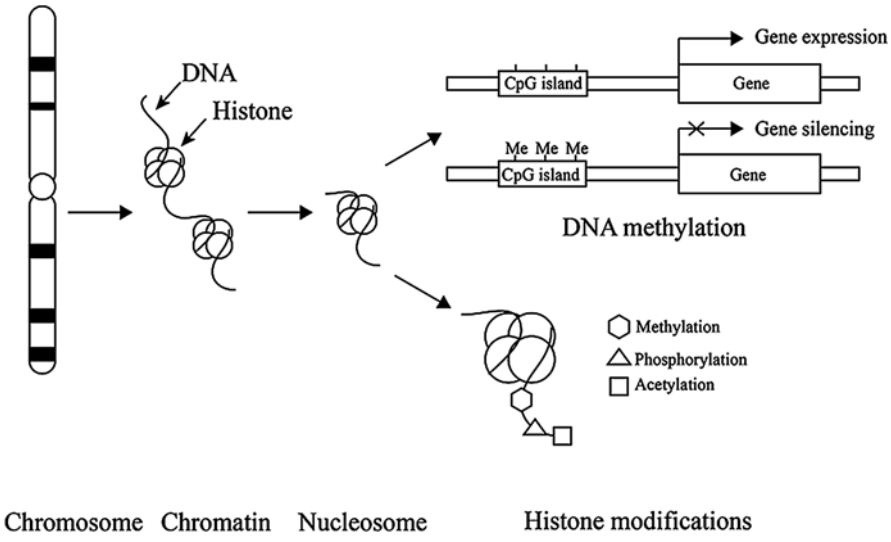

Fig. 6.1 Epigenetic mechanisms in RCC

structure and result in transcriptional activation or repression. For example, methylation of histone 3 lysine residues such as H3K4, H3K26, and H3K79 is associated with active transcription marks, whereas methylation at H3K9, H3K27, and H4K20 is associated with repressive marks [25]. Regulation following histone modifications could manifest as a change in chromatin structure directly or by affecting recruitment of numerous chromatin-modifying complexes.

In RCC, certain histone modifications are associated with clinicopathologic characteristics and overall survival. Emerging studies have analyzed global changes in acetylation and methylation in RCC samples as predictive indicators of progression-free survival and outcome. For example, a decrease in global levels of H3K4me2 (dimethyl) and H3K18Ac (acetylation) in localized RCC was linked to an overall poor prognosis, including a shorter period of progression-free survival [26]. Another study reported decreased global levels of H3K18Ac to be an independent predictor of RCC progression following surgery [27]. Additionally, low levels of histone methylation at H3K9 (me1, me2, me3) and H4K20 were significantly correlated to high tumor stage and grade [28], with decreased global levels of H3K9me2 (dimethyl) predictive of poor outcome in kidney cancer [29]. Decreased global levels of H3Ac [27, 30] and H3K9Ac [31] were reported in RCC patients with high Fuhrman grade and advanced stage disease. Decreased global H3K27 was also associated with high-grade and recurrence in patients with RCC [28].

Aberrant promoter hypermethylation and resulting silencing of gene expression can provide a selective advantage to preneoplastic lesions and could, therefore, contribute to initiation of cancer formation. In the case of RCC, VHL promoter hypermethylation occurs in 10 % of the cases and is not associated with tumor grade or

stage suggesting that this is an early event in tumor formation (Fig. 6.1). In addition to investigating VHL promoter hypermethylation, a number of other tumor suppressors have been identified in RCC with hypermethylated promoters. Although the genes methylated in RCC are found across the genome, most of these genes mapped to the frequently deleted chromosome 3p. These genes included tumor suppressors *RASSF1* (mapped to chromosome 3p21); *TU3A* (mapped to chromosome 3p21); *FHIT*, involved in purine metabolism (mapped to chromosome 3p14); and *RARB*, regulator of cell proliferation and differentiation (mapped to chromosome 3p24) [32]. Methylated tumor suppressors offer options as biomarkers for prediction and prognostic purposes in cancers including RCC [33]. Interestingly, the promoters of PBRM1, BAP1, SETD2, KDM5C, and KDM6A, tumor suppressors frequently mutated in RCC, were reported to be unmethylated in RCC tumors [34].

Epigenetic Modifiers in RCC

PBRM1 (Polybromo-1)

The *PBRM1* gene locus is located on chromosome 3p21, and mutational screening revealed PBRM1 mutations in 41 % of ccRCC cases [35], highlighting the importance of these mutations in RCC pathology. PBRM1 mutations resulting in the loss of PBRM1 protein are commonly observed secondary to loss of VHL [35–37]. Loss of PBRM1 is infrequently associated with nonrenal cancers, although there is one study that reported PBRM1 truncation mutations in breast cancer samples [38]. Mutations of PBRM1 include most commonly truncations but may also include missense and in-frame deletions [39]. Although earlier studies showed that PBRM1 mutations were associated with invasiveness and poor overall survival when compared to wild-type PBRM1 [37, 40], recent reports suggest that mutations in PBRM1 are not predictive of worse outcome [41, 42].

PBRM1 encodes the polybromo 1 protein (BAF180), which functions as the chromatin-targeting subunit of the SWItch/Sucrose NonFermentable (SWI/SNF) chromatin remodeling complex [43]. SWI/SNF complexes are ATP-dependent complexes that alter accessibility of DNA by modulating nucleosome occupancy and ultimately modulate transcription [44]. BAF180 contains six tandem bromodomains implicated in chromatin binding and recognition of acetylated lysine residues on histones [45]. In addition, BAF180 carries two bromo-adjacent homology domains (BHD) with potential protein-protein interacting function and a high mobility group (HMG) domain, which may bind nucleosomal DNA [43]. The exact mechanism of how BAF180 acts as a tumor suppressor is not completely understood but may be related to its role in modulating nucleosome occupancy and gene expression.

BAF180 has been reported to be critical for coronary vesicle formation [46] and cardiac chamber maturation [38], in addition to its role in regulating p53 during replicative senescence in human cells [47]. BAF180 has also been localized to the kinetochore [48], although its function at this structure is not known. However, a

role for BAF180 during mitosis could explain the resulting chromosomal instability observed in RCC. BAF180 was suggested to function as a repressor of the immunoregulatory cytokine IL-10 in Th2 cells, suggesting that the differential recruitment of diverse SWI/SNF subtypes could directly affect gene transcription and cell fate in T cells [49].

BAP1 (BRCA1-Associated Protein-1)

BAP1 mutations occur in 15 % of ccRCC patients [36], and the BAP1 gene locus is on chromosome 3p21 [50]. *BAP1* (BRCA1-associated protein 1) is a tumor suppressor gene with mutations seen in RCC and in a diverse array of solid tumor types [51–55] including lung and breast cancer [56, 57]. The gene undergoes frequent copy number loss and loss of heterozygosity in cancers [50, 58]. Mutations of *BAP1* include missense, nonsense, and out-of-frame insertions or deletions throughout the open reading frame [59]. BAP1 is a 729 amino acid protein that localizes to the nucleus and functions as a deubiquitinating enzyme [60]. Ubiquitination is a post-translational modification of proteins required for their proteasome-mediated degradation. Although primarily linked to protein turnover, depending on the extent of modification, ubiquitination can also regulate non-proteasome-mediated protein functions in the cell. Importantly, ubiquitination is a reversible modification conducted by ubiquitin proteases, such as BAP1, called deubiquitinases (DUBs). BAP1 belongs to the UCH (ubiquitin C-terminal hydrolase) family of DUBs [61]. This enzyme was first identified as binding BRCA1 in a yeast two-hybrid screen, with mild synergistic effects on BRCA1-mediated growth suppression [56]. BAP1 contains an N-terminal catalytic domain that contains the ubiquitin-binding site, a C-terminal extension required for protein-protein interaction, and two NLS (nuclear localization signals). Mutations in either the ubiquitin-binding site or the NLS are often associated with cancer [50].

Mass spectrometry analysis revealed that BAP1 interacts with proteins involved with chromatin modification and transcription response, although little is known about its enzymatic properties or substrate specificity. BAP1 does not deubiquitinate BRCA1 [62], and it appears that BAP1 may function in BRCA1-mediated processes and may also have BRCA1-independent functions [50]. Currently, targets for BAP1 activity are few, with BAP1 binding and deubiquitinating transcription regulator host cell factor (HCF-1), which modulates transcription via alteration of chromatin structure by recruitment of histone-modifying enzymes. BAP1 binds HCF-1 in ccRCC and is required for BAP1 suppression of cell proliferation [36]. Interaction between BAP1 and HCF-1 may be important for the growth effects of HCF-1 responsible for progression of cells through the G1/S boundary [60, 63, 64], thereby linking BAP1 to cell cycle [64–66] modulation. Recently, a report showed BAP1 interacting with ASXL2 (additional sex combs-like), a putative polycomb group protein, to ultimately deubiquitinate H2A in vitro [67]. Moreover, BAP1 reintroduction into BAP1-deficient RCC cell lines reduced the global levels of ubiquitinated

H2A (H2AK119Ub), although no direct correlation was observed between H2AK119Ub and BAP1 mutations in RCC [36]. In addition, BAP1 may be involved in the cellular DNA damage repair response as it becomes phosphorylated when DNA is damaged [68, 69], although BAP1s exact role in this process is not known.

A recent report [42] used two independent cohorts of ccRCC samples in a retrospective study and found that *BAP1* mutations carried a higher overall risk and were associated with worse overall survival (1.9–4.6 years) as compared to *PBRM1* mutations that had a more favorable risk and overall survival (5.4–10.6 years). *BAP1* mutations showed pathological features suggestive of more aggressive disease, although the mechanism of how this translates to a worse prognosis is not known. Moreover, this study also investigated the gene expression profiles/signatures from individuals with just *BAP1* mutations or just *PBRM1* mutations and found the signatures to be distinct between the two, indicating a nonoverlapping biology. In addition to BAP1 mutations, BAP1 loss by immunohistochemistry was associated with adverse clinicopathologic variables such as high Fuhrman grade, advanced T stage, necrosis, and the presence of sarcomatoid dedifferentiation which may reflect a different disease biology [70]. Loss of BAP1 caused a significant reduction in the expression of genes that constitute the identifying signature, and this was attributed to the function of BAP1 as a deubiquitinase, which in the absence of BAP1 would lead to enhanced ubiquitination and proteasomal degradation of these proteins [71]. *BAP1* and *PBRM1* mutations are mutually exclusive, although in rare instances do occur together and are associated with worsened disease severity and prognosis [42].

SETD2 (SET Domain-Containing Protein-2)

SET domain-containing protein 2 (SETD2), also known as Huntingtin-interacting protein B (HYPB), is a high molecular weight 230 kDa protein [72, 73], which serves as a histone methyltransferase (HMT) associated with the posttranslational modification of histone H3 at lysine 36 (H3K36). The mammalian HYPB/SETD2 is a nonredundant enzyme that mediates trimethylation but not mono- or dimethylation of H3K36 and does not affect histone acetylation across coding regions [73]. Methylation at H3K36 is usually associated with transcriptional activation and is more abundant in the exons compared to the introns from the same gene. SETD2 has a conserved SET domain that mediates H3K36 HMT activity [72, 74], a C-terminal Set2-Rbp1 interacting (SRI) domain that mediates interaction with RNAPII [72, 75], and a WW domain that likely mediates protein-protein interactions [76].

SETD2 is a tumor suppressor commonly mutated in 15 % of ccRCC [77], with the *SETD2* gene locus located on the frequently deleted chromosome 3p21. A meta-analysis shows that SETD2 mutations in RCC are twice as high in tumors with PBRM1 [77]. Loss of SETD2 results in embryonic lethality, as it is required for embryonic vascularization [78]. Besides its role in RCC, SETD2 levels were reported to be lower in metastatic high-grade breast cancer compared to disease free

samples [79] and matched normal tissue [80]. Additionally, SETD2 mutations occurred with a 15 % frequency in high-grade pediatric gliomas and an 8 % frequency in high-grade adult gliomas [81].

In its role as an HMT, SETD2 plays a crucial role in chromatin remodeling during transcriptional elongation, attributed to its physical association with hyperphosphorylated RNA polymerase II [82] (RNAPII). SETD2 also interacts with p53 and enhances p53 transcriptional activation to regulate expression of select p53 targets [83]. H3K36me3 is tightly linked to actively transcribed genome regions where the trimethyl mark prevents transcription initiation from cryptic gene promoters in the wake of elongating RNAPII and can also direct alternative splicing events. More recently, SETD2 was implicated to have a role in mRNA splicing where H3K36me3 marking by SETD2 was proportional to transcriptional activation, with the methyl mark being much more prevalent in the exons in contrast to the introns of the same gene [84]. SETD2 recruits the histone chaperone FACT (a complex termed after its ability to "Facilitate Chromatin Transcription"), which is crucial to reassembly of nucleosomes in the wake of RNAPII elongation, thereby repressing cryptic intragenic transcription initiation [85].

Loss of trimethyltransferase-specific SETD2 displays microsatellite instability and an elevated spontaneous mutation frequency, arising from lack of H3K36me3 marks to recruit mismatch repair proteins to the chromatin [86]. Mutations in *SETD2*, a nonredundant H3K36 methyltransferase, were associated with increased loss of DNA methylation at non-promoter regions. This discovery is consistent with the emerging view that H3K36 trimethylation may be involved in the maintenance of a heterochromatic state, whereby DNA methyltransferase 3A (DNMT3A) binds H3K36me3 and methylates nearby DNA [21]. Thus, reductions of H3K36me3 through *SETD2* inactivation could lead indirectly to regional loss of DNA methylation.

JARID1C/KDM5C

JARID1C is a lysine-specific demethylase, encoded by the KDM5C gene, and considered a tumor suppressor in the setting of VHL-null RCC [87]. JARID1C truncating mutations have an occurrence of 3 % frequency in RCC [88] and are generally associated with VHL mutations. RCC tumors with these mutations have a characteristic hypoxia signature and, in fact, JARID1C is transcriptionally induced by HIF2α under hypoxic conditions [89–91]. The gene expression signature, although unique, was restricted to only 18 genes showing altered expression in tumors with JARID1C mutations [88].

The *JARID1C* gene encodes a protein of 1,560 aa that belongs to a family of histone demethylases mainly involved in demethylation of tri- and dimethylated H3K4 (histone H3 lysine 4) [92, 93]. JARID1C, as an H3K4 trimethyl histone demethylase, catalyzes demethylation of H3K4me3 to H3K4me1 but has no effect on methylation at other lysine residues, including H3K9, -27, -36, and H4K20 [92].

The JARID1C protein contains several conserved DNA motifs, such as a Jumonji (JMJ) N domain, an ARID/BRIGHT domain, a JMJC domain, a C5HC2 zinc finger domain, and PHD zinc finger domains [94]. Mutations in the *JARID1C* gene have also been linked to XLMR (X-linked mental retardation) [94–96].

Other Modifiers

Several other chromatin-modifying enzymes are emerging as players in the pathology of RCC. For example, consistent overexpression of JMJD1A/KDM3A is reported in RCC [90, 91]. The H3K27 methyltransferase EZH2 (HMT), implicated in aggressiveness in breast and prostate cancer [97], is also overexpressed in renal tumors [98], and suppression of EZH2 improves RCC cell viability in vitro [99]. Conversely, histone demethylases (HDMT), UTX, and JMJD3 are also overexpressed in RCC, corresponding to a decrease in methylation at H3K27 [100] and is associated with high-grade RCC.

Conclusions

The discovery of mutations in histone-modifying genes in a significant percentage of RCC has led to a search for the functional significance of these alterations. The analysis of epigenetic signatures defined by genome-wide approaches, such as chromatin immunoprecipitation sequencing and DNA methylation data, may yield novel targets outside the traditional treatment RCC paradigm of immunotherapy, angiogenesis inhibitors, or mTOR inhibitors. Loss of function mutations in histone-modifying enzymes are challenging to target; however, epigenetic modifications can be reversed, and chromatin-modifying agents (CMAs), such as DNA-demethylating agents and HDAC inhibitors, are already approved for treatment in other malignancies [101]. Strategies to combine CMAs with different epigenetic targets would need to be balanced with increased toxicity and side effects. Other potential targets include upstream regulators of enzymatic activity or "readers" of these various histone marks.

The impact of these alterations on the cancer phenotype is challenging to define and likely to cooperate with other genomic changes in RCC. Although next-generation sequencing platforms are attractive for genotyping, traditional IHC may serve as a cost-effective alternative to identify loss of expression of BAP1, PBRM1, or SETD2 in clinical specimens. Although these assays can potentially define poor oncologic outcomes, it is unclear whether genotyping would add additional information to established adverse clinicopathologic features such as pT, sarcomatoid dedifferentiation, high Fuhrman grade, or necrosis. The mutations or loss of expression by IHC may be more useful in defining poor outcomes in the cohort of patients with low-risk clinicopathologic features. If validated, the assay would generate a risk

recurrence score where clinicians could use the information to either pursue adjuvant clinical trials or alter the schedule of restaging studies for patients with a poor prognosis. The elucidation of distinct epigenetic subtypes of ccRCC may further define molecular pathways that modify the RCC phenotype.

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Chapter 7
Genetic Heterogeneity in Renal Cell Carcinoma: Clinical Implications?

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Tumor Heterogeneity

Tumor heterogeneity is a problem that has been frequently associated with treatment failure. An evaluation of mechanisms underlying disease relapse has led to analyzing resistance to chemotherapy that is generally attributed to either "acquired" or "intrinsic" resistance. While several studies on "acquired" resistance using preclinical models or patient tumor samples have identified defined changes that can be linked to the expression of resistance, "intrinsic" resistance has never been satisfactorily addressed except within the context of tumor heterogeneity. While selection of tumor subpopulations that differ in sensitivity to treatment is frequently observed, rapid advances in genomic profiling have provided a new window of opportunity to analyzing resistance to therapy within the context of tumor heterogeneity.

Histological differences in tumors have led to classification of tumors originating from different sites/organs. Further, techniques such as DNA ploidy analysis and karyotyping have elegantly identified tumor heterogeneity and in particular genetic heterogeneity that is frequently observed with most tumors. During the last decade, efforts in genomic profiling and more importantly the availability of high throughput genomic analysis have provided considerably new opportunities to address genetic heterogeneity within the context of inter- and intratumor heterogeneity as well as to focus with greater precision on molecular heterogeneity [1]. Ultimately, the focus on molecular profiling of tumors has led to the era of "personalized therapy," and in this

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review we will discuss genetic heterogeneity in renal cell carcinoma with a focus on clear cell renal cell carcinoma.

Renal cell cancer, an epithelial tumor, is a heterogeneous disease both in cellular morphology and clinical course of the disease. Hereditary renal cell carcinoma syndromes are associated with specific genetic alterations and account for a small percentage of all renal cell carcinomas. The histologic classification of renal cell carcinoma based on the cell of origin includes the following subtypes: clear cell, papillary, chromophobic, oncocytic, and collecting duct. In this review, we will focus on sporadic clear cell renal cell carcinoma since it is the most prevalent type, and studies on the biology and genetics of this subtype of renal carcinoma have led to the development of targeted therapy. Genetics of sporadic clear cell renal cell carcinoma has almost exclusively focused on the *von Hippel-Lindau* (*VHL*) gene based on its central role in deregulating several key pathways related to angiogenesis as well as the high percentage of tumors of this subtype that demonstrate mutations in the *VHL* gene.

VHL Genotype and Sporadic Clear Cell Renal Cell Carcinoma

Mutations in the von Hippel-Lindau (*VHL*) tumor suppressor gene are associated with hereditary and sporadic forms of clear cell renal carcinoma. The product of the *VHL* gene forms a heterodimeric complex with elongin C, elongin B, Cul2, and RBX1 and targets the hypoxic inducible factors (HIF1α and HIF1β) for ubiquitin-mediated degradation. Mutation of the *VHL* gene in clear cell kidney cancer prevents the VHL complex from targeting HIFs for degradation, resulting in their accumulation. Increased levels of HIF result in increased transcription of downstream targets including vascular endothelial growth factor (VEGF) and angiogenic pathways [2]. Over the last few years, therapy targeting VEGF, VEGF receptor (VEGFR), and the angiogenic pathway has made a major impact in the standard of care for patients with advanced clear cell renal cell carcinoma [3]. These include bevacizumab [4], sunitinib [5], axitinib [6], pazopanib [7], and sorafenib [8].

We had previously reported the possible impact of *VHL* gene mutation and promoter hypermethylation on the outcome to VEGF-targeted agents in 123 patients with advanced CCRCC [9]. Sixty patients had mutations in VHL (Table 7.1).

Table 7.1 Frequency and characteristics of VHL mutations in a retrospective series of 123 patients with clear cell renal cell carcinoma [9]

Mutation type	Number (%)
Frameshift	29 (48)
Nonsense (stop)	6 (10)
In-frame deletion or insertion	7 (12)
Splice	5 (8)
Missense	13 (22)
Total	60 (100)

Mutations occurred in exon 1 (42 %), exon 2 (32 %), and exon 3 (27 %). Twelve patients (10 %) exhibited promoter methylation. Overall, the response rate for patients with a *VHL* mutation was not significantly different compared to patients with wild-type *VHL*. Mutations that were predicted to result in loss of *VHL* function (LOF), frameshift, in-frame deletions and insertions, splice mutations, and nonsense mutations comprised 78 % of the mutations identified in this cohort. Patients with LOF mutations in *VHL* had a better response rate than those with wild-type VHL (52 % vs. 31 %; $p=0.04$). However, survival was not significantly different. Twenty-two percent of the mutations identified in the study were missense. At the time of publication of this report [9], the significance of missense mutations on *VHL* function was not known. A recent study has demonstrated that missense mutations can be segregated into "driver and passenger" mutations, where certain missense mutations had functional significance and others did not [10]. In this study [10], 65 missense mutations were identified in 256 clear cell renal cell carcinoma samples. The thermodynamically destabilizing mutations were found in exon 1, while mutations in exon 3 affected elongin B and C protein interaction. After stably introducing *VHL* missense mutations into *VHL*-null cell lines, the authors identified three categories of missense mutations: those which destabilized *VHL*; those which did not destabilize *VHL* but affected *VHL's* interaction with HIFα, elongin B, and elongin C; and those with activity similar to wild-type *VHL*. These data may partly explain the variation of responses of *VHL* patients to therapy that is directed downstream of *VHL*.

Genetic Heterogeneity Between Primary and Metastatic Tumors and Intratumor Heterogeneity

While genetic and epigenetic alterations in *VHL* occur in most cases of clear cell renal cell carcinoma [11], the lack of a clear association between *VHL* mutation status of the primary tumor and clinical outcome following VEGF-/VEGFR-targeted therapy [9] could possibly be due to inter-tumor and intratumor genetic heterogeneity as outlined in Table 7.2 with a summary of some of the published reports [12–17]. Since VEGF-/VEGFR-targeted therapy is generally focused on metastatic renal cell cancer, we hypothesized [17] that the lack of association with *VHL* mutation status could be due to *VHL* genotype differences between the primary and metastatic tumor. Also, the impact for such heterogeneity in clear cell renal carcinoma, while not addressing *VHL* mutations directly, has been previously reported [13, 14, 18]. This suggests that inter- and/or intratumor heterogeneity within a patient may respond differently to treatment that is designed to act on pathways affected by mutant *VHL*. To directly address possible potential differences in *VHL* genotype between the primary and metastatic tumor in a patient, we sequenced the *VHL* gene in paired primary and metastatic tumor specimens from ten patients [17]. As outlined in Table 7.3, in 40 % of the patients, the *VHL* gene status differed between the paired primary and metastatic lesion. The complexity for such differences in *VHL* genotype was further exemplified by differences in tumor from

Table 7.2 Genetic heterogeneity of *VHL*

Study	Year	Patients	Samples	Focus	Results
Nenning [12]	1997	22			Intratumoral heterogeneity DNA distribution 45 % of tumors based on analysis of 7 different regions in same tumor
Moch [13]	1998	53	18 Clear cell and 2 tubulo-papillary	Genetic heterogeneity VHL	VHL deletion CCRCC not papillary. 8p22 and 17p13 deletions linked to VHL deletion. Differing chromosome 3 counts in same tumor
Phillips [14]	2001		22 Primary, 10 mets	VHL heterogeneity	Cytogenetic heterogeneity of multiple lesions from same kidney
Jones [15]	2005			Metastatic gene signature	Metastatic gene expression profile can be identified in primary tumors
Jones [16]	2005	22	Clear cell and papillary	Clear cell and sarcomatoid morphology	Allelic loss multiple chromosomal regions in clear and sarcomatoid components of same patient
Dalgliesh [19]	2010	101	CCRCC	3,544 Protein-coding genes	Genetic heterogeneity dominated by mutations in a single gene
Vaziri [17]	2012	10	42 (18 P, 14 M, 10 N)	VHL heterogeneity	40 % of patient's VHL status differed between primary and metastatic tumors

separate kidneys and between metastatic lesions in the same patient. Interestingly, when the *VHL* gene was mutated in the paired primary and metastatic lesion, the detected mutation was found to be identical [17]. Given the intratumoral heterogeneity in the *VHL* gene [13], it is more than likely that observed differences in *VHL* genotype between paired primary and metastatic lesion may indeed be due to differing populations harboring wild-type and mutant *VHL* in a single primary tumor.

VHL Genotype and Metastasis

In addition to the initiating role of the *VHL*-HIF pathway in clear cell renal cell carcinoma, other studies are investigating new roles of *VHL* to identify additional therapeutic targets. Dalgliesh et al. [19] sequenced 101 cases of clear cell renal cell carcinoma for over 3,500 coding genes and identified mutations in histone-modifying genes, SETD2 and JARID1C, in addition to the previously reported UTX gene [20]. Mutations in NF2 were found only in samples with no *VHL* mutation. Thus aberrations in histone modification may be associated with

Table 7.3 Heterogeneity in *VHL* genotype in paired primary and metastatic tumors [17]

Patient ID	Primary	VHL genotype	Metastatic	VHL genotype
Category 1. Patients with identical primary and metastatic VHL genotype				
4	Left kidney	WT	Lymph node	WT
5[a]	Left kidney	478 del G, ex3	Lymph node	478 del G, ex3
8	Right kidney	Methylated promoter	Adrenal	Methylated promoter
9	Left kidney	232 del A, ex1	Lymph node	232 del A, ex1
	Left kidney	232 del A, ex1		
11[a]	Left kidney	349 del T, ex1	Small bowel	349 del T, ex1
13[a]	Right kidney	WT	Left adrenal	WT
Category 2. Patients with different primary and metastatic VHL genotypes in tumors				
6[a]	Left kidney	407insATATATAT, ex2	Fallopian tube	WT
			Fallopian tube	WT
			Fallopian tube	407insATATATAT, ex2
7	Left kidney	WT	Colon	G463C, ex2
10[a]	Right kidney	WT	Lymph node	C33G, ex1
	Right kidney	C33G, ex1, spatially separated		
	Left kidney	C33G, ex1, relation to capsular margin		
	Left kidney	C33G, ex1, relation to parenchymal margin		
12[a]	Right kidney	del 31 bp, intron 1, 9 bp before exon 2	Lung	WT
	Right kidney	del 31 bp, intron 1, 9 bp before exon 2	Lung	WT
	Right kidney	del 31 bp, intron 1, 9 bp before exon 2	Lung	WT
	Left kidney	WT		
	Left kidney	WT		

[a]Patients with one or more normal tissues evaluated for VHL genotype. All normal tissue were wild-type for VHL

VHL-dependent renal cell carcinoma. A compelling new finding for inactivation of VHL comes from the recent report by Vanharanta et al. [21] demonstrating that during progression of clear cell carcinoma, epigenetic activation of the *VHL*-HIF pathway specific to metastatic cells correlates with poor clinical outcome. In metastatic cells, HIF target genes including CXCR4 associated with invasion and CYTIP associated with protection from death cytokine signals were overexpressed. The authors from this study suggest that the *VHL*-HIF pathway may indeed be expanded and altered in a subpopulation of metastatic cells that ultimately drive renal cancer. These previously unreported associations between metastatic phenotype and tumor initiating pathways may present new therapeutic targets and further our understanding of the metastatic process in clear cell renal cell carcinoma. A potential role for pVHL in cytokinesis that is important for chromosome stability and independent of

its role in ubiquitin ligase activity has also been reported by Sinha et al. [22]. The impact on clear cell renal cell carcinoma development in patients' needs to be further studied.

Functional Role for VHL Mutations

Identification of VHL mutant cells in tumor tissues may help to study tumor heterogeneity in disease as well as monitor tumor treatment. Boysen et al. [23] reported that *pVHL*-dependent expression of cell surface glycoproteins, specifically AXL and CD10, could serve as markers for therapeutic targeting of *VHL* tumors. Here, using cell surface capturing technology (CSC), the authors screened and identified cell surface N-glycoproteins in *VHL*-negative and *VHL*-positive cell lines. The abundance of 23 N-glycoproteins changed in a *VHL*-dependent manner. Human tumor tissue analysis showed a correlation between epithelial AXL expression and an aggressive phenotype. Functional assays showed that CD10 inhibitor, thiorphan, reduced "penetrating behavior" in cell invasion assays in *VHL*-negative cells. Based on results from this study, VHL-dependent cell surface glycoproteins may serve as diagnostic markers in renal cell carcinoma patients for monitoring disease progression and treatment effects.

Targeted Therapy and Implications of VEGF/VEGFR2 SNPs

Since the angiogenesis pathway and its deregulation due to inactivation of *VHL* have been central to development of targeted therapy for clear cell renal carcinoma, we directed efforts on evaluating inherited single nucleotide polymorphisms (SNPs) in VEGF/VEGFR [24] to determine their role in outcome in patients treated with sunitinib, as well as potential alteration of SNPs in the renal tumor. Rationale for the studies was based on reports defining a role for inherited single nucleotide polymorphisms (SNPs) in the VEGF and VEGFR genes in the incidence of toxicities associated with therapy targeting VEGF/VEGFR pathways [25, 26]. Using a panel of SNPs [24] representative for VEGF and VEGFR, we probed germline genomic DNA isolated from peripheral blood lymphocytes in patients with metastatic clear cell carcinoma treated with sunitinib. While the VEGF SNPs analyzed were not in the coding region, the VEGF −634 genotype was significantly ($p<0.05$) associated with the prevalence and duration of sunitinib-induced hypertension (Fig. 7.1). In the same study, while no single VEGF or VEGFR genotype was correlated with outcome, the combination of VEGF SNP 936 and VEGFR2 SNP 889 (Fig. 7.2) was found to be associated with overall survival ($p=0.03$). While other studies [27–29] have also identified SNPs associated with outcome or toxicity in patients with clear cell renal cell carcinoma treated with sunitinib or pazopanib, the role of *VHL* genotype in the patient cohort has not been addressed. In our study we did not correlate these results with *VHL* mutation status due to the small sample size (63 patients) but found that in

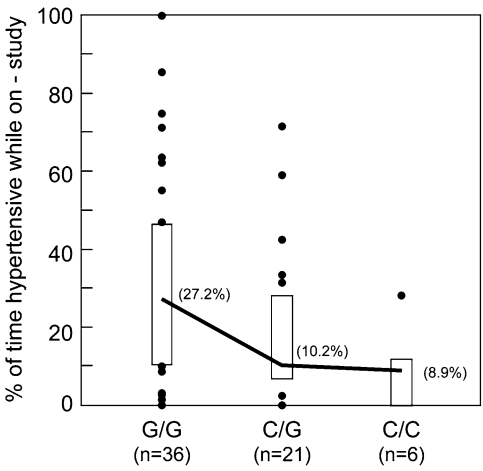

Fig. 7.1 VEGF SNP −634 G/C (rs2010963) is associated with the duration of hypertension during treatment with sunitinib in patients with MCCRCC; $p = 0.01$ (Reproduced with permission from *Cancer.* Apr 1 2012;118(7):1946–1954)

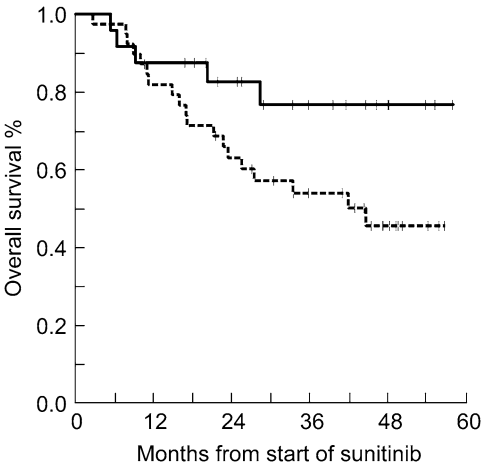

Fig. 7.2 Overall survival and correlation with VEGF 936 C/T combined with VEGFR2 889 G/A SNPs in MCCRCC patients treated with sunitinib. All other genotypes ($n = 24$); VEGF 936 C/C and VEGFR2 G/G ($n = 39$), $p = 0.03$ (Reproduced with permission from *Cancer.* Apr 1 2012;118(7):1946–1954)

30 patients with available tumor tissue the VEGF SNP genotypes identified were >98 % correlated with the paired lymphocytic DNA. While the precise target governing the clinical efficacy of targeted therapy for clear cell renal cell carcinoma is yet to be established, an integration of data from genomic profiling of angiogenic pathways with the *VHL* genotype of the tumor site being treated (primary or metastatic) will aid in identifying relevant correlations with outcome and toxicity.

Other Relevant Genes and Pathways

While the development of targeted therapy in clear cell kidney cancer has focused on angiogenic pathways deregulated by mutant *VHL*, the identification of *VHL-independent* pathways and their role in renal cancer suggests that the extent of the impact of *VHL* inactivation on sporadic clear cell renal cell carcinoma is yet to be determined. Gene expression profiling studies by Beleut et al. [30] and Vasselli et al. [31] of clear cell renal cell carcinoma patients have revealed a lack of correlation with *VHL* mutational status or gene expression patterns related to the HIF pathway. Beleut et al. [30] integrated gene expression data from primary renal cell carcinoma, metastatic renal cell carcinoma, and renal cancer cell lines using non-supervised hierarchical clustering in order to propose a new clinically relevant renal cell carcinoma classification model independent of histology. Observed genome-wide expression signatures were divided into three distinct molecular subgroups that had prognostic significance as shown by tissue microarray analysis using group-specific markers on 176 samples. The correlation between overall survival and molecular group was however found to be independent of tumor stage and grade. The groupings also did not correlate with pathologic criteria, not linked to copy number alterations or influenced by expression of genes in the *VHL*-HIF axis. Loss of chromosome 14q, where HIF1α resides, has been reported to occur in over 50 % of 112 archived clear cell renal cell carcinoma specimens and found to be independent of *VHL* chromosome loss [32].

Summary

The role and impact on therapy of the *VHL* genotype in clear cell renal cell carcinoma is certainly complex and still emerging. Recent studies indicating that mutation status of *VHL* may cause epigenetic changes independent of the HIF pathway provide much needed information in understanding implications in tumor metastasis and progression. Since *VHL* does not exhibit hotspot mutations and observed mutations appear to be patient unique, studies on identifying the functional impact of the mutations on pVHL function will continue to be important and informative in disease prognosis. Correlative clinical studies also need more emphasis analyzing metastatic tumors since they represent the target for the current standard of care, and a comprehensive understanding of genetic and epigenetic profiling within the context of intratumor and inter-tumor heterogeneity could aid in the challenges facing the design of individualized therapy.

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Chapter 8
First-Generation Tyrosine Kinase Inhibitors: Clinical Results

Han Hsi Wong and Tim Eisen

Introduction

The role of angiogenesis in RCC development was highlighted by the discovery of the von Hippel-Lindau (*VHL*) gene, which is inactivated or silenced in up to 91 % of clear cell RCC [1–6]. The loss of VHL causes the accumulation of the hypoxia-inducible factor-α (HIF-α), resulting in the expression of angiogenic proteins such as the vascular endothelial growth factor (VEGF). Secreted VEGF binds to VEGF receptors (VEGFRs), triggering receptor dimerization, activation of its intracellular tyrosine kinase domain, and phosphorylation of downstream signaling proteins resulting in the mitogenic and angiogenic phenotype of cancer. The understanding of this has led to the development of the TKIs to target this molecular aberration.

The emergence of the TKIs has completely revolutionized the management of advanced RCC, a disease that is resistant to standard chemotherapy. Immunotherapy, for example, with IFN-α or with high-dose IL-2 used to be the mainstay of treatment, but IL-2 is associated with significant toxicities, while IFN-α monotherapy was characterized by slow partial response of short duration in a minority of patients [7–9]. The orally active TKIs, on the other hand, have proven beneficial in terms of their ease of administration and their treatment efficacy and tolerability, both in the first-line setting and after previous cytokine therapy. The first-generation of TKIs, sorafenib and sunitinib, are now widely used in the treatment of advanced RCC since the publication of their phase III trial results in 2007 [10, 11]. They have also set the standards against which new drugs are measured. Sunitinib is currently the standard first-line agent, although local preferences in various countries mean that sorafenib and, increasingly, pazopanib are seen as attractive alternatives.

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Molecular Targets and Early Development

Sorafenib (Nexavar®, Bayer and Onyx Pharmaceuticals; previously known as BAY 43-9006) and sunitinib (Sutent®, Pfizer; previously known as SU11248) both have activities against the VEGFRs, platelet-derived growth factor receptors (PDGFRs), CD117 (c-Kit), Fms-like tyrosine kinase-3 (FLT-3), and the receptor encoded by the proto-oncogene *RET*. Sorafenib can also inhibit Raf, while sunitinib is also active against the colony-stimulating factor 1 receptor (CSF1R). The potency and selectivity of an agent against its targets is often denoted by its low 50 % maximal inhibitory concentration (IC_{50}) in laboratory tests, i.e., the concentration of drug required to inhibit its desired biological process (e.g., enzyme activation, cell proliferation) by half. However, the different types of assays used to measure this (e.g., biochemical or cellular assay, inhibition of receptor phosphorylation, ligand-dependent proliferation of cells or receptor blockade, etc.) make direct comparison of IC_{50} between agents difficult. Table 8.1 summarizes the IC_{50} of different targets for sorafenib and sunitinib based on biochemical, cell-free kinase assays from available data.

Although these two drugs are TKIs that ultimately act on the inhibition of tumor, endothelial, and stromal cells, it is clear that they have similar as well as unique molecular targets. Sorafenib was initially developed as an inhibitor of Raf—this is important as the Ras-Raf-MAPK signaling pathway mediates a number of processes involved in tumorigenesis, including cell proliferation, survival, and motility [12, 13] (Fig. 8.1). Subsequent laboratory work showed that sorafenib can also inhibit the VEGFRs—it thus has both antitumor proliferative and antiangiogenic activities [14]. This led to a phase I trial of patients with solid tumors, for which a dose of 400 mg

Table 8.1 Receptor tyrosine kinase inhibitory activity (cell-free kinase assay) of sorafenib and sunitinib

	IC_{50} (nmol/L)	
Targets	Sorafenib [14, 110]	Sunitinib [23, 110–114]
VEGFR-1	–	21
VEGFR-2	90	34
VEGFR-3	20	3
PDGFR-α	–	–
PDGFR-β	57	75
CD117 (c-Kit)	68	40
FLT-3	58	(250)[a]
RET	47	224
B-Raf	22	–
C-Raf	6	–
CSF1R	107	7

IC_{50} half maximal inhibitory concentration, *VEGFR* vascular endothelial growth factor receptor, *PDGFR* platelet-derived growth factor receptor, *FLT-3* Fms-like tyrosine kinase-3, *RET* rearranged during transfection, *CSF1R* colony-stimulating factor 1 receptor

[a]Inhibition of phosphorylation in cellular assay

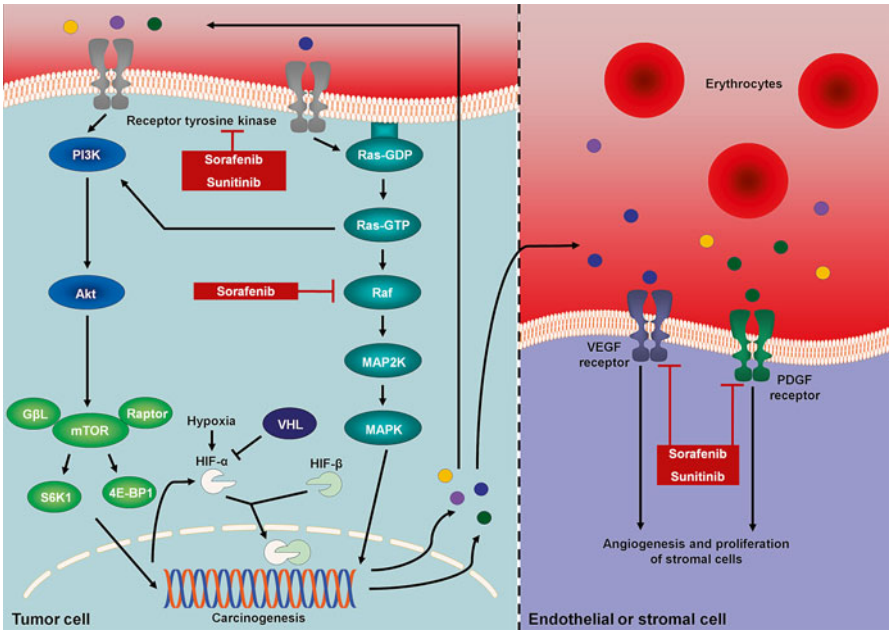

Fig. 8.1 Molecular targets of sorafenib and sunitinib. Binding of ligand to the growth factor receptor results in the activation of its intracellular tyrosine kinase. This phosphorylates and activates the PI3K-Akt-mTOR and the Ras-Raf-MAPK signaling pathways, which result in carcinogenic responses including increased cell survival, proliferation, motility, and angiogenesis. In normoxic condition, HIF-α is hydroxylated and subsequently recognized by the VHL complex which targets HIF-α for proteasomal degradation. However, during hypoxia or in the absence of VHL protein (secondary to gene mutation or silencing), HIF-α accumulates and forms a complex with HIF-β. These processes result in the transcription of growth factors which act on surrounding stromal or endothelial cells, leading to their proliferation and angiogenesis. They can also exert their effect via an autocrine fashion by binding to receptors on cancer cells. Sorafenib and sunitinib are TKIs that act primarily on the inhibition of endothelial cells. Sorafenib also inhibits Raf. The effect of these drugs on the autocrine receptor pathway is less clear. *4E-BP1* eukaryotic translation initiation factor 4E-binding protein 1, *GDP* guanosine diphosphate, *GTP* guanosine triphosphate, *HIF* hypoxia-inducible factor, *MAP2K* mitogen-activated protein kinase kinase, *MAPK* mitogen-activated protein kinase, *mTOR* mammalian target of rapamycin, *PDGF* platelet-derived growth factor, *PI3K* phosphatidylinositol 3-kinase, *S6K1* S6 kinase 1, *TKI* tyrosine kinase inhibitor, *VEGF* vascular endothelial growth factor, *VHL* von Hippel-Lindau

twice daily was found to be tolerable [15]. Its unique property of also inhibiting Raf has resulted in its use in the treatment of hepatocellular carcinoma [16].

Sunitinib was originally developed by Sugen (later acquired by Pfizer) along with two other TKIs, SU5416 and SU6668 [17]. Development of the latter two drugs was subsequently abandoned due to toxicities and inadequate pharmacokinetic properties, respectively. Other than the VEGFRs, sunitinib also inhibits CD117, PDGFRs, and RET, which has made it effective against imatinib-resistant gastrointestinal stromal tumors and pancreatic neuroendocrine tumors in randomized phase III trials [18, 19]. However, it has proved unsuccessful in early clinical trials

of advanced breast cancer in which CSF1R may be dysregulated [20–22]. In RCC, and in contrast to sorafenib, sunitinib appears to act primarily on the inhibition of angiogenesis rather on cell proliferation [23]. As shown in Table 8.1, it is more potent against the VEGFRs compared to sorafenib. It remains unclear whether the inhibition of receptor tyrosine kinases in RCC tumor cells, rather than on endothelial cells, plays a major role on the effect of sunitinib. Nonetheless, after promising laboratory results, a subsequent phase I study identified a tolerable dose of 50 mg/day for 4 weeks, followed by 2 weeks of rest [24]. This has now become the standard dose after its efficacy was shown.

Sorafenib

In an early attempt to ascertain the efficacy of sorafenib, Ratain et al. published a phase II randomized discontinuation trial in 2006 [25]. They recruited 202 patients with metastatic RCC, for which 84 % have had prior systemic anticancer therapy, including 76 % with previous IL-2 or IFN-α. The majority of patients have clear cell carcinoma (75 %), and most were in the low-to-intermediate-risk groups according to the Memorial Sloan Kettering Cancer Center (MSKCC) prognostic score [26] (94 %). At the start of the trial, all patients received sorafenib 400 mg twice daily for 12 weeks. After this period the patients were divided into three groups based on disease response on imaging: (a) 65 patients with <25 % change in tumor size (i.e., stable disease) were randomly assigned to sorafenib ($n=32$) for another 12 weeks or placebo ($n=33$) (this is the true experimental arm), (b) 73 patients with ≥25 % tumor shrinkage were continued on sorafenib, and (c) patients with disease progression (>25 % growth) were discontinued treatment. The primary end point was the percentage of the randomly assigned patients that remained progression-free at 24 weeks—this is to determine whether the stable disease achieved in the first 12 weeks was the result of sorafenib or due to slow-growing tumors. At 24 weeks, 50 % (16/32) of patients on sorafenib were progression-free compared to 18 % (6/33) in the placebo group ($P=0.007$). The respective median progression-free survival (PFS) was 24 weeks and 6 weeks ($P=0.0087$). Sorafenib was given again for 28 patients whose disease progressed on placebo, and they continued on this for a median of 24 weeks before further progression.

Since the publication of these promising results, a number of trials have been performed using sorafenib in the first- or subsequent-line setting for patients with metastatic RCC.

Sorafenib for Treatment-Naïve Patients

Sorafenib Monotherapy

A number of first-line randomized phase II trials which compared sorafenib alone or in combination with other therapies are summarized in Table 8.2.

Table 8.2 Randomized phase II trials of sorafenib as first-line treatment

Study	Patient group	Treatment	Number of patients	ORR (%)	Median PFS (months)
Escudier et al. 2009 [27]	Metastatic clear cell RCC	Sorafenib vs	97	5.2	5.7
		IFN-α	92	8.7	5.6
Jonasch et al. 2010 [34]	Metastatic clear cell RCC	Sorafenib vs	40	30	7.39
		sorafenib + IFN-α	40	25	7.56
ROSORC (Procopio et al. 2011) [35]	Metastatic RCC (11 % had non-clear cell histology)	Sorafenib vs	62	14.5	6.9
		sorafenib + subcutaneous IL-2	66	27.3	7.6
Rini et al. 2012 [36]	Metastatic clear cell RCC	Sorafenib + placebo vs	51	25	9.0
		sorafenib + trebananib (3 mg/kg) vs	51	37	8.5
		sorafenib + trebananib (10 mg/kg)	50	38	9.0

ORR objective response rate, *PFS* progression-free survival

A phase II trial by Escudier et al. demonstrated that sorafenib was not superior to IFN-α when given in the first-line setting [27]. In this study, 189 patients with unresectable and/or metastatic clear cell RCC were randomly assigned to receive sorafenib 400 mg twice daily or subcutaneous IFN-α 9 MIU three times weekly. At progression, patients on sorafenib were dose escalated to 600 mg twice daily, while those on IFN-α were switched to sorafenib 400 mg twice daily. There was no difference in PFS between the sorafenib and the IFN-α arms (5.7 and 5.6 months, respectively), although more patients in the sorafenib group had tumor shrinkage (68.2 vs. 39.0 %) and significantly higher disease control rate (stable disease for ≥6 weeks or confirmed partial or complete response) (79.4 vs. 64.1 %, $P=0.006$). They also reported fewer symptoms, better quality of life, and greater treatment satisfaction. For those who crossed over from IFN-α, sorafenib reduced tumor size in 76.2 % of patients, with a median PFS of 5.3 months. 41.9 % of those who received sorafenib 600 mg twice daily on disease progression had tumor shrinkage, and although there were no objective responses according to Response Evaluation Criteria in Solid Tumors (RECIST), 39.5 % had stable disease. Dose escalation resulted in an additional PFS of 3.6 months.

Tivozanib (AV-951; AVEO Oncology) is a third-generation TKI that is potent and specific for VEGFR-1 to VEGFR-3. Its development is aimed at maximizing the on-target antiangiogenic effects while minimizing off-target toxicities. In the phase III TIVO-1 (Tivozanib vs. Sorafenib in first-line Advanced RCC) trial, 517 patients with advanced clear cell RCC were randomized to receive either tivozanib 1.5 mg daily for 3 weeks followed by 1 week rest or sorafenib 400 mg twice daily continuously [28] (Table 8.3). The results demonstrated a significant benefit with

Table 8.3 Results of randomized phase III trials of sorafenib in advanced clear cell RCC

Trial	TARGET (Escudier et al. 2007, 2009) [10, 44]	AXIS (Rini et al. 2011) [29]	TIVO-1 (Motzer et al. 2012) [28]	AGILE 1051 (Hutson et al. 2013) [30]
Total number of patients	903	723	517	288
Patient group	Advanced clear cell RCC which progressed after first-line cytokine therapy	Advanced clear cell RCC which progressed after first-line therapy with sunitinib (54 %), bevacizumab plus IFN-α (8 %), temsirolimus (3 %), or cytokines (35 %)	Advanced clear cell RCC—treatment-naïve (70 %) or no more than one prior systemic therapy for RCC (but not VEGF or mTOR targeted) (30 %)	Treatment-naïve metastatic clear cell RCC
Treatment	Sorafenib vs. placebo	Sorafenib vs. axitinib	Sorafenib vs. tivozanib	Sorafenib vs. axitinib (1 : 2)
Number of patients in each arm	451 vs. 452	362 vs. 361	257 vs. 260	96 vs. 192
ORR	10 vs. 2 % ($P<0.001$)	9 vs. 19 % ($P=0.0001$)	23 vs. 33 % ($P=0.014$)	15 vs. 32 % ($P=0.0006$)
Median PFS (months)	5.5 vs. 2.8 (HR = 0.44, $P<0.01$)	4.7 vs. 6.7 (HR = 0.665, $P<0.0001$) (6.5 vs. 12.1 for previous cytokines. HR = 0.464, $P<0.0001$) (3.4 vs. 4.8 for previous sunitinib. HR = 0.741, $P=0.0107$)	9.1 vs. 11.9 (HR = 0.797, $P=0.042$) (9.1 vs. 12.7 for treatment-naïve patients. HR=0.756, $P=0.037$)	6.5 vs. 10.1 (HR = 0.77, $P=0.038$)
Median OS (months)	17.8 vs. 14.3[a] (HR = 0.78, $P=0.029$)	–	–	–

[a]After censoring of patients who crossed over to the sorafenib arm

ORR objective response rate, *PFS* progression-free survival, *OS* overall survival

tivozanib compared to sorafenib in terms of response rate and median PFS. The benefit in PFS was even clearer in treatment-naïve patients (12.7 and 9.1 months for tivozanib and sorafenib, respectively, HR = 0.756, $P=0.037$). However, a subgroup analysis revealed that tivozanib only performed better than sorafenib in the following group of patients: Eastern Cooperative Oncology Group Performance Status (ECOG PS) of 0, favorable MSKCC score, time of diagnosis to study entry of 1 year or more, no prior systemic therapy, had two or more metastatic lesions, Caucasian patients, and those from North America/Western Europe; there was no significant

difference between the two drugs in the other patient subgroups. Tivozanib resulted in less diarrhea, hand-foot syndrome, and alopecia, but has significantly more frequent hypertension, dysphonia, and biochemical hypothyroidism compared to sorafenib.

Axitinib is another third-generation TKI that can block VEGFR-1 to VEGFR-3 at sub-nanomolar concentrations and has weaker activity against PDGFRs and CD117. The AGILE 1051 randomized phase III trial enrolled 288 treatment-naïve metastatic RCC patients and was powered to show a 78 % improvement in PFS with axitinib (5 mg twice daily) compared to sorafenib. This trial was designed after the efficacy of axitinib was found to be superior to that of sorafenib in the second-line treatment of RCC in 2011 (see AXIS trial below) [29]. Results announced recently showed that the median PFS for the axitinib arm was higher than that of the sorafenib arm (10.1 vs. 6.5 months, HR = 0.77, $P = 0.038$), although the difference failed to meet the trial's prespecified significance level of $P = 0.025$ [30] (Table 8.3). Subgroup analysis suggested a PFS benefit of axitinib over sorafenib in patients with an ECOG PS of 0 (13.7 vs. 6.6 months, HR = 0.64, $P = 0.022$), but there was no survival difference when the ECOG PS was 1 (6.5 vs. 6.4 months, HR = 0.93, $P = 0.38$). Patients with prior nephrectomy also did better with axitinib than sorafenib (PFS of 10.3 vs. 6.4 months, HR = 0.67, $P = 0.009$). Although sorafenib resulted in more frequent hand-foot syndrome, rash, erythema and alopecia, axitinib caused more diarrhea, hypertension, weight loss, anorexia, dysphonia and hypothyroidism.

Sorafenib and Cytokine Combination

A phase II trial by Gollob et al. involved 40 patients with metastatic RCC receiving sorafenib and IFN-α 10 MIU three times a week followed by a 2-week break [31]. Patients were allowed to receive additional cycles of treatment until disease progression. The ORR was 33 %, including 5 % of patients with a complete response. These include treatment-naïve patients as well as those who had previous IL-2. Median PFS was 10 months. However, the toxicities from combination treatment were higher than expected with either drug alone, including more fatigue, anorexia, anemia, diarrhea, hypophosphatemia, and rash. Dose reductions were needed in 65 % of patients. In another study published at the same time, the Southwest Oncology Group treated 62 treatment-naïve advanced clear cell RCC patients with the same dose of sorafenib and IFN-α [32]. The confirmed ORR was 19 %, with an additional 11 % of patients having unconfirmed partial response. The median PFS was 7 months. Again, high frequencies of adverse events were noted, with 77 % of patients experienced ≥grade 3 toxicities. Most patients were unable to tolerate the full dose of IFN-α. In the phase II RAPSODY trial, 101 treatment-naïve patients were randomized to receive sorafenib with either IFN-α 9 MIU three times a week or IFN-α 3 MIU five times a week [33]. The results appeared to be in favor of frequent low-dose IFN-α, with median PFS of 8.6 months (vs. 7.9 months for the 9 MIU group, $P = 0.049$) and ORR of 34 % (vs. 17.6 %, $P = 0.058$). Nonetheless, no

conclusion could be drawn from these studies as to whether combination regimen is superior to sorafenib monotherapy.

Low-dose IFN-α in combination with sorafenib was found to be of similar efficacy to sorafenib alone in a trial by Jonasch et al., where 80 patients with metastatic clear cell RCC were randomized to receive either sorafenib alone or sorafenib with IFN-α 0.5 MIU twice daily (Table 8.2) [34]. There was no significant difference in ORR and PFS.

The ROSORC phase II trial randomized patients with metastatic RCC to received sorafenib alone or sorafenib in combination with subcutaneous IL-2 4.5 MIU five times per week for 6 in every 8 weeks [35] (Table 8.2). The dose of IL-2 was reduced after the first 20 patients in the combination arm were treated, due to the onset of adverse events, mainly asthenia. Although ORR was higher in the combination arm compared to sorafenib alone, there was no difference in median PFS (7.6 vs. 6.9 months, respectively, $P=0.109$). In patients who received a higher dose of IL-2, PFS was longer at 9.9 months compared to 7.1 months for those receiving the lower dose.

Sorafenib and Trebananib Combination

Trebananib (also known as AMG 386) is a selective angiopoietin 1/2-neutralizing peptibody (a peptide-Fc fusion protein). This inhibits the interaction between angiopoietins and Tie2 receptor, which are involved in tumor angiogenesis. In a phase II trial of treatment-naïve metastatic clear cell RCC, 152 patients were randomized to receive either sorafenib with intravenous trebananib at 10 or 3 mg/kg or placebo once weekly [36] (Table 8.2). There was no difference in median PFS between the arms, namely, 9.0, 8.5, and 9.0 months, respectively. Patients who received sorafenib and placebo were allowed to get trebananib 10 mg/kg following disease progression—of the 30 patients, the ORR was 3 % and 31 % had reduction in tumor burden. The authors concluded that dual inhibition of VEGF and angiopoietin/Tie2 may be of future interest in the treatment of metastatic RCC and that a higher dose of trebananib might be necessary. A phase II trial of trebananib (at 10 or 15 mg/kg dose) in combination with sunitinib is still ongoing, but preliminary result suggested a potential benefit with the higher dose [37].

Sorafenib and Chemotherapy Combination

The Spanish Oncology Genitourinary Group (SOGUG)-02-06 phase II trial studied the combination of sorafenib, gemcitabine, and metronomic capecitabine [38]. Forty treatment-naïve patients with metastatic clear cell RCC were given intravenous gemcitabine (1,000 mg/m^2 on days 1 and 8), oral capecitabine (500 mg/m^2 twice daily on days 1–14), and sorafenib (400 mg twice daily on days 1–21) for six cycles, followed by sorafenib monotherapy. The median PFS achieved was 11.1 months, which was much longer than previously observed with gemcitabine and capecitabine [39–41] or sorafenib monotherapy [27, 34–36]. Half of the patients had a partial response, and 42.5 % had stable disease.

Sorafenib and mTOR Inhibitor Combination

The simultaneous blockade of the VEGF and mammalian target of rapamycin (mTOR) signaling pathways has the possible advantage of synergistic antitumoral activity and abrogating the development of treatment resistance. However, a phase I study of patients with solid malignancies showed that the combination of sorafenib at 400 mg twice daily and intravenous temsirolimus at 25 mg weekly resulted in significant mucocutaneous toxicity [42]. Sorafenib and the oral mTOR inhibitor everolimus could only be tolerated at 50 % dose of the latter drug, as shown in a phase I study of patients with metastatic clear cell RCC [43]. In these studies, no drug-drug interaction was observed.

Sorafenib as a Second-Line Treatment

Promising phase II results led to the design of the large Treatment Approaches in Renal Cancer Global Evaluation Trial (TARGET). In this large phase III trial, 903 patients were randomized to receive sorafenib 400 mg twice daily or placebo (Table 8.3) [10, 44]. Patients selected had metastatic clear cell RCC which had progressed after one systemic treatment within the previous 8 months, had good ECOG PS, and were in the low-to-intermediate MSKCC risk groups. The median PFS was 5.5 months in the sorafenib group compared to 2.8 months in the placebo group (HR = 0.44, $P < 0.01$). Partial response was noted in 10 and 2 % of patients, respectively ($P < 0.001$). Only one complete response was noted in the sorafenib and none in the placebo group. In this trial, 48 % of patients in the placebo arm crossed over to sorafenib on disease progression, resulting in similar overall survival (OS) (17.8 vs. 15.2 months, respectively, HR = 0.88, $P = 0.146$). However, after censoring this crossover, the median OS was found to be 17.8 and 14.3 months for sorafenib and placebo, respectively (HR = 0.78, $P = 0.029$). Prespecified subgroup analysis showed benefit with sorafenib over placebo across all subgroups regardless of age, MSKCC score, whether patients have had previous cytokine therapy, the presence of lung or liver metastasis, and the time from diagnosis to treatment.

A subsequent subgroup analysis was done to compare older (age ≥70 years, $n = 115$) and younger (age <70 years, $n = 787$) patients who took part in the TARGET trial [45]. For patients treated with sorafenib, the median PFS was similar (26.3 and 23.9 weeks in the older and younger groups, respectively), and clinical benefit rates (84.3 and 83.5 %, respectively) were superior to those in the placebo groups (62.2 and 53.8 %, respectively).

Sorafenib has also been compared to axitinib in the phase III AXIS (Axitinib vs. Sorafenib) trial [29]. In the AXIS trial, 723 patients with metastatic clear cell RCC who had progressed after first-line treatment with sunitinib, bevacizumab plus IFN-α, temsirolimus, or cytokines were randomized to receive axitinib or sorafenib (Table 8.3). The median PFS was longer in the axitinib group compared to the sorafenib group (6.7 vs. 4.7 months, HR = 0.665, $P < 0.0001$), with ORR of 19 and 9 %, respectively. The benefit was higher in patients previously treated

with cytokines (12.1 vs. 6.5 months) compared to those treated previously with sunitinib (4.8 vs. 3.4 months). Diarrhea, hypertension, and fatigue were more common in the axitinib arm, but there were less hand-foot syndrome and alopecia compared to sorafenib, suggesting that some toxicities were partly due to different target specificity of the two TKIs.

The recently reported INTORSECT phase III trial included 512 RCC patients who had progressed after first-line sunitinib and who had ECOG PS of 0 or 1 [46]. Patients were randomized to receive either temsirolimus or sorafenib. The median PFS was similar between the arms (4.3 vs. 3.9 months for temsirolimus and sorafenib, respectively, HR = 0.87, $P = 0.19$), but OS was longer with sorafenib (12.3 vs. 16.6 months, HR = 1.31, $P = 0.01$). Diarrhea, rash, hand-foot syndrome, and anorexia were the most common adverse events with sorafenib. Based on these results, it was concluded that temsirolimus was not superior to sorafenib after first-line VEGF-targeted therapy.

Expanded-Access Trials

Because of stringent inclusion criteria, many patients with metastatic RCC were excluded from clinical trials. To collect data for the role of sorafenib in everyday clinical practice, two major expanded-access studies, named ARCCS (Advanced Renal Cell Carcinoma Sorafenib), were performed in Europe (EU-ARCCS) [47] and North America (NA-ARCCS) [48]. The EU-ARCCS include 1,150 patients with advanced RCC treated with sorafenib. These include 15 % with ECOG PS of 2, 23 % of age ≥70 years, 21 % with non-clear cell RCC, 2 % with brain metastases, and 67 % with previous cytokine therapy. The overall median PFS was 6.6 months and ORR was 4 %. Not unexpectedly, subgroup analysis showed that patients with ECOG PS of 0 and just one site of disease survived significantly longer than 6.6 months, whereas those with ECOG PS of 2, clear cell histology with sarcomatoid features, non-clear cell cancer, liver metastases, and three or more sites of disease did worse. No significant difference in PFS was seen between younger and older patients, or whether they had brain metastases or prior cytokine therapy. Sorafenib was well tolerated regardless of age, ECOG PS, histology, prior therapy, and site or number of metastases.

In the NA-ARCCS, a total 2,504 patients were treated. These also include 29 % of age ≥70 years, 8 % with non-clear cell RCC, 3 % with brain metastases, and 49 % with previous cytokine therapy, in addition to 12 and 1 % who had prior bevacizumab and sunitinib, respectively. For the evaluable patients, the overall ORR was 4 %. When sorafenib was approved 6 months after the study initiation, the expanded-access program was closed, and the 328 patients who had non-clear cell histology or who had been treatment-naïve were enrolled into an extension protocol for 6 months of treatment. In this subgroup, a median PFS of 8.3 months was achieved. Again, the incidence and severity of adverse events were similar across all subgroups.

These trials confirmed the activity of sorafenib in the treatment of metastatic RCC in a heterogeneous patient population, with efficacy and safety profile that were in line with the TARGET trial.

Dose Escalation

It remains uncertain if dose escalation of sorafenib would definitely confer better antitumoral efficacy. In the phase II trial by Escudier et al., 41.9 % of the 43 patients who received dose escalation of 600 mg twice daily on disease progression had tumor shrinkage (although no response as per RECIST), and 39.5 % had stable disease, resulting in an additional PFS of 3.6 months [27].

George et al. performed a retrospective study of 14 patients who had their dose increased above the standard 400 mg twice daily [49]. Seventy-one percentage tolerated a dose of 600 mg twice daily, while four of six patients tolerated a subsequent escalation to 800 mg. Higher dose of sorafenib resulted in eight stable diseases. In a phase II dose-escalation trial of 44 patients with metastatic RCC (22 had no prior treatment), sorafenib was increased to 600 mg twice daily in 42 patients, with 31 of these had further escalation to 800 mg [50]. An ORR of 48 % was reported, and 64 % had a PFS of ≥6 months. In another study of treatment-naïve patients with metastatic clear cell RCC, patients were started on the standard dose, with escalation to 600 mg and then 800 mg twice daily if there were no dose-limiting toxicities (DLTs) at the end of each cycle [51]. Of the 13 patients treated so far, preliminary results showed that dose escalation was possible in 70 % of the patients. The main toxicity limiting dose escalation was hand-foot syndrome. As yet, no enhanced efficacy with increasing treatment dose was observed.

Mancuso et al. performed a dose-escalation study on 19 patients with metastatic RCC who had previously been treated with a VEGF-targeted TKI [52]. Patients who progressed on the standard 400 mg dose were put on the 600 mg twice-daily dose—three out of six patients benefited with a PFS of >3 months. Gore et al. evaluated 67 intention-to-treat population in a phase II trial, where sorafenib was given at 400 mg twice daily for 4 weeks, followed by increment to 600 mg for 4 weeks, then to 800 mg from 3 months onwards [53]. Only 18 patients tolerated dose escalation as per protocol, while 49 patients had dose changes as tolerated throughout the study. Of the patients in the 400 mg ($n=25$), 600 mg ($n=12$), and 800 mg ($n=20$) subgroups, ORR and median PFS were 4, 16.7, and 35 % and 3.7, 7.4, and 8.5 months, respectively.

Although dose escalation of sorafenib appeared to be feasible and might be associated with better outcome, further studies are needed to clarify whether this approach has a role in the management of patients with metastatic RCC.

Sunitinib

Sunitinib as a First-Line Treatment

Sunitinib is approved by the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA) as a front-line therapy for patients with advanced RCC. Its role as a first-line treatment was studied in a landmark multinational phase III trial, where 750 treatment-naïve patients with metastatic clear cell

Table 8.4 Results of randomized phase III trials of sunitinib as first-line treatment in advanced clear cell RCC

Trial	NCT00083889 (Motzer et al. 2007, 2009) [11, 54]	COMPARZ (Motzer et al. 2012) [56]
Total number of patients	750	1,100
Patient group	Treatment-naïve metastatic clear cell RCC	Treatment-naïve advanced clear cell RCC
Treatment	Sunitinib vs. IFN-α	Sunitinib vs. pazopanib
Number of patients in each arm	375 vs. 375	553 vs. 557
ORR	47 vs. 12 % (P <0.001)	25 vs. 31 % (P = 0.032)
Median PFS (months)	11.0 vs. 5.0 (HR = 0.42, P <0.001)	9.5 vs. 8.4 (HR = 1.047)
Median OS (months)	26.4 vs. 20.0[a] (HR = 0.808, P = 0.036)	29.3 vs. 28.4 (HR = 0.91, P = 0.275)

ORR objective response rate, *PFS* progression-free survival, *OS* overall survival

[a]After censoring 25 patients who crossed over from the IFN-α arm to the sunitinib arm

RCC were randomly assigned to receive either repeated 6-week cycles of sunitinib (50 mg orally once daily for 4 weeks, followed by 2 weeks off treatment) or IFN-α (9 MIU subcutaneously thrice weekly) (Table 8.4) [11, 54]. Other eligibility criteria include the presence of measurable disease, ECOG PS of 0 or 1, and the absence of brain metastases. The majority of patients (93 %) were in the favorable and intermediate MSKCC prognostic groups. The results demonstrated that sunitinib conferred a survival benefit compared to IFN-α in the first-line setting, with a median PFS of 11 vs. 5 months (HR = 0.42, P < 0.001) and an ORR of 47 and 12 % (P < 0.001), respectively. An OS of 26.4 and 21.8 months (HR 0.818, P = 0.049) were observed by stratified log-rank test for the sunitinib arm and IFN-α arm, respectively. When 25 patients who crossed over from the IFN-α arm to the sunitinib arm were censored, a clear OS benefit was noted (26.4 vs. 20 months, HR = 0.808, P = 0.036) [54]. When analysis was limited to patients who did not receive post-study cancer treatment (n = 193 in the sunitinib group and n = 162 in the IFN-α group), the median OS with sunitinib was twice that of IFN-α (28.1 vs. 14.1 months, HR = 0.647, P = 0.003). Grades 3–4 fatigue was more common with IFN-α, whereas patients on sunitinib experienced more diarrheas.

Pazopanib is a second-generation TKI that targets VEGFRs, PDGFRs, CSF1R, and CD117. Following the publication of the VEG105192 trial showing its efficacy in treatment-naïve or cytokine-pretreated clear cell RCC [55], the phase III COMPARZ (Comparing the Efficacy, Safety and Tolerability of Pazopanib vs. Sunitinib) trial was performed [56]. This study is the largest trial yet in the history of RCC and involved 1,100 patients with locally advanced RCC. The results suggested that there was no difference in terms of efficacy between pazopanib and sunitinib—ORR of 31 and 25 % (P = 0.032), median PFS of 8.4 and 9.5 months (HR = 1.05), and median OS of 28.4 and 29.3 (HR = 0.91), respectively.

A randomized phase II trial has recently reported on the use of sunitinib in treatment-naïve patients. The TORAVA trial aimed to compare standard VEGF-targeted therapy with the combination of bevacizumab, a monoclonal antibody

directed against VEGF-A, and temsirolimus [57]. A total of 171 patients with metastatic RCC were randomized 2:1:1 to receive either bevacizumab (10 mg/kg intravenous every 2 weeks) and temsirolimus, sunitinib, or bevacizumab and IFN-α (9 MIU subcutaneous thrice weekly). The respective median PFS was 8.2, 8.2, and 16.8 months. The authors concluded that there was no benefit in combining bevacizumab and temsirolimus in the treatment of metastatic RCC. However, unexpectedly the ORR (24 vs. 39 %) and PFS (8.2 vs. 16.8 months) obtained with sunitinib was much lower than that with bevacizumab and IFN-α. A post hoc analysis demonstrated some major differences in patient characteristics between the two arms, in favor of bevacizumab and IFN-α, which could explain the findings: disease-free interval of >12 months (29 vs. 39 %), good MSKCC risk score (31 vs. 39 %), Fuhrman grade of 1–2 (32 vs. 38 %), liver metastases (19 vs. 14.6 %), and high serum lactate dehydrogenase (17.1 vs. 7.9 %) [58].

Expanded-Access Trials

After sunitinib became widely available, an expanded-access trial was performed by Gore et al. to assess the efficacy of sunitinib in patients excluded from the phase III trial [59]. A total of 4,564 patients were recruited, with 4,371 included in a modified intention-to-treat population. These include patients with brain metastases (7 %), ECOG PS of ≥2 (13 %), non-clear cell histology (13 %), and those aged >65 years (32 %). Of the evaluable patients, the respective ORR was 12, 9, 11, and 17 %. The ORR, median PFS, and OS for the whole cohort were 17 %, 10.9 months, and 18.4 months, respectively. The median PFS and OS for patients with brain metastases were 5.6 and 9.2 months, respectively [60], while these were 5.1 and 6.7 months, respectively, for those with ECOG PS of ≥2 [59]. The median PFS and OS in elderly patients (>65 years) were 11.3 and 18.2 months, respectively. These were similar to those found on other studies [61, 62]. In a study by De Giorgi et al., 185 patients of age ≥70 years were treated with sunitinib in the first-line setting for metastatic RCC [61]. A median PFS and OS of 11.0 and 25.5 months, respectively, were observed. A retrospective, multicenter study in Italy identified 68 treated elderly patients with a median age of 74 years—the PFS and OS obtained were 13.6 and 18.3 months, respectively [62]. All these results are in line with data from the sunitinib phase III trial (PFS and OS of 11.0 and 26.4 months, respectively) [11, 54].

In another study of 21 patients with non-clear cell RCC, an ORR of 14.3 % and clinical benefit rate of 52.4 % were observed, resulting in median PFS and OS of 4.1 and 14.6 months, respectively [63]. These data further support the use of sunitinib in all patients with metastatic RCC.

Dose and Regimen

Based on the phase III trial data, the recommended sunitinib dose is 50 mg daily for 4 weeks followed by 2 weeks off treatment, and the cycle is repeated [11, 54]. Evidence suggests that the ability to maintain the full dose of sunitinib is associated

with better efficacy, and as such the effective management of treatment toxicities is essential [64]. Houk et al. performed a pharmacokinetic/pharmacodynamic meta-analysis of sunitinib studies, including 192 metastatic RCC patients from two phase II and one phase III trials [65]. The sunitinib dose ranged from 25 to 150 mg. They found that increased exposure to sunitinib is associated with higher probability of achieving a partial response, longer time to progression, and improved OS. In addition, pharmacokinetic modeling studies have predicted that 50 mg of sunitinib results in greater tumor shrinkage than 25 mg [66]. With regard to safety, the meta-analysis demonstrated that higher sunitinib exposure is associated with only a slight increase in the incidence (but not severity) of fatigue and minimal change in blood pressure and neutrophil count [65].

In the phase II Renal EFFECT trial, 292 patients with advanced clear cell RCC were randomized to receive either sunitinib at the standard dose and schedule or sunitinib at 37.5 mg on the continuous daily dosing (CDD) schedule [67]. The median time to tumor progression was 9.9 months for the standard arm compared to 7.1 months for the CDD arm (HR = 0.77, $P = 0.09$), with no difference in the estimated PFS (8.5 vs. 7.0 months, HR = 0.77, $P = 0.07$) and OS (23.1 vs. 23.5 months, HR = 1.09, $P = 0.615$). ORR was 32 and 28 %, respectively. However, the standard schedule was superior in time to deterioration, a composite end point of death, progression, and disease-related symptoms ($P = 0.034$). There was no significant difference in any grade adverse events. As such, the 50 mg (4 weeks on, 2 weeks off) regimen remains the standard practice. A dose of less than 25 mg or exceeding 75 mg is not normally recommended. Nonetheless, maintaining the standard 50 mg dose but in a different schedule might benefit patients who could not tolerate the continuous 4-week treatment. In a small study of 31 patients, sunitinib given on a 2-week-on and 1-week-off regimen appeared well tolerated, with an ORR of 42 % and disease stabilization in 32 % of patients [68]. An earlier pharmacokinetic study with this regimen has shown no significant drug accumulation [69]. It is important to note that with the 4-week-on and 2-week-off schedule, tumors often regrow during the break period, and altering this might prove beneficial. An ongoing phase II study in Korea, which compares the 2/1 regimen with the standard 4/2 regimen, will provide more evidence as to which one of these is more effective.

As with all targeted therapies, sunitinib is normally given until the patient encounters unacceptable toxicities or disease progression. The randomized phase II/III STAR trial aims to compare standard treatment strategy (continuation until disease progression) with temporary cessation of sunitinib at the time of maximal radiological response, in the first-line treatment of advanced RCC [70].

Combination Treatment with Sunitinib

Sunitinib given with cytokine is of no proven role and is limited by frequent toxicities. In a phase I trial of 25 treatment-naïve patients with metastatic clear cell RCC, treatment was started at a standard sunitinib dose of 50 mg and IFN-α at 3 MIU three times weekly, with weekly dose escalation to 9 MIU as tolerated [71]. All patients experienced ≥ grade 3 adverse events, the most common being neutropenia,

thrombocytopenia, and fatigue. Overall, 72 % of patients required a dose reduction due to toxicities, and as a result, the ORR was only 12 % compared to 47 % observed in the phase III trial [54].

In another phase I trial of nine metastatic RCC patients who had not received previous systemic therapy, patients were given the standard dose of sunitinib in combination with an escalating dose of recombinant IL-21 given subcutaneously (planned dose levels were 3, 10, 30, and 100 μg/kg) [72]. Even at a dose of 10 μg/kg, two DLTs occurred in four patients, consisting of grade 3 thrombocytopenia and grade 4 neutropenia. The dose of 3 μg/kg was considered too low to be therapeutically effective and led to the early termination of the trial.

Feldman et al. performed a phase I study with bevacizumab intravenously 10 mg once every 2 weeks, together with escalating doses of sunitinib [73]. The maximum tolerated dose was 50 mg of sunitinib and 10 mg of bevacizumab, but chronic therapy resulted in grades 3–4 hypertension and hematologic and vascular adverse events. Overall, 48 % of patients had to discontinue treatment due to toxicities.

Phase I studies found that the combination of sunitinib and mTOR inhibitors was too toxic for further development. Even a lower starting dose of temsirolimus at 15 mg/week (standard dose is 25 mg) and sunitinib at 25 mg (half the normal dose), DLTs were observed in 2 out of 3 patients [74]. The combination of everolimus with sunitinib was associated with significant acute and chronic grades 3–4 toxicities, even at attenuated doses [75].

Sunitinib as a Second-Line Treatment

Since the publication of the phase III trial results, sunitinib (and pazopanib) has largely replaced IL-2 and IFN-α as the treatment of choice [11, 54]. Therefore its role in the second-line setting is becoming less relevant. In a trial by Motzer et al., 63 patients with metastatic RCC which has progressed after first-line cytokine therapy were recruited in the study [76]. Eighty-seven percentage of them had clear cell histology. The ORR achieved was 40 %, with 27 % of patients had stable disease that lasted ≥3 months. The overall median time to progression was 8.7 months. Following this, another phase II study was reported by the same group, but this was on 106 patients with cytokine-refractory metastatic RCC of clear cell histology only [77]. ORR was 34 % and the median PFS was 8.3 months. The PFS obtained with these two trials appears higher than that of sorafenib in the TARGET trial (5.5 months) [10].

Sequential Therapies with Sorafenib and Sunitinib

In the era when the use of cytokines is superseded by targeted agents, the availability of second-line treatment after progression of first-line targeted therapy is of utmost importance. As mentioned above, the AXIS trial has demonstrated a survival benefit

with axitinib in patients who had previously been treated with VEGF-targeted therapies. In the RECORD-1 (Renal Cell Cancer Treatment with Oral RAD001 Given Daily) trial, 416 patients with metastatic RCC who had progressed on or within 6 months of stopping sunitinib, sorafenib, or both were randomized 2:1 to receive everolimus 10 mg daily or placebo [78, 79]. The results showed a median PFS that was in favor of everolimus (4.9 vs. 1.9 months, $P<0.001$). The ongoing RECORD-3 phase II trial is evaluating the sequential treatment of sunitinib followed by everolimus and vice versa in treatment-naïve patients.

A number of studies have looked at the sequential use of sorafenib and sunitinib on disease progression. Studies with survival data are summarized in Table 8.5. These seem to indicate that the sequence of sorafenib to sunitinib confers a longer survival advantage. A recent meta-analysis incorporating 853 patients demonstrated that the sequential use of sorafenib followed by sunitinib resulted in a median combined PFS of 15.4 months, compared to 12.1 months with sunitinib to sorafenib ($P=0.0013$) [80]. Although there was no significant difference in the PFS from first-line treatment using either of the drugs, sunitinib in the second-line treatment resulted in an average increase in PFS of 2.66 months compared to sorafenib.

It is difficult to make any definite conclusion from these studies, which are disadvantaged by their small, retrospective, and nonrandomized nature. It is hoped that the ongoing SWITCH randomized phase III trial, which aims to compare sorafenib followed by sunitinib versus the opposite sequence in treatment-naïve advanced RCC patients, will shed some light on this issue [81].

Toxicities and Their Management

The prevention, early detection, and active management of treatment-related toxicities are essential to keep patients on the optimum dose of the drug for as long as possible in order to achieve the best therapeutic efficacy. Before commencing on the TKIs, patient's age, risk factors, and comorbidities that could compromise the treatment should be assessed and addressed as early as possible. These can include the treatment of hypertension, cardiological assessment, and stopping/changing drugs with potential interactions. It is important that patients are educated about the potential toxicities and how these could be managed and that they are provided with accessible support such as contact numbers and frequent clinic reviews.

Most treatment-related toxicities can be managed successfully [64]. Symptoms such as fatigue, alopecia, anorexia, nausea, stomatitis, and dermatological toxicities are managed by patient education and prophylactic or early interventions. Avoidance of excessive trauma and application of emollients are effective preventative measures for hand-foot syndrome. Routine monitoring of thyroid function is required, and replacement therapy needs to be started when necessary. Finally, if adverse events become unacceptable despite active management, dose reduction and interruption have to be considered.

Hypertension and cardiotoxicity are known toxicities with all TKIs, including sorafenib and sunitinib [82]. As such, blood pressure is routinely monitored and

Table 8.5 Studies of sequential treatment with sorafenib followed by sunitinib (SoSu) or vice versa (SuSo)

Study	Study type	Treatment	Number of patients	PFS with first treatment (months)	PFS with second treatment (months)	Overall PFS (months)
Eichelberg et al. [115]	Retrospective	SoSu	30	8.7	10.3	19
Tamaskar et al. [116]	Retrospective	SoSu	4	4.4[a]	7.8[a]	12.2[a]
		SuSo	5	8.6[a]	5.9[a]	14.5[a]*
Di Lorenzo et al. [117]	Prospective	SuSo	52	-	Median TTP = 3.7	Median OS = 7.4
Dudek et al. [118]	Retrospective	SoSu	29	Median TTP = 4.8 vs. 5.7	Median TTP = 13.2 vs. 2.8	Median TTP = 18.0 vs. 8.5
		SuSo	20			Median OS = 23.5 vs. 10.4
Sablin et al. [119]	Retrospective	SoSu	68	6.0	6.5	12.5 (median OS = 31.1*)
		SuSo	22	5.1	3.9	9.0 (median OS = 18.9*)
Zimmermann et al. [120]	Prospective	SoSu	22	11.5	5.0	16.5
Porta et al. [121]	Retrospective	SoSu	90	8.4	7.9*	16.3
		SuSo	99	7.8	4.2*	12.0
Richter et al. [122]	Retrospective	SoSu	5	7.9[a]	9.8[a]	17.7[a]
		SuSo	5	8.5[a]	8.9[a]	17.4[a]
Buchler et al. [123]	Retrospective	SoSu	122	–	–	18.8
		SuSo	138			17.7
Calvani et al. [124]	Retrospective	SoSu	15	6.0	11.0*	20.0*
		SuSo	18	7.5	3.0*	10.0*
Ambring et al. [125]	Retrospective	SoSu	43	–	–	~13.0
		SuSo	54			~11.0

Shown are median PFS unless indicated
PFS progression-free survival, *TTP* time to progression, *OS* overall survival
*$P < 0.05$
[a]Mean PFS

treated with antihypertensives when necessary. Echocardiogram and ECG are usually performed in elderly patients, in patients with cardiovascular risk factors such as previous history and diabetes, both before and, if clinically indicated, during treatment. A left ventricular ejection fraction of ≥50 % is normally considered safe to commence treatment, whereas care must be taken when there are significant ischemic changes, arrhythmias, or prolonged QT interval on ECG, and referral to a cardiologist may be indicated. These drugs can also cause QT prolongation and may

Table 8.6 Drugs that affect hepatic CYP3A4

CYP3A4 inhibitors (may increase sorafenib and sunitinib levels)	
Strong inhibitors	Moderate inhibitors
Clarithromycin	Aprepitant
Indinavir	Ciclosporin
Itraconazole	Diltiazem
Ketoconazole	Erythromycin
Nelfinavir	Fluconazole
Ritonavir	Grapefruit juice
Voriconazole	Verapamil
CYP3A4 inducers (may decrease sorafenib and sunitinib levels)	
Carbamazepine	
Dexamethasone	
Efavirenz	
Nevirapine	
Phenytoin	
Prednisolone	
Rifampicin	
St. John's wort	

lead to ventricular arrhythmias including torsades de pointes. Other concomitant drugs that may cause QT prolongation should be used in caution, including ciprofloxacin, clarithromycin, erythromycin, domperidone, ondansetron, and the selective serotonin reuptake inhibitor (SSRI) antidepressants.

As both sorafenib and sunitinib are metabolized by hepatic cytochrome P_{450}3A4 (CYP3A4), other drugs that inhibit this enzyme could increase the TKI plasma levels and, therefore, toxicities and should be avoided (Table 8.6). Potent CYP3A4 inducers, on the other hand, could reduce drug concentrations and potentially treatment efficacy.

Sorafenib

Adverse events published in the three phase III trials (TARGET, AXIS, TIVO-1) are summarized in Table 8.7. The most frequent toxicities associated with sorafenib treatment were diarrhea, hand-foot syndrome, and alopecia. Indeed the frequency of these events were significantly higher than the third-generation TKIs axitinib and tivozanib [28, 29, 83]. Subgroup analysis from the TARGET, EU-ARCCS, and NA-ARCCS trials showed that there was no difference in the type and toxicity rates between older and younger patients [45, 47, 48]. However, older patients tend to have poorer ECOG PS and more comorbidities that might necessitate a lower starting dose.

The recently concluded PREDICT (Patient Characteristics in RCC and Daily Practice Treatment with Sorafenib) observational study was carried out in 18 countries

Table 8.7 Toxicity profiles of sorafenib from phase III trials

	Frequency of toxicity (%) – all grade (grades 3 and 4)		
	TARGET ($n=451$) [10, 44]	AXIS ($n=362$) [29]	TIVO-1 ($n=257$) [28, 83]
Adverse events			
Hypertension	17 (4)	29 (11)	34 (18)
Dysphonia	–	14 (0)	5 (0)
Back pain	–	–	8 (2)
Fatigue	29 (3)	32 (5)	16 (4)
Asthenia	–	–	17 (3)
Mucositis (oral)	5 (0)	12 (1)	9 (1)
Nausea	19 (<1)	22 (1)	7 (<1)
Diarrhea	48 (2)	53 (7)	33 (7)
Anorexia	14 (<1)	29 (4)	9 (1)
Weight loss	8 (1)	21 (1)	21 (4)
Hand-foot syndrome	33 (6)	51 (16)	54 (17)
Rash	41 (1)	32 (4)	–
Alopecia	31 (0)	32 (0)	21 (0)
Dyspnea	14 (4)	–	9 (2)
Hypothyroidism	–	8 (0)	7
Laboratory abnormalities			
Alanine aminotransferase elevation	–	–	34 (4)
Aspartate aminotransferase elevation	–	–	51 (4)
Amylase elevation	–	–	53 (7)
Lipase elevation	–	46 (15)	64 (24)
Hypophosphatemia	–	50 (16)	71 (26)
Proteinuria	–	–	73 (3)
Anemia	–	52 (4)	49 (3)
Neutropenia	–	8 (1)	11 (2)
Thrombocytopenia	–	14 (0)	12 (0)

in Europe, Asia, and South America. The primary end point was tumor status after approximately 3, 6, 9, and 12 months on sorafenib treatment. Other endpoints were treatment duration, safety, PFS, status of metastases, and change in performance status. The final results are still awaited, but the available subgroup analysis data showed that adverse events were more common in patients with comorbidities (hypertension and diabetes—69 and 66.3 % compared to 56.9 % in the total population) and brain metastases (63.6 % compared to 56.9 % in the total population), although in the latter no cerebrovascular events were reported [84, 85]. The median treatment duration of ≥6 months was similar regardless of the presence of comorbidities or site of metastases, although patients with only one disease site were on treatment longer (8.4 months) compared to those with more than one site of metastasis (6.2 months). In other studies in Asia, the incidence of hand-foot syndrome with sorafenib treatment appeared to be more frequent in the Asia-Pacific population

compared to Caucasians [86, 87]. A slightly longer median PFS (7.4–14 months) compared to the TARGET trial has also been observed in Chinese and Japanese patients, although these would require further clarification [88–91].

Sorafenib appears to be safe to use in patients with renal insufficiency. A small retrospective study of 14 patients with mild to moderate renal impairment (creatinine clearance (CrCl) from 32 to 60 mL/min) showed no significant difference in efficacy compared to those with normal renal function, although they appeared to have more frequent diarrhea, hand-foot syndrome, and dose interruption and reduction [92]. Sorafenib has also been used in patients undergoing hemodialysis [93]. Pharmacokinetic data from the use of sorafenib in hepatocellular carcinoma suggested that no dose reduction is required for patients with mild (Child-Pugh A) or moderate (Child-Pugh B) liver impairment [94]. To provide guidance to clinicians treating these group of patients, the Cancer and Leukemia Group B (CALGB) 60301 phase I study looked at the pharmacokinetic and tolerability of sorafenib in patients with hepatic or renal dysfunction [95]. DLTs were defined as significant increase in liver enzymes, reduction in CrCl, grade 3 or worse non-hematological toxicities, or grade 4 neutropenia and thrombocytopenia. Based on the frequency of DLTs, they recommended the following starting doses:

(a) CrCl between 40 and 59 mL/min—400 mg twice daily
(b) CrCl between 20 and 39 mL/min—200 mg twice daily
(c) Hemodialysis—200 mg daily
(d) Bilirubin of > upper limit of normal (ULN) but ≤1.5× ULN and/or aspartate aminotransferase of >ULN—400 mg twice daily
(e) Bilirubin of >1.5× ULN to ≤3× ULN—200 mg twice daily
(f) Bilirubin of >3× ULN—<200 mg every other day
(g) Albumin of <2.5 mg/dL—200 mg daily

Sunitinib

In the phase III trial with IFN-α, the most common all-grade adverse events (reported in >50 % of patients) were diarrhea, fatigue, and nausea (Table 8.8) [54]. Compared to IFN-α, diarrhea, nausea and vomiting, hypertension, hand-foot syndrome, leukopenia, and thrombocytopenia were more common with sunitinib. The incidence of hematological toxicity is clearly higher than that of sorafenib. Compared to the Western world, the Asia-Pacific population appeared to experience higher incidence of grades 3–4 toxicities resulting in more frequent dose reduction [96, 97].

Hutson et al. performed a pooled analysis of sunitinib treatment from six clinical trials ($n = 1{,}059$) [98]. This showed that elderly patients (age ≥70 years) had similar median PFS (11.0 vs. 9.9 months, $P = 0.2629$) and OS (23.6 vs. 25.6 months, $P = 0.5442$) compared to younger patients, although some adverse events were statistically more common in the older age group. These include fatigue (69 vs. 60 %),

Table 8.8 Toxicity profiles of sunitinib from phase III trials

	Frequency of toxicity (%) – all grade (grades 3 and 4)	
	NCT00083889 (Motzer et al. 2007, 2009) [11, 54] ($n=375$)	COMPARZ (Motzer et al. 2012) [56] ($n=548$)
Adverse events		
Hypertension	30 (12)	41 (15)
Dysgeusia	46 (<1)	36 (0)
Fatigue	54 (11)	63 (17)
Asthenia	20 (7)	–
Stomatitis	30 (1)	–
Nausea	52 (5)	46 (2)
Diarrhea	61 (9)	57 (7)
Anorexia	34 (2)	37 (3)
Weight loss	12 (<1)	–
Hand-foot syndrome	29 (9)	50 (11)
Rash	24 (1)	–
Alopecia	12 (0)	–
Hair color changes	20 (0)	10 (<1)
Dyspnea	10 (2)	–
Hypothyroidism	14 (2)	–
Laboratory abnormalities		
Alanine aminotransferase elevation	51 (2)	43 (4)
Aspartate aminotransferase elevation	56 (2)	60 (3)
Bilirubin elevation	20 (1)	27 (2)
Hypoalbuminemia	–	42 (2)
Lipase elevation	56 (18)	–
Creatinine elevation	70 (<1)	46 (<1)
Uric acid elevation	46 (14)	–
Hypophosphatemia	31 (6)	52 (8)
Hyperglycemia	–	57 (4)
Anemia	79 (8)	60 (7)
Lymphopenia	68 (18)	55 (14)
Neutropenia	77 (18)	68 (20)
Thrombocytopenia	68 (9)	34 (16)

decreased appetite (29 vs. 13 %), cough (29 vs. 20 %), peripheral edema (27 vs. 17 %), anemia (25 vs. 18 %), and thrombocytopenia (25 vs. 16 %). Hand-foot syndrome, however, was more common in younger patients (32 vs. 24 %). In practice, due to other comorbidities and ECOG PS, a lower starting dose is often used in older patients.

A phase I trial showed that the pharmacokinetics of sunitinib in subjects with severe renal impairment were similar to those with normal renal function [99]. In a retrospective study of 19 patients (10 were undergoing dialysis, 9 had CrCl of <30 mL/min), sunitinib at doses of 25–50 mg daily for 4 weeks followed by a

2-week break appeared safe and had similar efficacy in those with normal renal function [100]. Pharmacokinetic study also showed that after a dose of 50 mg, there was no difference in drug levels in patients with normal, mild (Child-Pugh A), or moderate (Child-Pugh B) hepatic impairment [101]. In all cases, close monitoring of renal and liver functions are essential.

Dose reduction of sunitinib from its standard regime of 50 mg daily for 4 weeks followed by 2 weeks off is sometimes required. This could be done at a dose level of 37.5 mg and if needed, to 25 mg daily for 4 weeks with a 2-week break [102]. A schedule of 2 weeks treatment followed by 1 week off has also been used—this regimen has recently been studied retrospectively by Najjar et al., where they identified 30 patients who had changed from the standard 4/2 schedule to this 2/1 schedule due to intolerable side effects (97 % of patients had grades 3–4 toxicities) [103]. After switching regimen, only 27 and 0 % experienced grades 3 and 4 toxicities, respectively, with significantly less frequent fatigue and hand-foot syndrome. The median overall treatment duration was 12.6 months (range 1.2 months to 5.1 years) for the 4/2 schedule, whereas this was 11.9 months (range 0.9+ to 73.3+ months) for the 2/1 schedule.

In the COMPARZ trial, sunitinib has a lower incidence of derangement in liver function tests, hair color change, alopecia, and weight loss compared to pazopanib, but has a higher incidence of fatigue, hand-foot syndrome, mucositis, hypothyroidism, and bone marrow suppression [56]. Quality of life assessment suggested that patients experienced more fatigue and mouth/throat/hands/feet soreness with sunitinib [104]. The PISCES (Patient Preference Study of Pazopanib Versus Sunitinib in Advanced or Metastatic Kidney Cancer) trial randomized 169 patients to receive first-line treatment with either of the drugs for 10 weeks followed by a 2-week washout period [105]. This was followed by treatment with the other remaining drug for another 10 weeks. In the primary analysis ($n = 114$), 70 % of patients preferred pazopanib, 22 % preferred sunitinib, and 8 % had no preference. The main reasons for pazopanib were the better quality of life and less fatigue. The tolerability of pazopanib resulted in fewer dose reductions (13 vs. 20 %) and interruptions (6 vs. 12 %) compared to sunitinib while maintaining equivalent efficacy (ORR of 19 vs. 21 %, respectively). The main criticism of these trials is the time at which patient's quality of life (day 28 of each cycle in COMPARZ) and preference (week 22 in PISCES) was assessed. This was unfavorable to sunitinib as it coincided with day 28 of its treatment cycle, when the frequency of toxicities is expected to be the highest [106].

Summary

Sunitinib is currently the standard first-line treatment for patients with metastatic RCC, a role also shared by the second-generation TKI pazopanib. Phase III trials estimated the median PFS with sunitinib treatment to be between 9.5 and 11 months [11, 54, 56].

Studies are currently ongoing to further improve its efficacy, including the use of different dose and schedule regimens and combination or sequential treatments.

TKIs might also have a role in the adjuvant setting, given that a large proportion of patients will relapse after initial curative surgery [107, 108]. At present, adjuvant treatment is not indicated outside the trial setting, as previous studies using immunotherapy, vaccine, chemotherapy, and hormonal therapy have failed to show any clinical benefit [109]. The role of adjuvant therapy after surgical resection of high- or intermediate-risk primary RCC is currently being investigated in a number of trials, including SORCE (sorafenib/placebo), S-TRAC (sunitinib/placebo), and ASSURE (sunitinib/sorafenib).

Combination treatments so far have been disappointing, although current trials are investigating the novel combination of sunitinib with vaccines.

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Chapter 9
Second-Generation Tyrosine Kinase Inhibitors (Pazopanib) in Renal Cell Carcinoma: Current Status

Linda Cerbone and Cora N. Sternberg

Pazopanib: A Second-Generation Tyrosine Kinase Inhibitor

Pazopanib (Votrient®), a synthetic indazolylpyrimidine, is an oral TKI with highly selective activity against VEGFR 1, 2, and 3, PDGFR-α and β, and c-kit and modest activity against FGFR 1, 2, and 3 and c-fms. Differences in the kinase profile of pazopanib and that of sunitinib and sorafenib may partly explain differences in the efficacy and safety profiles of these agents and justify the nomenclature of first- and second-generation TKIs [3]. In a comparative analysis of molecular specificity, pazopanib was less active in blocking Flt-3 receptor than sunitinib or sorafenib which may explain the reduced effects on bone marrow suppression [4]. The differences in selectivity of pazopanib and sunitinib were also observed in a quantitative analysis of kinase inhibitor selectivity. For high-affinity interactions ($K_d < 100$ nM), the selectivity score was higher for pazopanib than for sunitinib. Sunitinib interacted with five times more kinases than pazopanib with a $K_d < 100$ nM [5]. The concentrations required to produce 50 % inhibition (IC50) of human VEGFR-2 kinase activity was 0.03 μM for pazopanib, compared to 0.09 μM of sorafenib and 0.009 of sunitinib [3, 6].

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Current Status in the Treatment of Metastatic Renal Cell Carcinoma

Phase I/II Trials

Pazopanib was investigated in a multicenter, open-label, phase I dose-escalating trial (VEG10003) to determine the maximum tolerated dose (MTD). Sixty-three patients with solid tumors (13 with RCC), refractory to standard therapy, were enrolled and received different schedules and doses of oral pazopanib (50 mg and 100 mg three times weekly; 50–2,000 mg daily; 300 mg and 400 mg twice daily). The MTD was not reached in this trial but pazopanib was well tolerated by the majority of patients. The most common adverse events (AEs) were increase in transaminases (ALT 38 %, AST 24 %), hypertension (33 %), diarrhea (33 %), hair depigmentation (32 %), nausea (32 %), and fatigue (24 %). Pazopanib produced clinical benefit in 17 patients overall, and in four with RCC, two achieved a partial response and two had stable disease for more than 6 months. This trial established an 800 mg daily dose of pazopanib based on the study-state exposure as an increase in dose did not result in additional benefit [7]. Pazopanib should be taken on an empty stomach. Systemic exposure is increased twofold with food. Because it is metabolized by CYP3A4, it should be used with caution with concomitant therapies that inhibit or induce CYP3A4. Pazopanib inhibits UGT1A1, and patients with UGT1A1 polymorphism are susceptible to develop hyperbilirubinemia [8].

A multicenter, phase II trial (VEG102616) with pazopanib enrolled 225 patients with metastatic RCC who were treatment naive (69 %) or who had received one prior line of therapy (31 %). It was initially designed as a randomized discontinuation trial, but due to the safety profile and impressive efficacy results at the 12-week interim analysis (38 % RR), it was amended to an open-label trial (VEG107769) to provide pazopanib for subjects in the placebo arm. The primary endpoint was overall response rate (RR), and secondary endpoints were duration of response and progression-free survival (PFS). RR was 35 % regardless of the status of previous treatment. The median duration of response was 68 weeks. Median PFS was 11.9 months in the pazopanib arm and 6.2 months (p, 0.013) in the placebo arm.

The most frequent all-grade AEs were diarrhea (63 %), hair depigmentation (43 %), hypertension (41 %), nausea (42 %), fatigue (46 %), and AST and ALT elevation (54 %, 53 %). The most common grade 3 or higher AEs were hypertension (8 %), diarrhea (4 %), fatigue (4 %), and AST and ALT increase (3 %, 5 %).

This phase II trial showed interesting data based on biomarker analysis with circulating levels of VEGF, serum VEGFR 1–2 (sVEGFR), and von Hippel-Lindau (VHL) gene variations in tumor tissue. VHL gene variations were observed in 90 % of patients with the following response distribution compared to patients with lack of VHL variation: 30 % PR vs. 38 %, 46 % SD vs. 25 %, and 9 % PD vs. 13 %. A decrease of sVEGFR2 at week 12 was significantly correlated with tumor response (p, 0.00002). No correlations were found between sVEGFR1, VEGF, and patient outcome suggesting that sVEGFR2 could be considered as a surrogate of response [9].

Phase III Trials

Pivotal Trial

In a phase III double-blind, placebo-controlled, randomized trial (VEG105192), 435 patients, with clear-cell advanced RCC, measurable disease, and no prior treatment or one prior cytokine-based treatment, were enrolled and stratified (ratio 2:1) to pazopanib (800 mg daily) or placebo. The primary endpoint was PFS, and secondary endpoints included overall survival, response rate, and tolerability. Fifty-four percent of patients were treatment-naïve and 46 % had received prior cytokine therapy. More than half of the population (54 %) randomized to placebo crossed over to the active treatment arm in a companion trial (NCT00334282) at progression, many as early as 6 weeks. Pazopanib significantly improved PFS compared to placebo (9.2 months vs. 4.2 months, $p<0.0001$; HR 0.46). This benefit was confirmed in both groups and was most evident in treatment-naïve patients. In patients who had not received prior therapy, PFS was 11.1 months vs. 2.8 months, $p<0.0001$, HR 0.40, and in patients who had received prior cytokine therapy, it was 7.4 months vs. 4.2 months, $p<0.001$, HR 0.54. Overall objective responses were 30 % vs. 3 % ($p<0.001$) in the overall population, 32 % vs. 4 % ($p<0.001$) in treatment-naïve patients, and 29 % vs. 3 % ($p<0.001$) in cytokine-pretreated patients. The median duration of response in the pazopanib arm was 58.7 weeks, while median OS in the pazopanib arm was 22.9 months compared with 20.5 months in the placebo group (HR, 0.91; p, 0.224); however these results reflected the high crossover rate [10].

The most common AEs were diarrhea (52 %), hypertension (40 %), hair color changes (38 %), nausea (26 %), anorexia (22 %), and vomiting (21 %). Grade 3/4 adverse reactions were abnormal liver aminotransferases (ALT/AST) (12 %), diarrhea (4 %), and hypertension (4 %). A low incidence of grade 3/4 hematological events was reported [11].

The FDA approved pazopanib for advanced RCC in October 2009 based on the results of this trial.

A retrospective analysis of this trial was conducted to investigate the correlation between plasma concentrations of selected cytokines and angiogenic factors (interleukin 6 and 8, osteopontin, hepatocyte growth factor (HGF), tissue inhibitor of metalloproteinases (TIMP)-1) and clinical outcomes. These markers had a significant prognostic value which may add to the clinical classification normally used. Patients treated with pazopanib and with high concentrations of IL-8, osteopontin, HGF, and TIMP-1 had a shorter PFS. High levels of IL-6 correlated with improvement in PFS [12]. Another interesting retrospective study analyzed correlation between single-nucleotide polymorphisms (SNPs) of angiogenesis-related genes and OS. Xu et al. showed that SNPs in IL-8, FGFR2, and NR1/2 were independent predictors of OS. Patients carrying the wild type of these genes often had a better OS [13]. Prospective trials are needed to validate these findings.

COMPARZ Trial

The COMPARZ trial (NCT00720941) was a non-inferiority phase III randomized study comparing pazopanib vs. sunitinib as first-line treatment in patients with mRCC. The primary endpoint was non-inferiority of PFS, and secondary endpoints were OS, objective RR, safety, and quality of life. Of the 1,110 patients enrolled, 557 were randomized to receive pazopanib (800 mg daily) and 553 to sunitinib (50 mg daily 4/6 weeks). Pazopanib was shown to be non-inferior to sunitinib in terms of efficacy. Results presented in 2012 at the ESMO congress were welcomed as this is the first head-to-head trial comparing two TKIs approved as first-line therapy. Median PFS of pazopanib was 8.4 months vs. 9.5 months for sunitinib (HR, 1.047). Overall response rates were 31 % with pazopanib and 25 %, with sunitinib. Median OS, at the interim analysis, was 28.4 months for patients treated with pazopanib and 29.3 s for sunitinib-treated patients. Tolerability data confirmed the known different toxicities of the two drugs. Most common all-grade AEs reported with pazopanib were increase of transaminases (31 % vs. 18 %) and hair color change (30 % vs. 10 %). Patients treated with sunitinib reported more fatigue (63 % vs. 55 %), hand-foot syndrome (50 % vs. 29 %), taste alteration (36 % vs. 26 %), and thrombocytopenia (34 % vs. 10 %). Quality of life was better with pazopanib confirming its improved toxicity profile [14, 15].

PISCES Trial

The PISCES trial (NCT01064310) was a randomized double-blind, crossover, phase III trial investigating patient preferences between pazopanib and sunitinib. One hundred sixty-nine patients with locally advanced RCC or mRCC, who were not previously treated, were randomized in a 1:1 ratio. The study consisted of two periods of 10 weeks with a 2-week washout period in between. Patients received pazopanib and sunitinib treatment sequentially in a double-blind manner. In case of significant radiologic response (>50 % reduction in tumor size) after the first period, patients had the option to be unblinded and to continue the same treatment without crossover. The primary endpoint was to assess the patient's preference between the first and second drug administered, tolerability, quality of life, and safety of pazopanib as compared to sunitinib. Secondary endpoints were to evaluate the reasons for patient preference and to evaluate fatigue, dose modification, and safety. Results of this trial were reported at the ASCO meeting in 2012. Patients reported that they preferred pazopanib in 70 % of cases and sunitinib in 22 %, and 6 % had no preference (difference 49.3 %, $p<0.001$). The main reasons in favor of pazopanib were better QoL, less fatigue, fewer changes in food taste, less soreness in mouth, less hand-foot syndrome, and less nausea. Physicians also preferred pazopanib in 61 % of cases, while 22 % preferred sunitinib and 17 % had no preference. Dose modifications occurred in 20 % of patients during sunitinib treatment vs. 13 % during pazopanib. Although this trial was not designed to compare efficacy of these TKIs, ORR was 21 % for sunitinib and 19 % for pazopanib. The most common AEs were

Table 9.1 Overview of results of major trials with pazopanib

Trial	No. of patients	Arm(s)	Objective response	Median PFS (months)	Median OS (months)
Phase I (Hurwitz et al. [7])	63	Pazopanib (50 mg 3 times weekly, 100 mg 3 times weekly, 50–2,000 mg daily, 300 mg BID, 400 mg BID)	NR	NR	NR
Phase II (Hutson et al. [9])	225	Pazopanib (800 mg QD) vs. placebo	35 %	11.9 vs. 6.2 (p, 0.013)	NR
Pivotal phase III (Sternberg et al. [10])	435	Pazopanib (800 mg QD) vs. placebo	30 % vs. 3 % ($p<0.001$)	9.2 vs. 4.2 ($p<0.0001$)	22.9 vs. 20.5 (p, 0.224)
COMPARZ phase III (Motzer et al. [12])	1,110	Pazopanib (800 mg QD) vs. sunitinib (50 mg QD 4/6 weeks)	31 % vs. 25 % (p, 0.032)	8.4 vs. 9.5 (HR 1.047)	28.4 vs. 29.3 (p, 0.275)
PISCES phase III non-inferiority trial (Escudier et al. [13])	169	Pazopanib (800 mg QD) → sunitinib (50 mg QD 4/6 weeks) vs. sunitinib → pazopanib	19 % vs. 21 %	NR	NR

NR not reported

diarrhea (42 % vs. 32 %), increase of transaminases (39 % vs. 28 %) with pazopanib and dysgeusia (27 % vs. 16 %), HFS (26 % vs. 16 %), mucositis (22 % vs. 16 %), anemia (25 % vs. 11 %), leukopenia (49 % vs. 15 %), and thrombocytopenia (47 % vs. 15 %) with sunitinib [16].

Results of the trials described above are summarized in Table 9.1 and AEs in Table 9.2.

Current Status in Adjuvant Setting

PROTECT Trial

The PROTECT trial (NCT01235962) is a randomized, double-blind, placebo-controlled phase III study designed to evaluate efficacy and safety of pazopanib as adjuvant therapy in high-risk nonmetastatic patients post nephrectomy. Therapy is randomized 1:1 between oral pazopanib and placebo for 1 year. The planned primary endpoint is disease-free survival (DFS), and secondary endpoints include safety and quality of life. Translational research on tumor tissue and plasma samples will investigate potential molecular mechanisms of response or resistance to pazopanib. Approximately 1,500 patients with localized or locally advanced RCC after nephrectomy will be randomized. The starting dose of pazopanib has been

Table 9.2 Overview of tolerability of pazopanib in clinical trials

	Phase I	Phase II	Pivotal phase III		COMPARZ		PISCES	
Toxicity (all grades %)	Pazopanib	Pazopanib	Pazopanib	Placebo	Pazopanib	Sunitinib	Pazopanib	Sunitinib
Diarrhea	33	63	52	9	63	57	42	32
Fatigue	24	46	19	8	55	63	29	30
Nausea	32	42	26	9	45	46	33	30
Stomatitis	NR	NR	NR	NR	NR	NR	16	22
Vomiting	17	20	21	8	NR	NR	14	16
Hypertension	33	41	40	10	46	41	23	26
Hand-foot syndrome	NR	11	NR	NR	29	50	16	26
Hair discoloration	33	43	38	3	30	10	17	14
Hypothyroidism	NR	NR	4	NR	NR	NR	NR	NR
Leukopenia	35	35	37	6	43	78	15	49
Anemia	16	26	NR	NR	31	60	11	25
Thrombocytopenia	30	26	32	5	10	34	15	47
Increase AST	24	54	53	19	31	18	39	28
Increase ALT	38	53	53	22	31	18	39	28
Increase bilirubin	13	28	36	10	36	27	24	12

NR not reported

reduced to 600 mg from 800 mg daily due to AEs reported in the first patients enrolled. Physicians are allowed to increase the dose to the standard 800 mg daily after 8–12 weeks of treatment based on tolerability [17]. The precise reason of the high levels of AEs is as yet unclear but may be related to a higher VEGF inhibition of normal vessels due to the absence of tumor VEGF or could be related to lower tolerability in the adjuvant setting in patients "cured" by surgery. Similar reductions have been required in all adjuvant therapy TKI trials. This trial is still recruiting.

Ongoing Clinical Trials

The efficacy and safety of pazopanib in combination with immunotherapy is currently being studied in the neoadjuvant setting in poor-risk patients and in sequential trials with mTOR inhibitors. These trials are summarized in Table 9.3.

Table 9.3 Ongoing clinical trials with pazopanib in renal cell carcinoma [17]

Trial	Arm (s)	Primary and secondary endpoints
Pazopanib before surgery in treating patients with kidney cancer Phase II (NCT01158521)	Pazopanib 800 mg QD for 18 weeks	Rate of partial nephrectomy in pts otherwise requiring radical nephrectomy, tumor shrinkage, surgical morbidity
Neoadjuvant pazopanib in renal cell carcinoma Phase II (NCT01361113)	Pazopanib 800 mg QD for 12 weeks	RR RFS, predictive molecular biomarkers, surgical morbidity
First-line pazopanib in poor-risk patients with metastatic renal cell carcinoma Phase IV (NCT01521715)	Pazopanib 800 mg QD	Rate of poor-risk pts free of disease progression after 6 months of pazopanib PFS, ORR, safety, and correlation between biomarker and outcome
Pazopanib vs. temsirolimus in poor-risk clear-cell renal cell carcinoma Phase II (NCT01392183)	Pazopanib 800 mg QD vs. temsirolimus 25 mg every week (ev)	PFS
Trebananib with or without pazopanib, bevacizumab, sorafenib, or sunitinib in treating patients with advanced kidney cancer Phase II (NCT01664182)	Trebananib (AMG386) ± pazopanib, sunitinib, sorafenib, or bevacizumab	ORR, PFS, safety

(continued)

Table 9.3 (continued)

Trial	Arm (s)	Primary and secondary endpoints
Nivolumab in combination with pazopanib, sunitinib, or ipilimumab in subjects with metastatic renal cell carcinoma Phase I (NCT01472081)	Nivolumab (BMS936558) + pazopanib or sunitinib or ipilimumab	Safety and tolerability Antitumor activity
Pazopanib in combination with interferon alfa 2-A, in patients with advanced renal cell carcinoma Phase I/II (NCT01513187)	Pazopanib + INFa2	MTD, RR, efficacy PFS, OS, toxicity
START: sequential two-agent assessment in renal cell carcinoma therapy Phase II (NCT01217931)	Six different 2-drug "sequences" of everolimus, bevacizumab, or pazopanib	Time to overall treatment failure
SWITCH II: phase III sequential open-label study to evaluate the efficacy and safety of sorafenib followed by pazopanib vs. pazopanib followed by sorafenib in the treatment of advanced/metastatic renal cell carcinoma Phase III (NCT01613846)	Sorafenib → pazopanib vs. pazopanib → sorafenib	PFS TTP, OS, ORR
ROPETAR: rotating pazopanib and everolimus to avoid resistance Phase II (NCT01408004)	Bimonthly rotation of pazopanib and everolimus	PFS Time to second progression, change in QoL, OS

Two phase II trials are investigating pazopanib before surgery. NCT01158521 is evaluating the rate of partial nephrectomy in patients otherwise requiring radical nephrectomy after 18 weeks of pazopanib at standard dose. In trial NCT01361113, pazopanib will be administered for 12 weeks before surgery. The primary endpoint is response rate, and secondary endpoints are recurrence-free survival (RFS), predictive molecular markers, and evaluation of an altered surgical approach.

In poor-risk patients, pazopanib has been evaluated in a phase IV trial (NCT01521715) as first-line monotherapy for mRCC and in a randomized phase II trial (NCT01392183) vs. temsirolimus. PFS, ORR, safety, and correlation between biomarker and clinical outcome are the endpoints of both trials.

Pazopanib, in combination with immunotherapy and other investigational drugs, is being studied in phase I/II trials. Trebananib (AMG 386) is an investigational drug that is being combined in a phase II randomized trial (NCT01664182) with or without bevacizumab, sorafenib, sunitinib, or pazopanib. The primary endpoint is ORR. The secondary endpoints are PFS and safety. Nivolumab (BMS-936558) is a novel promising immunotherapy with established antitumor activity in RCC. A phase I (NCT01472081) trial is investigating safety and tolerability in combination

with sunitinib, pazopanib, or ipilimumab in mRCC. Efficacy, MTD, and RR are the primary endpoints of a phase I/II trial (NCT01513187) investigating pazopanib in combination with INFα2 in advanced RCC.

One of the important challenges in the management of mRCC with the approval of many new agents is to find the optimal sequence and to avoid resistance to therapy. The START trial (NCT01217931) is a phase II randomized study comparing six 2-drug sequences of pazopanib, everolimus, and bevacizumab. The SWITCH II trial (NCT01613846) is an open-label, randomized, phase III study comparing sorafenib followed by pazopanib vs. pazopanib followed by sorafenib. The primary endpoint is PFS. Secondary endpoints are TTP, OS, and ORR. Another phase II single-arm, open-label trial (NCT01566747) will evaluate PFS with pazopanib as second-line therapy in mRCC after failure of sunitinib, everolimus, or temsirolimus. The ROPETAR trial (NCT01408004) is a phase II study investigating whether alternating treatment between pazopanib and everolimus postpones or prevents drug resistance in patients with renal cancer. The primary outcome measure is PFS with time to second progression as a secondary endpoint [17].

Conclusions

Pazopanib is a novel second-generation TKI that has been shown to be effective in patients with advanced RCC. Data from the pivotal phase III trial showed a significant improvement in PFS. OS was confounded by frequent and prolonged crossover from placebo to pazopanib [10]. Safety and improved quality of life with this drug has been extensively investigated. Its favorable toxicity profile with non-inferior efficacy compared to first-generation TKIs has been recently confirmed in two phase III trials (COMPARZ and PISCES). Pazopanib is currently under investigation in the neoadjuvant and adjuvant settings, in combination with investigational drugs and as sequential therapy with other approved TKIs and mTOR inhibitors. Results of these trials will provide much needed information on the therapeutic use of pazopanib in RCC.

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Chapter 10
Third-Generation TKIs (Axitinib, Tivozanib) in RCC: Enhanced Efficacy and Diminished Toxicity?

Hui Zhu and Brian I. Rini

Introduction

Sorafenib (Nexavar®), an oral small molecule inhibitor of Raf kinase and vascular endothelial growth factor receptor (VEGFR) tyrosine kinases, was approved in late 2005 by the FDA for treating advanced renal cell carcinoma (RCC), and since then anti-VEGF-targeted therapy has become the standard care for patients with metastatic or unresectable localized kidney cancer. VEGFR signaling pathway plays an essential role in regulating all three key tumor processes: growth, vascular angiogenesis, and metastasis [1]. VEGFR-1 is involved in angiogenesis and tumor growth; VEGF-2 is involved in endothelial cell proliferation, migration and survival, and angiogenesis; and VEGF-3 is involved in lymphagiogenesis [2, 3].

The first generation (sorafenib and sunitinib) and second generation (pazopanib) of VEGFR tyrosine kinase inhibitors (TKIs) are multi-kinase inhibitors: Sorafenib targets the intracellular kinase BRAF (wide type and mutant) and CRAF, as well as cell surface kinase VEGFR-1, VEGFR-2, VEGF-3, platelet-derived growth factor receptor beta (PDGFR-β), epidermal growth factor receptor (EGFR), Fms-like tyrosine kinase-3 receptor (FLT3), and c-KIT [4]; sunitinib inhibits VEGFR-1, VEGFR-2, and VEGFR-3, PDGFR-α and PDGFR-β, FLT-3, c-KIT and fibroblast growth factor receptor 1 (FGFR1) [5]; while pazopanib targets VEGFR-1,

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Table 10.1 Target specificity and affinity of various VEGF TKIs in proliferation cell-based assays (IC_{50}) [4, 6, 7, 25, 35–38]

IC_{50} (nM)	Sorafenib	Sunitinib	Pazopanib	Axitinib	Tivozanib
VEGFR-1	NR	2	10	0.1	0.2
VEGFR-2	90	10	30	0.2	0.2
VEGFR-3	20	17	47	0.1–0.3	0.2
PDGFR-β	57	8	84	1.6	1.7
Raf-1	6	NR	NR	NR	NR
c-KIT	68	10	74	1.7	1.6
FLT-3	58	14	NR	>1,000	422
FGFR1	580	880	14	231	299
EGFR	58	880	NR	NR	NR

NR not reported

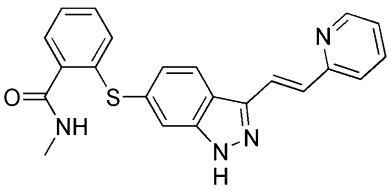

Fig. 10.1 Chemical structure of axitinib

VEGFR-2, and VEGFR-3, PDGFR-α and PDGFR-β, and c-KIT [6] (see Table 10.1). Theoretically, the nonspecific TKIs can be highly effective in treating cancer by targeting multiple oncogenic pathways. On the other hand, their clinical use can also be limited secondary to their broad range of toxicities. The lack of specificity for VEGFRs is manifested in the occurrence of several toxicities that are seemingly unrelated to blockage of the VEGF signaling pathway of multi-targeted TKIs including fatigue, diarrhea, and hand-foot syndrome. The newly developed third-generation VEGFR TKIs (axitinib and tivozanib) have demonstrated high potency and selectivity against VEGFR-1, VEGFR-2, and VEGFR-3 both in vitro and in vivo. Clinically, axitinib and tivozanib have significant antitumor activity and a favorable toxicity profile, making them the potential drug of choice for both treatment-naïve patients and patients who developed resistance to other VEGFR TKI agents.

Axitinib (AG-013736, INLYTA®, Pfizer Inc., New York, USA)

Molecular Structure and Targets

Axitinib is a small molecule TKI with the chemical formula $C_{22}H_{18}N_4OS$ and a molecular weight of 386.47 Da. It is a substituted indazole derivative chemically known as *N*-methyl-2-[3-((*E*)-2-pyridin-2-yl-vinyl)-1*H*-indazol-6-ylsulfanyl]-benzamide (see Fig. 10.1).

Axitinib inhibits VEGFR-1, VEGF-2, and VEGFR-3 tyrosine kinases by competitively binding to the intracellular ATP-binding domain. The structure-based drug design of axitinib allows tight fit of axitinib into the kinase domain of VEGFRs resulting high potency and selectivity [7]. In vitro, axitinib inhibits VEGFR-1, VEGFR-2, and VEGFR-3 autophosphorylation at picomolar concentrations. It inhibits PDGFRs and c-KIT at roughly tenfold higher concentration in proliferation cell-based assays. Flt-3, FGFR-1, and EGFR are not substantially inhibited by axitinib (see Table 10.1).

Preclinical Antitumor Activity

In vitro, axitinib dose-dependently inhibits endothelial cell proliferation, survival, and three-dimensional tube formation. In vivo, axitinib markedly reduced tumor vascular angiogenesis and the growth of human colorectal cancer, renal cell carcinoma, and melanoma in xenograft mouse models [7]. Its antitumor effect was associated with a significant decrease in microvessel density and increased tumor necrosis.

Pharmacokinetics, Pharmacodynamics, and Safety of Axitinib: A Phase I Study

The pharmacokinetics and safety of axitinib was investigated in a phase I multicenter clinical trial of 36 patients with various refractory tumors, including six patients with advanced RCC [8]. The dose escalation study dosed patients from 5 mg to 30 mg on a twice-daily schedule. Dose-limiting toxicities (DLTs) were realized at dose of 10 mg twice daily or higher. Fatal hemoptysis was observed in two patients with non-small cell adenocarcinoma of the lung who initially received 20 mg twice daily and subsequently reduced to 10 mg twice daily. Other dose-limiting toxicities included hypertension, stomatitis, and diarrhea, which were observed primarily at higher dose levels. The common toxicities of axitinib were similar to other antiangiogenic agents, including hypertension, fatigue, nausea, diarrhea, stomatitis, asymptomatic proteinuria, and transaminase elevation. Dose-dependent hypertension, as the leading common side effect, can be most often controlled with standard antihypertensive agents at 5 mg twice-daily dose range. Therefore, 5 mg twice daily was selected as maximum tolerated dose (MTD).

Axitinib is rapidly absorbed after oral administration, with peak plasma concentrations occurring at 1–2 h after dosing in a fasted state and 2–6 h after dosing in a fed state [8]. Its plasma concentrations decline with a terminal plasma half-life between 2 and 5 h. A steady-state plasma concentration was reached in 15 days of continuous dosing without unexpected accumulation. Food affected the bioavailability of axitinib: Dosing axitinib in a fasted state increased the plasma exposure by a median of 49 %.

There is no appreciable difference in plasma half-life in fed and fasted states. The presence of the potent proton-pump inhibitor (PPI) rabeprazole decreased the rate of absorption without affecting the extent of absorption. Therefore, the dose of 5 mg twice daily in the fasted state, given continuously for a 28-day cycle was recommended for further clinical development of axitinib. The major rout of elimination of axitinib was through systemic metabolism, with less than 1 % of the drug eliminated in the urine at its original form. It primarily undergoes hepatic metabolism via cytochrome P450 (CYP) 3A4 isozyme. Ketoconazole, a CYP3A inhibitor, significantly increased the peak plasma concentration (C_{max}) and the area under the concentration-time curve from 0 to 24 h ($AUC_{0\text{-}24}$) [8, 9]; on the other hand, reduced C_{max} and $AUC_{0\text{-}24}$ was observed in patients taking CYP 450 inducers, such as rifampin or phenytoin [8, 10].

Three out of the 36 patients enrolled in the phase I study achieved a confirmed Response Evaluation Criteria in Solid Tumors (RECIST)-defined partial response (PR) to axitinib. Two of them had advanced RCC, and one had adenoid cystic carcinoma. Three additional patients (including one with RCC) had decreases in tumor burden that did not meet the RECIST criteria for response. Additionally, this trial assessed changes in tumor blood flow associated with axitinib by dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) [11]. Reliable data were achieved from 17 predetermined patients at baseline and treatment day 2, in which a rapid decrease in tumor vascular parameters was observed on day 2 after axitinib administration. The study also revealed a linear correlation between DCE-MRI variables and axitinib drug levels, indicating a greater effect on tumor vasculature with increasing axitinib drug exposure.

Clinical Efficacy of Axitinib in Advanced RCC: Single-Arm Phase II Studies

Axitinib was studied in three phase II, single-arm, multicenter, clinical trials to investigate its efficacy and safety as second-line treatment for advanced RCC. Two of them were conducted in the United States [12, 13], and the third one was conducted in Japan [14]. The two US-based phase II trials will be discussed in this chapter, and a direct comparison of the efficacy of the two trials is summarized in Table 10.2.

Efficacy in Cytokine-Refractory RCC

The first phase II study of axitinib in advanced RCC was carried out in patients who were refractory to cytokine treatment but anti-VEGF therapy naïve [12]. 52 patients were enrolled and eligible for evaluation. All but two of them had prior nephrectomy, and all were clear cell type except one. In this study, axitinib was given as 5 mg twice daily in a fasted state in 28-day treatment cycles until RECIST-defined disease progression or unacceptable toxicity. The primary end point of the study

Table 10.2 Efficacy of axitinib in phase II studies of advanced RCC: cytokine refractory vs. sorafenib refractory [12, 13, 24]

Key enrollment criteria	Cytokine refractory	Sorafenib refractory (prior cytokine and sunitinib exposure allowed)
Number of patients	52	62
Dose	5 mg twice-daily fixed dose	5 mg twice daily then modify to 2–10 mg twice daily
ORR	44.2 %	22.6 %
CR	4 %	NR
PR	40 %	22.6 %
SD	42 %	17.7 %
Median response duration (months)	23	17.5
PFS (months)	15.7[a]	7.4
OS (months)	29.9	13.6

[a]The result is TTP instead of PFS

was objective response rate (ORR). Two complete responses (CRs) and 21 partial responses (PRs) were observed, which gave an ORR of 44.2 % (95 % CI 30.5–58.7) and median response duration of 23.0 months (95 % CI 20.9–not estimable). Additionally 22 patients had a best response of stable disease (SD). Median time to progression (TTP) was 15.7 months (95 % CI, 8.4–23.3) and median overall survival (OS) was 29.9 months (95 % CI, 20.3–not estimable). A subset of the study patients ($n = 13$) underwent CT perfusion imaging at baseline and after 2 months of therapy to assess tumor blood flow and revealed a correlation between clinical response to axitinib and reduction of tumor blood by axitinib [15].

Efficacy in Sorafenib-Refractory RCC

The efficacy in patients who developed resistance to first-line VEGFR TKIs was evaluated in a phase II study of axitinib in sorafenib-refractory metastatic RCC patients [13]. The primary end point was ORR as well. A total of 62 patients who had prior failed treatment with sorafenib were enrolled in this study; 25.8 % had received two or more prior systemic treatments (cytokine and sunitinib). All of the patients had prior nephrectomy; all except three had clear cell type. Axitinib was started at 5 mg twice daily with food and then increased in a step-wise fashion to 7 mg twice daily then 10 mg twice daily if lack of severe drug-induced toxicity. Axitinib dose was allowed to decrease to 3 mg twice daily then to 2 mg twice daily in patients who experienced severe to serious adverse events (AEs). Overall 53.2 % of patients were dose escalated to greater than 5 mg twice daily, and 17.7 % of patients required dose modification to less than 5 mg. A total of 14 patients achieved a PR, with ORR of 22.6 % (95 % CI 12.9–35.0 %), and median duration of response

was 17.5 months (95 % CI 7.4–not estimable). Median PFS and OS were 7.4 months (95 % CI, 6.7–11.0) and 13.6 months (95 % CI, 8.4–18.8), respectively. This study revealed significant activity of axitinib in patients with prior VEGFR TKIs exposure, which supported the presence of incomplete cross resistance among agents that target the VEGF pathway [16, 17].

Safety of Axitinib in Phase II Studies

Axitinib was well tolerated in both phase II studies, with most side effects being grade 1 or 2, including fatigue, hypertension, diarrhea, nausea, dysphonia, and hand-foot syndrome (HFS). Grade 3 or 4 events included hypertension (15 % and 16 %, respectively), fatigue (8 % and16%, respectively), diarrhea (10 % and 15 %, respectively), and HFS (0 % and 16 %, respectively). The HFS was more common in the sorafenib-refractory study that was taking escalated dose of axitinib. No severe myelosuppression observed in either study. Most AEs were manageable by dose reductions or interruptions and by standard medical intervention.

Comparative Efficacy of Axitinib Versus Sorafenib as Second-Line Therapy in Advanced RCC: A Phase III Study (AXIS Trial)

AXIS trial was the first randomized phase III trial that directly compared two VEGFR targeted therapies in metastatic RCC patients. 723 patients with advanced RCC with one prior systemic therapy failure were randomly assigned to axitinib (5 mg twice daily) or sorafenib (400 mg twice daily). Axitinib dose increases to 7 mg and then to 10 mg twice daily were allowed for those patients without hypertension or other severe AEs. All these patients had received one prior systemic treatment, including sunitinib (54 % of patients), cytokines (35 %), bevacizumab (8 %), and temsirolimus (3 %). The primary end point of the study was independently assessed progression-free survival (PFS). Median PFS was 6.7 months (95 % CI, 6.3–8.6 months; $n=361$) for axitinib and 4.7 months (95 % CI, 4.6–5.6 months; $n=362$) for sorafenib, with HR of 0.665 (95 % CI, 0.544–0.812, $p<0.0001$) [18]. The preplanned subgroup analyses showed a significant superiority of axitinib over sorafenib in both sunitinib-refractory and cytokine-refractory subgroups. In patients with prior exposure to cytokine, median PFS was 12.1 months for axitinib group and 6.5 months for sorafenib, HR was 0.464 (95 % CI 0.318–0.676, $p<0.0001$); in patients previously treated with sunitinib, median PFS was 4.8 months for axitinib and 3.4 months for sorafenib, HR was 0.741 (95 % CI 0.573–0.968, $p=0.0107$) (see Fig. 10.2). The shorter median PFS observed in both treatment arms in sunitinib-refractory patients relative to those who received cytokines is suggestive of at least partial cross-resistance with sequential VEGF-targeted therapy. The ORR assessed by masked independent radiology review committee was 19 % for axitinib

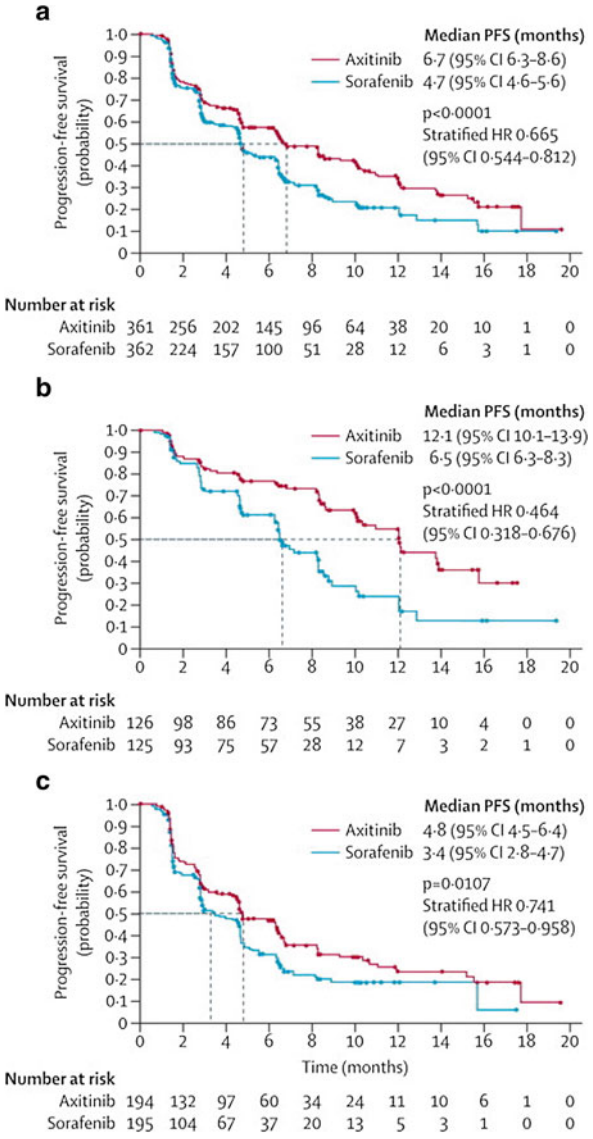

Fig. 10.2 Kaplan-Meier estimated median PFS in patients who received axitinib or sorafenib as second-line therapy for metastatic renal cell carcinoma in AXIS trial [18]: (**a**) all patients, (**b**) cytokine-refractory patients, and (**c**) sunitinib-refractory patients

and 9 % for sorafenib ($p = 0.0001$), with a median duration of response of 11 months (95 % CI 7.4–not estimable) for axitinib and 10.6 (8.8–11.5) for sorafenib.

Axitinib displayed a similar, yet distinct safety profile to sorafenib. Axitinib-treated patients more commonly reported hypertension, dysphonia, and hypothyroidism, but lower incidence of anemia, hand-foot syndrome, rash, and alopecia, which were characteristic toxicity for multi-targeted TKIs (see Table 10.3). This discrepancy could potentially attribute to the more specific VEGFR inhibition.

Table 10.3 Summary of common treatment-emergent AEs and laboratory abnormalities in AXIS trial: axitinib vs. sorafenib [18]

More prevalent in axitinib	Axitinib (all grades/≥grade 3)	Sorafenib (all grades/≥grade 3)
Hypertension	40 %/16 %	29 %/11 %
Dysphonia	31 %/0	14 %/0
Hypothyroidism	19 %/<1 %	8 %/0
More prevalent in sorafenib		
Anemia	35 %/<1 %	52 %/4 %
HFS	27 %/5 %	51 %/16 %
Rash	13 %/<1 %	32 %/14 %
Alopecia	4 %/0	32 %/0
Other common AEs		
Diarrhea	55 %/11 %	53 %/7 %
Fatigue	39 %/11 %	32 %/5 %
Anorexia	34 %/5 %	29 %/4 %
Thrombocytopenia	15 %/<1 %	14 %/NA

NA not available

Association of Hypertension and Clinical Outcomes with Axitinib

The occurrence of elevated blood pressure (BP) is one of the most frequently reported AEs associated with VEGF-targeted agents including axitinib [8, 12, 13, 17, 18]. The incidence of hypertension observed in clinical trials appears to correlate with the potency against VEGFR-2 [19]. This correlation was supported by the recent finding that inhibition of VEGF signaling pathway would lead to increased vascular resistance by reduced NO and prostacyclin production, as well as decreased number of small arteries and arterioles [19]. Therefore, it was hypothesized that VEGFR TKI-induced BP elevation may be an indicator of drug activity at VEGF receptor, serving as a surrogate of VEGF pathway inhibition.

A retrospective meta-analysis was carried out to evaluate if axitinib-induced BP elevation is a marker of efficacy [20]. The study included 230 patients with normal pretreatment BP (allow antihypertensive medication) from five phase II single-agent axitinib trials of four different tumor types, including two trials of advanced RCC [12, 13, 21–23]. Axitinib dose escalation in the absence of elevated BP (defined as BP ≥ 150/90 mmHg) or other treatment-related AEs was allowed. At least one in-clinic measurement of diastolic BP (dBP) ≥90 mmHg was used to stratify patients into dBP ≥90 group ($n = 130$) and dBP <90 group ($n = 100$). Median age and gender were similar between the two groups. The dBP ≥90 group had significantly higher baseline dBP than dBP <90 group. In pooled analyses of OS ($n = 140$), there was a significantly lower relative risk of death for patients in the dBP ≥90 group compared to the dBP <90 group, with median OS of 25.8 months and 14.9 months, respectively, and hazard ratio (HR) of 0.55 (95 % CI, 0.39–0.77, $p < 0.001$). Longer median PFS (10.2 months vs. 7.1 months) and ORR (43.9 % vs. 12.0 %, $p < 0.001$) were observed in the dBP ≥90 group as well (see Table 10.4).

Table 10.4 Median OS and PFS estimated from landmark analysis and ORRs in the dBP < 90 and ≥ 90 mmHg groups [20]

Study tumor type	*n*	dBP (mmHg) group	Median OS (months)	Median PFS (months)	ORR (%)
Melanoma	29	<90	4.6	2.3	20.0
		≥90	8.7	5.9	21.4
mRCC (sorafenib refractory)	61	<90	11.6	5.8	9.7
		≥90	14.7	5.7	36.7
NSCLC	30	<90	12.8	5.0	5.9
		≥90	NR	2.2	15.4
mRCC (cytokine refractory)	51	<90	18.4	7.9	10.5
		≥90	28.1	21.4	65.6
Thyroid cancer	59	<90	NR	14.5	16.7
		≥90	29.2	13.1	48.8
Pooled analysis	230	<90	14.9	7.1	12.0
		≥90	25.8	10.2	43.9
			HR 0.55 (95 % CI, 0.39–0.77, $p < 0.001$)	HR 0.76 (95 % CI, 0.54–1.06, $p = 0.107$)	$p < 0.001$

NR not reached

Multivariate analysis revealed that dBP was an independent predictor of OS, with an HR of 0.676 (95 % CI 0.470–0.972, $p = 0.036$). This study supported further evaluation of dBP as a biomarker of clinical outcome in patients receiving axitinib.

Frontline Axitinib with or Without Dose Titration: An Ongoing Phase II RCT

Based on the above findings, a prospective study was initiated to evaluate the correlation of treatment-induced hypertension, drug pharmacokinetics, and clinical outcomes in metastatic RCC patients treated with first-line axitinib (AGILE 1046). This phase II double-blind, placebo-controlled, randomized trial (RCT) was designed to evaluate the impact of axitinib dose escalation to outcome in patients who are able to tolerate standard dose of axitinib without developing treatment-related hypertension (see Fig. 10.3). A total of 213 patients were enrolled and 203 patients continued after the initial 4-week lead-in period. Out of these patients, 91 patients who did not meet the randomization criteria continued at 5 mg twice daily or a lower dose as tolerated (Arm C). 112 patients met the randomization criteria (and thus potentially inadequately dose at 5 mg BID). They were randomized at 1:1 ratio to active titration arm (arm A) and placebo-titration (control) arm (arm B). Patients on the active titration arm received standard dose of axitinib (5 mg twice daily) plus axitinib dose titration up to 10 mg axitinib twice daily total. Patients on control arm received standard dose of axitinib (5 mg twice daily) plus up placebo. A subset of patients had pharmacokinetic studies on day 15 of their cycle 1 (lead-in period).

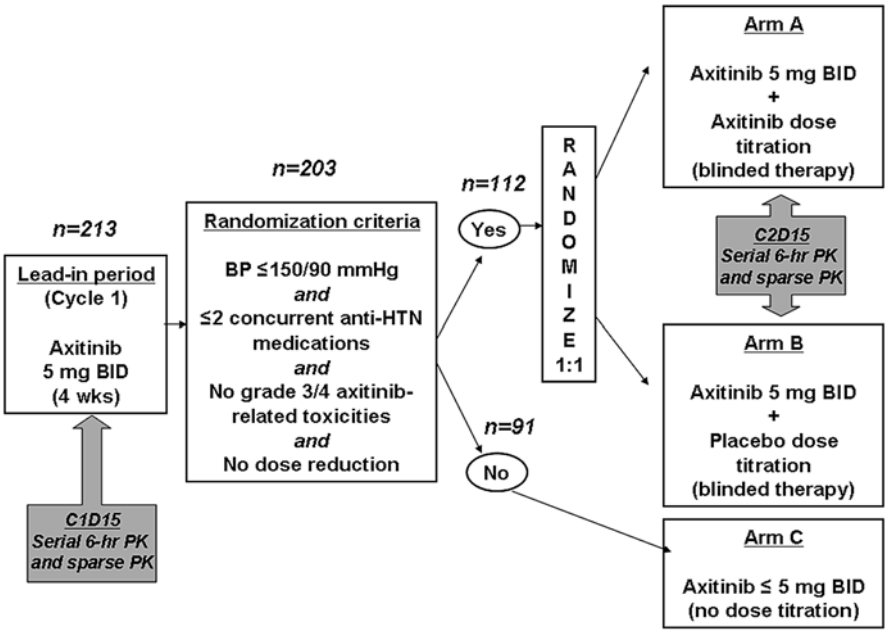

Fig. 10.3 Trial design of AGILE 1046: A randomized, double-blind, phase 2 study is investigating axitinib with or without dose titration in patients with metastatic renal cell carcinoma

Patients on arm A and B will have an additional pharmacokinetic study on day 15 after dose escalation (cycle 2).

The preliminary pharmacokinetic study result indicated that arm assignment reflected serum level of drug: The average AUC_{12} on C1D15 was 234 ng·h/ml in arm C compared to 99 ng·h/ml in arm A + B. This is expected given that patients without hypertension or other toxicity are, on average, under dosed and may require dose escalation for clinical efficacy. As of April 30, 2012, the preliminary clinical data revealed a trend of improved clinical outcome in patients with treatment-induced hypertension [24]: The median ORR of arm C (likely adequate dosing) was 59 %, which was superior to 43 % achieved by arm A + B (inadequate dosing), with median PFS of 16.4 months in arm C and 14.5 months in arm A + B. The leading total adverse events and serious adverse events were hypertension (63 %), followed by diarrhea (58 %), and fatigue (48 %).

Frontline Axitinib for Advanced RCC: An Ongoing Phase III Trial

A phase III study to demonstrate the superiority of axitinib over sorafenib in delaying tumor progression as first-line treatment for in patients with metastatic RCC (AGILE1051) finished data collection. This multicenter, randomized, open-label

study was designed to recruit 492 metastatic RCC patients with no prior systemic therapy or one systemic therapy of either sunitinib or cytokines. The active comparator is sorafenib. Preliminary data was announced by Pfizer Inc. in October of 2012 that in the 280 patients who are treatment-naïve, axitinib failed to show a statistically significant improvement in median PFS. The final data are being evaluated in specific subpopulation to determine the value of axitinib as frontline therapy for advanced RCC.

Other Ongoing Clinical Trial of Axitinib Monotherapy in RCC

Several ongoing trials are evaluating the efficacy of axitinib in treating RCC at different clinical settings, including neoadjuvant therapy (NCT01263769), adjuvant therapy (NCT01599754), and treating recurrent RCC therapy after adjuvant sunitinib or sorafenib (NEXT trial, NCT01649180).

Tivozanib (AV-951, AVEO Pharmaceuticals, Inc., Cambridge, Massachusetts, USA; KRN-951, Kyowa Hakko Kirin Co Ltd., Tokyo, Japan)

Molecular Structure and Target

Tivozanib is quinolone-urea-derived compound chemically known as 1-{2-Chloro-4-[(6, 7-dimethoxyquinolin-4-yl)oxy]phenyl-3-(5-methylisoxazol-3-yl)}urea (see Fig. 10.3). It is a small molecule TKI with the chemical formula $C_{22}H_{19}ClN_4O_5$ and a molecular weight of 454.86 Da.

As a potent and selective VEGFR TKI, tivozanib can inhibit the ligand-induced phosphorylation of VEGFR-1, VEGFR-2, and VEGFR-3 at picomolar concentrations and inhibits angiogenesis and vascular permeability in tumor tissue [25]. Preclinical testing showed the highest potency for VEGFR-2 (IC_{50} = 0.16 nmol/L), followed by VEGFR-1 and VEGFR-3 (IC_{50} = 0.21 nmol/L and 0.24 nmol/L, respectively) in proliferation cell-based assay. Inhibition of c-KIT and PDGFR-β was about an order of magnitude less potent (1.63 and 1.72 nmol/L, respectively). Its activity panel is comparable to axitinib regarding potency and selectivity (see Table 10.1).

Preclinical Activity

Tivozanib has shown antitumor effects in the human breast, colon, liver, lung, ovary, pancreas, prostate, brain, and RCC xenograft rat models [25, 26]. In this setting, daily oral dosing of tivozanib significantly inhibited growth of all tumors, whereas

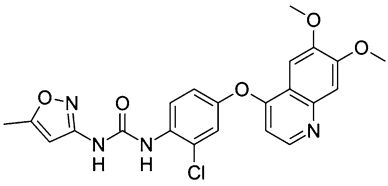

Fig. 10.4 Figure 10.3: Chemical structure of tivozanib

tumor regression was observed in the human breast and colon adenocarcinoma cell xenograft rat models. DCE-MRI analysis detected a significant change in tumor angiogenesis and vascular permeability after 3 days of treatment. The minimum effective blood level of tivozanib was approximately 70 ng/ml (140 nmol/L). This estimate is much higher than the in vitro concentrations required to inhibit VEGF signaling and is likely due to plasma protein binding of tivozanib (Fig. 10.4).

Pharmacokinetics and Safety of Tivozanib: A Phase I Study

A phase I study tivozanib in 41 patients, including nine with metastatic RCC was conducted [27]. Tivozanib dose levels of 2 mg ($n=7$), 1 mg ($n=18$), and 1.5 mg ($n=16$) were explored on a daily schedule continuously for 28 days followed by 14 days off medication. One case of DLT (uncontrolled hypertension) was observed in the initial six-patient cohort at the 1.5 mg level. Subsequently ten more patients were added at 1.5 mg dose level for expanded safety assessment. The reported DLTs in 16 patients consisted of two episodes of asymptomatic and reversible grade 3 and 4 transaminase elevation, one episode of uncontrolled hypertension, and one episode of grade 3 fatigue and dyspnea. None of the 18 patients on the 1.0 mg dose scale developed any DLTs. Therefore, dose of 1.5 mg was determined to be the MTD of tivozanib. Other frequently observed AEs were manageable hypertension, fatigue, hoarseness, and diarrhea, which have been frequently observed in clinical studies with other VEGFR tyrosine kinase inhibitors.

After single and multiple dosing, the overall rate of absorption was slow. The peak serum concentration was 2–24 h with substantial individual variability, and pharmacokinetics analyses revealed a mean half-life of 4.7 days (range 1.3–9.7 days), suitable for daily dosing. AUCs on day 28 were higher than those on day 1 because of expected accumulation. For the majority of patients, there was continuous systemic drug exposure even during the 14-day dosing break between cycles.

Tivozanib induced a rapid dose-dependent surge of serum levels of VEGF-A and concomitant fall of serum VEGFR-2, which persist throughout the treatment cycle and returned to near-baseline after 14 days off medication. Eight patients underwent DCE-MRI analysis and revealed a trend to diminishing internal vascularization of tumors over time. One patient (RCC) had a decrease in tumor vascularization accompanied by a decrease in tumor size, indicating likely antiangiogenic effects underlying an observed clinical response.

Overall 35 % of patients enrolled in this study demonstrated various degrees of tumor shrinkage during treatment, and 55.2 % of patients had a best response of

SD. There is no correlation between dosing and response rate. The phase I study demonstrated that tivozanib can be well tolerated at 1.5 mg daily dose level continuously for 4 weeks and followed by 2 weeks off and indicated its promising clinical antitumor activity.

Efficacy and Safety in Anti-VEGF Naïve RCC: A Phase II RDT

Giving the clinical response to tivozanib in RCC patients in the phase I study, a phase II randomized discontinuation trial (RDT) was designed to access the clinical activity and safety of tivozanib in advanced RCC [28]. The primary end points were safety and ORR. 272 patients with locally advanced or metastatic RCC were enrolled, 83 % were clear cell type, and 73 % received prior nephrectomy. 54 % of patients were treatment naïve, and the remaining patients received previous treatment with cytokines, vaccines, chemotherapy, or other agents. In the study design, all qualified patients entered a 16-week initial phase where patients received open-label tivozanib 1.5 mg daily at a 3-week on followed by 1-week off schedule. At the end of the fourth cycle, patients with radiographic response (≥25 % tumor shrinkage) proceeded on open-label tivozanib therapy, whereas patients with PD (≥25 % tumor growth) discontinued protocol treatment. Those patients falling between the cutoffs (SD) were randomized in a 1:1 double-blinded fashion to receive either tivozanib or placebo for the next 12 weeks. Crossover was allowed at the end of the study.

Throughout the study, the confirmed ORR was 24 % (95 % CI, 19–30 %) with median PFS of 11.7 months (95 % CI 8.3–14.3 months). In the 12-week double-blind phase, 49 % of patients on the treatment arm were progression free at the end of the study compared to 21 % on the placebo arm. The PFS was significantly higher on the treatment arm (10.3 months) compared to placebo arm (3.3 months).

The most common all-grade treatment-related AEs were hypertension (45 % all grade and 12 % grade 3/4) and dysphonia (22 % all grade and 0 grade 3/4). There was a low incidence of diarrhea (12 % all grade and 2 % grade 3/4), asthenia (10 % all grade and 3 % grade 3/4), fatigue (8 % all grade and 2 % grade 3/4), stomatitis (4 % all grade and <1 % grade 3/4), and hand-foot syndrome (4 % all grade and <1 % grade 3/4). The most common laboratory abnormalities were γ-glutamyl transpeptidase (GGT) elevation (66 % all grade and 17 % grade 3/4). There was low incidence of severe elevation of aminitransferase (1 %). The treatment was well tolerated with low rate of dose reduction (8 %) and dose interruption (4 %) [28].

Comparative Efficacy of Tivozanib in Selected Anti-VEGF and Anti-mTOR Pathway-Naïve Patients (TIVO-1): A Phase III Trial

The phase III randomized TIVO-1 trial compared tivozanib to sorafenib in patients with metastatic RCC never exposed to prior anti-VEGF or mTOR-directed agents (up to 1 prior nontargeted therapy was allowed) [29]. Furthermore, participants

were required to have clear cell histology and prior nephrectomy. Patient were randomized in a 1:1 fashion to receive either tivozanib at 1.5 mg daily at 3-week on/1-week off schedule or sorafenib at 400 mg oral twice daily continuously. Patients who had disease progression on sorafenib were allowed to cross over to tivozanib. The primary objective of the study was to determine superiority of tivozanib as compared to sorafenib in terms of PFS. Ultimately 517 patients were enrolled and 70 % of patients were treatment naïve. A total of 259 patients received tivozanib and 257 patients were assigned to sorafenib. By independent reviewer, there was a statistically significant improvement in PFS with tivozanib therapy compared to sorafenib (11.9 months vs. 9.1 months, HR 0.797, 95 % CI 0.639–0.993, $p = 0.042$) [30] (see Fig. 10.5a). The treatment-naïve patients had a slightly longer PFS in tivozanib arm (12.7 months vs. 9.1 months, HR 0.756, 95 % CI 0.580–0.985, $p = 0.037$) (see Fig. 10.5b). The development of hypertension was again noted associated with increased efficacy in TIVO-1 study. Patients with a dBP greater than 90 mmHg had an improved PFS compared to dBP less than 90 mmHg in patients with tivozanib (18.3 months vs. 9.1 months). Hypertension, diarrhea, and dysphonia were the most common AEs noted with tivozanib; hand-foot syndrome, hypertension, and diarrhea are the most common AEs with sorafenib. Overall, tivozanib was better tolerated, with less off-target AEs and fewer dose adjustments. The final survival data, however, revealed a non-significant advantage to sorafenib resulting in regulatory dissapproval.

Discussion and Future Direction

Role of Axitinib and Tivozanib in Sequential Therapy

In most patients receiving frontline VEGFR TKI therapy, resistance develops within a year after the initiation of the therapy, which presented with disease progression. Sequential therapy with targeted agents, either another VEGFR TKI or an mTOR inhibitor agent, is the current standard of care in advanced and metastatic RCC. Axitinib demonstrated superior antitumor activity as second-line agent. Tivozanib has not been extensively studied in the second-line setting. The relative value of axitinib and tivozanib as frontline agents and in sequence requires further study. Whether starting with the most biochemically potent VEGFR TKI or "saving" such therapy until later is better is not settled. Given the inherent VEGF-driven biology of clear cell RCC, likely more potential initial therapy would produce enhanced clinical effects (as supported by the PFS findings from the TIVO1 study).

Axitinib as Second-Line Therapy in Cytokine-Refractory Patients

At present, axitinib and sorafenib are the only targeted agents to have been directly studied in a head-to-head randomized controlled trial (AXIS trial) in cytokine-refractory RCC patients [18]. Sunitinib and pazopanib were tested in

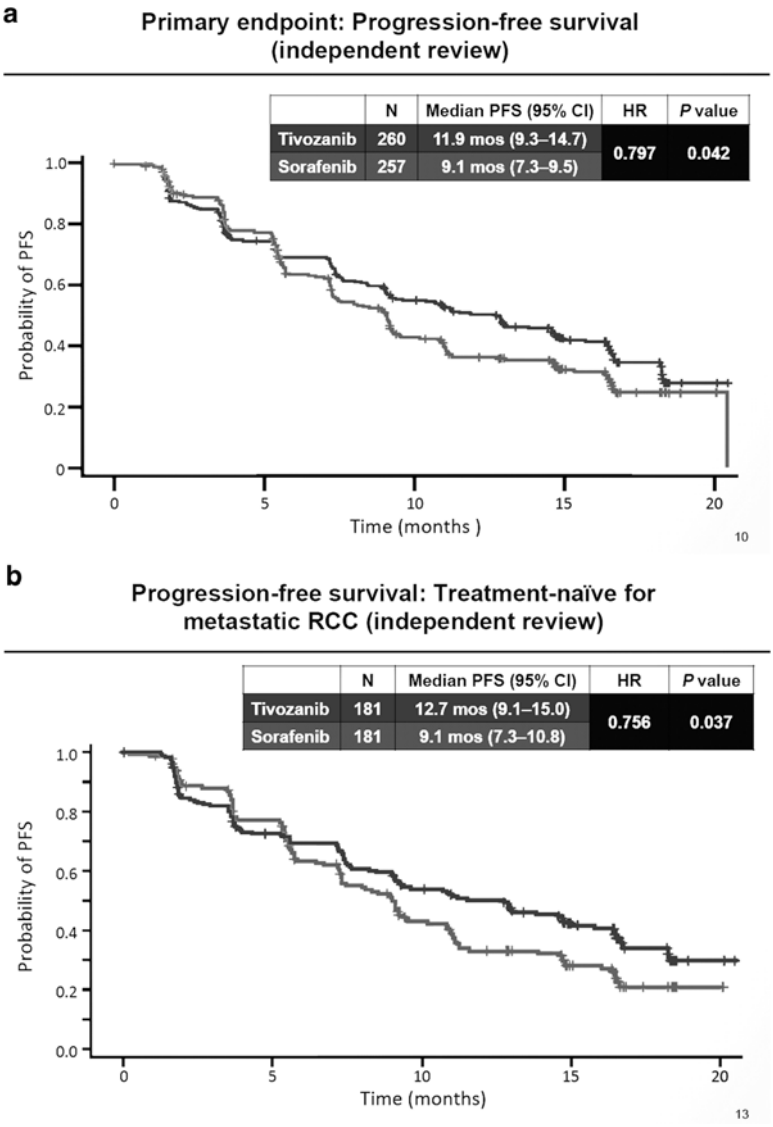

Fig. 10.5 Kaplan-Meier estimated median PFS in patients who received tivozanib or sorafenib for metastatic renal cell carcinoma in TIVO-1 trial [29]: (**a**) all patients and (**b**) treatment-naïve patients

cytokine-refractory patients in single-arm phase II trials [31, 32] and placebo-controlled phase III trial [33]. There is no data available in patients treated with tivozanib after progression on cytokine. Indirect comparisons of the limited data suggested axitinib associated with the best outcome with 12.1 months of PFS, followed by

sunitinib (PFS of 8.3 months), pazopanib (PFS of 7.4 months), and sorafenib (PFS of 6.5 months). Therefore, axitinib has the strongest clinical data in patients who failed frontline cytokine therapy.

Axitinib as Second-Line Therapy in Anti-VEGF-Refractory Patients

Efficacy of sequential VEGFR TKI therapies has been evaluated in numerous retrospective and prospective studies. Clear clinical benefit was observed in patients receiving sequential VEGF TKIs, which indicated an incomplete cross resistance among the VEGF-targeted agents. The magnitude of benefit achieved by the second-line VEGF TKI may depend on its relative potency and selectivity compared to the first-line agent.

Axitinib is approved for patients who received prior VEGFR TKI therapy based on RCT data. The phase III AXIS trial subgroup analyses in patients progressed on sunitinib revealed a moderate superior clinical outcome in disease control compared to sorafenib (PFS 4.8 month vs. 3.4 months) [18], which is comparable to everolimus. Everolimus has been widely used in patients who failed first-line VEGFR TKIs based on the result of RECORD-1 trial [34], which revealed a PFS of 4.9 months (95 % CI, 3.7–5.5) vs. 1.9 months (95 % CI, 1.8–1.9) in patients who were refractory to sunitinib, sorafenib, or both. Additionally, the single-arm phase II axitinib trial in patients who were refractory to sorafenib revealed a median PFS of 7.4 months [13]. In spite of lacking head-to-head comparison, axitinib seems to have at least equivalent clinical outcome compared to mTOR inhibitors as second-line treatment in patients who failed first-line anti-VEGF agent. Therefore, in patients who are refractory to frontline sunitinib or sorafenib, either axitinib or everolimus are the treatment of choice with supporting clinical data. More head-to head comparison study are needed for developing a sequential therapy algorithm in advanced RCC.

Dose Titration of Axitinib Based on Treatment-Induced Hypertension

Treatment-induced hypertension is the leading toxicity in patients treated with selective VEGFR TKIs (axitinib and tivozanib) and associated with improved clinical outcome [20, 24, 29]. A prospective study of axitinib (AGILE1046) suggested that developing hypertension at the early course of treatment associated with adequate serum drug level improved response rate and superior disease control. Anti-VEGF-induced hypertension may serve as a reliable biomarker of drug exposure for individual patient [24]. The final result of the randomized placebo-controlled axitinib dose titration of AGILE1046 trial is not available yet, but axitinib dose escalation should be recommended to patients who tolerate axitinib well in standard practice. Conversely, oncologists are encouraged to manage treatment-induced hypertension proactively to improve treatment adherence and maximize clinical benefit.

Combination Therapy

Combining targeted agents with different mechanism of action may provide additive or synergistic activity as a result of a more complete blockade of aberrant signaling, which may improve antitumor activity and overcome development of resistance of single agents. This strategy has been explored in VEGFR TKIs, especially combining anti-VEGF agents with mTOR inhibitors, without success (42–44). As a new generation of highly selective VEGFR TKIs, axitinib, and tivozanib demonstrated a distinct toxicity profile with less off-target toxicity (less skin toxicities and anemia), making them more tolerable when administrated with mTOR inhibitors. Both axitinib and tivozanib are being tested in phase I trial in combination with everolimus.

In a phase Ib open-label study in patients with anti-VEGF-refractory metastatic RCC [27], both tivozanib 1.5 mg daily, 3 weeks on and 1 week off, and temsirolimus 25 mg weekly were tolerated without dose-limiting toxicities. Severe AEs are observed including fatigue (most common), stomatitis, and thrombocytopenia. A promising 28 % PR and 64 % SD was observed in the study. Expanded phase II trial will further explore the clinical efficacy and safety of the combination therapy.

Conclusions

Axitinib and tivozanib are potent selective VEGFR TKIs with superior tolerability and enhanced antitumor activity. Axitinib is a preferred therapy in mRCC patients who failed any prior systemic treatment. Axitinib and tivozanib are being evaluated as frontline therapies with the potential for a more favorable efficacy/toxicity balance over earlier generation VEGFR inhibitors.

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Chapter 11
Anti-VEGF and VEGFR Monoclonal Antibodies in RCC

Bernard Escudier and Laurence Albiges

VEGF and the Role of VHL Mutation in RCC

VEGF is a growth factor produced by a variety of cells and exerts its biological effects primarily on vascular endothelial cells [7–9]. Upon ligation to its receptor, VEGF receptor-2, VEGF can induce growth, proliferation, and migration of endothelial cells and promote the survival of immature endothelial cells via inhibition of apoptosis. It also increases vascular permeability. As a key pro-angiogenic molecule, VEGF plays an important role in a number of physiological processes such as embryogenesis, skeletal growth, and wound healing [10–13].

VEGF is also a key mediator of angiogenesis in cancer [14]. During tumorigenesis, tumor growth reaches a growth-limiting step where oxygen and nutrient levels are insufficient to continue proliferation. As a result, tumors tend to become hypoxic. The normal cellular response to hypoxia is to produce growth factors such as VEGF, transforming growth factor-α and platelet-derived growth factor that stimulate neoangiogenesis. Production of these growth factors is controlled by hypoxia-inducible transcription factors such as via hypoxia-inducible factor-α (HIFα) [15]. VEGF is continuously expressed throughout the development of many tumor types and is the only angiogenic factor known to be present throughout the entire tumor life cycle [16]. Although the resulting tumor-associated vasculature is abnormal and inefficient, it is essential for tumor growth and metastasis.

The development of RCC is directly linked to VEGF overexpression and angiogenesis [17]. This is evidenced by the high degree of vascularization and high expression of VEGF in RCC tumors [18, 19]. The VHL protein regulates the normal cellular responses to hypoxia via HIFα [20, 21]. When the oxygen levels are normal,

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the oxygen content in the blood regulates the formation of VHL protein complexes which target HIFα for degradation by proteasomes. Thus, pro-angiogenic factors are not released. However, mutation or inactivation of the VHL protein, which is common in clear cell RCC [4, 5], disrupts the ability to degrade HIFα in the presence of normal oxygen levels [17]. This leads to an excess accumulation of HIFα resulting in overproduction of pro-angiogenic factors such as VEGF and ultimately leading to increased angiogenesis and tumor growth. Targeting VEGF as a means of preventing angiogenesis in RCC led to the development of the direct anti-VEGF inhibitor, bevacizumab, as well as small molecules blocking VEGF receptors, called tyrosine-kinase inhibitors.

This chapter will focus on VEGF antibodies, mainly bevacizumab, but also VEGF trap.

Bevacizumab Development

Bevacizumab (Avastin®) is a recombinant humanized monoclonal immunoglobulin G 1 (IgG1) antibody developed from murine anti-VEGF monoclonal antibody (mAb) A4.6.1. The murine mAb A4.6.1 is specific for human VEGF, binding to all isoforms of the ligand and preventing it from binding to VEGF receptors on vascular endothelial cells [22]. Although effective in suppressing the growth of tumor xenografts in animal models [23, 24], as a foreign protein A4.6.1 is not appropriate for use in humans because it provokes an immune response, limiting its bioavailability and half-life and potentially causing allergic reactions. In 1997, the murine anti-VEGF mAb A4.6.1 was humanized by site-directed mutagenesis [25], resulting in the production of bevacizumab. Bevacizumab is 93 % human and 7 % murine and recognizes all major isoforms of human VEGF with a binding affinity of $K_d = 8 \times 10^{-10}$ M (similar affinity to the murine antibody). Bevacizumab's VEGF binding ability is restricted to human, nonhuman primate, and rabbit VEGF. It has a terminal half-life of 17–21 days, with no dose-limiting toxicity as a single agent.

Sustained inhibition of VEGF with bevacizumab results in the regression of existing tumor microvasculature, normalization of surviving tumor vasculature, and inhibition in the formation of new vasculature [25, 26]. It may also revert tumor-associated immune suppression and improve concomitant drug delivery into the tumor [27–29]. Furthermore, withdrawal of anti-VEGF therapy has been shown to result in rapid regrowth of tumor vasculature, suggesting that anti-VEGF therapy should be continued until disease progression [30].

The pharmacokinetics for bevacizumab are well described by a two-compartment model and are characterized by a low clearance (0.195 L/day), a limited volume of the central compartment (2.98 L), and a long terminal half-life (~20 days), as seen with endogenous IgG antibodies. The pharmacokinetics of bevacizumab were linear within the dose range of 1–10 mg/kg/week. Steady state is reached at approximately 100 days with multiple dosing. The low inter-patient variability and the modest effects of covariates on the clearance and volume of distribution of bevacizumab support the current

strategy of dosing bevacizumab based on a body weight-adjusted dose (mg/kg). Due to its long terminal half-life, bevacizumab can be administered every 2–3 weeks depending on the chemotherapy schedule with which it is combined [31].

Results of the analysis of blood samples taken from patients in the AVOREN trial (Avastin and Roferon in Renal Cell Carcinoma [BO17705]) showed that the pharmacokinetics of bevacizumab during concomitant IFN administration in patients with advanced and/or metastatic RCC were comparable to that previously seen in oncology patients with different cancer types [32].

Clinical Experience with Bevacizumab in RCC: Phase II Trials

There are two key phase II trials of bevacizumab in RCC.

Efficacy

The first trial, AVF0890s, was a randomized, placebo-controlled, double-blind trial of bevacizumab monotherapy conducted in patients with metastatic RCC who were not optimal candidates for IL-2 therapy or had previously not responded to this therapy [33]. Between October 1998 and September 2001, 116 patients were randomized to one of three treatment arms: placebo ($n=40$), bevacizumab 3 mg/kg ($n=37$), or bevacizumab 10 mg/kg ($n=39$). Patients were stratified according to whether or not they had previously received IL-2 therapy. Efficacy was assessed in this trial by measuring the time to disease progression (TTP) and OS.

Data from this trial showed that the median TTP was significantly longer for the bevacizumab 10 mg/kg arm than the placebo arm (4.8 vs. 2.5 months; hazard ratio [HR]=2.55; $p<0.001$). The median TTP for the bevacizumab 3 mg/kg arm was 3.0 months and was not significantly greater than the placebo arm (HR=1.26; $p=0.053$). Four (10 %) patients in the 10 mg/kg arm had PRs, which were of variable duration (6, 9, 15, and >39 months, respectively). No patients in the 3 mg/kg arm had a PR. OS was not significantly different between the three arms ($p>0.20$ for all comparisons), which could well be due to the crossover of the placebo patients. This trial has been the rationale for further using bevacizumab 10 mg/kg in RCC.

A substantial number of patients receiving bevacizumab in this trial had mixed tumor responses [34]. Due to the use of strict criteria for progression, in which a 25 % increase in the perpendicular diameters of any lesion constituted progression, patients may have stopped therapy while still obtaining its benefit. The tumor burden compared to baseline for individual patients with mRCC was calculated during the course of treatment to investigate tumor burden at progression. The data showed that when patients stopped receiving bevacizumab 10 mg/kg, the tumor burden was less than what they started with at baseline. This was mainly due to mixed responses

where progression occurred only in a minority of their lesions. Interestingly, a subset of patients receiving bevacizumab 3 mg/kg also showed net tumor stability while on the drug. These findings support the concept of continuing bevacizumab despite limited progression.

Long-term efficacy and safety of bevacizumab was assessed in four patients who had received bevacizumab treatment for 3–5 years [34]. Of these four patients, one patient had a PR, and one had a minor response and had not progressed after 2 years of bevacizumab 10 mg/kg therapy. Two other patients (one in each of the bevacizumab arms) had stable disease (SD) at 2 years and remained stable for >4 years. Long-term therapy with bevacizumab was well tolerated, and proteinuria was the only significant adverse event (AE) attributable to bevacizumab therapy; normal renal function was maintained in these patients. These observations suggest that it is feasible to treat patients with bevacizumab for >4 years.

Epidermal growth factor receptor (EGFR) and VEGF are overexpressed in many tumors, and VEGF has been implicated in anti-EGFR resistance [35]. Therefore, there was a rationale to combine bevacizumab with the anti-HER1/EGFR small-molecule tyrosine-kinase inhibitor (TKI) erlotinib (Tarceva®). The phase II RACE trial evaluated bevacizumab in combination with erlotinib in patients with mRCC [36]. It was a randomized, double-blind, placebo-controlled trial conducted at 21 sites in the USA. Patients were enrolled from March 2004 through to October 2004 to receive bevacizumab 10 mg/kg every 2 weeks and either erlotinib 150 mg orally or placebo daily. Treatment continued for a maximum of 24 months or until toxicity or disease progression. This trial had two co-primary efficacy end points, progression-free survival (PFS) and ORR (CR plus PR). Secondary efficacy parameters were the duration of objective response, OS, and time to symptom progression.

The median PFS was not significantly improved by the addition of erlotinib to bevacizumab, which was 8.5 months with bevacizumab plus placebo versus 9.9 months with bevacizumab plus erlotinib (HR 0.86; 95 % confidence interval [CI] 0.50–1.49). Also, the addition of erlotinib to bevacizumab resulted in similar ORR which was 13 % with bevacizumab plus placebo versus 14 % with bevacizumab plus erlotinib. One CR was noted in the bevacizumab plus erlotinib arm, and this patient completed 2 years of therapy. The addition of erlotinib to bevacizumab did not result in an improvement in duration of objective response (6.7 vs. 9.1 months) or time to symptom progression (HR 1.172; $p=0.5076$). The median OS with bevacizumab plus placebo had not been reached at the time of this analysis: the median survival duration with bevacizumab plus erlotinib was 20 months. There was a higher use of second-line therapies in the bevacizumab-only arm versus the bevacizumab plus erlotinib arm (32 % and 14 %, respectively).

Tolerability

Bevacizumab at a dose of up to 10 mg/kg every 2 weeks was generally well tolerated and was associated with manageable side effects. Increases in the incidence of grade 2 or 3 hypertension, proteinuria, malaise, and epistaxis were seen in patients

receiving bevacizumab 10 mg/kg. Of these, hypertension was the most common side effect (36 % bevacizumab 10 mg/kg arm vs. 5 % placebo arm; $p<0.05$) and was managed with oral antihypertensive medication. Proteinuria was the second most common AE (64 % [56 % grade 1/2] bevacizumab 10 mg/kg arm vs. 38 % [all grade 1/2] placebo arm; $p<0.05$). Both hypertension and proteinuria improved after treatment was stopped.

The most common grade 3/4 AE with bevacizumab plus erlotinib was grade 3 hypertension, which occurred in 16 (31 %) patients versus 14 (26 %) patients in the bevacizumab plus placebo arm. Other grade 3 AEs of note in the bevacizumab plus erlotinib arm were rash (16 %) and diarrhea (7.8 %). These events did not occur in the bevacizumab plus placebo arm. Grade 3/4 hemorrhage occurred in 3.8 % of patients in the bevacizumab plus placebo arm and 5.9 % of patients in the bevacizumab plus erlotinib arm.

Clinical Experience with Bevacizumab Plus Interferon (IFN) in RCC (Table 11.1)

AVOREN Trial

AVOREN was a phase III trial evaluating the efficacy and safety of adding bevacizumab to IFN in the treatment of mRCC [37]. Bevacizumab was combined with IFN because at the time of trial design, IFN was the standard of care for the treatment of advanced RCC and was better tolerated than high-dose IL-2 therapy. Furthermore, clinical trials to date had shown that bevacizumab could be combined with other therapies without exacerbating their tolerability [31, 45, 46]. The proposed

Table 11.1 Efficacy of bevacizumab[a] + interferon in mRCC (phases II and III)

Trial name	Design	Number of patients	IFN dose[a]	Control arm	PFS (mos)	OS (mos)	References
AVOREN	Phase III	325	9 MIU ×3/week	IFN + placebo	10.2	23.3	[37, 38]
CALGB	Phase III	369	9 MIU ×3/week	IFN	8.5	17.3	[39, 40]
TORAVA	Phase II[b]	41	9 MIU ×3/week	Sunitinib temsirolimus + bevacizumab	16.8	NA	[41]
BEVLiN	Phase II	147	3 MIU ×3/week	NA	15.6	NA	[42]
INTORACT	Phase III	391	9 MIU ×3/week	Temsirolimus + bevacizumab	9.3	25.5	[43]
RECORD-2	Phase II[b]	182	9 MIU ×3/week	Everolimus + bevacizumab	10.2	25.6	[44]

[a]Bevacizumab was delivered at 10 mg/kg in all the trials
[b]Randomized

mechanisms of action of bevacizumab and IFN suggested that these two agents may have complementary and synergistic effects when combined [47, 48], therefore maximizing patient outcomes.

Between June 2004 and October 2005, the trial enrolled 649 patients at 101 trial sites in 18 countries. Patients were randomized on a 1:1 basis to receive IFN 9 MIU three times a week plus placebo or bevacizumab 10 mg/kg every 2 weeks plus IFN. Stratification criteria were by country and MSKCC risk group. After 52 weeks of treatment, IFN was stopped, and patients could continue to receive single-agent bevacizumab/placebo. The primary objective of this trial is OS. Secondary end points include PFS, TTP, and objective response rate.

Overall Efficacy

The final analysis of PFS was performed at the scheduled interim analysis of OS (after 505 progression events and 251 deaths) and showed that PFS was significantly extended by the addition of bevacizumab to IFN, from 5.4 to 10.2 months (HR = 0.63; $p = 0.0001$) [37]. This represents an 89 % improvement in median PFS with bevacizumab + IFN. The addition of bevacizumab to IFN also improves ORRs compared with IFN plus placebo (31 vs. 13 %). Increase in PFS was seen with bevacizumab plus IFN alfa irrespective of risk groups or whether reduced-dose IFN alfa was received [49].

The final analysis showed that median OS was 23.3 months with bevacizumab plus IFN and 21.3 months with IFN plus placebo (unstratified hazard ratio [HR] = 0.91; 95 % CI, 0.76–1.10; $p = 0.3360$; stratified HR = 0.86; 95 % CI, 0.72–1.04; $p = .1291$) [38]. In patients whose IFN doses were reduced to 6 or 3 MIU three times a week to manage IFN-related AEs, the OS benefit (as well as the PFS benefit) of bevacizumab plus IFN was maintained (median OS 26.0 months).

Efficacy in Patient Subgroups

Analyses of patient subgroups suggest that the addition of bevacizumab to IFN improves PFS in all subgroups analyzed [49, 50]. Improvements in PFS were observed in both favorable ($n = 180$) and intermediate ($n = 363$) MSKCC risk categories (median PFS 12.9 vs. 7.6 months, HR = 0.60; median PFS 10.2 vs. 4.5 months, HR = 0.55, respectively). Patients in the poor MSKCC risk category ($n = 54$) had improved PFS (HR = 0.81; $p = 0.457$), although this did not reach the predefined significance levels stated in the statistical analysis plan.

PFS improvement was not affected by tumor histology or age. PFS benefit was observed in patients receiving bevacizumab + IFN with either clear cell RCC histology ($n = 564$; median PFS 10.2 vs. 5.5 months, HR = 0.64; 95 % CI 0.53–0.77) or mixed RCC histology ($n = 85$; median PFS 5.7 vs. 2.9 months, HR = 0.60; 95 % CI 0.33–0.85). Patients aged ≥65 years ($n = 239$; HR = 0.77; 95 % CI 0.58–1.03) and <65 years ($n = 410$; HR = 0.54; 95 % CI 0.43–0.68) had significant PFS improvement.

In addition, PFS did not appear to be affected by reduced kidney function, as assessed by creatinine clearance (CLcr) or VEGF levels. Patients with either high/normal CLcr ($n=131$) or low CLcr ($n=191$) benefited from bevacizumab + IFN (HR = 0.60 [95 % CI 0.46–0.79]; HR = 0.65 [95 % CI 0.51–0.82], respectively). Baseline VEGF levels were established upon recruitment, and improvements in PFS were observed in patients with VEGF levels below the median baseline level (HR = 0.44; 95 % CI 0.32–0.64) and also above the median (HR = 0.66; 95 % CI 0.49–0.93).

Patients with either single or multiple metastatic sites all obtained PFS benefit with the addition of bevacizumab to IFN. Upon enrolment to the trial, baseline measurements of target lesions were determined. PFS benefit was observed regardless of whether the sum of the baseline measurements was above or below the median (below the median $n=292$, HR = 0.65; 95 % CI 0.49–0.85; above the median $n=297$, HR = 0.60; 95 % CI 0.47–0.77).

Combining bevacizumab and IFN led to an increase in response rate predominantly in patients in the favorable and intermediate MSKCC risk categories compared with IFN plus placebo (32 vs. 11 % and 36 vs. 14 %, respectively); these data are consistent with the overall population. Response rates were similar with bevacizumab + IFN and IFN plus placebo in patients in the poor MSKCC risk group (10 % bevacizumab + IFN vs. 8 % IFN plus placebo).

The addition of bevacizumab to IFN also slightly prolonged the overall median duration of response compared with IFN plus placebo (13.5 vs. 11.1 months). As with response rates, the median duration of response was longer in patients in the favorable and intermediate MSKCC risk categories in the bevacizumab arm than the placebo arm (13.6 vs. 11.1 months and 12.0 vs. 10.6 months, respectively). Also, the overall median duration of SD was longer in the bevacizumab arm than the placebo arm (10.1 vs. 7.2 months). Improved median durations of SD were seen in patients in the favorable and intermediate MSKCC risk groups of the bevacizumab arm when compared to placebo (12.9 vs. 10.1 months and 8.8 vs. 5.7 months, respectively).

Overall Tolerability

Both bevacizumab and IFN have well-characterized tolerability profiles. The tolerability profile for bevacizumab + IFN in the AVOREN trial is consistent with the side effects previously reported for both agents. The dose intensity (percentage of planned total dose) of bevacizumab/placebo and IFN was similar in the two arms (92 % bevacizumab + IFN vs. 96 % IFN plus placebo for the bevacizumab/placebo arms and 91 % bevacizumab + IFN vs. 96 % IFN plus placebo for the IFN arms). The addition of bevacizumab to IFN increased the incidence of grade 3/4 events associated with IFN therapy from 15 to 23 %. This may be attributable to the longer duration of IFN therapy in the bevacizumab + IFN arm compared to the control arm (7.8 vs. 4.6 months). It is of note that the median duration of bevacizumab treatment in the bevacizumab + IFN arm was almost double that of the placebo arm

(9.7 months vs. 5.1 months). The incidence of grade 3/4 events associated with bevacizumab therapy included hypertension (7 %), proteinuria (4 %), bleeding (3 %), arterial and venous thromboembolic events (3 %), gastrointestinal perforation (1 %), and wound healing complications (<1 %).

Tolerability in Patient Subgroups

The dose intensity of IFN in both treatment arms was lower in patients with low CLcr (IFN plus placebo 92 %; bevacizumab + IFN 78 %) and ≥65 years (IFN plus placebo 92 %; bevacizumab + IFN 82 %) than in those with normal/high CLcr (IFN plus placebo 99 %; bevacizumab + IFN 87 %) and <65 years (IFN plus placebo 99 %; bevacizumab + IFN 92 %). Dose intensity of bevacizumab was similar in subgroups defined by CLcr and by age.

The analysis of patient subgroups showed the incidence of grade ≥3 AEs (mean number of AEs per patients) in patients receiving bevacizumab in the favorable and intermediate MSKCC risk categories was 1.3 and 1.2, respectively, compared to 1.0 and 0.8 in the placebo arm. Patients in the poor MSKCC risk category showed no difference in the incidence of grade ≥3 AEs. Patients aged ≥65 years had a higher incidence of grade ≥3 AEs in both arms (bevacizumab 66 %; IFN 48 %) compared to patients <65 years (bevacizumab 58 %; IFN 45 %), with a higher incidence of fatigue and asthenia in patients aged ≥65 years in the bevacizumab arm. Additionally, there was a similar increase in incidence of grade ≥3 AEs in both patients with low (bevacizumab 18 %; IFN 2 %) and high/normal CLcr (bevacizumab 16 %; IFN 2 %) receiving bevacizumab; however, the incidence of bevacizumab-associated AEs was similar in both subgroups.

CALGB 90206

The Cancer and Leukemia Group B (CALGB) 90206 trial is another pivotal phase III randomized, open label trial comparing the efficacy and safety of bevacizumab to IFN with IFN alone in patients with mRCC [39]. Patients were assigned to receive either bevacizumab (10 mg/kg intravenously every 2 weeks) plus IFN (9 million IU subcutaneously three times weekly) for 369 patients or the same dose and schedule of IFN monotherapy for 363 patients. Bevacizumab plus IFN had a higher efficacy through a superior ORR as compared with IFN (25.5 % [95 % CI, 20.9–30.6 %] vs. 13.1 % [95 % CI, 9.5–17.3 %]; $p < 0.0001$). The median PFS was 8.5 months in patients receiving bevacizumab plus IFN (95 % CI, 7.5–9.7 months) versus 5.2 months (95 % CI, 3.1–5.6 months) in patients receiving IFN alone (log-rank $p < 0.0001$). The adjusted hazard ratio was 0.71 (95 % CI, 0.61–0.83; $p < 0.0001$). The final analysis showed that the median OS time was 18.3 months (95 % CI, 16.5–22.5 months) for bevacizumab plus IFN-alpha and 17.4 months (95 % CI, 14.4–20.0 months) for IFN-alpha monotherapy (unstratified log-rank $p = 0.097$).

Adjusting on stratification factors, the hazard ratio was 0.86 (95 % CI, 0.73–1.01; stratified log-rank $p = 0.069$) favoring bevacizumab plus IFN-alpha [40].

In December 2007, bevacizumab combined with IFN received the EMEA approval as a first-line treatment for patients with advanced and/or metastatic RCC. Since that time, bevacizumab plus IFN-alpha is considered as an option for first-line treatment in every published guideline, including the most recent one [51].

Further Studies Evaluating Bevacizumab Plus IFN-Alpha in RCC

Since 2008, bevacizumab plus IFN, at the standard dose tested in both AVOREN and CALGB trials (IFN 9 MIU three times a week plus bevacizumab 10 mg/kg every 2 weeks), has been evaluated in numerous phase II and III. This regimen has been considered as the standard control arm in 2 phase II and one large phase III.

The TORAVA [41] was a randomized phase II evaluating the efficacy of bevacizumab plus temsirolimus as first-line treatment in mRCC (the rationale for this combination will be discussed further). Control arms for this study were sunitinib and bevacizumab plus IFN. In this study, 41 patients received bevacizumab plus IFN, and median PFS was 16.8 months (6.0–26.0) with a response rate of 43 %.

More recently two large trials also addressing the issue of mTOR inhibitor and bevacizumab combination, RECORD-2 and INTORACT, were reported.

RECORD-2 was a large randomized phase 2 comparing bevacizumab plus everolimus and bevacizumab plus IFN [44]. In this study, 365 patients have been randomized. Median PFS was 10.2 months for the bevacizumab plus IFN arm (vs. 9.3 months in the experimental arm), response rate 27.9 %, and overall survival above 26 months (median not reached at the time of presentation).

INTORACT was a large randomized phase 3 comparing bevacizumab plus temsirolimus and bevacizumab plus IFN [43]. In this study, 791 patients have been randomized. This study failed to show any improvement for the experimental arm. In the bevacizumab plus IFN arm, median PFS was 9.3 months with 28 % response rate and overall survival 25.5 months.

Bevacizumab with Low-Dose IFN

Based on the results and the observation of the AVOREN trial, patients with reduced dose of IFN had a better PFS than the whole population [49]. A large phase 2 was performed to evaluate the efficacy of bevacizumab combined with 3 million IU three times a week [42]. In this study, 147 patients received the above regimen. Median PFS was 15.6 months and response rate 22 %. The incidence of side effects was lower than that in the AVOREN trial. Unfortunately, this was not a randomized

study, and comparing data with this regimen to the AVOREN regimen would be misleading. Ideally, a randomized study would be warranted to further evaluate this promising regimen.

Conclusions on Efficacy of Bevacizumab Plus IFN

Bevacizumab plus IFN has demonstrated efficacy in 3 large phase 3 as well as in various phase 2. The median PFS in phase 3 range from 8.5 to 10.2, which is in the range of sunitinib and pazopanib, which are the other recommended first-line options. However, this regimen is poorly used in routine practice, because of several issues:

- Toxicity of IFN, which provides a bad reputation to this treatment
- Disadvantage of IV and SQ administrations for bevacizumab and IFN, respectively, especially by comparison with easy use of TKIs
- Cost of bevacizumab

Bevacizumab in Combination with TKIs

Based on the rationale that blocking both VEGF receptors and its ligand, VEGF, might be more potent than blocking only one of them, trials combining TKIs and bevacizumab have been launched. Overall, combinations of bevacizumab plus sunitinib or sorafenib appear to have poor tolerability and require reduced doses of one or both regimens [32, 52–54]. The cause of the increased toxicity with two indirect VEGF inhibitors is unknown but could be due to an overlap of inhibition of eukaryotic translation initiation factor 4B (eIF4B), which plays a critical role in recruiting ribosomes to mRNA. The phosphorylation of this factor, which is physiologically significant, is controlled by both the PI3K/Akt pathway, which is inhibited by mTOR inhibitors, and the MAPK pathway, which is inhibited by sorafenib [55].

In a phase I dose-finding trial of patients with mRCC, the maximum tolerated dose (MTD) of bevacizumab plus sunitinib was determined to comprise full doses of both agents [52]. A high tumor response rate was seen, but accompanying chronic and late toxicity (e.g., hypertension, proteinuria, and thrombocytopenia), not addressed in the definition of a dose-limiting toxicity (DLT) in this trial, was also observed. Similar results were observed in a randomized phase II study (SABRE-R trial—NCT00491738) of sunitinib with or without bevacizumab in first-line patients with mRCC, which was stopped early due to poor tolerability. Of note, both trials reported two cases of grade 3/4 microangiopathic hemolytic anemia (MAHA) with reverse posterior leukoencephalopathy syndrome. In addition, approximately half of the patients required treatment interruption or discontinuation due to AEs. In contrast, another phase I trial of sunitinib plus bevacizumab in 32 patients with advanced solid tumors appeared to be better tolerated [32], probably due to schedule-dependent reasons.

A combination of sorafenib plus bevacizumab also appeared to be active and was better tolerated than the sunitinib combination, but VEGF-related (e.g., hypertension, proteinuria) and non-VEGF-related (e.g., hand-foot syndrome and fatigue) toxicity was increased compared with monotherapy [53]. These data are consistent with a similar trial of this combination in patients with various solid tumors [54].

Finally, recently the BEST trial has been reported [56]. This trial randomized first-line RCC patients between 4 arms, one of them being combination of sorafenib and bevacizumab. Efficacy was confirmed in terms of PFS but without any survival advantage compared with bevacizumab and also increased toxicity.

Finally, the combination of bevacizumab with other TKIs has been tested such as pazopanib combined with bevacizumab [57]. Preliminary results showed that the MTD of the combination of pazopanib and bevacizumab was 400 mg/day and 7.5 mg/kg, respectively, in nephrectomized patients, which will preclude this combination further in RCC.

Despite signals of activity, these combinations are clearly endowed by relevant toxicities, which are likely due to an overlap in mechanisms of action. Different treatment approaches may theoretically improve tolerability including reduced doses as well as intermittent and/or alternating dosing.

Bevacizumab in Combination with an mTOR Inhibitor (Table 11.2)

According to early data, bevacizumab appeared to be a promising combination partner for the two mTOR inhibitors temsirolimus and everolimus [58, 59] in both treatment-naïve and previously treated patients, with a good tolerability profile at full approved doses and an incidence of AEs largely consistent with that of monotherapy, thus suggesting that the relative mechanisms of action do not overlap.

However, such encouraging preliminary data were not confirmed by the first results of the TORAVA trial, a randomized 3-arm, phase II trial, where the combination of bevacizumab and temsirolimus was compared to bevacizumab plus IFN or sunitinib as a first-line therapy for mRCC patients [60].

Table 11.2 Trials of bevacizumab + mTOR inhibitors in first-line RCC patients

Design	Bevacizumab	mTOR inhibitor	Number of patients	PFS	OS	References
Phase 2	10 mg/kg	Everolimus 10 mg daily	50	9.1	21.3	[59]
Phase 2[a]	10 mg/kg	Temsirolimus 25 mg weekly	88	8.2	NA	[60]
Phase 2[a]	10 mg/kg	Everolimus 10 mg daily	183	9.3	NA	[44]
Phase 3	10 mg/kg	Temsirolimus 25 mg weekly	400	9.1	25.8	[43]

[a]Randomized

Indeed, this trial showed—someway surprisingly—a superior antitumor activity (in terms of overall response rate), as well as a longer PFS, in favor of the bevacizumab plus IFN arm, as compared to either sunitinib or the combination of bevacizumab plus temsirolimus (39.0 % vs. 23.8 % vs. 27.3 % and 16.8 months vs. 8.2 vs. 8.2, respectively). More importantly, this trial demonstrated that the combination of bevacizumab and temsirolimus had a very poor safety profile; indeed, 26.1 % and 12.5 % of the patients treated with bevacizumab and temsirolimus experienced grade 3 or 4 AEs (as compared to 11.9 and 2.4 for sunitinib and 20.0 and 7.5 for bevacizumab plus IFN) with 3.4 % of treatment-related deaths; furthermore, 40.9 % of the patients in the experimental arm discontinued the treatment due to toxicity. As a whole, the primary objective of the study, i.e., a 48-week nonprogression rate >50 % for the combination of bevacizumab and temsirolimus, was not reached, and the treatment demonstrated a higher than expected toxicity profile [60].

Even though this relatively small, randomized phase II trial clearly did not support the use of bevacizumab together with temsirolimus, 2 large phase II and III studies exploring similar combinations have been initiated and recently reported [43, 44]. The RECORD-2 trial [44] compared everolimus plus bevacizumab to the standard combination of bevacizumab plus interferon in 365 patients. Both PFS (9.3 months) and OS were similar to the control arm, and toxicity was higher. Similarly, INTORACT was a large phase 3 which compared temsirolimus plus bevacizumab to the same control arm in 791 patients [43]. The median PFS in patients treated with temsirolimus/bevacizumab ($n=400$) versus IFN/bevacizumab ($n=391$) was 9.1 and 9.3 months, respectively (hazard ratio [HR], 1.1; 95 % CI, 0.9–1.3; $p=0.8$). There were no significant differences in overall survival (25.8 vs. 25.5 months; HR, 1.0; $p=0.6$) or objective response rate (27.0 % vs. 27.4 %) with temsirolimus/bevacizumab versus IFN/bevacizumab, respectively. Treatment-emergent, all-causality grade ≥3 adverse events more common ($p<0.001$) with temsirolimus/bevacizumab were mucosal inflammation, stomatitis, hypophosphatemia, hyperglycemia, and hypercholesterolemia, while neutropenia was more common with IFN/bevacizumab. The incidence of pneumonitis with temsirolimus/bevacizumab was 4.8 %, mostly grade 1 or 2.

Based on these large studies, combination of mTOR inhibitors and bevacizumab should not be anymore tested in RCC.

Bevacizumab in Combination with Erlotinib

Combination of bevacizumab and erlotinib has been initially evaluated in phase 1–2 [61], with encouraging efficacy. However, this combination was not more effective than bevacizumab alone in a large phase 2 [36]. Similarly, the combination of bevacizumab, erlotinib, and gefitinib did not reach enough efficacy to be further evaluated [62].

VEGF Trap

VEGF Trap (Regeneron Pharmaceuticals, Tarrytown, New York and Sanofi-Aventis, Bridgewater, New Jersey) is a fusion compound composed of the human VEGFR-1 (Flt-1) extracellular immunoglobulin domain number two and the VEGFR-2 (KDR) extracellular immunoglobulin domain number three, fused to the human IgGg1 Fc molecule. Therefore, this fusion protein acts as a soluble decoy receptor that binds to VEGF and prevents subsequent VEGF binding and signaling. VEGF Trap binds to VEGF with a great affinity ($K_d = 5.1$ pmol/L) and also binds to the placental growth factor, another angiogenic protein. In cultured endothelial cell assays, VEGF Trap showed inhibition of VEGF-induced VEGFR-2 phosphorylation and endothelial cell proliferation. In xenograft models, mice treated with VEGF Trap exhibited significant growth inhibition of different tumor subtypes. VEGF Trap activity has been assessed in phase I trials [63]. In two trials, patients presented with refractory solid tumors. In the first report, 38 patients, including nine with mRCC, received one or two subcutaneous doses of VEGF Trap, followed 4 weeks later with 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, although a maximum tolerated dose was not determined. No anti-VEGF Trap antibodies were detected. No objective responses were observed in this trial. Of the 24 assessable patients, 14, including five of six at the highest-dose level, maintained stable disease for 10 weeks. In the second trial, 30 patients were treated with intravenous VEGF Trap every 2 weeks at one of five different 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 mRCC maintained a stable disease for more than 11 months (at the 1.0 mg/kg dose level). Dynamic contrast-enhanced magnetic resonance vascular imaging performed at baseline and after 24 h indicated effective inhibition of tumor perfusion at the higher-dose levels (2.0 mg/kg). Complete binding of circulating VEGF was documented at higher-dose levels (2.0 mg/kg), with more free than bound VEGF Trap observed in the plasma. Further investigation is ongoing through an Eastern Cooperative Oncology Group (ECOG) phase II trial (ECOG-E4805, NCT00357760) that randomized 120 patients with mRCC to two different doses of VEGF Trap, with a primary end point of PFS at 8 weeks.

In conclusion, bevacizumab has efficacy in mRCC in combination with interferon in at least 3 large phase 3, justifying its use as first-line option in mRCC. Whether bevacizumab alone would have similar efficacy remains controversial, without any well-designed phase 3 comparing monotherapy to the IFN plus bevacizumab combination.

The combination of bevacizumab with other targeted agents such as TKIs or mTOR inhibitors is toxic, and none of the randomized studies have been able to demonstrate increased efficacy.

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Chapter 12
PI3-kinase, Akt, and mTOR Inhibitors in RCC

Daniel C. Cho and James W. Mier

Molecular Biology of the PI3-K/Akt/mTOR Pathway

The kinase mTOR is regulated in large part through the activity of phophatidylinositol 3-kinase (PI3-K) and Akt (protein kinase B). The PI3-K pathway regulates critical aspects of cell growth, metabolism, survival, and proliferation. In human malignancy, this pathway is one of the most frequently altered and plays a critical role in tumor cell growth, invasiveness, and metastatic behavior [1, 2].

Activation of PI3-K in RCC

Other than the clinical activity of mTOR inhibitors, one of the primary reasons for the interest in the PI3-K/Akt pathway in RCC is that it appears to be activated in a large percentage of RCC tumor specimens, and this activation is correlated with higher histologic grade and worse clinical outcomes [3]. Class IA PI3-K, the most relevant of the three classes of PI3-K to human cancer, are heterodimeric kinases consisting of a p85 regulatory subunit and a p110 catalytic subunit. There are three class IA p110 isoforms (α, β, and δ) encoded by three genes (*PIK3CA*, *PIK3CB*,

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and *PIK3CD*, respectively) and one related class IB p110 isoform (γ). Of these, the α and β isoforms are believed to be expressed ubiquitously, whereas the δ and γ isoforms are expressed only in the hematopoietic lineage [4]. While mutations in both the p110α subunit (*PIK3CA*) and p85 regulatory subunit have been described and can lead to constitutive activation of PI3-K, *PIK3CA* mutations are far more common [5, 6]. In renal tumors, *PIK3CA* mutations are quite rare [7], suggesting that the PI3-K activity observed in renal cell carcinoma (RCC) is due to alternate mechanisms.

The class IA PI3-K phosphorylate phosphatidylinositol-4,5-biphosphate (PIP2) generates phosphatidylinositol-3,4,5-triphosphate (PIP3). This activity is directly opposed by the tumor suppressor phosphatase and tensin homologue (*PTEN*), which dephosphorylates PIP3 to PIP2, reversing the activity of PI3-K. Therefore, another mechanism by which the PI3-K pathway can also be activated is by the loss of *PTEN* [8]. While somatic mutations in *PTEN* appear rare in RCC [9, 10], the expression of PTEN appears to be frequently downmodulated in RCC relative to normal renal tissue [11]. The molecular basis for this downmodulation is currently unknown. Regardless of mechanism, however, the relative absence of PTEN is thought to contribute to the PI3-K activity observed in these tumors.

Class IA PI3-K can also be activated through upstream signaling by receptor tyrosine kinases (RTK). Activation of RTKs, most commonly through growth factor signaling, results in the phosphorylation of the Y-X-X-M motif present in the cytoplasmic tail of the RTK, which then binds to the Src homology (SH2) domain of the p85 regulatory subunit of PI3-K. This results in a functional dissociation of the p85 subunit from the p110 subunit, augmenting kinase activity of the latter. Mutations in RTKs are exceedingly rare in RCC. However, it is likely that some RTKs may play a role in the basal PI3-K activity observed. For example, epidermal growth factor receptor (EGFR) is activated by transforming growth factor (TGF)-α and other EGFR ligands produced by the tumor cells in a hypoxia-inducible factor (HIF)-dependent manner [12]. It is likely that this autocrine loop contributes to the PI3-K activation frequently observed in RCC.

Activation of mTOR in RCC

mTOR exists in two functionally distinct complexes, TORC1 and TORC2, distinguished by their relative sensitivity to rapamycin. TORC1, a complex including mTOR and Raptor (regulatory-associated protein of mTOR), is sensitive to rapamycin and regulates many of the functions canonically associated with mTOR such as growth, proliferation, cap-dependent translation, and protein synthesis. The activity of TORC1 responds to numerous environmental signals, including the availability of oxygen, nutrients, ATP, and amino acids, some of which are transmitted through PI3-K. PI3-K mediates the activation of mTOR through its downstream effector, Akt, which phosphorylates tuberous sclerosis complex (TSC) 2 at multiple sites, causing it to disassociate from binding partner TSC1. The TSC1/TSC2 complex

functions as a GTPase-activating protein (GAP) for the G protein Rheb. PI3-K/Akt activation reduces the GAP activity of TSC1/TSC2 toward Rheb, allowing it remain in a GTP-bound state capable and activating TORC1. The activity of TSC1/TSC2 complex can be affected by numerous other pathways besides PI3-K/Akt including the AMP-LKB1 pathway and MAP-K [13, 14]. TORC1 activity is also regulated by TSC-independent inputs such as amino acid availability which modulates mTOR activity through the RAG GTPases [15]. Although mutations in various members of the mTOR regulatory pathways have been traditionally felt to be uncommon in RCC, data emerging from the Cancer Genome Atlas (TCGA) project will more accurately profile the frequency of these mutations in RCC tumor specimens. Indeed, the presence of activating mutations in mTOR and inactivating mutations in TSC1 and TSC2 has already been associated with prolonged responses to rapalogue therapy in patients with advanced RCC [16].

Biologic Consequences of PI3-K/Akt/mTOR Activation

Regardless of the mechanism by which it is activated, PI3-K signals to a vast network of kinases, transcription factors, and other proteins which promote cellular growth and proliferation. While the best described effector PI3-K is Akt (protein kinase B), there are several other PI3-K dependent pathways including those possibly relevant to cancer, such as serum and glucocorticoid kinases (SGKs) and Bruton tyrosine kinase (BTK) [17, 18]. However, Akt has traditionally been regarded as the primary executer of PI3-K and regulates the function of a broad array of proteins involved in cell growth, proliferation, motility, adhesion, neovascularization, and apoptosis [19]. Akt enhances cellular resistance to apoptosis by directly phosphorylating and inactivating several proapoptotic proteins, including procaspase 9, the bcl-2 family member BAD, and apoptosis signal-regulating kinase-1 (ASK1) [20–22]. Akt also differentially regulates transcriptional factors controlling expression of apoptotic genes, negatively regulating factors promoting expression of death associated genes (e.g., forkhead family members [FOXO]) and positively regulating genes promoting survival (NF-κB) [23, 24].

In addition to its pro-survival 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 enhances cyclin D1 levels through suppression of glycogen synthase kinase 3 (GSK3β) [25, 26]. GSK3β is also known to phosphorylate and promote the degradation of 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.

As discussed earlier, mTOR is activated downstream of Akt and executes its biologic functions in two distinct complexes, TORC1 and TORC2. TORC2, which

includes mTOR and Rictor (rapamycin-insensitive companion of TOR), is relatively insensitive to rapamycin and functions to enhance Akt activity by mediating its phosphorylation on the Ser473 residue. 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 activation of S6K by mTOR is critical for ribosomal biogenesis, cell growth, anti-apoptosis, and translation of structured 5′UTR containing mRNA species, while the phosphorylation (and inactivation) of 4E-BP1 promotes cap-dependent translation of nuclear mRNA by releasing the inhibition of eukaryotic translation initiation factor 4E (eIF4E).

In addition to stimulation of growth and proliferation, activation of the mTOR pathway may be of particular relevance to RCC because of its role in the regulation of the expression of both HIF-1α and HIF-2α. Inappropriate accumulation of HIF-1α and HIF-2α 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 [27, 28]. It has recently been suggested that the expression of HIF-1α is dependent upon the activity of both TORC1 and TORC2, while the expression of HIF-2α is dependent upon TORC2 activity alone [29]. While the overlap between the roles of HIF-1α and HIF-2α is poorly understood, it is generally accepted that HIF-2α is the more relevant HIF with respect to the development and progression of RCC [30, 31]. In fact, recent studies suggest that HIF-1α may function as a tumor suppressor in clear cell RCC [32]. Another recent study segregating *VHL*-deficient sporadic RCC into two subtypes, those expressing both HIF-1α and HIF-2α and those expressing HIF-2α alone, found no specimens expressing HIF-1α alone [33]. Thus the differential activation of TORC1 and TORC2 might play a critical role in RCC tumorigenesis and progression.

Clinical Results with mTOR Inhibitors

The rapalogues temsirolimus and everolimus have both demonstrated clinical efficacy in large randomized phase III trials in patients with advanced RCC. Temsirolimus is an intravenously administered analog of rapamycin. After showing promising activity in a phase II trial randomizing patients with metastatic RCC to three different doses [34], temsirolimus was assessed in a randomized three-arm phase III trial comparing temsirolimus alone versus IFN-α alone versus the combination [35]. As the phase II study suggested potentially unique efficacy in patients with poor prognostic features in a retrospective analysis, the phase III study enrolled only patients with metastatic RCC and ≥3 of 6 risk factors (5 MSKCC risk factors [Karnofsky PS <80, time from diagnosis to randomization <12 months, serum LDH >1.5 ULN, hemoglobin <LLN, corrected serum calcium >10 mg/dl] + >1 metastatic site). Overall, 626 previously untreated patients were enrolled and randomized in a 1:1:1 fashion to receive IFN-α alone (3 million units three times weekly), temsirolimus alone (25 mg IV weekly), or the combination (temsirolimus 15 mg weekly and

6 million units IFN-α three times weekly). The overall survival of patients treated with temsirolimus alone was statistically longer than those treated with IFN-α alone (7.3 versus 10.9 months; 0.73 hazard ratio, $p = 0.0069$). There was no statistical difference between patients treated with IFN-α alone and the combination of IFN-α and temsirolimus. Overall, temsirolimus was well tolerated with the most common adverse effects being asthenia, rash, anemia, nausea, peripheral edema, hyperlipidemia, and hyperglycemia. Based on these findings, temsirolimus was approved by the FDA for therapy in advanced RCC on May 30, 2007, and is now considered a standard therapeutic option in the first-line setting for patients with poor prognosis features.

Everolimus is an orally administered rapalogue and was assessed in a randomized, double-blind, placebo-controlled phase III trial in patients with advanced RCC who had failed prior treatment with either sorafenib, sunitinib, or both (other prior therapy also allowed) within the preceding 6 months (*RE*nal Cell cancer treatment with *O*ral *R*AD001 given *D*aily-1 [RECORD-1]) [36]. Overall, 416 patients were enrolled and randomized in a 2:1 fashion to receive either everolimus ($n = 277$) or placebo ($n = 139$) each together with best supportive care. The primary end point was PFS as randomization was unblinded at time of progression, and patients on placebo were allowed to crossover to open-label everolimus, confounding any potential differences in overall survival. The trial was halted at the second interim analysis after 191 progression events had been observed. At the final central radiology assessment, the median PFS for patients treated with everolimus was 4.88 months as compared with 1.87 months in the placebo group (hazard ratio 0.33, [95 % CI 0.25–0.43] $p < 0.0001$) [37]. Five patients (2 %) in the everolimus group experienced partial responses versus none in the placebo group. Similar to temsirolimus, the side effect profile of everolimus was favorable with most common adverse events with everolimus being stomatitis (40 %), rash (25 %), fatigue (20 %), hypercholesterolemia (76 %), hypertriglyceridemia (71 %), and hyperglycemia (50 %). Pneumonitis was observed in 22 patients (8 %) compared with 0 in the placebo group. Based on these findings, everolimus was approved by the FDA in March 2009 for the treatment of patients with advanced RCC who failed either sorafenib, sunitinib, or both and is now considered a standard second-line therapeutic option following the failure of VEGF-targeted TKI.

Special mention should be made of the potential efficacy of the rapalogues in non-clear cell RCC. Of the molecularly targeted agents, only temsirolimus has been studied in a randomized phase III trial allowing patients with non-clear cell histology [35]. Upon sub-analysis of this phase III trial, among the 73 patients with non-clear cell histology (75 % of which had the papillary subtype) randomized to receive either temsirolimus ($n = 36$) or IFN ($n = 37$), the median overall survival of patients was 11.6 months in the temsirolimus group versus 4.3 months in the IFN group [38]. For this reason, temsirolimus is the only agent given a category 1 recommendation by the National Comprehensive Cancer Institute (NCCN) for the treatment of patients with metastatic non-clear cell RCC. Studies comparing the efficacy of the rapalogues in comparison to VEGFR-TKI in patients with non-clear cell RCC are underway and should better characterize the efficacy of these agents in this group of patients.

Predictive Biomarkers

Unfortunately, only a subset of patients experience substantial clinical benefit from treatment with rapalogues. Therefore, the therapeutic index of this class of agents might be enhanced by the development of patient selection strategies to direct these drugs to the patients most likely to benefit. As with other molecularly targeted agents, however, there are currently no clinically validated predictive clinicopathologic features or biomarkers of benefit from therapy with mTOR inhibitors. Although temsirolimus has demonstrated specific efficacy in patients with poor-risk MSKCC features, the same finding has yet to be observed with everolimus, raising questions as to whether this is a class effect of all mTOR inhibitors. Several lines of evidence suggest, however, that treatment outcome is likely to be determined by the particular genetic alterations and signaling pathways activated in individual tumors.

Many studies have suggested the familiar paradigm that the pretreatment activation status of PI3-K/Akt/mTOR signaling may be a predictor of the likelihood of response to agents targeting this pathway [39]. For example, in a small study carried out in parallel with a recent phase II trial of temsirolimus in patients with RCC, a correlation between tumor cell Akt and S6 phosphorylation as defined by immunohistochemistry and clinical response was demonstrated [40]. The significance of this study is limited because of its retrospective nature and the small number of tumors examined. This study was also limited by the reliance on immunohistochemistry which is associated with an inherent subjectivity in interpretation and also dependent upon the availability of reliable antibodies against the substrates of interest. Many investigators are now moving toward genetic predictors of mTOR pathway activation as a more objectively determined biomarker. In a recent study reported by Voss et al., specimens from six patients treated with rapalogues who were felt to be robust responders were analyzed by directed sequencing [16]. It was subsequently found that two cases had mutations in TSC1, one case with a mutation in TSC2, and one case with a mutation in mTOR. Data from The Cancer Genome Atlas (TCGA) project will soon be emerging and will provide valuable information on the expected frequency of mutations expected to result in constitutive mTOR activation in RCC and shed light on the feasibility of the correlation of such mutations to clinical response in a broader sample of patients.

Novel Inhibitors of PI3-K, Akt, and mTOR

In addition to identifying predictive biomarkers, efforts to improve upon the therapeutic index of rapalogues have also focused on developing more effective drugs targeting this pathway. As discussed earlier, although mTOR is a validated therapeutic target in RCC, it is but one of many kinases governed by PI3-K and Akt, which activate several other downstream signaling pathways essential for energy generation, protein synthesis, proliferation, and cell survival. It is clear that there are several mechanisms by which TORC1 inhibition is felt to potentially result in the feedback activation of PI3-K and Akt, including via release a feedback loop involving

the IGF-1 receptor and derepression of TORC2 resulting in TORC2-mediated phosphorylation of Akt on Ser^{473} [41, 42]. The feedback activation of PI3-K may directly undermine the efficacy of TORC1 inhibitors by promoting the phosphorylation of eIF4E by Mnk1, thereby enhancing its affinity for the mRNA cap structure and activating cap-dependent translation [43]. Therefore, inhibition of PI3-K or Akt has emerged as a therapeutic strategy that may negate activation of this feedback loop and more effectively suppress the translation of critical mRNAs.

Another pharmacologic approach worthy of investigation is the direct inhibition of the catalytic domain of mTOR. Such an approach has the advantage of inhibiting the kinase activity of mTOR regardless of whether it is in a complex with Raptor (TORC1) or Rictor (TORC2). As noted earlier, the expression of HIF-2α (the dominant HIF in RCC) is largely dependent upon the activity of TORC2 and independent of TORC1 activity. As such, this therapeutic approach may have advantages to the allosteric inhibition of TORC1 alone and have particular relevance to RCC.

Many agents are currently in development which are pan-isoform inhibitors of PI3-K, inhibitors of Akt (both catalytic and allosteric), and dual inhibitors of PI3-K and mTOR. Preclinical studies with PI3-kinase inhibitors in RCC have supported the hypothesis that these agents may have activity in RCC. Inhibition of PI3-K/Akt signaling by PI3-K 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) [44]. Treatment of nude mice bearing RCC xenografts derived from the 786-O cells with LY294002 resulted in up to 50 % reduction in tumor size. Similarly, the treatment of nude beige mice bearing RCC xenografts with NVP-BEZ235, a dual inhibitor of PI3-K/mTOR, resulted in significantly greater suppression of tumor growth compared with either rapamycin or vehicle [45]. This suppression of tumor growth was correlated with reduced markers of proliferation (Ki67 staining) and modest induction of markers of apoptosis (cleaved caspase 3 staining), as well as suppression of the expression of HIF-2α and cap-dependent gene products such as cyclin D1. Together, these preclinical studies have suggested that PI3-K/Akt may be a relevant therapeutic target in RCC and provided the rationale for the clinical assessment of novel agents targeting this pathway.

One of the earliest such agents to be assessed in RCC was perifosine, an orally available alkylphospholipid which prevents Akt activation by blocking its pleckstrin homology domain-dependent recruitment to the cell membrane. Perifosine was recently assessed in two independent phase II trials in patients with advanced RCC who had failed prior targeted therapy [46]. In Perifosine 228, 24 patients with advanced RCC who had progressed after prior therapy with VEGF-targeted agents and/or cytokines were enrolled and treated with perifosine at 100 mg once daily. In Perifosine 231, 50 patients with advanced RCC were enrolled into two groups and treated with perifosine at a dose of 100 mg once daily. Group A included patients who failed a VEGFR-TKI but not on an mTOR inhibitor, whereas group B included patients who failed both targeted agents. In the combined analysis of 74 patients on both trials, six patients experienced a partial response (ORR 8 %), and the median PFS was 14 weeks [95 % CI (12.8, 20.0)]. The most common toxicities were fatigue, musculoskeletal pain, diarrhea, and nausea. Although perifosine had

Table 12.1 Ongoing trials with novel PI3-K/Akt inhibitors in RCC

Agent	Mechanism of action	Trial
MK2206	Allosteric inhibitor of Akt	A randomized phase 2 study of MK-2206 in comparison with everolimus in refractory renal cell carcinoma (NCT01239342)
GDC-0980	Pan-isoform inhibitor of PI3-K and catalytic inhibitor of mTOR	A phase II, open label, randomized study of GDC-0980 versus everolimus in patients with metastatic renal cell carcinoma who have progressed on or following VEGF-targeted therapy (NCT01442090)
BEZ-235	Pan-isoform inhibitor of PI3-K and catalytic inhibitor of mTOR	A phase 1b/2 study of BEZ235 in patients with advanced renal cell carcinoma (RCC) (NCT01453595)

clear clinical activity in RCC, it was felt that this activity was not superior to currently available agents, and this agent was not worthy of further development as a single agent in RCC.

The lack of robust clinical activity seen with perifosine has not muted the enthusiasm for PI3-K/Akt as a therapeutic target in RCC, however. Perifosine is an indirect inhibitor of Akt. As mentioned earlier, more reliable inhibition of this pathway may be achieved with the catalytic inhibitors of PI3-K/mTOR or with direct inhibitors of Akt (both catalytic and allosteric). Not surprisingly, several clinical trials with novel inhibitors of PI3-K/mTOR and Akt are underway as shown in Table 12.1. It is hoped that the results from these clinical trials may provide further validation of the PI3-K/Akt pathway as a therapeutic target in RCC.

Conclusion

The clear clinical activity of the rapalogues has established the relevance of the mTOR pathway in renal cell carcinoma. Enhanced understanding of the biology of this pathway has facilitated the identification of both potential predictive biomarkers of response and novel therapeutic strategies. Efforts must remain focused on identifying the subset of patients who derive the most clinical benefit from agents targeting this pathway. At the same time, agents that might prove superior to rapalogues are in active clinical development in RCC. The hope remains that the concurrent development of both patient selection strategies and better drugs will result in improved clinical outcomes for patients with advanced RCC.

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Chapter 13
Carbonic Anhydrase IX and Monoclonal Antibody G250: Relevance as a Clinical and Biologic Target in Renal Cell Carcinoma

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Introduction

Carbonic anhydrase IX (CAIX) is a member of the carbonic anhydrase group of enzymes, has a transmembrane as well as a cytosolic domain, and catalyzes the reaction: $CO_2 + H_2O \leftrightarrows HCO^-_3 + H^+$. These enzymes are critical in the regulation of proton flux in cells and thus in pH regulation [1]. In contrast to most carbonic anhydrases, the catalytic site of CAIX is located extracellularly, where it is involved in creating an acidic microenvironment [2]. This N-glycosylated single-pass transmembrane protein also contains an N-terminal proteoglycan-like domain that functions in cell adhesion and CAIX can be shed by stimulus-dependent activation of metalloproteinase activity [3–5].

Extensive molecular studies of the CAIX promoter region demonstrated that HIF-1α binding was an absolute requirement for CAIX expression [6, 7]. This finding uncovered a direct molecular link between the observed CAIX expression in clear cell renal cell carcinoma (ccRCC), the most prominent type of RCC, and the

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molecular events leading to ccRCC. Elegant molecular studies in families suffering from the von Hippel–Lindau (VHL) syndrome, an autosomal dominant disease, showed that defects in the VHL gene were responsible for tumor development [8]. These patients develop multiple tumors during their lifetime and almost invariably develop multiple ccRCC. Multiple studies have demonstrated that also in sporadic ccRCC, aberrations in VHL are present [9]. Aberrant VHL leads to upregulation of hypoxia-inducible genes as VHL acts as an E3 ubiquitin ligase catalyzing the polyubiquitylation of prolyl-hydroxylated HIF-1α for subsequent degradation via the 26S proteasome [10, 11]. If pVHL is mutated as in ccRCC, HIF-1α is not degraded but associates with the constitutively stable partner HIF-1β to form an active heterodimeric HIF-1 transcription factor, which binds to hypoxia-responsive elements located in the promoter/enhancer regions of numerous hypoxia-inducible genes. In view of the HIF-1α dependency of CAIX expression, the ubiquitous expression of CAIX is the functional consequence of a nonfunctional VHL gene product in ccRCC. CAIX expression in non-ccRCC malignancies also leads to CAIX expression, but there it is the consequence of hypoxia [12]. Besides by mutation, the VHL suppressor gene can also be silenced by promoter methylation [13].

It has become apparent that the genetic landscape of ccRCC is more complex and several new cancer-associated genes have been identified in ccRCC [14–16]. In addition, frequent mutations of genes encoding ubiquitin-mediated proteolysis pathway component have been identified in ccRCC [17]. Theoretically, these also lead to HIF1α stabilization and CAIX expression. Thus, CAIX expression can also occur in the absence of VHL mutations or VHL gene silencing.

Although one might extrapolate that all metastatic sites derived from CAIX-positive ccRCC express CAIX (as a molecular defect underlies CAIX expression), this may not be the case; lower CAIX staining levels in metastatic lesions relative to matched primary tumor specimens have been demonstrated [18]. The loss of CAIX is most likely a reflection of a more aggressive phenotype as tumors progress. Nevertheless, CAIX has emerged as an attractive target for ccRCC.

Monoclonal antibodies have revolutionized the clinical landscape of many diseases, and the list of therapeutic, diagnostic, and preventive mAbs is impressive. In an era where personalized medicine is the new norm, mAbs, with their predefined target specificity, are ideal to personalize therapy based on geno- or phenotype. The widespread use of mAbs is the culmination of the concept of Paul Ehrlich proposed at the end of the nineteenth century. He aimed to find chemical substances which have special affinities for pathogenic organisms, to which they would go, as antitoxins go to the toxins to which they are specifically related, and would be, as Ehrlich expressed it, “magic bullets” which would go straight to the organisms at which they were aimed.

In 1975 Kohler and Milstein described the first production of mouse hybrid cells producing mAbs [19]. A year earlier Schwaber and Cohen had already described the production of human–mouse hybrid cells [20]. In 1984 Kohler, Milstein, and Jerne shared the Nobel Prize in Physiology or Medicine for the discovery. The mAbs, with their predefined specificity, were heralded as the long sought after magic bullets described by Ehrlich. More than 200 mAbs are now on the market or under investigation,

a clear sign of the success of mAbs. The first FDA-approved therapeutic mAb was OKT3 (muromonab), a murine CD3-specific mAb used in organ transplant procedures.

The use of molecular biology technologies has led to a whole range of mAbs, ranging from murine mAbs (-oab), to antibodies where the constant region is replaced with the human form (chimeric mAbs, -ixmab), to antibodies where everything is replaced by human sequences except the complementary determining regions (CDRs), resulting in humanized mAb (-zumab) to partly chimeric and partly humanized antibodies (-xizu). Additionally pure human antibodies have become available (-umab). Moreover, various antibody formats are available, ranging from whole Ig to F(ab′)2, Fab′, single-chain Fv fragments (scFv), dimeric scFv, single-domain antibody, and trifunctional antibodies and bispecific T-cell engagers.

Already from the early days of mAb development, investigators have been highly interested in the development of mAbs with specificity for renal cell carcinoma (RCC) [21–25]. This interest was based on observations that (a) some RCC patients experienced spontaneous remission, (b) immunotherapy with high-dose interleukin-2 was effective in a subset of patients, (c) polyvalent sera could discriminate between normal kidney tissue and renal cancer, and (d) "RCC-specific" cytotoxic T cells could be isolated. Collectively these observations suggested the presence of (a) RCC-specific target(s) that might be used for diagnostic or therapeutic purposes. Moreover, effective therapy for metastatic RCC patients was lacking, and RCC-specific mAbs were seen as highly attractive new therapeutic modalities for this deadly disease. With few exceptions the described mAbs recognized kidney-associated molecules.

The most extensively studied mAb in RCC is mAbG250, initially described in 1985 [26]. The antibody was isolated from the spleen of mice immunized with a fresh human RCC, a procedure that differed from most other studies where mice were immunized with cell line homogenates. The target antigen of mAbG250 was not known, but the mAb showed tumor specificity, whereas the cross-reactivity with other tissues seemed to be restricted. Subsequent more detailed fine-specificity analysis revealed slight reactivity with somewhat more tissues, albeit that antigen expression was low [27]. The occurrence of the target antigen in RCC where no expression in normal kidney could be detected suggested that induction was inherently related to tumor development, possibly due to a common initiating event. In view of the expression in non-kidney tissues, the target was clearly not related to aberrantly expressed embryonic antigens.

Initially no association with a particular histological RCC subtype was noted, but now it has become clear that the antigen recognized by mAbG250 is almost ubiquitously expressed in ccRCC. In the initial analysis, non-RCC tumors revealed variable staining for which no satisfactory explanation was given other than that the event common to the initiation of the expression of the recognized G250 antigen in RCC might also play a role in these non-RCC tumors.

Importantly, the investigators showed that metastatic RCC still expressed G250 antigen. Albeit that the number of RCC metastases tested was limited, the study suggested that mAbG250 might have value for the diagnosis or therapy of RCC.

The general expression of G250 in RCC and its absence from normal kidney suggested that induction was inherently related to tumor development, possibly due

to a common initiating event [26]. Cloning of the antigen recognized by mAbG250 showed that mAbG250 recognized a conformational determinant of CAIX, a gene originally identified in Hela cells [3, 4].

CAIX as Biomarker

Several studies have shown that CAIX expression in ccRCC has prognostic value. Low CAIX expression in primary ccRCC was described as an adverse predictor of survival, and in the multivariate analysis, CAIX expression remained an independent prognostic parameter [18]. These findings were confirmed in an independent series showing that CAIX expression was an independent predictor of survival [28]. In yet another retrospective study, CAIX was identified as an independent prognostic marker, and additionally, high CAIX expression was correlated with a threefold higher response rate to immunotherapy [29]. When CAIX expression was combined with VHL mutation status, this allowed further stratification: patients with high CAIX expression combined with VHL gene mutation had the most favorable prognosis, whereas low CAIX expression without VHL gene mutation gave the most adverse prognosis [30].

In contrast to the former studies, Leibovich et al. were unable to confirm these results. Although low CAIX expression was associated with death from ccRCC in univariate analysis, this association failed to reach statistical significance in the multivariate analysis, when death from ccRCC and low CAIX expression were corrected for nuclear grade, tumor necrosis, and sarcomatoid differentiation [27, 31]. However, the patient population differed significantly: only 11 % mRCC patients were included, in contrast to 40–60 % mRCC patients in the other studies.

Recently, CAIX polymorphisms were analyzed in 54 mRCC tissue samples to investigate their possible value as biomarker. The single nucleotide polymorphism rs12553173 in CAIX and CAIX expression both proved to be independent prognostic factors for overall survival, and both were associated with a greater likelihood of response to IL-2 immunotherapy [32]. Collectively, many studies suggest that CAIX is an independent prognostic marker in RCC with a correlation between low CAIX expression and death from ccRCC.

In an effort to improve the nomogram for RCC patients, several investigators have studied a number of molecular markers, including CAIX [33, 34]. Inclusion of the biomarkers improved the prognostic accuracy as compared to the TNM ($C=0.79$ vs. $C=0.73$; $p<0.001$) and UCLA integrated staging system (UISS) alone [34] ($C=0.79$ vs. $C=0.75$; $p=0.038$). A nomogram designed to predict the survival of mRCC patients based on the combination of biomarkers and clinical parameters proved to be a more accurate prognostic model for mRCC patients as compared to the UISS staging system ($C=0.68$ vs. $C=0.62$; $p=0.0033$) [33].

Despite overwhelming evidence that CAIX can be used as a reliable biomarker, incorporation of CAIX expression in nomograms has been complicated due to the lack of commercial availability of the mAb M75, needed for the immunohistochemical

detection of CAIX in tissue samples. Commercially available mAb NB100-417 is reported to be a CAIX-specific mAb with similar staining characteristics as M75 [35], but at high dilutions, cross-reactivity with beta-tubulin was observed [36] making specific CAIX detection by mAb NB100-417 doubtful. Incorporation of CAIX expression in nomograms improves prognostic accuracy, but since mAb M75 is not commercially available, widespread use is not yet possible.

In 2005 Atkins et al. reported that CAIX expression could serve as a predictor of response to IL-2 immunotherapy. Of 66 patients who had received IL-2 immunotherapy, 21 of 27 (78 %) responding patients had high CAIX-expressing primary tumors compared with 20 of 39 (51 %) nonresponding patients ($p = 0.04$). Moreover, median survival was prolonged in the high CAIX expression group ($p = 0.03$) and survival >5 years was only seen in patients with high CAIX-expressing tumors [37]. These findings were confirmed in a group of 62 tumors of mRCC patients who underwent nephrectomy and were subsequently treated with IL-2. Besides a correlation between high CAIX expression and response to immunotherapy ($p = 0.04$), a significant association between response to immunotherapy and high COX-2 intensity and distribution in RCC ($p < 0.001$), normal hemoglobin ($p = 0.05$), and corrected serum calcium level ≤10 mg/dl ($p = 0.007$) was described. In another study, high CAIX expression (>85 % CAIX expression) was associated with IL-2 response, but the difference did not reach statistical significance ($p = 0.070$) [32]. Interestingly, all four complete responses were observed in patients with high CAIX. The correlation between high CAIX expression and greater IL-2 response rate could not be confirmed in the prospective, nonrandomized trial ("SELECT" trial) [38].

The implementation of various tyrosine-kinase inhibitors (TKI) and mammalian target of rapamycin (mTOR) inhibitors has led to studies investigating whether a correlation between CAIX expression and TKI response exists. Currently patient stratification for TKI/mTOR treatment is based on parameters determined in the cytokine era, and these may not be entirely applicable to mRCC patients who are receiving targeted therapy.

Evaluation of the possible prognostic utility of CAIX expression in patients treated with VEGF-targeted therapy showed that CAIX expression was not associated with a response to sunitinib or sorafenib treatment [39]. High tumor CAIX expression was associated (but not statistically significant) with a superior tumor shrinkage rate, and a predictive value of tumor CAIX expression for sorafenib therapy was suggested [39]. In a preliminary analysis, the authors suggested that a benefit of sorafenib in terms of tumor shrinkage and progression-free survival relative to placebo might exist for patients with high CAIX-expressing tumors. However, in the final study, CAIX expression was not predictive of clinical benefit in patients receiving sorafenib vs. placebo [40]. In a side study of a randomized phase II trial where patients received varying doses of temsirolimus [41], no correlation between CAIX or HIF-1α expression and response to temsirolimus treatment was seen [42]. These results need to be viewed with caution as this was a highly inhomogeneous patient population, with many patients with poor prognosis or with non-ccRCC. At this time it is unclear whether CAIX expression for the response to TKI or mTOR has

any value in the mRCC patient population, but thus far the evidence suggests that CAIX expression in the primary RCC does not correlate with clinical outcome.

RT-PCR analyses of patients' blood samples have shown the possibility to detect the presence of circulating CAIX-expressing cells [43]. Nested RT-PCR with improved primers, eliminating PCR signals in blood obtained from unaffected individuals, allowed detection of CAIX-positive cells, albeit that formal evidence that circulating tumor cells were detected was lacking [44]. The sensitivity (32/42, 76.2 %) and specificity (21/31, 77.8 %) of the CAIX RT-PCR were relatively low, and investigations along these lines have not been pursued.

Studies investigating the value of CAIX serum levels to monitor ccRCC are also limited. Serum CAIX levels are significantly higher in ccRCC patients than in non-ccRCC patients [45] and correlate with tumor size [46]. Not unexpected, mean serum CAIX levels have been shown to be significantly higher in patients with metastatic RCC than in those with localized disease. Importantly, recurrence-free survival of patients with localized disease and significantly higher serum CAIX level was lower than those with low serum CAIX levels, suggesting that serum CAIX levels increase as the tumor progression occurs [47]. However, serum CAIX levels did not correlate with survival [48]. Remarkably, CAIX serum levels of ccRCC and chromophobe RCC were similar [48], although CAIX expression in this RCC subtype has been reported to be absent [49]. Despite solid evidence that serum CAIX levels can be measured adequately and appear to correlate with tumor burden, studies investigating the correlation between CAIX serum levels in relation to TKI/mTOR treatment and response have not been performed yet.

CAIX Imaging

Conventional imaging methods (CT, MRI, and plain X-ray) are generally used to depict RCC, but reliable distinction of malignant and benign solid renal lesions by conventional imaging modalities is difficult, posing clinicians for diagnostic problems. With approximately 50 % of all renal lesions found incidentally, differentiation between benign and malignant lesions is even more crucial. Ultrasound (US)- and CT-guided biopsies have a relatively high sensitivity and specificity [50, 51], but these invasive procedures require careful observation of the patient. ^{18}F-Fluorodeoxyglucose-positron emission tomography (FDG-PET) of RCC is also difficult, because the excretion of FDG via the kidneys results in relatively high background activity. Moreover, FDG-PET imaging cannot distinguish ccRCC from angiomyolipoma, pericytoma, and pheochromocytoma [52]. Nevertheless, characterization of suspect lesions is essential to avoid invasive biopsies and superfluous surgery, both in localized and advanced disease.

The almost ubiquitous expression of CAIX in ccRCC was immediately recognized as an important asset for a potential diagnostic imaging agent. Already in the first phase I clinical trial with murine mAbG250, the clarity of mAbG250 imaging was noted, as well as its possibility to image primary as well as metastatic RCC [53].

Remarkably, in one patient, mAbG250 accumulation in a multicystic lesion was noted, whereas MRI did not indicate any malignancy. However, upon surgery, ccRCC was detected. In this protein dose-escalation study, the pharmacokinetics, toxicity, immunogenicity, and imaging characteristics of ^{131}I-mAbG250 were determined, and clear antibody targeting was observed in patients with CAIX-positive tumors at the optimum protein dose of 5–10 mg [53]. The mean %ID/g of liver uptake was dose dependent, and the liver uptake appeared to be saturable and did not hamper the image quality as with higher-protein doses liver uptake became invisible as only a small percent of the labeled material ended up in the liver compartment. This liver uptake was attributed to the CAIX expression in the larger bile ducts.

Although low amounts of circulating CAIX are present in ccRCC patients [46, 47], this did not appear to hamper ccRCC targeting. The mean levels of circulating CAIX are 100–200 pg/ml serum [46], albeit that levels in mRCC patients can be as high as 4,000 pg/ml serum [48]. Thus, microgram quantities of mAbG250 would be needed to completely deplete circulating CAIX. Moreover, part of the circulating CAIX is a shed form of CAIX, which is rapidly cleared [54]. Therefore, it is unlikely that part of the observed liver uptake was due to complexed antibody/antigen.

Human anti-mouse antibodies (HAMA) were detected in all from eight patients obtained 2 weeks to 5 months after infusion of the antibody, without any relation to protein dose and HAMA occurrence [53].

The vast majority of studies have been performed with a chimerized version of mAbG250, cG250 (girentuximab, Rencarex®), as administration of the murine mAb resulted in high human anti-murine antibody titers, preventing multiple administrations [55].

In phase I protein dose-escalation trial with ^{131}I-girentuximab, all patients with CAIX-expressing tumors showed excellent girentuximab targeting to all known tumor sites. Similar to murine mAbG250, previously undetected metastatic lesions (brain, bone, and soft tissue) were detected. The performance of girentuximab mimicked the murine mAbG250: the optimal protein dose was similar (5–10 mg) and very high focal uptake was observed (up to 0.52 %ID/g). The half-life ($t½$ β) of girentuximab was longer than murine mAbG250 (68.5 h vs. 47 h). ^{131}I-girentuximab uptake in non-tumor tissues remained low. Most importantly, chimerization greatly diminished the immunogenicity of the antibody: in only 2 of 15 patients, low levels of human anti-chimeric antibody (HACA) were observed. Thus, multiple administrations became feasible [56].

Several clinical trials have demonstrated the possibility to detect CAIX-expressing ccRCC lesions using girentuximab labeled with ^{131}I, ^{124}I, or ^{111}In (Table 13.1). Remarkably, direct ^{131}I-labeled girentuximab was inferior to FDG-PET in detecting ccRCC metastases [57]. Routine imaging analysis had revealed 79 metastases in 20 patients, and 33 previously unknown lesions were detected by FDG-PET plus girentuximab. Surprisingly, only 34/112 lesions were visualized by girentuximab, whereas FDG-PET detected 77/112 lesions. Particularly intrathoracic and intra-abdominal lesions were poorly visualized by girentuximab (13/69 lesions). This contrasts with all other performed trials that demonstrated adequate girentuximab targeting and

Table 13.1 Renal Cell Carcinoma Imaging Studies: G250 Monoclonal Antibody

Reference	Year	Agent	Number of patients	Patients	Outcome[a]	Duration of response	Remarks[b]
Oosterwijk et al. [53][b]	1993	^{131}I-mG250	15	Primary RCC	12/12 pts imaged	NA	Phase I, dose escalation
Steffens et al. [56][b]	1997	^{131}I-girentuximab	16	Primary RCC	13/13 pts imaged	NA	Phase I, dose escalation
Steffens et al. [91][b]	1999	^{131}I-girentuximab and ^{111}In-girentuximab	10	Primary RCC	10/10 pts imaged	NA	Dual-label study
Brouwers et al. [57][b]	2002	^{131}I-girentuximab vs. ^{18}F-FDG	20	M+RCC	^{131}I-girentuximab: 34/112 lesions detected ^{18}F-FDG: 77/112 lesions detected	NA	Comparative intrapatient study
Brouwers et al. [58][b]	2003	^{131}I-girentuximab and ^{111}In-girentuximab	5	M+RCC	^{111}In-girentuximab: 47 lesions detected ^{131}I-girentuximab: 30 lesions detected	NA	Comparative intrapatient study
Divgi et al. [59][b]	2007	^{124}I-girentuximab	26	Primary RCC	15/16 ccRCC imaged	NA	Prospective girentuximab immunoPET
Divgi et al. [61][b]	2013	^{124}I-girentuximab	226	Primary RCC	124/143 ccRCC imaged (sens. and spec. 86 %)	NA	Phase III, REDECT trial
Muselaers et al. [89][b]	2013	^{111}In-girentuximab	29	Primary RCC	15/16 ccRCC imaged	NA	^{111}In-girentuximab immunoSPECT
Divgi et al. [79][c]	1998	^{131}I-mG250	33	M+RCC	17 SD; 16 PD	2–3 mo	Phase I/II
Steffens et al. [92][c]	1999	^{131}I-girentuximab	12	M+RCC	1 PR; 1 SD; 10 PD	9+; 3–6 mo	Phase I activity dose
Divgi et al. [81][c]	2004	^{131}I-girentuximab	15	M+RCC	7 SD; 8 PD	2–11 mo	Phase I fractionated dose

Brouwers et al. [82][c]	2005	^{131}I-girentuximab Two doses	27	M+RCC	5 SD; 22 PD	3–12 mo	Phase I two high doses
Stillebroer et al. [86][c]	2012	^{177}Lu-girentuximab Multiple doses	23	M+RCC	1 PR; 17 SD	9+; 3+ mo	Phase I dose escalation
Davis et al. [72][c]	2007	Girentuximab	12	M+RCC	1 CR; 8 SD; 3 PD	6–66 wk	Phase I
Bleumer et al. [73]	2004	Girentuximab	36	M+RCC	1 CR; 1 PR; 8 SD; 26 PD	1–20+ wk	Phase II
ARISER [78][c]	2013	Girentuximab	864	High risk, after nephrectomy	No benefit[d]		Phase III
Davis et al. [74][c]	2007	Girentuximab + IL-2	9	M+RCC	2 SD; 7 PD	6, 12 wk	Phase I
Bleumer et al. [75][c]	2006	Girentuximab + IL-2	35	M+RCC	1 PR; 7 SD; 27 PD	95+; 24+ wk	Phase II
Siebels et al. [77][c]	2011	Girentuximab + IFN-2α	31	M+RCC	1 CR; 9 SD	17+; 24+ wk	Phase II

[a]Outcome refers to diagnostic accuracy, i.e., number of positive images over total number of images (imaging trials) or response (therapy trials)
[b]Imaging studies
[c]Therapy studies
[d]High-risk patients with high CAIX expression appeared to benefit
mG250 murine monoclonal antibody G250, *girentuximab* chimeric monoclonal antibody G250, *ccRCC* clear cell renal cell carcinoma, *M+RCC* metastatic renal cell carcinoma, *IL-2* interleukin-2, *IFN* interferon, *^{18}F-FDG* fluorine-18 fluorodeoxyglucose, *PET* positron emission tomography, *SPECT* single-photon emission CT, *CR* complete response, *PR* partial response, *SD* stable disease, *PD* progressive disease, *mo* months, *wk* weeks

excellent imaging of RCC irrespective of the anatomical site. Patient bias may explain this difference as many patients were heavily pretreated [57].

When radionuclides with more suitable imaging characteristics were used, ^{111}In-labeled girentuximab was superior to ^{131}I in terms of imaging characteristics and increased tumor-to-blood ratios: with the ^{111}In-labeled tracer, 47 lesions were detected, compared to 30 with the ^{131}I-labeled tracer [58].

In addition to SPECT tracers, the PET tracer ^{124}I-girentuximab was extensively investigated to study whether the presence of ccRCC could be predicted preoperatively. This is of particular importance because almost 50 % of renal lesions are detected incidentally in the course of nonrenal cancer-related imaging. Approximately 30 % of incidentalomas are benign and surgery is unnecessary. Adequate presurgical stratification of patients might therefore reduce unnecessary surgery. In the proof-of-concept phase I trial in 26 patients scheduled for nephrectomy, 15 of total 16 ccRCC lesions were detected, resulting in a sensitivity of 94 %, high specificity (100 %, CI 66–100), and high negative (90 %, CI 55–100) and positive (100 %, CI 78–100) predictive values. The authors concluded that ^{124}I-girentuximab immunoPET can identify ccRCC accurately and help in clinical decision-making when dealing with renal masses of uncertain origin [59]. The significant correlation between the PET measurements and autoradiography of the surgical specimens suggested that immunoPET may be useful in quantitatively assessing antigen targeting by girentuximab-based therapies [60]. In a large multicentre study comparing the diagnostic accuracy of ^{124}I-girentuximab, PET/CT with diagnostic CT for the detection of ccRCC in presurgical patients with renal masses has confirmed the high accuracy of ^{124}I-girentuximab as the reported sensitivity (86 %, CI 75–97) and specificity (86 %, CI 69–100) were markedly higher than those of conventional CT [61, 62]. ^{124}I-girentuximab imaging was performed 2–6 days after injection and CT scanning was performed within 48 h of ^{124}I-girentuximab PET/CT. Currently a new trial is planned that aims to demonstrate that surgery can be delayed or avoided based on negative girentuximab scans.

The better spatial resolution of PET as compared to SPECT may allow more exact visualization and visualization of smaller lesions with PET. However, the performance of ^{111}In-girentuximab and that of ^{124}I-girentuximab has not been directly compared to date. Animal data suggest better delineation of lesions is possible with ^{89}Zr-labeled girentuximab immunoPET as compared to ^{111}In-girentuximab immunoSPECT [63].

It has proven problematic to assess clinical responses induced by TKI and mTOR inhibitors by RECIST. In view of the excellent imaging capabilities of girentuximab, girentuximab imaging is now being evaluated as possible therapy evaluation modality (NCT01582204). Sorafenib treatment resulted in a markedly decreased uptake of ^{111}In-girentuximab (NCT00602862) [64]. This decrease was not related to decreased CAIX expression, as sorafenib treatment did not affect CIAX expression. This decrease is most likely caused by the destruction of the tumor vasculature, resulting in a reduced delivery and tumor penetration of the antibody. This conclusion was substantiated by animal studies demonstrating a dramatic decrease in antibody accumulation and tumor penetration after TKI treatment [65]. Although information about the

effect of other TKI on antibody distribution and accumulation in humans is lacking, it is not unlikely that a similar effect can be expected with sunitinib and pazopanib, in view of the same mode of action of these antiangiogenic drugs. Preclinical animal experiments suggest increased antibody uptake after discontinuation of sunitinib treatment, presumably due to rapid rebound neovascularization [65].

CAIX-Directed Therapy: Unmodified Girentuximab Studies

Numerous antibodies are now being used as therapeutic modality for malignancies as well as nonmalignant indications. They can trap circulating antigen and block receptor signaling, and mAbs can also be used to target tumor cells. The clinical landscape for metastatic RCC patients has altered dramatically in recent years. Implementation of tyrosine-kinase inhibitors (sunitinib, sorafenib, and pazopanib) and mammalian target of rapamycin (mTOR) inhibitors, aiming at the tumor vasculature, has resulted in an impressive increase in progression-free survival of mRCC patients [66], but true complete responses are rare. In general, the disease stabilizes or partial responses are observed. Unfortunately almost invariably, patients progress due to treatment resistance or vessel cooption, a phenomenon where tumor cells follow existing blood vessels [67]. This growth pattern permits tumor growth in the absence of sprouting angiogenesis; thus, antiangiogenic therapy becomes ineffective. Thus, there is still a need to improve treatment of mRCC patients.

CAIX was immediately recognized as an attractive target for therapy as it is specifically expressed on the malignant cells. If an appropriate balance can be found between targeting of ccRCC cells and non-ccRCC (normal) cells, CAIX-directed therapy might lead to durable and long-lasting responses. The advantage of mAbG250 as CAIX targeting agent might be the size of the molecule: ccRCC cells are expected to be more accessible than normal CAIX-expressing cells due to the leaky tumor vasculature, simplifying mAb extravasation in tumors [68]. In contrast, mAb extravasation in normal tissues is expected to be much lower as cells connect through tight junctions.

The therapeutic potential of CAIX targeting with mAbG250 has been studied in numerous clinical trials (Table 13.1). These can be divided into trials with unmodified antibody alone or in combination with cytokines and in imaging and radioimmunotherapy trials.

Antibodies can lyse target cells by complement activation or by antibody-dependent cellular cytotoxicity (ADCC). For girentuximab, in vitro studies established that CAIX-positive cells could be lysed through ADCC [69, 70]. Animal studies demonstrated significant tumor growth reduction in mice bearing human RCC xenografts treated with unmodified mAbG250 [71]. In view of the limited treatment possibilities at that time, a number of clinical trials have been performed investigating the potential of unmodified girentuximab in metastatic RCC (mRCC). In the first phase I study, escalating doses of 5–50 mg/m^2 of girentuximab were administered weekly for 6 weeks. Treatment up to the highest dose was safe and

well tolerated. Of the 11 mRCC patients treated, 1 patient showed a CR, and 9 patients had SD after one treatment cycle [72]. In the following phase II study, all patients received 50 mg of girentuximab weekly for 12 weeks. Before treatment, 80 % of patients were progressive and SD was observed in 28 % of these previously progressive patients for at least 6 months after one treatment cycle, suggesting a clinical benefit [73]. During follow-up, one CR and one PR were noted which lasted >1 year. The median survival was 15 months and 41 % of the 32 evaluable patients were still alive after 2 years.

A subgroup of patients received an additional 8 weeks of treatment with a median survival of 39 months, compared to 10 months in the discontinued group. Patients receiving extended treatment with girentuximab showed a significantly longer survival rate than the nonresponsive patients (70 % vs. 26 %).

Interleukin-2 (IL-2) can enhance ADCC of mAbs, and in vitro studies demonstrated increased girentuximab ADCC when cells from IL-2 treated patients were used, suggesting that the combination of girentuximab with IL-2 might be better than girentuximab alone [69]. In a small phase I trial, the safety of this approach was shown [74]. In a phase II trial where patients with progressive mRCC received weekly intravenous infusions of 50 mg of girentuximab and daily subcutaneous low-dose IL-2 for 11 weeks, clinical benefit was noted in 8 of 35 patients (23 %), with 1 long-lasting PR (>95 weeks), 6 long-lasting SD (>24 weeks), and a mean survival of 24 months with 45 % of the 30 evaluable patients still alive after 2 years. The extended treatment group (additional 6 weeks of treatment) showed a median survival of 41 months, compared with 13 months in the nonresponse group. Similar to the girentuximab alone trial, patients receiving extended treatment showed a significantly longer survival rate than the nonresponse patients (55 % vs. 25 %). The increased survival (as compared to historic controls) was attributed to girentuximab and not related to the IL-2, as a sixfold decrease of the normal IL-2 dose was used [75]. The synergistic effect of girentuximab and IL-2 as was observed in the in vitro studies might explain this favorable outcome. In vitro analyses of the whole blood demonstrated increased levels of effector cells (CD3−/CD16+/CD56+) during treatment, but lytic capacity per cell did not increase and ADCC and clinical outcome did not correlate.

Upregulation of CAIX expression can be achieved by exposure of RCC cells to interferons (IFNs) and interferon gamma (IFN-γ) enhances ADCC of girentuximab [70, 76]. The effect of girentuximab combined with IFN-2a was studied in a multicenter, open-label, prospective, single-arm phase I/II trial study in 32 patients with progressive mRCC [77]. Patients received 20 mg girentuximab weekly for 3 months, combined with IFN-2a, three MIU, three times per week subcutaneously. Two patients showed a PR and 14 patients SD in week 16. One patient experienced a PR for at least 8 months, and nine patients had long durable disease stabilization (≥24 weeks). The outcome of the trial compared favorably with historic controls: clinical benefit was observed in 42 % (11/26) of the patients, overall median survival was 30 months for the 31 patients treated with girentuximab plus IFN-2a, and 57 % of patients were still alive after 2 years. Patients receiving extended treatment showed an even higher median survival of 45 months compared with 10 months in

the non-extended group and survived significantly longer than the nonresponse patients (79 % vs. 30 %).

Collectively, the trials with unmodified girentuximab, alone or in combination with cytokines, suggested that the natural course of mRCC could be altered by girentuximab treatment. However, patients were not randomized and patient bias may have occurred. The natural disease course of mRCC is highly variable, and periods with stable disease and/or partial regression can occur, even in the absence of treatment. It is therefore difficult to judge the value of these observations. Randomized trials are needed to determine the true effect of girentuximab treatment in mRCC.

ARISER (*A*djuvant *R*ENCAREX® *I*mmunotherapy Phase III trial to *S*tudy *E*fficacy in non-metastatic *R*CC), an adjuvant double-blind, placebo-controlled phase III trial, was designed to show that girentuximab treatment could reduce the recurrence of the disease in nephrectomized RCC patients who had a high risk of relapse. The trial recruited 864 patients with prior nephrectomy of primary ccRCC. Patients received weekly infusion of girentuximab or placebo for 24 weeks. Patients receiving girentuximab received a loading dose of 50 mg in week 1 and weekly doses of 20 mg during weeks 2–24. Unfortunately, the trial did not meet its primary endpoint [78]. The analysis showed no improvement in median disease-free survival (approximately 72 months) following girentuximab treatment compared with placebo. However, a retrospective subanalysis appears to indicate that the treatment was more effective in patients with high CAIX-expressing ccRCC compared to low CAIX-expressing ccRCC. Disease-free survival was clinically and statistically significantly improved in the patient population with a high CAIX level treated with girentuximab compared to both placebo and patients with a low CAIX score [78]. Therefore, adjuvant immunotherapy with girentuximab might still be of value in a highly defined subpopulation. A follow-up trial is planned to study adjuvant girentuximab treatment in high-risk patients with high CAIX expression to test whether this can delay or circumvent disease recurrence.

CAIX-Directed Therapy: Girentuximab Radioimmunotherapy Studies

Radioimmunotherapy (RIT) with mAbs has been successful in the treatment of malignant non-Hodgkin's lymphoma: ^{90}Y-ibritumomab tiuxetan (Zevalin®) and ^{131}I-Tositumomab (Bexxar®) have been approved as treatment modalities. RIT in solid tumors has been less successful due to various parameters such as the presence of tissue barriers, presence of antigen-expressing normal tissues, poor vascularization, high interstitial tumor pressures, long diffusion distances, and antigen heterogeneity, leading to much lower exposure of solid tumors. The observations in biopsy trials that the absolute and relative amount of girentuximab guided to RCC lesions were at least an order of magnitude higher than those of other mAbs in solid tumors [56] stimulated studies on the development of RIT for ccRCC.

In the first dose-escalating radioimmunotherapy (RIT) trial with ^{131}I-labeled murine mAbG250, progressive patients with measurable, histologically proven ccRCC participated [79]. Hepatic toxicity was observed, most likely due to specific mG250 accumulation in the liver. In the protein dose-escalation study, liver uptake decreased at higher protein dose levels, suggesting saturation of G250 sites by the antibody [53], and the transient toxicity was attributed to radiation damage due to ^{131}I-mG250 uptake. The toxicity was transient and not dose limiting. As in all RIT studies with other radio-labeled antibodies, dose-limiting toxicity was hematopoietic. After determining the maximum tolerated dose (MTD) of ^{131}I-activity (3,330 MBq/m^2), 15 patients were treated at the MTD level to determine efficacy, and major responses were absent. However, overall survival of these patients seemed to be increased in comparison with that of historic control patients: 17/33 SD and two minor responses. The development of high HAMA levels in all patients precluded retreatment [55], and all subsequent trials were carried out with the chimerized version of mAbG250 (girentuximab).

In phase I ^{131}I-girentuximab activity dose-escalation study to establish dose-limiting toxicity similar to the mG250 trial, an imaging dose (222 MBq of ^{131}I-labeled to 5 mg of girentuximab) was included, before patients were allowed to advance to therapeutic dose (1,665–2,775 MBq of ^{131}I-labeled to 5 mg of girentuximab) to prevent infusion of high-dose ^{131}I-girentuximab in CAIX-negative patients [80]. Only patients showing targeting to tumor ($n=8$) received the therapeutic infusion of ^{131}I-girentuximab 1 week later. Unexpectedly, through the administration of the scout dose, liver toxicity was avoided, most likely because the liver compartment was saturated. Alternatively, as formal evidence is lacking, hepatic uptake of chimeric mAbG250 is lower than murine mAbG250. At equal doses, liver uptake of mG250 [53] was two to three times higher than the liver uptake of girentuximab [56]. Dose-limiting toxicity of ^{131}I-girentuximab was significantly lower than dose-limiting toxicity observed for the murine version at 2,775 MBq ^{131}I-girentuximab/m^2. The extended serum half-life is probably responsible for the enhanced hematopoietic toxicity, since this leads to extended radiation of the bone marrow compartment. An antitumor response was observed in 2/8 patients: one SD for 3–6 months and one partial response (PR) >9 months. Both patients were treated at the 2,220 MBq/m^2 dose level. However, quite disappointingly, all other patients progressed.

This first RIT trial with girentuximab clearly showed that increased doses of radioactivity to the tumors were required to achieve more complete and lasting responses. In an effort to increase RIT efficacy, a fractionated dose RIT was performed, based on whole-body radiation absorbed dose [81]. Fractionated RIT is more effective than a single large amount and is associated with a lower toxicity profile in animal models. The primary objective of this trial was to determine the maximum tolerated whole-body radiation-absorbed dose of fractionated ^{131}I-girentuximab. Fifteen patients with measurable mRCC were included. The majority of patients tolerated repeated injections with no change in kinetics, confirming the greatly reduced immunogenicity of girentuximab. The significant variation of whole-body and serum kinetics between patients (estimated biologic clearance half-times ranged from 3.2 to 7.5 days for the whole body and from 1.3 to >5 days

for serum β-half-life ($t½$ β)) was unexpected and may be due to interindividual variation in IgG catabolism. In 2 of 15 patients, HACA was observed which led to a faster serum clearance. Similar to single-dose girentuximab RIT, dose-limiting toxicity was hematopoietic. Moreover, total dose that could be delivered was low and efforts along these lines were abandoned.

In view of the minimal clinical response in single-dose girentuximab RIT, the effects of two sequential high-dose (at MTD) ^{131}I-girentuximab treatments in patients with progressive mRCC were studied [82]. After receiving a scout dose of 185 MBq/m^2 of ^{131}I-girentuximab to demonstrate tumor targeting, 29 patients with adequate girentuximab uptake received a therapeutic dose of 2,220 MBq/m^2 ^{131}I-girentuximab. In the absence of grade IV hematological toxicity, patients received a second cycle after 3 months, consisting of a diagnostic infusion and a second high-dose injection of ^{131}I-girentuximab, escalated from 1,110 to 1,665 MBq/m^2. The MTD of the second RIT was 1,665 MBq/m^2, with myelotoxicity as DLT. Of the 16 patients who completed the protocol at both MTDs, none demonstrated an objective response but 5 previously progressive patients had stabilization of their disease lasting 3–12 months. The low efficacy was partly attributed to the bulky disease in these end-stage patients. It was deemed unlikely that sufficiently high radiation doses of ^{131}I could be delivered to these large tumor masses. An inverse correlation between the size of metastases and radiation-absorbed dose was observed, and dosimetric analyses showed that therapeutic radiation doses (>50 Gy) were only guided to lesions smaller than 5 g. Therefore, it was suggested that future RIT with girentuximab should aim at treatment of small-volume disease or should be used in an adjuvant setting or other more potent radionuclides should be used [83].

Treatment of RCC-bearing mice with girentuximab labeled with more potent radionuclides (^{177}Lu, ^{90}Y, or ^{186}Re) for RIT showed that tumor growth was most effectively inhibited by ^{177}Lu-girentuximab, followed by ^{90}Y, ^{186}Re, and ^{131}I-girentuximab [84]. These more potent radionuclides have the additional advantage that after internalization of the mAb–antigen complex by the target cells, metabolites labeled with these metallic radionuclides are trapped in the lysosomes and residualize [85]. This contrasts with the fate of intracellular ^{131}I-labeled material: after degradation, tyrosine-^{131}I is rapidly excreted by the tumor cell upon internalization. These preclinical RIT studies clearly showed the superiority of ^{177}Lu- and ^{90}Y-based RIT, in line with other studies [85]. The dual-label clinical study [58] also supported that residualizing radionuclides were superior to the iodine. In view of this evidence, subsequent clinical studies have focused on the possibility to use ^{90}Y or ^{177}Lu in RIT.

In the phase I/II trial with ^{177}Lu-girentuximab [86], 23 patients with progressive mRCC with proven ccRCC received a diagnostic dose of ^{111}In-girentuximab (185 MBq), to establish adequate tumor accumulation followed by a dose of ^{177}Lu-girentuximab 1 week later. In the absence of grade IV toxicity, patients were eligible to receive a second (13/23 patients) and a third cycle (4/23 patients), at 75 % of the dose level of the previous injection. Hematopoietic toxicity was dose limiting and MTD was set at 2,405 MBq/m^2; ^{111}In-girentuximab images were super imposable on the ^{177}Lu-girentuximab images, validating the predictive value of

^{111}In-girentuximab for ^{177}Lu-girentuximab accumulation [87]. In one patient, grade IV toxicity was observed at the 1,850 MBq/m^2 dose level. The majority of patients responded by stabilization of the disease. In one patient (1,850 MBq/m^2 dose level), a PR was documented that lasted for 9 months. Dosimetric analyses indicated effective uptake after consecutive treatments. Observed hematologic toxicity, especially platelet toxicity, correlated significantly with the administered activity, whole-body absorbed dose, and red marrow dose. This trial was paralleled by a trial with ^{90}Y-girentuximab at Memorial Sloan Kettering Cancer Center, New York (clinicaltrials.gov/NCT00199875). The tumor-to-red marrow dose ratio was higher for RIT with ^{177}Lu-girentuximab than for RIT with ^{90}Y-girentuximab, indicating that ^{177}Lu has a wider therapeutic window for RIT with girentuximab than ^{90}Y and that in patients with mRCC, higher radiation doses can be guided to the tumors with ^{177}Lu-girentuximab than with ^{90}Y-girentuximab [87]. The authors concluded that RIT with ^{177}Lu-girentuximab may stabilize previously progressive metastatic ccRCC.

Conclusions and Future Prospects

The molecular link between genetic defects in ccRCC (VHL aberrations, VHL silencing, and ubiquitin-pathway defects) makes CAIX an attractive target for ccRCC. For diagnostic purposes, CAIX can easily distinguish ccRCC from other entities and as such can be helpful in the differential diagnosis of ccRCC. Inclusion of CAIX as biomarker in RCC nomograms may be helpful to improve prognostic accuracy, and commercially available CAIX-specific antibody that can also be used on FFPE tissues is needed to implement CAIX expression in nomograms. Serum assays may also be used, but whether these can be used to detect small ccRCC lesions and/or can be used to monitor disease progression and/or therapy response remains to be investigated.

Clinical studies have shown that CAIX targeting can be used to detect small ccRCC incidentalomas. In the future, this new imaging tool may help to prevent unnecessary surgery. Moreover, in fragile patients, girentuximab imaging may provide additional information that can guide clinical management. Trials are planned to show that surgery can be avoided in girentuximab-negative incidentalomas. Labeling with superior PET tracers such as zirconium may even improve the image quality. Currently efforts are also aimed at girentuximab labeling with bioluminescent dyes [88], which may be useful to detect and delineate ccRCC during surgery. One of the latest developments is CAIX imaging to monitor and detect recurrent disease (Fig. 13.1) [89], but further studies are needed to investigate the sensitivity and specificity of this approach.

Remarkably, treatment with unmodified girentuximab showed a clinical benefit for the treated patients, regardless of addition of cytokines. This suggests that girentuximab treatment can alter the natural course of ccRCC, but randomized trials are necessary to substantiate this finding. CAIX adjuvant treatment may be beneficial to high-risk patients. The subanalysis of adjuvant-treated high-risk

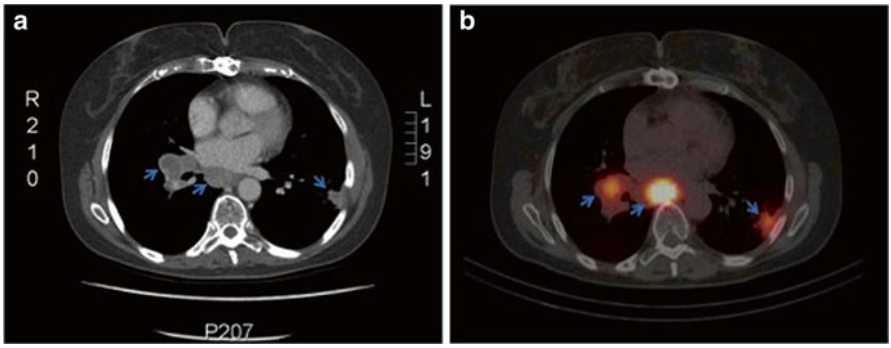

Fig. 13.1 Conventional CT (**a**) and ^{111}In-girentuximab immunoSPECT (**b**) images of a patient with metastatic ccRCC. Clear and preferential uptake of the radio-labeled antibody was observed in mediastinal and pleural lesions (*arrows*)

patients suggests that high-risk patients with high CAIX-expressing tumors may benefit from girentuximab treatment. A new trial is being planned to study whether delineation of high-risk patients according to CAIX expression levels leads to lower recurrence rates.

Recently, the construction of a girentuximab-TNF fusion protein was described [90]. Specific accumulation and retention of girentuximab-TNF in the tumor was observed, resulting in growth control of established RCC. Furthermore, a combination regimen with IFN-γ increased significantly the antitumor response in vivo. Whether such cG250-TNF-based immunotherapeutic approaches could be a valuable therapeutic option for mRCC patients needs to be established.

Despite the high accumulation levels of girentuximab, radioimmunotherapy trials have been disappointing. This may be related to the inherent radiotherapy resistance of ccRCC. Alternatively this treatment may be more suited in patients with less extensive disease burden. The current phase I/II trial investigating the effects of girentuximab labeled with more potent radionuclide Lutetium-^{177} may provide better results.

Whether girentuximab imaging can be used to monitor TKI response remains to be determined. Finally, whether girentuximab treatment can be combined with TKI needs to be established.

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Chapter 14
EGFR and HER2: Relevance in Renal Cell Carcinoma

Sarathi Kalra and Eric Jonasch

ErbB Receptor Family in RCC

The epidermal growth factor receptor belongs to the ErbB family of receptor tyrosine kinases, which comprises the epidermal growth factor (EGF) receptor (HER1/ErbB1), HER2/neu (ErbB2), HER3 (ErbB3), and HER4 (ErbB4) [1, 2]. Activation of ErbB receptors plays a vital role in mediating cell proliferation and differentiation. 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 a ligand, ErbB receptors reside within the membrane as inactive monomers. In the presence of a 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].

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

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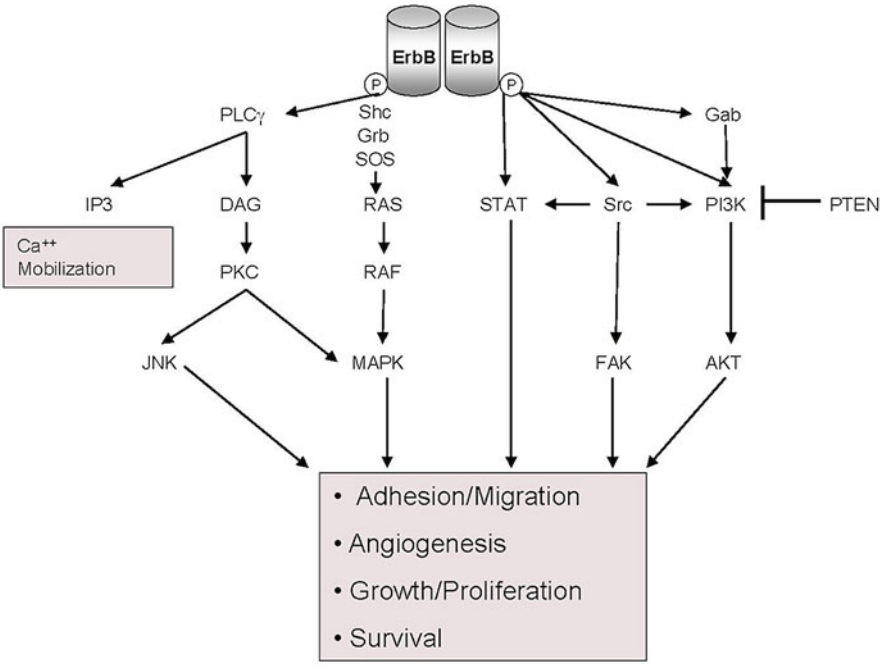

Fig. 14.1 Transphosphorylation of homo- and heterodimers

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 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. 14.1, 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 Silwkowski [2]). PI3K/AKT signaling is activated via docking of the SH2 domain of the p85 regulatory subunit of PI3K 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 adapters 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, 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 IP3, 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 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 that 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, ErbB3, and ErbB4 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.

ErbB Receptor Blockade: Strategies

Several different molecules have been developed to block ErbB signaling. The two major strategies have been to engineer small molecule inhibitors of ErbB1/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.

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, non-clear 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.

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 17,000 or more EGF receptors per cell. In contrast, the growth of tumors expressing 11,000 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].

Small Molecule Inhibitors

Gefitinib

The screening of a compound library using an EGF enzyme prepared from A431 cells identified a series of potent (IC50 <1 μM) 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 IC50 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 the 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 MAP kinase pathway and downregulated BCL-2 protein expression, in an AKT-independent fashion [57].

Gemmill and colleagues demonstrated that combined EGF receptor and mTOR inhibition synergistically impaired growth in a VHL-dependent manner. Gefitinib blocked ERK1/2 phosphorylation specifically in wtVHL 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.

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 IC50 of 2 nM and inhibited autophosphorylation in intact cells with an IC50 of 20 nM [58]. 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.

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, the HER2 overexpressing BT474, and the EGF receptor and HER2 overexpressing N87 cell lines demonstrated IC50 values for growth inhibition of less than 0.16 μM. 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].

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 renal cell carcinoma. As a whole, this strategy has not resulted in 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.

Cetuximab Studies

A phase II clinical trial using cetuximab in patients with treatment naïve renal cell carcinoma 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/m^2, followed by weekly maintenance doses at 250 mg/m^2. None of the patients treated with cetuximab achieved either a complete or partial response. The median time to progression was 57 days. This compared unfavorably to a median progression-free survival of 4.7 months seen in patients treated with interferon alpha and even more unfavorably to the vascular endothelial growth factor (VEGF) blocking agents, including sorafenib, bevacizumab, and sunitinib [63–66].

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 five tumor responses, including three partial responses and two minor responses, and disease stabilization occurred at 8 weeks in 44 patients [67]. The median progression-free survival (PFS) was 100 days. EGF receptor immunostaining was performed on 76 tumor biopsy specimens, and 69 of these (91 %) scored positive.

Gefitinib Studies

Drucker et al. published their experience with gefitinib in 18 patients with metastatic RCC. Eleven of 12 evaluable tumor specimens stained positive for the EGF receptor. Patients received gefitinib at 500 mg/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 from the start of therapy. VEGF and bFGF serum levels were tested before starting therapy and every 3 months on therapy, but there was no correlation between pretreatment level and time to progression. 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 eight patients (38 %). Median progression-free survival 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 versus 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 renal cell carcinoma. Twenty-eight patients were enrolled. Patients received oral gefitinib 500 mg/day. No responses were recorded, but stable disease was seen in 14 patients (53.8 %). Median time to progression was 110, and median overall survival 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].

In 2010, Motzer et al. reported a phase I/II study investigating sunitinib in combination with gefitinib. Eleven and 31 patients were enrolled in phase I and II studies, respectively. The phase I study identified the maximum tolerated dose of sunitinib as being 37.5 mg along with 250 mg of gefitinib. Of the patients treated at the maximum tolerated dose, 13 patients achieved a partial response (objective response rate: 37 %; 95 % CI: 22–55), and 12 had stable disease. Median progression-free survival was 11 months (95 % CI: 6–17). The study concluded that sunitinib plus gefitinib demonstrated an acceptable safety profile and a comparable efficacy to sunitinib monotherapy [71].

Shek et al. conducted a phase II trial of gefitinib and pegylated IFN-alpha (PEG-IFN-α) in patients who had been already treated for metastatic renal cell carcinoma. The optimal response considered was a progression-free survival rate of >50 % at 6 months. Two hundred and fifty milligram of gefitinib was given orally until patients developed intolerance or progression of their disease, whereas PEG-IFN-α was dosed subcutaneously once weekly for 12 weeks. The progression-free survival at 6 months was 29 % (95 % CI: 15–56 %). Median progression-free survival was 5.3 months (95 % CI: 3–10.1), and overall survival was 13.6 months (95 % CI 10.3–NA). Although well-tolerated, the gefitinib and PEG-IFN-α combination did not generate an efficacy signal in this study [72].

Erlotinib Studies

Hainsworth et al. reported the combination of bevacizumab and erlotinib in a paper 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 orally 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 %) had progressed at this time point. The median and 1-year progression-free survivals 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 mouth 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 progression-free survival (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 [73]. The conclusion from these two studies is that erlotinib did not add a significant PFS or OS benefit to bevacizumab therapy.

Gordon and colleagues conducted a phase II study of erlotinib in patients with locally advanced or metastatic papillary RCC. All patients received 150 mg po daily of erlotinib. The overall response rate was 11 % (95 % CI: 3–24), and the disease control rate was 64 % (24 had stable disease and 5 had a partial response). Median overall survival was an impressive 27 months (95 % CI: 17–42 %). These clinical data correlate to the observations made in preclinical models that tumors with wild-type VHL have a higher probability of exhibiting response to EGF pathway targeting agents [74].

Flaig et al. reported the safety and efficacy of combination erlotinib and sirolimus in 25 patients who had progressed after treatment with sunitinib and sorafenib. Patients were treated with 150 mg of erlotinib daily as well as 6 mg daily of sirolimus starting on day 1, followed by 2 mg daily. The median progression-free survival was 12 weeks (95 % CI: 5.9–18.1), and median overall survival was 40 weeks (95 % CI: 0–85.7). No confirmed complete or partial responses were observed, but stable disease was noted in 21.8 % (95 % CI 4.9–38.6) of patients. There was no correlation between erlotinib or sirolimus blood levels and progression-free survival or overall survival. There was no comparative significance of combination therapy of erlotinib and sirolimus over single-agent treatments available in the second-line setting [75].

Lapatinib

A phase 3 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 megestrol acetate. A total of 417 patients were randomized. The primary efficacy end point was time to progression (TTP), with secondary end points of overall survival. 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 overall survival was 46.9 weeks for lapatinib versus 43.1 weeks for medroxyprogesterone (HR = 0.88; $p = 0.29$) [76].

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 IHC), median TTP was 15.1 weeks for lapatinib versus 10.9 weeks for medroxyprogesterone (HR = 0.76; $p = 0.06$), and median overall survival was 46.0 weeks for lapatinib versus 37.9 weeks for medroxyprogesterone (HR = 0.69; $p = 0.02$) [76]. 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 ErbB 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.

Future Perspectives: Patient Selection and Mechanisms of Resistance

Renal cell carcinoma 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" [77, 78], 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 [77]. 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 [79]. 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 [80], 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 [81]. 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-2α 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 EFGR 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 non-clear cell, non-VHL-mutated histologies have shown promise in phase II 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 [82–85]. As shown in Fig. 14.1, PTEN opposes the action of PI3K, and loss of PTEN function can result in constitutive activation of PI3K/AKT signaling downstream of ErbB receptors [86–89]. 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 [90]. Mutations in PTEN are rare in RCC, although PTEN expression is frequently reduced in these tumors [85, 91] and correlates with increased AKT activity [92]. This suggests that with regard to patient selection, individuals with both VHL and PTEN alterations may be 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 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 [93–96]. This suggests that tumor-associated endothelial cells may also be targetable by ErbB inhibitors [97–99]. Elevated VEGF expression is associated with resistance to targeted EGF receptor

therapy in several tumor types [100, 101] 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 [73].

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 [102–108]. While EGF receptor mutations have been shown to predict patient response to gefitinib in NSCLC [109–113], EGF receptor mutations have not been identified in other tumor types that respond to EGFR-targeted therapy [114]. 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 marginal survival benefit if they received lapatinib versus placebo [76].

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. A small phase II study suggests there may be a role for EGF receptor blockade in the non-clear cell RCC patient population. As such, future directions in RCC research with ErbB blocking agents should include investigation of histologies that are not dependent on VHL mutation.

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Chapter 15
The Role of Hepatocyte Growth Factor Pathway Signaling in Renal Cell Carcinoma

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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 majority of patients with advanced RCC [1, 2]. Four main sporadic 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

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their underlying genetic defects and helped forge mechanistic links with sporadic RCC types with similar histologies [2]. For two prevalent RCC subtypes, clear cell and papillary type 1, these mechanistic links strongly implicate the 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 the liver, lung, kidney, and other tissues [5]. The cell surface receptor for HGF is the transmembrane tyrosine kinase encoded by the *MET* proto-oncogene [6]. The *MET* oncogene was isolated from a chemically mutagenized human osteogenic sarcoma cell line; its transforming activity was due to gene rearrangement where sequences from the *TPR* (translocated promoter region) locus on chromosome 1 fused to sequences from the *MET* locus on chromosome 7 (*TPR-MET*) [7]. Subsequent isolation of the full-length *MET* proto-oncogene coding sequence revealed structural features of a membrane spanning receptor tyrosine kinase (TK) [7]. The identification of HGF as the natural ligand for the Met receptor 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, *MET* is widely expressed early in development, deletion of the gene is lethal in mice, and widespread expression persists throughout adulthood [5, 8].

The HGF/Met Signaling Pathway: An Overview

Upon HGF binding, Met is autophosphorylated on two tyrosine residues (Y1234 and Y1235 per sequence for UniProt accession P08581) within the activation loop of the TK domain which significantly enhance kinase activity, while phosphorylation on two tyrosine residues near the carboxyl 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 Met signaling are Grb2, Gab1, phosphatidylinositol 3-kinase (PI3K), phospholipase C-gamma (PLCγ), Shc, Src, Shp2, Ship1, and STAT3 [7]. Gab1 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 Met through Y1356 links the receptor to the Ras/MAPK pathway regulating cell cycle progression [3, 7, 9]. Gab1/Met interactions initiate branching morphogenesis in several epithelial and vascular endothelial cell types. Gab1 is also highly phosphorylated by 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 *MET* itself [9], creating

the potential for Met overexpression in otherwise normal target cells through persistent ligand stimulation; indeed, Met overexpression is widely observed in cancers of epithelial origin [3].

HGF/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 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 Met expression and enhances HGF signaling in cultured cells and mouse tumor models [9].

The HGF/Met Signaling Pathway in Kidney

HGF Signaling in Kidney Development

The critical roles of HGF and 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 the 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 epithelium and the mesenchyme that they are invading is critical. Several soluble factors act in a complementary fashion either as pro- or anti-tubulogenic regulators, including members of the fibroblast growth factor, transforming growth

factor-beta 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].

HGF Signaling in Renal Homeostasis

HGF and *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 upon 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].

Dysregulated HGF Signaling in RCC

Most of the intracellular mediators and pathways activated by 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/Met signaling has been firmly established for hereditary papillary renal carcinoma (HPRC), where inherited missense mutations in the *MET* gene were first found; similar somatic mutations were also found in a small subset (13 %) of sporadic papillary renal carcinoma (PRC) tumor samples [18–21]. The biochemical and biological impacts 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/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].

HGF/Met Pathway Activation in HPRC and Sporadic PRC Type 1

Several missense mutations in *MET* have been identified in individuals with PRC type 1, HPRC, 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 papillary renal carcinomas [18]. Five germ line mutations and four somatic mutations were localized to the 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 impacts of these *MET* mutants were first investigated in NIH3T3 cell transfectants [22, 23, 30]. Mutant 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 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 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 al*lele occurred in 16 of 16 tumor samples, 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 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 *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 Met, the link to Gab1 and other effectors required phosphorylation of the carboxyl-terminal docking sites, as did other indices of cell transformation such as growth in soft agar [24]. Thus, the oncogenicity of Met mutants is mediated by many of the same receptor-proximal intracellular effectors involved in WT Met signaling, suggesting that interruption of key receptor-effector interactions at the carboxyl-terminal docking sites remains a viable strategy for blocking mutant 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 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 upon addition of HGF [25].

The role of ligand binding in the oncogenic potential of PRC-associated *MET* mutations was investigated further by Michieli et al. using cultured epithelial cells, which typically do not express HGF [26]. Met mutants reconstituted in MDCK epithelial cells required exogenously added ligand for colony formation in soft agar [26].

Met mutants 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 upon addition of a receptor-ligating monoclonal antibody [26]. Soft agar colony formation by NIH3T3 cells bearing Met M1268T could be blocked by coexpression of a soluble Met extracellular domain, an uncleavable form of HGF, or the HGF competitive antagonist HGF/NK4 [26]. Together these results revealed that ligand binding contributes 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 *MET* mutations might alter catalytic function, Miller et al. aligned the TK domain of Met with that of the insulin receptor by computer modeling [27]. The results showed that certain HPRC 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 [27–29]. 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 Met forms are more easily activated than WT Met and more likely to remain active, but did not 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 and colleagues later compared the autophosphorylation events in WT and mutant Met receptors expressed in cultured cells using phosphorylation site-specific antibodies and proposed that mutant receptors possessed a lower threshold for kinase activation [30]. HGF binding to WT 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 [30, 31]. A more recent study further showed that mutation in Y1235D reduced k_{cat} compared with the activated, autophosphorylated wild-type enzyme [32]. Unlike WT Met, the D1246H/N and M1268T Met mutants did not undergo Y1234 phosphorylation and were not catalytically impaired by F substitutions at that site [30]. Thus, these mutants were not constitutively active, but mutation overcame the normal requirement for a second phosphorylation step leading to kinase activation [30]. Importantly, the apparent need for ligand activation of HPRC and PRC-associated Met mutant forms suggests that therapeutic strategies aimed at ligand blockade remain viable possibilities for these patient populations.

HGF/Met Signaling in Clear Cell RCC

von Hippel-Lindau (VHL) syndrome is an autosomal dominant hereditary neoplastic disorder [3, 33, 34]. VHL-associated clear cell renal cell carcinoma (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 proteasomal 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 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 proteasomal 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 an 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]. A later report demonstrated that Jade-1, acting downstream of VHL, binds the oncoprotein-β-catenin directly in Wnt-responsive fashion [49]. Interestingly, VHL-mediated ubiquitylation of Jade-1

stabilizes the Jade-1 protein, and VHL loss in clear cell RCC is accompanied by Jade-1 loss through proteasomal degradation [50]. In the presence of VHL, Jade-1 ubiquitylates both phosphorylated and non-phosphorylated-β-catenin and therefore regulates canonical Wnt signaling in both Wnt-off and Wnt-on phases [49]. pVHL downregulates β-catenin in a Jade-1-dependent manner and inhibits Wnt signaling, supporting a role for Jade-1 loss and Wnt signaling in renal tumorigenesis [49]. Together these findings identify β-catenin as a potential target for drug development for *VHL*-negative clear cell RCC.

Independent of its role in HGF/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 [51–53], and no inactivating mutations in *APC* or *axin* have been reported in RCC [54, 55]. 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 [56]. Further evidence for the activation of Wnt signaling pathway in RCC comes from a recent article [57] which describes the homozygous deletion of CXXC4, a gene coding for Idax (an inhibitor of Wnt signaling) in aggressive RCC. The secreted Frizzled receptor proteins, Dickkopf 2 and Wnt inhibitory factor 1, are Wnt antagonists, and expression of these genes is also silenced by aberrant hypermethylation in RCC [58–61]. The persistent expression of Wnt family members from kidney development through adulthood [5, 12, 62] 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.

Xp11 translocation RCC is a newly identified RCC variants added to the WHO 2004 classification [63]. The *ASPL-TFE3* fusion arising from a t(X;17)(p11.2;q25.3) characterizes a subset of pediatric renal adenocarcinomas [64]. Tsuda et al. [65] discovered that ASPL-TFE3 binds to the *MET* promoter and activates it. Induction of *MET* by ASPL-TFE3 results in an apparent increase in Met protein autophosphorylation and activation of downstream signaling in the presence of HGF. In malignant cell lines containing endogenous TFE3 fusion proteins, inhibiting *MET* expression by RNA interference or inhibition of Met protein by the inhibitor PHA665752 abolishes HGF-dependent Met activation, resulting in decreased cell growth. Met may therefore be an additional therapeutic target in tumors with *TFE3* fusions, and these results provide a rationale for clinical trials of Met-targeted therapy in this tumor group [65].

Cancer Drug Development: Targeting the HGF/Met Pathway

Our present understanding of oncogenesis mediated by 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 [66]. In addition, combinations of conventional and Met-targeted therapies may offer promise for specific cancers [67].

Antagonism of ligand binding is a logical therapeutic strategy for a majority of carcinomas where paracrine HGF signaling and Met overexpression result in aberrant pathway activation, including PRC and clear cell RCC. Agents currently under development as HGF/Met pathway inhibitors directed against ligand-receptor binding include competitive molecular analogs of HGF, decoy Met, and monoclonal antibodies directed against either HGF or Met. 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 to the development of HGF/NK4, a larger, more completely antagonistic HGF fragment [68], and to an uncleavable form of pro-HGF [69], both of which block tumor growth and metastasis in animal models. Similarly, the early development of a Met ectodomain/IgG fusion protein with HGF-neutralizing activity preceded the engineering of a soluble Met ectodomain fragments with pathway neutralizing and antitumor activities [70, 71]. Neutralizing mouse monoclonal antibodies against human HGF have also been shown to be effective antitumor agents in animal models [72–74]. Rilotumumab (AMG 102) is a fully human monoclonal antibody with HGF-neutralizing activity. It was evaluated in a phase II clinical study including patients with all histologic subtypes of advanced RCC and did not select patients based on evidence of Met pathway activation. Only a single partial response was seen in the 61 patients treated at two dose levels [75]. Although rilotumumab is unlikely to offer clinical benefit as a single agent in unselected patients, further evaluation of Met pathway antagonists in tumors with known pathway activation is warranted (NCT00422019).

Recent successes in the treatment of cancers using TK inhibitors strongly support the potential efficacy this therapeutic strategy for targeting Met in RCC. Early work with the nonselective staurosporine-like alkaloid K252a showed that it could inhibit Met autophosphorylation, MAPK, and Akt activation and revert the transforming potential of the *TPR-MET* oncogene [76]. Other early TK antagonists that exhibited more selective, but by no means exclusive, activity against Met, such as SU11274 and PHA665752, showed similar preclinical anti-oncogenic potential and revealed that HPRC-associated Met mutations could impact drug sensitivity [77–79]. More selective and potent synthetic inhibitors of Met ATP binding have been developed and tested in various model systems [78, 79]. Most Met TKIs competitively antagonize occupancy of the intracellular ATP binding site, preventing TK activation and downstream signaling. Among these, foretinib targets Met, VEGFR2, Axl, Ron, and Tie-2 with high affinity. In the largest clinical trial devoted to papillary renal cell carcinoma, foretinib demonstrated antitumor activity, modulation of several target indicator plasma proteins, and a manageable toxicity profile [80]. Unlike previous trials of Met pathway antagonists, this trial was restricted to patients with papillary histology (both type 1 and 2 histologies were included). In addition, patients enrolled on this trial were stratified based on the presence of indications of Met pathway activation to determine if Met status impacted response to the agent [80]. The overall response rate in the trial was 13.5 %, and the median duration of response was 18.5 months. The median progression-free survival (PFS) was

9.6 months for the whole study population. When analyzed by dosing cohort, the intermediate dosing group treated with 240 mg/day on days 1–5 of a 14-day cycle had a slightly longer progression-free survival (PFS) at 11.6 months than patients treated with continuous dosing of 80 mg/day at 9.1 months. Fifty out of the 68 evaluable patients had some degree of tumor shrinkage, although most did not meet the criteria for partial response by RECIST. Remarkably, five out of ten patients with germ line *MET* mutations had a partial response. The other five had stable disease for at least 6 weeks, and four of them had more than 10 % tumor shrinkage but less than the 20 % necessary for a partial response [81].

Tivantinib is the only Met-directed TK inhibitor currently in human clinical trials that is not ATP competitive; it reportedly binds to the Met TK domain near the ATP binding site and acts allosterically [82]. A phase II, multicenter, single-arm study assessing the safety and efficacy of tivantinib monotherapy in adolescent and adult patients with metastatic or surgically unresectable microphthalmia transcription factor (MITF)-associated (MiT) tumors, including translocation-associated RCC (tRCC), was recently completed. Median progression-free survival was 1.9 months in tRCC, and tivantinib was safe and tolerable in patients with MiT tumors, but antitumor activity was modest [83]. A randomized phase II clinical trial is recruiting patients with metastatic or locally advanced kidney cancer that cannot be removed by surgery. The primary objective is to assess the response rate (confirmed complete and partial response) of patients with locally advanced or metastatic pRCC treated with either tivantinib or tivantinib combined with erlotinib hydrochloride (NCT01688973).

A cross-tumoral phase II clinical study is recruiting patients to study the antitumor activity of crizotinib across predefined tumor types harboring specific alterations in ALK and/or Met. One arm of the study will test crizotinib in PRC type 1 at doses of 500, 400, or 250 mg/day, depending on toxicity. A phase I/II multiple ascending dose study of BMS-777607 in subjects with advanced or metastatic gastroesophageal cancer, hormone refractory prostate cancer, head and neck squamous cell carcinoma, and PRC type 1 has been completed and results are awaited (NCT00605618). Preliminary analysis of an ongoing phase I clinical trial testing cabozantinib in 25 patients with metastatic clear cell renal cancer showed a median progression-free survival (PFS) of 14.7 months. Of 21 patients evaluable for response, 7 had a partial response by modified RECIST criteria, 13 had stable disease, and 1 had progressive disease. Interestingly, the investigators saw responses in patients who had prior anti-VEGF therapy, suggesting that the combination of Met-VEGF inhibition is therapeutically valuable. Further trials in this disease setting are planned (NCT01100619) [84, 85].

The requirement of the carboxyl-terminal docking site for WT or mutant 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 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 [86]. 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 [87]. Beyond effector targeting, compounds that block HSP90/client interactions, such as geldanamycin [88], also potently block Met oncogenic signaling [89, 90]. Human clinical trials of geldanamycin-related compounds are underway for a variety of cancers where the Met pathway is active, including RCC.

While the potential efficacy of HGF/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/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-/Met-targeted therapeutics, assessing drug activities in tumor tissues, and predicting the potential toxicity of long-term pathway blockade.

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Chapter 16
Development of Resistance to Targeted Therapy: Preclinical Findings and Clinical Relevance

James W. Mier, Rupal S. Bhatt, David J. Panka, and Michael B. Atkins

Introduction

Five VEGF-targeted therapies—the humanized anti-VEGF monoclonal antibody bevacizumab and four small molecule VEGF receptor 2 (VEGFR2) tyrosine kinase inhibitors (sorafenib, sunitinib, pazopanib, and axitinib)—are now approved by the FDA for the treatment of patients with metastatic RCC [1–3], and several others are in the developmental pipeline. As a group, these VEGF-targeted tyrosine kinase inhibitors (TKIs) have had the greatest impact on the treatment of RCC to date. The progression-free survival of RCC patients treated with either sunitinib or pazopanib as first-line therapy, for example, is in excess of 11 months (reviewed in Chaps. 9–11). However, despite these encouraging results, at least a minority of RCC appear to be innately resistant to VEGF-targeted therapies, and the overwhelming majority of RCC initially responsive to these drugs later progress despite continued treatment [4, 5]. The failure of these drugs to induce durable or complete responses and the limited number of therapeutic options available to RCC patients once TKI resistance develops have led investigators to redouble their efforts to acquire a more thorough understanding of the molecular mechanisms by which TKI resistance develops. This chapter will review the various experimental models that have informed our current view of this problem, some of which have provided insights into possible therapeutic solutions.

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Although there is an abundant literature on the various evasive mechanisms utilized by tumors to escape the effects of angiogenesis inhibitors (summarized in [6]), most of the experimental data concerning resistance to VEGF-targeted therapies has been generated from xenograft models. Very little of the information that has molded our current understanding of this subject has been derived from analyses of tumor tissue obtained from RCC patients. Furthermore, most of the xenograft studies designed to investigate the problem of TKI resistance have been carried out in tumor types other than RCC (e.g., lung and islet cell carcinomas), and it is possible that the conclusions from these studies may not pertain to a VEGF-driven malignancy such as VHL-deficient RCC. Many of these xenograft studies have made use of agents highly specific for the VEGF signaling pathway (e.g., neutralizing anti-VEGF or anti-VEGFR2 antibodies) and do not take into account the fact that the various FDA-approved TKIs now in common use (e.g., sunitinib, pazopanib) target other kinases (e.g., the PDGF receptors, c-kit) in addition to VEGFR2. Although these previous studies have been informative, many of the candidate resistance mechanisms they have identified involve cytokines (e.g., PlGF, PDGF-C) and signaling pathways known to be blocked by the less specific TKIs. These pathways may therefore not factor into the development of resistance against more broadly targeted TKIs. Many of the mechanisms of TKI resistance discussed in this chapter should therefore be viewed as provisional pending validation in studies based on serial tumor biopsies from patients with RCC.

Adaptation to Stress Induced by Hypoxia and Nutrient Deprivation: HIF, AMPK, p53, and the UPR

Treatment with a VEGFR-targeted angiogenesis inhibitor results in the transient de-endothelialization of the tumor and a reduction in tumor blood flow [7]. This decrease in tumor perfusion causes worsening hypoxia and nutrient deprivation, which in turn trigger adaptive responses in the surviving cells that enhance survival and the ability to tolerate hypoxia and other forms of cellular stress (see Fig. 16.1). These adaptive responses are driven in part by the hypoxia-sensing transcription factors HIF-1 and HIF-2. HIF-2 and in some instances HIF-1 levels are constitutively elevated in clear cell RCC as a result of the loss of VHL function and both are further increased by hypoxia. This increase in HIF transcriptional activity results in the increased expression of HIF-dependent genes, several of which promote angiogenesis and maintain anaerobic glycolysis as the dominant mechanism of energy production. Both of these effects would be predicted to enhance survival in the setting of hypoxia.

The activation of the AMP kinase pathway, the unfolded protein response (UPR), and other stress pathways (Fig. 16.2) may contribute as well to the ability of tumor cells to survive the effects of angiogenesis inhibition. Each of these cellular adaptations is associated with the increased production of not only VEGF but also other angiogenesis factors, the effects of which would not be blocked by VEGFR2-targeted TKIs. These non-VEGF factors could facilitate the restoration

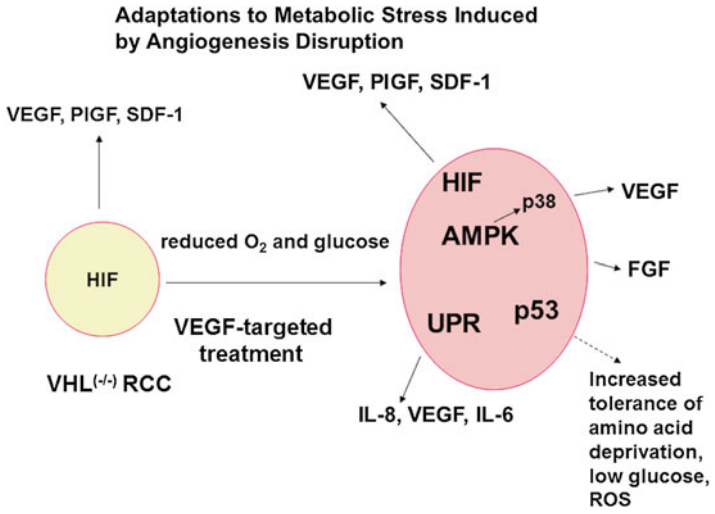

Fig. 16.1 Signaling pathways that contribute to stress tolerance and tumor angiogenesis in the setting of VEGFR blockade. Treatment with a VEGF-targeted therapy induces a transient involution of the tumor microvasculature, resulting in worsening hypoxia and diminished access to glucose. This metabolic stress increases HIF activity and augments the production of VEGF, PlGF, SDF-1, and other HIF-dependent proangiogenic cytokines. The reduced production of energy results in increased AMP levels and the activation of AMPK, which further enhances the production of VEGF independently of HIF. The accumulation of misfolded protein in the endoplasmic reticulum (ER) during hypoxia activates the unfolded protein response (UPR), which selectively increases the translation of mRNAs encoding several angiogenesis factors including VEGF, IL-8, and IL-6. The signaling pathway involved in the increased FGF production in this setting is unclear. The activation of p53 in response to hypoxia increases the ability of cells to tolerate ROS, low glucose, and the absence of certain amino acids (e.g., serine). p53 activation in this setting is apt to be transient and must ultimately be disabled before tumor cell proliferation can resume. The emergence of tumor cells resistant to VEGF-targeted therapy is thought to arise as a result of a selection process favoring tumor cells in which these adaptive pathways are particularly robust

of the tumor microcirculation essential for the resumption of tumor growth in the setting of drug-induced VEGFR2 blockade. Treatment-induced hypoxia, for example, is known to increase the production of placental growth factor (PlGF) and stromal-derived factor-1 (SDF-1, CXCL12) by tumor cells and associated stromal elements. The biological activities of neither PlGF nor SDF-1 would be affected by an anti-VEGF antibody, and since their proangiogenic effects are largely due to their engagement of VEGFR1 (flt-1) and CXCR4, respectively, they would not likely to be affected by an anti-VEGFR2 antibody. Thus, either of these two cytokines could theoretically drive angiogenesis and the development of resistance in those situations in which treatment involves either an anti-VEGF or anti-VEGFR2 antibody.

Hypoxia and the lack of glucose limit ATP production, which results in the accumulation of AMP. Increased AMP levels in turn activate AMPK, which phosphorylates numerous substrates that promote energy production and inhibit anabolism. The activation of AMPK retards cell proliferation through its effects on p53 and

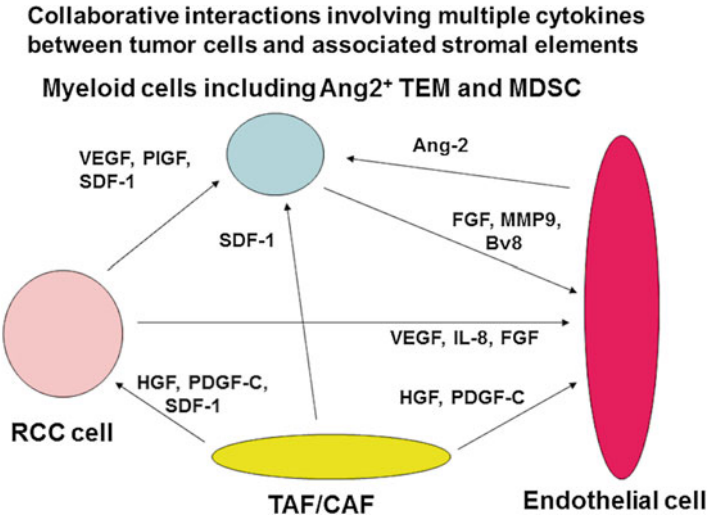

Fig. 16.2 Collaborative interactions between tumor cells, tumor-associated fibroblasts (TAF/CAF), endothelial cells (EC), and various bone marrow-derived myeloid cells present within the tumor infiltrate, including Tie-2-expressing macrophages (TEM) and myeloid-derived suppressor cells (MDSC). Myeloid cells are stimulated by numerous cytokines derived from virtually all of the tumor cellular compartments. They, in turn, produce the angiogenic factors FGF and Bv8 as well as large amounts of MMP9, which drives angiogenesis by liberating VEGF immobilized in the tumor extracellular matrix

mTORC1 but preserves cell viability by increasing glucose import and promoting fatty acid oxidation [8]. Although it reduces global protein synthesis through the suppression of the mTORC1 pathway (see Chap. 13), AMPK activation increases the translation of a select group of mRNAs, many of which encode proangiogenic factors [9]. It is therefore possible that AMPK activation in response to metabolic stress assists in the recovery of the microvasculature from the initial effects of VEGF/VEGFR blockade.

Proper protein folding in the ER involves the function of numerous ER-based chaperones, the introduction of disulfide bridges, and the N′-linked attachment of complex mannose-containing branched oligosaccharide chains in order to ensure proper catalytic function of the proteins and to prevent self-association and precipitation of newly synthesized protein within the ER. Each of these processes requires energy, oxygen, and glucose. The metabolic stresses resulting from angiogenesis inhibition can lead to protein misfolding in the ER (i.e., ER stress) and the activation of the unfolded protein response (UPR), an integrated adaptation that enhances protein folding capacity and facilitates the degradation of misfolded protein in the ER [10, 11]. Several of the proteins (e.g., PERK, IRE-1, ATF4, XBP-1) that mediate various aspects of the UPR are absolutely required for cell survival in the setting of hypoxia [12–14]. These same proteins would be predicted to play a similar prosurvival role in tumor cells subjected to the effects of an angiogenesis inhibitor. One of the downstream effects triggered by the UPR is the increased production of angiogenesis factors such as VEGF, IL-8, and IL-6 [15, 16], the latter two of which would

be expected to drive angiogenesis independent of the VEGF signaling and to substitute for that cytokine in the restoration of the tumor vasculature during TKI treatment. The AMPK signaling pathway and the UPR are HIF-independent mechanisms activated by hypoxia that could contribute to the reconstitution of a vasculature depleted by VEGF-targeted therapy.

Hypoxia is known to activate the tumor suppressor p53. At least two of the kinases that phosphorylate p53 in response to DNA damage and enhance its stability are redox sensitive and activated by hypoxia [17]. Panka et al. recently showed that p53 is activated in RCC xenografts in response to treatment with sunitinib [18], presumably as a result of the hypoxia induced by the diminution in tumor perfusion. Although generally regarded as antiproliferative and antiangiogenic, p53 activation can in some circumstances promote cell survival. For example, p53 activation protects cells from the effects of low glucose and enhances cellular tolerance of ER stress [19]. p53 is required for cells to survive in the absence of the amino acid serine [20] and has an antioxidant effect that increases the ability of cells to tolerate ROS [21]. It is therefore possible that the p53 activation induced early in response to an angiogenesis inhibitor has a transient protective effect against some of the metabolic stresses encountered during treatment. These salutary effects of p53, however, cannot be durable or substantial since the absence of p53 does not sensitize either RCC or CRC xenografts to VEGFR2-targeted drugs—in fact, it has the opposite effect of rendering these agents ineffective [18, 22]. p53 can mediate programmed cell death in response to hypoxia, and it appears that this effect trumps the cytoprotective effects of p53 in the setting of TKI treatment.

Of the various adaptations to metabolic stress, HIF activation may be the only one that promotes angiogenesis without suppressing anabolic pathways in the tumor cell. AMPK activation and the UPR, for example, are both associated with increased production of proangiogenic factors. However, they both limit tumor cell proliferation through their effects on protein translation via TORC1 and eIF-2α phosphorylation, respectively. Although p53 may protect against some forms of metabolic stress, p53 activation is both antiproliferative and antiangiogenic. Thus, although these stress-activated signaling pathways may confer a survival advantage on metabolically stressed tumor cells, their persistent activity is not compatible with tumor growth. The increase in p53 transcriptional activity induced in RCC xenografts during sunitinib treatment, for example, is quite transient [18]. Although p53 persists in tumor cells during treatment, the expression of p53-dependent genes is brief and lost prior to the onset of TKI resistance. In fact, it is possible that the development of resistance to VEGF-targeted therapies requires the subversion of p53 function.

Developmental Pathways Involved in Pathologic Angiogenesis: Potential Value of Notch and BMP Blockade as a Means of Preventing TKI Resistance

Several signaling pathways essential for embryonic vascular development play a prominent role in pathologic angiogenesis as well. Although they are not necessarily hyperactivated in response to metabolic stress as is the case with the UPR,

AMPK, and other prosurvival pathways discussed in the preceding section, they are essential for tumor angiogenesis and their disruption augments the effects of VEGF-targeted agents. The Notch pathway, for example, is required for tumor microvessel development, and its inhibition enhances the sensitivity of tumor microvessels to the effects of VEGF-targeted therapies [23, 24]. The engineered overproduction of the Notch ligand DLL4 by glioma cells results in large, abnormal tumor vessels that are highly resistant to the effects of VEGF neutralization by bevacizumab or VEGF receptor inhibition [23]. As expected, blockade of the Notch pathway with a γ-secretase inhibitor dibenzazepine blocked drug resistance in these tumors. In another study, neutralization of DLL4 with a specific antibody was shown to give rise to prematurely branched, malformed capillaries that limit tumor perfusion and growth [24]. These microvessels were hypersensitive to VEGFR inhibitors. In fact, DLL4 neutralization was able to render TKI-resistant tumors sensitive to these agents. Despite these encouraging results, however, protracted DLL4 blockade was associated with the involution of the thymus, abnormalities in the hepatic sinusoids, and the development of subcutaneous tumors of vascular origin [25]. These effects suggest that inhibition of the Notch pathway might be fraught with too many side effects to be considered a safe strategy for averting TKI resistance in RCC.

BMP (bone morphogenic proteins)-9 and BMP-10 are ligands for activin-like kinase-1 (ALK-1) and endoglin (CD105, ENG). ALK-1, a type II TGF-β receptor, and its coreceptor ENG are expressed on endothelial cells and are known to regulate angiogenesis. ALK-1- and ENG-deficient mice die of vascular defects early during embryonic development [26, 27]. Patients with mutations in ENG or ALK-1 have the autosomal dominant disorder hereditary hemorrhagic telangiectasia (HHT) [28, 29]. HHT-1 has been attributed to mutations in ENG and HHT-2 to mutations in ALK-1. These disorders are characterized by abnormal vessel development characterized by the formation of telangectasias on the skin and arteriovenous malformations which are predisposed to bleed. It has been shown that BMP-9 and BMP-10 via binding to ALK-1 and ENG mediate angiogenesis in vitro and in vivo [30, 31]. Preclinical studies support the idea that both ALK-1 and ENG may be attractive targets for angiogenesis inhibition. Inhibition of either of these receptors has additive antiangiogenic effects with VEGFR inhibitors [32, 33], suggesting that agents that block BMP-9/BMP-10 signaling may be useful as a means of forestalling TKI resistance.

Reversibility of Resistance to VEGF-Targeted Therapy

The development of resistance to drugs that target receptor tyrosine kinases is not unique to agents that block VEGF signaling. Resistance to drugs that block the mutated EGF receptor in NSCLC, for example, or the Bcr-Abl fusion protein in CML is common—even inevitable—and is often attributable to secondary

mutations in the genes encoding the targeted kinases. In some cases, it is due to additional mutations involving genes that encode tyrosine kinases other than the one originally targeted (e.g., c-met) [34–36]. Resistance to VEGFR antagonists, on the other hand, does not appear to have a genetic basis and at least in some circumstances is readily reversible. Zhang et al., for example, have shown that human RCC xenografts that become resistant to sorafenib reacquire their initial sensitivity to the drug when cells from resistant tumors are disaggregated and reimplanted into mice [37]. These sorafenib-resistant xenografts can, in fact, be serially reimplanted and each new implant retraces the growth curve of the first implant, responding initially to the drug and then becoming resistant. Hammers et al. described a similar reversible phenotype in an aggressive RCC that had developed resistance to sunitinib after an initial response to the drug [38]. When implanted into nude mice, this tumor lost many of its aggressive features and acquired a more epithelial phenotype as well as its original sensitivity to VEGF-targeted treatment.

Reversible resistance to VEGFR-targeted therapies is quite familiar to most clinicians who treat RCC patients. It is well known that patients who fail sorafenib or bevacizumab can respond to other VEGFR antagonists such as sunitinib, although the PFS of these patients (5.8 months) is generally less than that reported for patients receiving sunitinib as first-line therapy (11 months) [39]. Patients who develop resistance to sunitinib can even be retreated later with the same agent with some degree of success. In fact, approximately one in four such patients respond to sunitinib "rechallenge" [40]. These observations all attest to the potential reversibility of resistance to VEGF-targeted therapies. They suggest that the underlying mechanism(s) may involve an adaptation to hypoxia and other metabolic stresses and the progressive selection of tumor cells in which the adaptive responses may be particularly robust. It is possible that these adaptations may place the tumor cells at a proliferative disadvantage once the stress is removed—hence the reversion to the initial "sensitive" phenotype when drug treatment is terminated. Why resistance to EGFR inhibitors is permanent and genetically based whereas that which develops to VEGFR antagonists is reversible is unclear. One plausible explanation for this difference is the genetic stability of the cell targeted by drug. The targets of VEGFR antagonists are endothelial cells, which are not particularly prone to mutation, whereas the cells targeted by EGFR inhibitors are genetically unstable tumor cells.

The various mechanisms by which tumors develop resistance to VEGF- or VEGFR-targeted therapies are presented in two sections of this chapter, the first of which reviews the contribution of specific cytokines such as HGF and FGF and the second of which discusses the stromal and myeloid cell types that infiltrate tumors and induce resistance through the production of several mediators. Although much of the material presented in this chapter is derived from xenograft studies (mostly non-RCC), the resistance models reviewed involve stereotypical mechanisms by which tumor (and even normal) cells respond to hypoxic stress. The data presented are therefore likely to apply to RCC during treatment with VEGF-targeted therapies.

Enhanced Production of Alternative Proangiogenic Growth Factors

As mentioned previously, the adaptation to treatment-induced hypoxia involves the activation of HIF, the UPR, and the AMPK pathway and the increased production of factors capable of promoting the restoration of blood flow. Several investigators have, for example, demonstrated increased levels of VEGF and PlGF in the blood of patients undergoing treatment with VEGFR-targeted drugs [41]. These proangiogenic factors were first thought to be produced by ischemic tumor tissue. More recent studies, however, have refuted this notion since the same increase in proangiogenic cytokine levels is observed in tumor-free mice treated with these agents [42]. Others have shown an increase in FGF or IL-8 production by tumor cells and/or their associated stromal elements during treatment with VEGFR antagonists [43, 44]. These two cytokines are of particular interest since, unlike PlGF and VEGF, they activate endothelial signaling pathways not likely to be affected by the VEGFR2 inhibitors currently used to treat RCC. They could therefore promote angiogenesis in the presence of drugs such as sunitinib or pazopanib. Furthermore, their increased production during treatment has been shown to correlate with the development of resistance to VEGF-targeted therapy. The following is a brief review of the various angiogenesis factors that have been shown to contribute to the development of resistance to VEGF-targeted therapies (see Fig. 16.2).

Interleukin-8

Chemokines are 8–12 kDa proteins produced primarily by inflammatory cells but other cell types, including most tumor cells, are capable of producing them in some circumstances [45]. Chemokines regulate several aspects of leukocyte biology including chemotactic responses, respiration, and metabolism. Some have the ability to promote or suppress angiogenesis in addition to their effects on inflammatory cells. The chemokines can be categorized into four subgroups based on the specific arrangement of certain cysteines within the proteins (i.e., CC, CXC, C, and CX_3C), and those with the CXC motif can be further divided into two classes based on whether they have a specific glutamine-leucine-arginine (ELR) motif. Interleukin-8 (IL-8) is one of several ELR(+) CXC chemokines capable of binding to the G protein-coupled receptor CXCR2 present on the endothelium and promoting angiogenesis [46]. Its expression is readily induced by hypoxia, proinflammatory cytokines, and other stimuli [47], and it is thought to contribute to the development of the tumor microcirculation through the recruitment of inflammatory cells and endothelial progenitors into tumor tissue.

One of the first observations implicating IL-8 in the development of resistance to primary antiangiogenic therapy was that of Mizukami et al., who demonstrated that IL-8 production could compensate for the loss of HIF-1 in DLD-1 colon cancer

xenografts [48]. In this study, tumors in which HIF-1 had been knocked down were shown to be well vascularized despite the loss of a transcription factor thought by most investigators to be essential to the generation and maintenance of the tumor microcirculation. These HIF-1-deficient tumors produced large amounts of IL-8, the neutralization of which reduced the tumor microvessel density and retarded tumor growth. This study clearly showed that IL-8 production could maintain the tumor microvasculature in the absence of HIF-1-dependent angiogenesis factors (e.g., VEGF).

The question of whether IL-8 mediates the resistance to sunitinib that inevitably develops in RCC was recently addressed by Huang et al. [44]. These investigators measured the levels of some 89 proangiogenic factors in plasma samples from mice harboring sunitinib-responsive and sunitinib-resistant RCC xenografts and found elevated levels of IL-8 in the mice with resistant tumors. To determine if the increased IL-8 levels were functionally significant, they treated the mice with resistant tumors with either a murine antihuman IL-8 monoclonal antibody, sunitinib alone, or both sunitinib and the anti-IL-8 antibody. Although the antibody alone had no significant antitumor effect, it was able to restore the responsiveness to sunitinib. The sunitinib/anti-IL-8 antibody combination not only inhibited tumor growth but reduced tumor microvessel density, suggesting that the primary effect of IL-8 neutralization was the suppression of tumor angiogenesis. Finally, to determine if these observations were relevant to human RCC, they analyzed IL-8 expression in primary RCC specimens and demonstrated that IL-8 expression and the response to sunitinib treatment were inversely correlated. These data provide the most convincing evidence to date that IL-8 production is an important escape mechanism for RCC subjected to the stress of VEGFR blockade.

Not all CXC chemokines have the ability to promote angiogenesis—in fact, the non-ELR-containing CXC chemokines CXCL9 (Mig), CXCL10 (IP-10), and CXCL11 (ITAC) actually suppress tumor neovascularization [49]. These three chemokines CXCL9–11 are induced by interferon and their production accounts for a substantial portion of the antiangiogenic effects of that cytokine. They all bind the G protein-coupled receptor CXCR3 on endothelial cells and inhibit endothelial proliferation and motility. To determine how sunitinib administration might affect the expression of these angiostatic chemokines, Bhatt et al. analyzed lysates from RCC xenografts by western blot and found that sunitinib treatment down-modulated the expression of these interferon-inducible chemokines as well as that of the interferon-γ receptor [50]. To determine the functional significance of these data, they injected recombinant CXCL9 directly into RCC xenografts and noted that, although the injections had little effect on tumor growth by themselves, they delayed the onset of sunitinib resistance. Intratumoral CXCL9 augmented the ability of sunitinib to reduce tumor microvessel density and perfusion, suggesting that the enhanced therapeutic effect of the combination was due to the inhibitory effects of CXCL9 on tumor angiogenesis. It is unknown whether the down-modulation of these non-ELR chemokines that occurs during sunitinib treatment contributes to the development of drug resistance. However, one might predict that the disappearance of these angiostatic chemokines from the tumor might lower the threshold of

response to the stimulatory effects of IL-8 and other proangiogenic factors and thereby promote angiogenesis (and the development of resistance) indirectly through this mechanism.

Fibroblast Growth Factor

One of the first studies to address the mechanism of acquired resistance to VEGF-targeted therapy was performed by Casanovas et al. using the RIP-Tag2 spontaneous islet cell carcinoma model [43]. This tumor is known to respond to agents that block signaling through VEGFR2, but not VEGFR1. Treatment of mice bearing these islet cell tumors with the rat anti-murine VEGFR2 antibody DC-101, for example, induced partial tumor regression accompanied by a marked reduction in tumor vascularity. These antitumor effects, however, were associated with increased tumor aggressiveness manifested as increased invasion by tumor cells into the tumor capsule and infiltration into the surrounding normal pancreatic tissue. This disturbing observation was one of the first to suggest that the hypoxia induced by antiangiogenic therapy might result in a more malignant tumor phenotype.

In the aforementioned Casanovas study, the islet cell carcinomas developed resistance to the DC-101 anti-VEGFR2 antibody within a few weeks despite continued treatment. The resumption of tumor growth was associated with the restoration of the vasculature and the increased expression of several proangiogenic factors including members of the ephrin, angiopoietin, and FGF families. To determine the cellular origin of these factors, cells derived from the tumors were fractionated and the tumor cells and stromal elements were analyzed separately by RT-PCR for mRNAs encoding these proteins. The epithelial tumor cells were shown to produce increased amounts of FGF1, FGF2, FGF7, FGF9, Ephrin A1, and angiopoietin-2 (Ang-2) with the onset of resistance. Tumor-associated endothelial cells expressed an overlapping array of transcripts, including those encoding FGF-1 and FGF-2 as well as angiopoietin-1 and angiopoietin-2. The increased expression of FGF-2 and Ang-1 was also observed at the protein level. Many of these gene products could be induced in islet cell carcinoma cells in vitro by hypoxia, suggesting that the enhanced expression of these genes observed during the course of DC-101 treatment might have been due to the hypoxia that results from the attenuation of the vasculature. To determine if the development of resistance to the DC-101 antibody might have been due to increased production of FGF family members, mice were treated with a soluble form of the FGF receptor FGFR-2 (FGF trap), a protein that binds FGF1, FGF3, and FGF7. The neutralization of these FGFs by the FGF trap significantly delayed the development of resistance to the DC-101 antibody. These studies were the first to implicate FGF production as a strategy by which tumor cells might evade the biological effects of VEGFR2-targeted therapy.

In a related in vitro study with cultured endothelial cells, Welti et al. showed that FGF2 was able to induce endothelial cell proliferation and tubule formation in the presence of the VEGFR antagonist sunitinib [51]. They also showed that human renal

cell carcinoma specimens strongly and consistently express FGF2. Together, these observations suggest that tumor cell FGF2 production might be able to override the inhibitory effects of VEGFR2 antagonists on tumor angiogenesis and that FGF2 expression might therefore play a role in the development of resistance to VEGFR2 antagonists. This hypothesis has since been corroborated by xenograft studies examining the antitumor activity of small molecule inhibitors of both VEGFR2 and the FGFR. For example, E-3810, a potent inhibitor of VEGFR-1, VEGFR-2, and VEGFR-3 and FGFR-1 and FGFR-2 tyrosine kinase activities, induces tumor regression in numerous xenograft models, including A498 human RCC xenografts that had become resistant to sunitinib [52]. Collectively, these studies demonstrate that the production of FGF2 enables tumors to maintain their vasculature and to thrive despite treatment with VEGFR antagonists.

Hepatocyte Growth Factor (HGF)

HGF is produced primarily by the non-endothelial stromal elements within tumors rather than by the tumor cells or associated microvasculature and is readily detectable in most tumors [53, 54]. Its receptor, c-met, is present on some tumor cells but is particularly well expressed by tumor vascular cells including endothelial cells and pericytes. When bound by its ligand HGF, c-met autophosphorylates on certain tyrosine residues, after which numerous adaptor proteins and downstream signaling molecules are recruited to its cytosolic domain. This activates several canonical pathways (e.g., MAPK, PI3-K) shared by other receptor tyrosine kinases that serve to promote proliferation, motility, and survival [55].

Several previous observations have suggested the involvement of the HGF/c-met signaling pathway in the development of resistance to VEGFR antagonists. For example, Shojaei et al. showed that HGF is more abundant in sunitinib-resistant than in sunitinib-responsive tumors [54]. They also demonstrated that the administration of recombinant HGF reduces the antitumor and antiangiogenic effects of sunitinib in otherwise sensitive tumors. Finally, they showed that the concurrent administration of the selective c-met inhibitor PF-04217903 amplified the antitumor activity of sunitinib. These two drugs individually and in combination retarded the growth of cultured endothelial cells but had no effect on the proliferation of tumor cells in vitro, suggesting that the antitumor activity of the drug combination might be due to the additive effects of the drugs on tumor angiogenesis. It should be kept in mind that these studies were carried out in melanoma and lymphoma cells and their negative conclusions regarding the potential direct effects of c-met inhibition on tumor cells may not apply to RCC. Several studies have, in fact suggested that RCC cells may rely on c-met signaling to maintain their oncogenicity. For example, a synthetic lethal shRNA screen designed to identify kinases whose absence was selectively toxic for $VHL^{(-/-)}$ RCC (but not RCC in which VHL function had been restored) specifically identified c-met as one of the kinases essential for the viability of the VHL-deficient tumor cells [56]. It therefore appears almost a foregone

conclusion that a c-met inhibitor would have at least some intrinsic antitumor activity in RCC independent of its antiangiogenic effects. Regardless of whether HGF has direct prosurvival effects on RCC cells or functions solely as an angiogenesis factor, the findings discussed above suggest that the HGF/c-met signaling pathway can be exploited by tumor cells to escape from the effects of VEGF-targeted therapies and that inhibitors of this pathway might prove to be useful adjuncts to VEGFR antagonists in the treatment of RCC.

Epidermal Growth Factor Receptor (EGFR) Ligands

The EGFR ligands TGF-α and amphiregulin as well as the EGF receptor (EGFR, erbB1) are abundantly expressed by RCC [57]. This observation suggests that EGF family-EGFR interactions might contribute to the proliferation, invasiveness, or metastatic behavior of RCC cells. This hypothesis was in fact validated in studies by Weber et al., who demonstrated that the development and growth of RCC bone metastases in an orthotopic (i.e., tibial implant) xenograft model were partly dependent on EGFR activation [58]. Given its central role in the biology of RCC, the question has arisen whether EGFR signaling might contribute to the development of resistance to VEGF-targeted therapy. The idea that EGFR ligands might be able to substitute for VEGF in the promotion of angiogenesis was first proposed by Cascone et al. [59]. Using a lung cancer xenograft model, these investigators observed that both de novo and acquired resistance to bevacizumab was associated with reduced endothelial cell apoptosis despite persistent inhibition of VEGFR2 signaling (i.e., absent endothelial cell VEGFR2 phosphorylation). Using species-specific gene expression profiling, they showed that the cells whose gene expression was most affected with the development of resistance were stromal and that many of the changes observed were consistent with enhanced EGFR signaling. They subsequently demonstrated increased EGFR phosphorylation on the endothelial cells of tumors with de novo resistance to bevacizumab and on the pericytes of tumors that developed resistance during treatment. Finally, they showed that concurrent EGFR and VEGFR blockade with either a combination of erlotinib and bevacizumab or with the dual EGFR/VEGFR inhibitor vandetanib yielded superior antitumor activity compared with that induced by VEGFR blockade alone. These data establish a role for EGFR ligands in both primary and acquired resistance to bevacizumab in lung cancer.

Whether EGFR ligands play a similar role in the resistance to VEGFR-targeted therapy that develops in RCC is, however, unclear. There are virtually no studies that support the view that the EGFR is critically involved in the biology of RCC in humans. The PFS of RCC patients receiving the EGFR monoclonal antibody ABX-EGF, for example, was only 3 months and only 3 of 88 patients had major responses to the antibody [60]. The gene encoding the EGFR ligand TGF-α is HIF dependent and constitutively overexpressed in VHL-deficient RCC. If TGF-α/EGFR signaling were able to override the effects of VEGFR blockade, the biological consequences

of producing large amounts of TGF-α would be apparent at the outset of treatment as most RCC would be resistant de novo to sunitinib and related TKIs. In a recent randomized, double-blinded, phase II clinical trial comparing bevacizumab alone with a bevacizumab/erlotinib combination in RCC patients, the combination arm was not found to be superior [61]. The PFS of the combination vs. bevacizumab alone arms was 9.9 and 8.5 months, respectively, and the response rates (complete plus partial) were 14 and 13 %. Neither of these differences was statistically significant. The failure to demonstrate that erlotinib could augment the clinical efficacy of bevacizumab in this clinical trial casts doubt on the importance of EGFR signaling as a mechanism of resistance to VEGFR antagonists in RCC.

Angiopoietin-2

Angiopoietin-2 is produced primarily by hypoxic tumor-associated endothelial cells. This cytokine engages a tyrosine kinase receptor (Tie-2) through which it augments several of the effects of VEGF on the endothelium. Several lines of evidence suggest that its production may limit the effectiveness of VEGFR-targeted therapies and predispose to drug resistance. For example, Hashizume et al. showed in Colo-205 colon carcinoma xenografts that the inhibition of Ang-2 enhanced the antitumor and antiangiogenic effects of an anti-VEGF antibody [62]. In a subsequent study, Falcon et al. showed that Ang-2 inhibition could "normalize" the vasculature of these Colo-205 xenografts [63]. Specifically, the Ang-2 inhibitor was shown to increase the extent of endothelial pericyte coverage and the expression of VE cadherin at endothelial junctions, both of which are indicative of vascular maturation. These effects were antagonized by the concurrent administration of a selective Ang-1 inhibitor.

Independent of its direct effects on endothelial cells, at least some of the effects of Ang-2 appear to be mediated through the activation of a population of Tie-2-expressing monocytes/macrophages (TEM) that infiltrate tumor tissue. For example, Mazzieri et al. showed that the effects of Ang-2 inhibition on the growth of murine mammary and islet cell carcinomas could be attributed to the loss of TEM function as these cells failed to associate with the tumor endothelium in the absence of Ang-2 signaling [64]. The role played by Ang-2 and TEM in the development of resistance to VEGF-targeted therapies is further discussed below in the section on "Tumor-Associated Macrophages".

Bioactive Lipids

Sphingosine-1-phosphate (S1P) is a bioactive sphingolipid produced by sphingokinases 1 and 2. The biological effects of S1P are mediated through the engagement of membrane-associated G protein-coupled S1P receptors (S1PR) as well as through

the binding to various intracellular targets. The S1PR mediate many cellular functions including angiogenesis, cell proliferation, autophagy, and apoptosis. SPHK is known to be regulated by hypoxia, likely via HIF-1 and HIF-2. SPHK1 expression has been demonstrated in several tumor types and is associated with poor prognosis [65]. It is possible that SPHK/S1P signaling is an adaptation to hypoxia. For example, Wang et al. have shown that SPHK is increased in a murine model of resistance to antiangiogenic therapy—likely secondary to treatment-induced hypoxia [33]. Three classes of S1P pathway inhibitors are currently being explored. SPHK1 inhibitors decrease S1P production by hypoxic tumor cells, blocking S1P-mediated tumor cell survival and angiogenesis [66, 67]. S1P-neutralizing molecules can sequester S1P and prevent its binding to S1PRs. This approach has been shown to have antiangiogenic activity in colon cancer models [68]. S1PR inhibitors are also potentially beneficial therapeutic agents, preventing tumor growth in preclinical models [69].

Cyclooxygenase-2 (COX-2) has been shown to play a role in inflammation, tumor growth, invasiveness, metastasis, angiogenesis, and survival [70]. COX-2 catalyzes the production of prostaglandin E2 (PGE2) from arachidonic acid. Inhibition of COX-2 has been shown to be a promising antitumor and antiangiogenic strategy in several tumor types including RCC [71–74]. In preclinical models, COX-2 inhibition has an activity as a single agent as well as in combination with immunotherapy and chemotherapy [75–77]. Clinical testing of COX-2 inhibitors has been performed in many tumor types. Although initial reports suggested improved response rates for patients bearing tumors expressing COX-2, a subsequent study of the combination of a COX-2 inhibitor and interferon alpha confined to these patients did not demonstrate a significant benefit for the combination relative to interferon alone. Wang et al. found that COX-2 expression in RCC models was elevated in areas of hypoxia induced by VEGFR inhibition. In RCC xenografts generated both from established cell lines and from fresh patient-derived tumors, the concurrent administration of the selective COX-2 inhibitor celecoxib with sunitinib delayed the emergence of treatment resistance longer than that achieved with sunitinib alone [78], suggesting that COX-2 inhibition might be a useful strategy to prevent TKI resistance.

Contribution of Bone Marrow-Derived Myeloid Cells and Other Stromal Elements to the Development of Resistance to VEGFR Antagonists

Tumor-Associated Fibroblasts (TAF)

Several studies suggest that tumor-associated fibroblasts (TAF) stimulate tumor growth and angiogenesis and contribute to the development of resistance to VEGF-targeted therapy. Olumi et al., for example, showed that fibroblasts obtained from prostate

carcinomas promote the growth of "primed" (i.e., SV40 large T antigen-immortalized) prostate cells both in vitro and in vivo [79]. This growth promotion could not be induced with fibroblasts isolated from normal prostatic tissue and appeared to depend on the "priming" effect of the SV40 large T antigen since the TAF had no demonstrable effect on the proliferation of normal prostate epithelial cells. Crawford et al. observed a similar tumor cell-fibroblast symbiotic interaction in their studies with EL4 and TIB6 tumors [80]. In their studies, however, the tumor-promoting effect of TAF was observed only with fibroblasts isolated from tumors resistant to VEGF-directed therapy (e.g., EL4) and not with either normal skin-derived fibroblasts or TAF derived from tumors that were responsive to anti-VEGF treatment (e.g., TIB6). TAF from the resistant EL4 tumors promoted the growth of TIB6 tumors even in the setting of VEGF blockade and roughly doubled the tumor microvessel density. Furthermore, they showed by confocal microscopy that the admixture of EL4-derived TAF with TIB6 tumor cells increased the number of vascular branch points in the resulting tumors up to sixfold and increased vessel area and volume by approximately 2.5-fold. The EL4-derived TAF, but not normal skin fibroblasts or TAF from TIB6, were capable of supporting angiogenesis in implanted Matrigel plugs, indicating that TAF did not require the continued presence of tumor cells to retain their ability to promote angiogenesis. The genetic or epigenetic alterations in the TAF that maintain their proangiogenic phenotype in the absence of an ongoing inductive effect from tumor cells were not characterized in this study.

The tumor-promoting effects of TAF have been ascribed to their ability to produce cytokines such as TGF-β [81], HGF [54], and SDF-1 [82]. Gene expression profiling of EL4-derived TAF as well as direct cytokine measurements suggested that the production of the PDGF isoform PDGF-C might also contribute to their proangiogenic effects [80]. This hypothesis was validated in subsequent studies in which the ability of EL4-derived TAF to generate a microvasculature in Matrigel plugs was shown to be suppressible with a neutralizing anti-PDGF-C antibody. This antibody also suppressed the growth of EL4 tumors and augmented the growth inhibitory effects of an anti-VEGF antibody. The anti-PDGF-C antibody, however, did not suppress the growth of TIB6 tumors, which were responsive to the anti-VEGF antibody. Collectively, these findings indicate that the production of the novel PDGF isoform, PDGF-C, by TAF promotes tumor angiogenesis and is responsible for the primary resistance to anti-VEGF therapy observed in some tumors. The collaborative interactions between TAF-generated PDGF-C and the other cytokines produced by stromal cells that infiltrate tumor tissue are depicted in Fig. 16.2.

Although the Crawford data discussed above clearly demonstrate that TAF—in particular, those obtained from tumors that are resistant to VEGF-targeted therapies—are able to promote tumor growth and angiogenesis through mechanisms that are largely independent of VEGF, it should be kept in mind that these studies were not done with RCC and the extent to which these data are applicable to RCC is not known. The individual PDGF polypeptides are encoded by four genes, and five different homo- and heterodimers can be assembled from these gene products [83]. All of these PDGF dimers, including the PDGF-CC isoform implicated as a resistance

factor in the Crawford study, signal through PDGFR-α and PDGFR-β receptor tyrosine kinases. Many of the VEGFR2-targeted small molecule inhibitors (e.g., sunitinib, pazopanib) also efficiently block the PDGF receptors so it appears unlikely that the production of PDGF-C would provide a means of escape from the TKIs currently used to treat RCC.

The ability of TAF to promote tumor growth and angiogenesis is not peculiar to prostate and lung carcinoma. Breast carcinoma-associated fibroblasts (CAF) have similar biologic properties [82]. Orimo et al., for example, has shown that CAF, but not normal fibroblasts, cocultivated with human breast cancer (MCF7) cells prior to implantation accelerate the growth and enhance the vascularity of the resulting xenografts [82]. Fibroblasts isolated from normal breast tissue had no effect on tumor growth or vascularity. Tumors derived from the CAF-MCF-7 cell mixture contained far more $Sca1^{+}CD31^{+}$ endothelial progenitor cells (EPC) than those generated from MCF-7 cells alone or cells mixed with normal fibroblasts. These EPC were also much more abundant in the circulation of mice harboring tumors derived from the cocultivation of MCF-7 cells with CAF than in control mice. This observation suggests that the relative hypervascularity of the CAF-MCF-7 tumors was due in part to the enhanced EPC mobilization from the bone marrow. This suspicion was confirmed in a subsequent MCF-7 xenograft study involving H2k-d mice injected with H2k-b bone marrow-derived $Sca1^{+}CD31^{+}$ cells. In this study, the tumor endothelial cells stained positively for H2k-b by immunofluorescence, corroborating their bone marrow origin. RT-PCR analyses of the breast carcinoma CAF revealed abundant mRNA encoding the chemokine SDF-1, and ELISA of the CAF-conditioned media demonstrated abundant SDF-1 secretion. A similar high degree of SDF-1 expression was observed in the α-smooth muscle actin (SMA)-staining fibroblasts present within invasive human breast carcinomas. To determine the functional significance of this SDF-1 production, mice bearing MCF-7 xenografts were treated with a neutralizing anti-SDF-1 antibody. The intraperitoneal instillation of this antibody suppressed the growth and vascularity of the tumors and reduced the number of $Sca1^{+}CD31^{+}$ cells present within the tumor infiltrate [82].

The gene encoding the chemokine SDF-1 and that encoding one of the SDF-1 G protein-coupled receptors CXCR4 are both HIF dependent and abundantly expressed by VHL-deficient RCC. It is likely that tumor-associated fibroblasts and other stromal elements also contribute to the SDF-1 produced by these tumors, especially in the setting of hypoxia induced by treatment with VEGF-targeted agents. In a SCID mouse model of human RCC, CXCR4 expression was shown to correlate with increased metastatic behavior and the neutralization of SDF-1 shown to reduce metastases [84]. It is therefore likely that the SDF-1/CXCR4 axis plays a role in the growth and hypervascularity of human RCC, regardless of the identity of the cells from which the SDF-1 is derived. To the extent that this is the case, one would expect that treatment strategies that incorporate the neutralization of SDF-1 or blockade of CXCR4 function (e.g., with AMD3100) might serve as useful adjuncts to VEGFR-targeted therapies in the treatment of RCC [85].

Tumor-Associated Macrophages (TAM)

Among the most abundant leukocytes infiltrating tumors, macrophages accumulate in hypoxic/necrotic areas where they scavenge dead cells and promote tissue remodeling through the secretion of VEGF, MMP-9, and other factors [86]. These cells recognize and respond to a wide variety of chemoattractants including endothelin (ET), the chemokine CCL5, VEGF, and PlGF. Approximately 20 % of macrophages (as well as circulating monocytes) express the tyrosine kinase receptor Tie-2 and respond to the Tie-2 ligands Ang-1 and Ang-2 as well [87]. These Tie-2$^+$ macrophages also express the generic leukocyte marker CD45 and the myeloid marker CD11b, but not the neutrophil marker Gr-1 or any of the well-characterized markers found on endothelial progenitor cells such as CD34 or CD146.

Of all of the myeloid cell subpopulations, Tie-2$^+$ monocyte/macrophages are particular adept at promoting tumor growth and angiogenesis. For example, cocultivation of glioma cells with Tie-2$^+$CD14$^+$ monocytes (TEM), but not their Tie-2$^-$ counterparts, prior to implantation into nude mice has been shown to enhance the growth and vascularity of the resulting tumors [88]. The proangiogenic effect of TEM was at least in part due to their production of FGF [89]. Exposure to angiopoietin-2 enhanced the ability of TEM to promote angiogenesis further by suppressing their production of the angiostatic cytokine Interleukin-12 (IL-12) [90]. The importance of these cells was further illustrated in studies involving transgenic mice carrying the gene encoding thymidine kinase (TK) placed under the control of the Tie-2 promoter/enhancer. These mice express the enzyme in their Tie-2$^+$ cells, rendering the TEM of these mice vulnerable to the antiviral prodrug ganciclovir [89]. In these studies, bone marrow from the transgenic mice was adoptively transferred to recipient mice that were later implanted with mammary carcinoma or glioma cells and then treated with ganciclovir to ablate the TEM population. Ganciclovir treatment reduced the size and vascularity of the tumors growing in these mice, indicating that TEM contribute to the development of the tumor microcirculation.

The VEGF family member PlGF is another cytokine that activates macrophages. It stimulates the revascularization of ischemic tissue through its interaction with VEGFR1 and neuropilins 1 and 2 [91]. PlGF is produced by both tumor cells and stromal elements in response to hypoxia and high levels can be detected in the plasma of patients undergoing treatment with VEGFR antagonists such as sunitinib [41]. In a series of studies examining the role of PlGF in tumor growth and angiogenesis, Fischer et al. demonstrated that the neutralization of the cytokine with an anti-PlGF antibody resulted in the regression of several tumors, including some (e.g., the colon carcinoma CT26) resistant to treatment with an anti-VEGFR2 antibody [92]. This antitumor effect was additive to that of an anti-VEGFR2 antibody. One of the most obvious histologic effects of PlGF neutralization was a marked reduction in tumor-infiltrating macrophages. Macrophage depletion by treatment with clodronate liposomes reduced tumor growth, but this effect was not additive to that of the anti-PlGF antibody [92, 93]. These data show that tumor-infiltrating macrophages enhance tumor growth and angiogenesis and that PlGF is one of the cytokines

responsible for their recruitment into tumor tissue. The data, in fact, suggest that the promotion of macrophage recruitment may be the dominant mechanism by which PlGF stimulates tumor growth.

It is unknown whether PlGF-induced macrophage recruitment is a contributing factor in the development of TKI resistance by RCC. The fact that PlGF neutralization inhibits the growth of tumors resistant to VEGF-targeted therapy and enhances the efficacy of an anti-VEGFR2 antibody in other tumor models supports this hypothesis, as does the detection of high circulating PlGF levels in RCC patients undergoing sunitinib treatment [41]. However, many of the small molecule VEGFR2 inhibitors FDA-approved for use in RCC (e.g., sunitinib, pazopanib) also potently block VEGFR1, which should incapacitate PlGF-mediated signaling in endothelial and other cell types. It is therefore possible that the increased production of PlGF during treatment and the PlGF-dependent recruitment of macrophages into tumors contributes to the development of resistance only to VEGF-specific therapies (e.g., bevacizumab), but not to agents that block both VEGFRs 1 and 2 (e.g., sunitinib, pazopanib).

Gr1⁺CD11b⁺ Marrow-Derived Myeloid Suppressor Cells (MDSC)

Several studies have implicated a heterogeneous population of bone marrow-derived myeloid cells that express both the granulocyte phenotypic marker Gr-1 and the macrophage marker CD11b in the development of resistance to VEGF-targeted therapies [94–96]. MDSC are responsive to several cytokines and chemokines, some of which (e.g., SDF-1) are produced in hypoxic areas within the tumor through a HIF-dependent mechanism. Kioi et al., for example, showed that the recruitment of MDSC into irradiated glioblastoma xenografts was driven predominantly by the chemokine SDF-1. Tumor infiltration by these cells could be prevented by the administration of AMD3100, a drug that blocks the function of the SDF-1 receptor CXCR4 [85]. These cells are also responsive to the chemokines CXCL5 [97], CXCL1, and CXCL2 [98] and to GM-CSF and TNF [99, 100]. Finally, in a study by Chan et al., the recruitment of these cells into tumors was found to be mediated by IL-8 and angiogenin through an NF-κB-dependent mechanism [101]. Thus, it appears that there a number of factors that regulate the influx of these MDSC into tumor tissue, some, but not all, of which are hypoxia (HIF) dependent.

Regardless of the specific chemotactic factors responsible for MDSC recruitment, the influx of these cells into tumor tissue is governed by the p53 status of the stromal elements within the tumor. Guo et al., for example, recently demonstrated that melanoma cells elicit a much more intense MDSC infiltrate when implanted into $p53^{(-/-)}$ mice than in p53-WT mice [102]. This observation suggests that the production of chemotactic factors by the tumor stroma is suppressed by even baseline p53 activity in these cells. In a related study, Panka et al. demonstrated that the infiltration of $CD11b^{+}Gr\text{-}1^{+}$ MDSC into RCC xenografts is augmented by antiangiogenic therapy

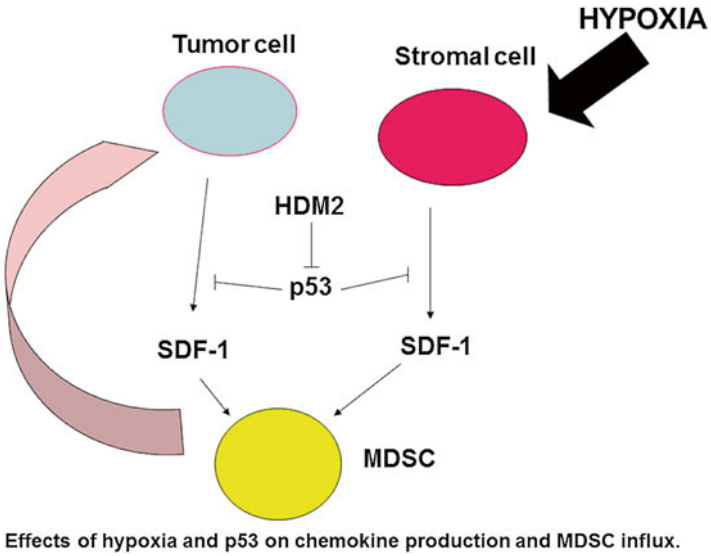

Fig. 16.3 VHL-deficient RCC are known to produce SDF-1 constitutively. The gene encoding this chemokine is regulated by HIF and its expression is induced by hypoxia—as occurs, for example, in tumor stromal cells during treatment with VEGF-targeted drugs. SDF-1 is one of several chemokines that regulate MDSC trafficking into tumors. In this model, the induction of SDF-1 by hypoxia results in the recruitment of MDSC, whereas the suppression of SDF-1 production by p53 limits the influx of these cells

(i.e., sunitinib) and that this enhanced influx can be prevented by the concurrent administration of an HDM2 antagonist (MI-319, Sanofi-Aventis) [18]. MI-319 increases p53 levels and p53-dependent gene expression by blocking the interaction between p53 and its dominant E3 ligase HDM2. In this study, MI-319 was also shown to suppress the increase in SDF-1 production within the tumor induced by sunitinib treatment, suggesting that this chemokine might be responsible for the MDSC recruitment. The interactions between p53, SDF-1, and MDSC are depicted in Fig. 16.3. The addition of MI-319 to sunitinib markedly extended the interval during which the growth of the RCC xenografts was constrained by sunitinib. These data suggest that the ability of HDM2 antagonists to suppress MDSC influx might be exploitable as a means of preventing TKI resistance.

Through a mechanism that is not entirely understood, MDSC are able to confer on adjacent tumor cells the ability to tolerate cellular stress and to render the tumor cells resistant to many forms of treatment, including even chemotherapy. MDSC are relatively abundant, especially in tumors refractory to anti-VEGF therapies, and their depletion by treatment with an anti-Gr1 antibody has been reported to increase tumor responsiveness to treatment with an anti-VEGF antibody [94]. Yang et al. showed that the implantation of an admixture of tumor cells with $Gr1^{+}CD11b^{+}$ MDSC resulted in tumors that grew faster than control tumors and had increased microvessel density and reduced areas of necrosis [95]. These proangiogenic effects

of MDSC were attributed to their ability to produce the matrix metalloproteinase MMP9 as the deletion of the gene encoding this matrix metalloproteinase from the MDSC rendered them unable to promote tumor vascularity. MMP9 is thought to stimulate angiogenesis through the liberation of high molecular weight isoforms of VEGF immobilized in the extracellular matrix. In other studies, Bv8 (prokineticin), a secreted protein generated in response to tumor-derived cytokines such as G-CSF, was found to be the dominant proangiogenic factor produced by $Gr1^+CD11b^+$ cells [95, 103]. Shojaei et al., for example, showed that forced Bv8 expression by tumor cells increased tumor angiogenesis [103]. In addition to the production of MMP9 and Bv8, at least a subset of $Gr1^+CD11b^+$ cells has the capacity to insinuate into the developing tumor endothelium and to contribute structurally to the developing tumor microcirculation. This extent to which the incorporation of these cells into the tumor microvasculature contributes to their proangiogenic agenda is unclear.

As implied in the acronym, $Gr1^+CD11b^+$ MDSC are immunosuppressive as well as proangiogenic. These cells express arginase and deplete the microenvironment of arginine, which results in the accumulation of uncharged arginine-tRNA and the activation of the eIF2α kinase GCN2 [104]. The translational arrest induced by the activation of eIF2α results in the selective down-modulation of the TCR-ζ chain, the src-related kinase $p56^{lck}$, as well as the components of the NF-κB family in T lymphocytes and the loss of T cell function [104]. MDSC produce immunosuppressive cytokines such as TGF-β. They also express high levels of iNOS, which enables them to generate large amounts of peroxynitrite, a radical that directly nitrosylates the TCR, rendering it incapable of recognizing antigens [105]. Thus, there are several means by which MDSC undermine the immune response to tumor-associated antigens. Whether these immunosuppressive effects of MDSC contribute to the development of resistance to VEGF-targeted therapy is less clear.

Clinical Trials of Regimens Designed to Delay/Prevent Resistance to VEGF-Targeted Therapy

Several clinical trials have been conducted or are now underway in which a VEGFR2-targeted drug is administered in combination with second agent chosen on the basis of its ability to block one or more of the signaling pathways suspected of playing a role in the development of TKI resistance. Similar studies involving single agents that target both VEGFR2 and a non-VEGFR signaling pathway implicated in TKI resistance are also underway. The preclinical and clinical data available for some of the agents involved in these trials are reviewed in detail in other chapters of this book and will therefore be discussed here only as they relate to the problem of TKI resistance.

As discussed above, the HGF/c-met pathway is one of several suspected of providing a means of escape from the effects of VEGFR2 blockade [54]. Cabozantinib (XL184), an agent active against both c-met and VEGFR2, has been tested in a small phase II trial in patients with metastatic RCC. Twenty-five patients were

enrolled, 17 of whom had received more than two prior agents including 13 whose disease had progressed following receipt of a VEGF pathway and an mTOR pathway inhibitor. Tumor responses were seen in 7 patients (28 %) and an additional 13 patients exhibited disease stabilization. Responses were seen in multiple disease sites including in four patients with bone metastases. Median PFS was 14.7 months, which was quite impressive given the heavy prior treatment of the patient population. This encouraging data has formed the basis of an Alliance Cooperative Group randomized phase II trial comparing cabozantinib to sunitinib in VEGFR TKI-naïve patients as well as an industry-sponsored phase III trial comparing cabozantinib to everolimus in patients whose disease has progressed on one of more VEGF pathway inhibitors [106].

Several reversible ATP-competitive TKIs that block FGFR-1 as well as VEGFR-2 and PDGFR-β with IC_{50} values of <100 nM are active in various murine tumor models, and phase I studies with two such agents—dovitinib (Novartis) and E7080 (Eisai)—have been completed and found to have antitumor activity. The ability of dovitinib to inhibit FGFR-1 appears to be a crucial component of the drug's activity since in two preclinical studies, the antitumor effects of the drug were shown to correlate with FGFR expression and/or the presence of an activating FGFR mutation in the tumor cells [107–109]. The other agent, E7080, shares with sunitinib and pazopanib the ability to inhibit c-kit, and its antiangiogenic effects in at least some tumors (e.g., human H146 SCLC xenografts) may depend on this activity [110]. The extent to which its ability to block FGFR signaling contributes to its antitumor effect is unknown. E7080 is currently being tested in a randomized phase II trial comparing its efficacy to everolimus in the VEGFR TKI refractory population of patients with metastatic RCC. In addition, dovitinib is being compared to sorafenib in a randomized phase III trial involving patients whose disease has progressed following both VEGFR TKI and mTOR inhibitor therapies. This latter trial has completed accrual with results anticipated shortly. These clinical trials should hopefully clarify the role played by FGFR signaling in the development of resistance to VEGFR2 antagonists.

Amgen has developed a soluble Tie2-Fc "peptibody" AMG386 that blocks the interaction of angiopoietin-1 and angiopoietin-2 with their tyrosine kinase receptor Tie-2. This drug has marked antitumor activity in tumor xenograft models [111] and potent antiangiogenic activity in some tumors as determined by dynamic contrast-enhanced magnetic resonance imaging [112]. Although the dual nature of this drug precludes an analysis of the individual effects of Ang-1 and Ang-2 neutralization, at least one study suggests that both may contribute to the antitumor activity of AMG386 [113]. A phase I clinical trial examined AMG386 in combination with either sorafenib or sunitinib in patients with metastatic RCC [112]. The combination was fairly well tolerated with toxicity primarily attributed to the VEGFR TKI. Further, significant antitumor activity was noted with tumor responses seen in 5 of 17 patients treated with AMG386 and sorafenib and 8 of 15 patients treated with the agent and sunitinib. Subsequently a randomized, phase II, placebo-controlled clinical trial was performed examining sorafenib with or without AMG386 administered at either 3 or 10 mg/kg intravenously every week. Although the response rate was

higher in both of the AMG 386 arms (37 and 38 %) than that seen with sorafenib alone (24 %), there was no difference in median progression-free survival. In addition, a multi-institutional phase II clinical trial of standard dose and schedule sunitinib in combination with AMG386 at either 10 or 15 mg/kg has also been performed. Results showed response rates for the two cohorts of 58 % and 63 %, respectively, and median PFS of 13.6 and 16.3 months and very little toxicity that was not attributable to sunitinib [114]. These results appear to be potentially superior to sunitinib alone but are difficult to reconcile with the failure of AMG386 to prolong median PFS in combination with sorafenib in the randomized phase II trial mentioned previously. A randomized trial of sunitinib ± AMG386 at the 15 mg/kg dose would seem indicated, but at the present time is not being considered. The Pfizer Ang-2 inhibitor PF-04856884 (CVX-060) is also undergoing clinical evaluation. Unlike AMG-386, however, this agent selectively blocks Ang-2 and has no effect on the other angiopoietins. PF-04856884 has been shown to enhance the antitumor activity of sunitinib and bevacizumab in tumor xenografts, and a phase I trial of the drug in combination with axitinib in patients with previously treated RCC has been planned. Based on trials to date, the extent to which Ang-2 production contributes to the development of resistance to VEGFR-targeted therapies remains to be firmly established.

Although there is no evidence that the BMP-9 and BMP-10 activin receptor-like kinase-1 (ALK-1) signaling pathway is upregulated in response to treatment with a VEGF-targeted therapy, this pathway is essential for vascular remodeling and pathologic angiogenesis [31], and agents that block this pathway may be exploitable in delaying or preventing resistance to VEGFR antagonists. Two drugs that block this pathway—an ALK-1-Fc fusion protein (Acceleron) and an ALK-1-specific antibody (Pfizer)—have been shown to have antitumor and antiangiogenic effects in xenograft models [32, 115]. The anti-ALK-1 antibody has, in fact, been shown to cooperate with the VEGFR2 inhibitor axitinib in a melanoma xenograft model [116], suggesting that these agents might be useful as adjuncts to VEGFR2 antagonists as a means of delaying the emergence of drug resistance. A phase I trial to determine the antitumor activity of the ALK-1-Fc fusion protein administered in combination with axitinib in previously treated RCC patients was recently launched.

HIF-1 and HIF-2 are regulated by mTORC1 and mTORC2, respectively, and agents that block these signaling complexes (or the upstream kinase PI-3K) would be expected to suppress the production of numerous HIF-dependent proangiogenic factors such as VEGF. It is therefore possible that the use of an mTOR or PI3-K inhibitor in conjunction with a VEGF-targeted therapy would delay or prevent the development of resistance. Unfortunately, such combinations have been poorly tolerated and have necessitated significant reductions in the doses of the VEGF pathway inhibitor. Of note, despite some encouraging early data [117], randomized studies of either bevacizumab or sorafenib with temsirolimus have produced more toxicity and less activity than single agent VEGF pathway inhibitors alone [118, 119].

Finally, it may be possible to delay the emergence of resistance and enhance the PFS of drugs such as sunitinib by the concurrent administration of conventional chemotherapy. The combination of sunitinib with gemcitabine, for example, is active even in RCC patients whose tumors have become refractory to single agent

sunitinib [120]. Although the mechanism has not been verified experimentally, interference with the recruitment of various proangiogenic myeloid cells into the tumor may account for this additive effect.

Conclusions

The cytokines, cell types, and signaling pathways that have been proposed to mediate the development of resistance to VEGF-targeted therapies are numerous and diverse. Our inability to identify a particular single cytokine or factor that is consistently responsible for TKI resistance attests to the complexity of the cellular response to the hypoxia and nutrient deprivation induced by drugs whose primary mode of action is the disruption of the tumor microvasculature. The sheer number of signaling pathways that enhance the ability of tumor cells to tolerate hypoxia and other forms of metabolic stress and facilitate the restoration of the microcirculation in the setting of VEGF/VEGFR blockade tends to undermine the notion that the blockade of any one additional cytokine (e.g., HGF) or signaling pathway would solve the problem of TKI resistance for all tumors. The success or failure of the upcoming clinical trials with drug combinations that target VEGFR2 and either c-met or the FGFR, for example, will determine whether such therapeutic nihilism is justified. A careful delineation of the roles played by the various stress-induced signaling pathways (e.g., HIF, APMK, the UPR) activated in the setting of hypoxia and the development of agents that block these pathways may yield a solution to the problem of sunitinib resistance that cannot be solved through the continued focus on individual tyrosine kinases. Drugs that block the expression or activity of HIF-2, for example, might prove extremely useful as adjuncts to VEGF-targeted therapies. Several investigators are in fact in the process of developing agents with this capability [121]. An effort to better define the contribution of tumor-infiltrating myeloid cells to the problem of TKI resistance might also prove useful as would an analysis of the factors that regulate their trafficking. HDM2 antagonists and drugs that block the interaction between the chemokine SDF-1 and its receptor CXCR4 may be effective when used in combination with TKIs because of their ability to regulate MDSC influx.

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Chapter 17
Development of Combination Therapy with Targeted Agents

C. Lance Cowey and Thomas E. Hutson

Introduction

The management of metastatic renal cell carcinoma (mRCC) has changed dramatically over the last decade. For many years, the use of immune-based therapies was standard as conventional cytotoxic chemotherapy was ineffective for the disease [1, 2]. Unfortunately, these cytokine-based immunotherapies, such as interferon (IFN) and interleukin-2 (IL-2), have had limited success due to low rates of response and high toxicity rates [3]. These therapies as single agents have failed to produce improvements in progression-free survival (PFS) and overall survival (OS) endpoints. Fortunately, improved understanding of the molecular pathogenesis of RCC led to the development and approval of several molecularly targeted therapies [4] such as the vascular endothelial growth factor (VEGF) inhibitors and mammalian target of rapamycin (mTOR) inhibitors. Defects in the von Hippel-Lindau (VHL) gene occur at a high frequency in sporadic clear cell RCC (reviewed in a separate chapter) resulting in increased levels of the transcription factor, hypoxia-inducible factor (HIF), and thus upregulation of a variety of hypoxia-inducible genes involving angiogenic (e.g., VEGF), growth, and survival pathways [5].

Both VEGF and mTOR pathway inhibitors have received FDA approval based on their ability to prolong either progression-free survival (PFS) or overall survival (OS) in large randomized trials. VEGF pathway inhibitors which have shown a high level of clinical evidence for use in metastatic RCC include sunitinib [6, 7], sorafenib [8, 9], pazopanib [10], axitinib [11], tivozanib [12], and bevacizumab [13–16]. mTOR pathway inhibitors which have a high level of evidence include temsirolimus [17] and everolimus [18, 19]. These agents have all been approved as single agents

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with the exception of bevacizumab, which has been approved in combination with interferon, and tivozanib which is pending FDA approval as a single agent. Although these molecularly targeted therapies appear to have better toxicity profiles and better survival advantages compared to previous cytokine therapies, major responses are still seen in the minority of patients, complete responses are rare, and survival improvements are measured in months.

These limitations have led to many attempts to combine available agents together for possible synergistic or additive effects. The goal of combination therapy over single-agent therapy is to induce greater responses with hopes of prolonged survival outcomes. Several barriers exist to combination therapies including potentially increased toxicities, more complex management, and cost of therapy. From a molecular pathway standpoint, there are two strategies with combination therapies: (1) vertical inhibition, which targets two different molecules in the same pathway, and (2) horizontal inhibition, which targets molecules in separate molecular pathways (Fig. 17.1). Both of these strategies have been attempted in advanced RCC and will be reviewed in this chapter.

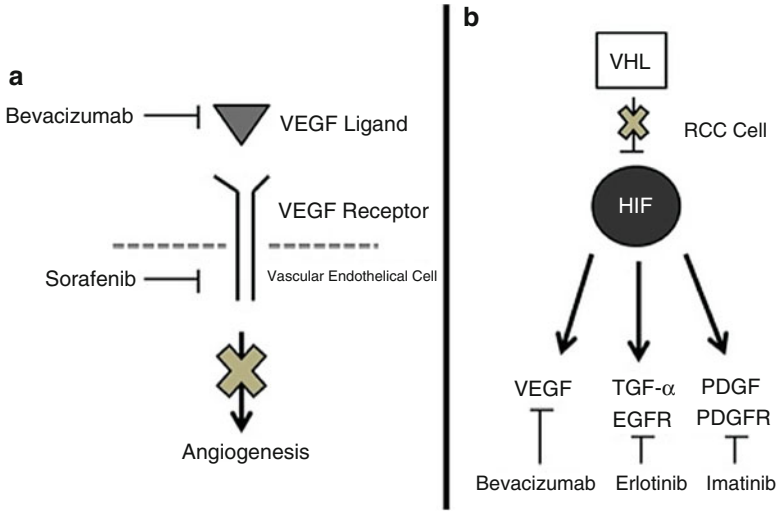

Fig. 17.1 Combination approaches include vertical or horizontal pathway inhibition. (**a**) Vertical inhibition. This illustration gives an example of vertical inhibition of a molecular pathway. In this example, both the vascular endothelial growth factor (VEGF) ligand and the VEGF receptor on a vascular endothelial cell are being inhibited. Bevacizumab is a monoclonal antibody which binds VEGF. Sorafenib is a multi-targeted VEGFR tyrosine kinase inhibitor. Both agents act to inhibit VEGF-directed angiogenesis. (**b**) Horizontal inhibition. This illustration gives an example of horizontal inhibition of different molecular pathways. In the renal cell carcinoma (RCC), the von Hippel-Lindau (VHL) tumor suppressor function is lost allowing for increased levels of the transcription factor, hypoxia-inducible factor (HIF). HIF transactivates a variety of pro-tumor genes such as VEGF, transforming growth factor-α (TGF-α), and platelet-derived growth factor (PDGF). Bevacizumab, erlotinib, and imatinib are drugs which inhibit each of these diverging molecular pathways, respectively

Combining Molecularly Targeted Agents with Immunotherapy

One of the more extensively studied areas in combinational therapies for RCC has been combining cytokines with VEGF pathway inhibitors. With cytokine therapies being the standard treatment for RCC for years, the advent of VEGF inhibitors naturally led to strategies to combine these agents (Table 17.1). The most commonly explored cytokines used in combination has been interferon and interleukin-2. These agents have several proposed mechanisms of activity against RCC including activation of T lymphocytes and natural killer cells as well as possible antiangiogenic effects [20]. Interestingly, the only FDA-approved combination therapy for RCC is the use of bevacizumab and interferon together which has shown benefit in two large randomized phase III trials.

Bevacizumab and Interferon

Based on earlier trials demonstrating single-agent activity of bevacizumab in RCC [21, 22], two large combination trials with interferon were undertaken. The Cancer and Leukemia Group B (CALGB) trial 90206 was a multicenter, phase III randomized trial comparing the combination of bevacizumab plus interferon with single-agent interferon [15]. There were 732 patients with metastatic RCC enrolled into this study and were stratified based on prior nephrectomy status and number of risk factors. The primary endpoint was overall survival (OS) with secondary endpoints including progression-free survival (PFS), objective response rate (ORR), and safety. The median PFS for the combination arm was 8.5 months compared to 5.2 months for single-agent interferon (HR 0.71, $P<0.0001$). Additionally, the ORR was higher for the bevacizumab-containing arm compared to interferon alone (25.5 % vs. 13.1 %, $P<0.0001$). The primary endpoint of overall survival was not statistically different between the two groups (bevacizumab/interferon 18.3 months vs. interferon 17.4 months, $P=0.097$), although it favored the combination treatment group when adjusting for stratification factors (HR=0.86, $P=0.069$) [16]. As with other randomized phase III trials which have failed to show a survival advantage, a large percentage of patients in the comparator arm went on to receive targeted therapies with known clinical benefit.

The second phase III multicenter, randomized study to be performed evaluating the combination of bevacizumab and interferon was the AVOREN (Avastin and Roferon in Renal Cell Carcinoma) trial [13]. This trial also evaluated the combination of bevacizumab and interferon compared to interferon and placebo at the same dosing administration as in the CALGB trial. The primary endpoint was OS and secondary endpoints were PFS and safety. In this study, 649 patients with treatment-naïve RCC were randomized. The median OS was 23.3 months in the combination arm compared with 21.3 months with the IFN arm (HR=0.91, $P=0.3360$). The secondary endpoint of PFS was significantly different between the two arms with

Table 17.1 Studies evaluating VEGF inhibitor plus immune therapy combinations

Combination	Phase	*N*	Population	ORR	PFS (months)	OS (months)	Common adverse events	Reference
Sorafenib + IFN	Phase I	12	Refractory	8 %	NR	NR	Fatigue, diarrhea, nausea, alopecia, and HFS	[23]
Sorafenib + IFN	Phase I	18	Treated or untreated/Japanese nationality	27.8 %	NR	NR	Fatigue, fever, thrombocytopenia, leukopenia, anemia, weight loss, and anorexia	[24]
Sorafenib + IFN	Single-arm phase II	40	Treated or untreated	33 %	10	NR	Fatigue, anorexia, diarrhea, rash, weight loss, and anemia	[25]
Sorafenib + IFN	Single-arm phase II	62	Treatment naïve	19 %	7	NR	Fatigue, anorexia, anemia, diarrhea, nausea, rigors/chills, leukopenia, fever, and transaminase elevation	[26]
Sorafenib + IFN	Randomized phase II	80	Treatment naïve	25 %	7.56	27.04	Fatigue, diarrhea, and HFS	[28]
Sorafenib + IFN	Randomized phase II (2 different doses of IFN: 9 MU three times weekly, 3 MU three times weekly)	101	Treatment naïve	17.6 % (9 MU) /34 % (3 MU)	7.9 (9 MU)/8.6 (3 MU)	NR	HFS, stomatitis, diarrhea, pyrexia, and fatigue	[27]

Bevacizumab + IL2	Single-arm phase II	51	Treatment naïve	28 %	9	24.6	Hypotension, creatinine elevation, hyperbilirubinemia, fatigue, lymphopenia, thrombocytopenia, and proteinuria	[32]
Bevacizumab + IFN	Randomized phase III	369	Treatment naïve	26 %	8.5	18.3	Hypertension, anorexia, fatigue, and proteinuria	[14, 15]
Bevacizumab + IFN	Randomized phase III	327	Treatment naïve	31 %	10.4	23.3	Fatigue, asthenia, proteinuria, and hypertension	[12, 13]
Sorafenib + IL-2	Randomized phase II	128	Treatment naïve	27.3 %	33 weeks	NR	HFS, stomatitis, diarrhea, pyrexia, and fatigue	[31]
Sunitinib + tremelimumab	Phase I	28	One prior treatment	43 %	NR	NR	Renal failure, colitis, and fatigue	[33]

N number of patients in trial or arm, *ORR* overall response rate, *PFS* progression-free survival, *OS* overall survival, *IFN* interferon-α, *IL-2* interleukin-2, MU million units, *HFS* hand-foot syndrome, *NR* not reported

the bevacizumab combination having a superior outcome (10.2 months compared to 5.4 months for interferon-alone arm, HR 0.63, $P=0.0001$). Based on improved PFS outcomes with bevacizumab and interferon, this regimen was FDA approved in 2009. The side effects commonly seen with this combination in these studies included fatigue, hypertension, anorexia, and proteinuria. One major question that remains with the bevacizumab and interferon combination is whether it is better than single-agent bevacizumab as there has not been a large randomized phase III trial comparing these two approaches.

Sorafenib Plus Interferon

The combination of sorafenib and interferon has been studied in numerous phase I and II clinical trials. A phase I dose-escalation trial by Escudier et al. demonstrated that both agents could be used at full doses together (sorafenib 400 mg twice daily plus interferon 9 million units [MU] three times weekly) [23]. In this study 12 metastatic RCC patients whose diseases were refractory to standard therapy were treated. There were 1 partial response and 8 patients with stable disease, and side effects were tolerable. In a more recently reported phase I trial in Japanese patients, even higher activity was seen. In this study of 18 patients treated in three dose-escalating cohorts, there were 5 partial responses (ORR 27.8 %) and 11 with stable disease [24].

In a single-arm phase II study of sorafenib plus interferon, Gollob et al. treated 40 patients with sorafenib 400 mg twice daily and interferon 10 MU three times weekly [25]. The ORR for this trial was 33 % with a median PFS of 10 months. Common side effects included fatigue, anorexia, diarrhea, rash, weight loss, and anemia. A separate phase II study of 62 patients evaluating the same dosing of the two agents as the Gollob trial demonstrated a 19 % ORR and 50 % stable disease rate [26]. The median PFS was slightly lower at 7 months with a similar side effect profile. Although the combination of these two agents appeared superior to single-agent interferon from prior studies, it was still unclear if this combination was superior to single-agent sorafenib.

A phase II study evaluating two different interferon dosing levels in combination with sorafenib was recently reported by Bracarda et al. [27] In this "pick-the-winner" randomized, non-comparative trial, 101 patients were randomized 1:1 to receive sorafenib 400 mg twice daily plus either interferon 9 MU three times weekly or 3 MU three times weekly. Primary endpoints included PFS and safety. There was a significant difference in median PFS between the two arms with the 3 MU interferon showing a better outcome (8.6 months vs. 7.9 months, $P=0.049$). Additionally, there was a better ORR for the 3 MU arm (34 % vs. 17.6 %), including 6 % ($n=3$) with a complete response.

Perhaps the most definite evaluation of this combination was performed in a randomized phase II trial which compared single-agent sorafenib to the sorafenib and interferon combination [28]. This trial did use an atypical dosing of interferon (0.5 MU twice daily) with standard dosing of sorafenib (400 mg twice daily). A total

of 80 patients were randomized with a primary endpoint of overall survival and safety. While safety outcomes were similar between the two arms, there did not appear to be a difference between the sorafenib and sorafenib plus interferon arms in terms of ORR (30 % vs. 25 %) and median PFS (7.39 months vs. 7.56 months).

In summary of the available data, while the sorafenib and interferon combination appears tolerable and perhaps superior to interferon, it does not appear to be more efficacious than other frontline options such as sunitinib or pazopanib in cross-trial comparisons.

Other Cytokine-Based Combinations

The evaluation of other cytokine regimens has been explored to some extent as well. For example, interferon has also been combined with sunitinib in a phase I study [29]. In this trial, treatment-naïve metastatic clear cell RCC patients were treated with either sunitinib 37.5 or 50 mg daily (4 weeks on, 2 weeks off) plus dose-escalated interferon (up to 9 MU three times weekly). There were 25 patients treated, and all experienced ≥grade 3 events which included fatigue, thrombocytopenia, and neutropenia. The ORR was 12 % with an additional 80 % having stable disease after a median of four cycles. Based on poor tolerability, this combination has not gone forward in further clinical studies.

The mTOR inhibitor, temsirolimus, has also been evaluated in combination studies with interferon. In a phase I/II trial, patients were treated in a dose-escalation fashion with temsirolimus (5–25 mg weekly) plus interferon (6–9 MU three times weekly) [30]. The chosen dose for this combination was 15 mg of temsirolimus with 6 MU of interferon. A total of 39 patients received this dose level including those in the phase II expanded cohort. Of these patients, 8 % had a partial response and 36 % had stable disease as best outcome. The median PFS was 9.1 months for all patients treated. The most common side effects included leukopenia, hypophosphatemia, asthenia, anemia, and hypertriglyceridemia. Interestingly, patients who seemed to have the most benefit with temsirolimus were poorer-risk patients. This combination was later evaluated in a pivotal phase III randomized trial comparing the combination of temsirolimus plus interferon to temsirolimus alone and interferon alone [17]. This trial randomized 626 patients with treatment-naïve, poor-risk metastatic RCC to one of these three arms. The dosing of the combination arm was temsirolimus 15 mg weekly plus interferon 6 MU three times weekly. The primary endpoint of the trial was OS. The study showed that single-agent temsirolimus was superior to interferon in terms of efficacy (both median OS and PFS) and equivalent to the temsirolimus plus interferon combination. Because the addition of interferon to temsirolimus did not improve OS, temsirolimus was FDA approved as a single agent in 2007.

The combination of VEGF inhibitors and IL-2 immunotherapy has also been explored in a few studies. In a randomized phase II trial, sorafenib plus IL-2 was compared to sorafenib alone [31]. The dosing of sorafenib was 400 mg twice daily,

and the dosing of IL-2 was 4.5 million international units (MIU) subcutaneously 5 days per week (6 weeks on, 2 weeks off). Due to the development of a high rate of asthenia (55 % ≥grade3), the protocol was amended after the first 40 patients to an IL-2 dose of 3 MIU subcutaneously 5 days per week (2 weeks on, 2 weeks off). The primary endpoint of the trial was PFS with secondary endpoints of ORR, OS, and safety. There was no significant difference in median PFS between the two arms with the combination arm having a median PFS of 33 weeks and single-agent sorafenib of 30 weeks ($P = 0.109$). Additionally, evaluation of the low-risk subgroup in each arm yielded a similar PFS outcome (47 weeks for combination and 41 weeks for sorafenib alone). In the combination arm the ORR was 27.3 % and was 14.5 % in the sorafenib-alone arm. Common side effects of the combination included hand-foot syndrome (HFS), stomatitis, diarrhea, pyrexia, and fatigue.

Another attempt to combine IL-2 with a VEGF pathway inhibitor was reported by Dandamudi et al and the Cytokine Working Group [32]. In a phase II multicenter trial, high-dose IL-2 (600,000 IU/kg intravenous every 8 h × maximum of 28 doses over two 5-day treatment periods) and bevacizumab (10 mg/kg every 2 weeks) were administered to patients with metastatic clear cell RCC with primary endpoints of PFS and ORR. At the time of the report, there were 51 patients enrolled with 49 evaluable. The median PFS for the evaluable group was 9 months. There were 8 % of patients with a complete response, 20 % of patients with a partial response, and 42 % with stable disease. The ORR of 28 % is similar to that previously seen with IL-2 alone, and the stable disease rate is similar to what is seen with bevacizumab alone. This suggests that efficacy of these two agents together is more additive than synergistic. Toxicities with the combination were deemed similar to that seen with either agent alone and manageable.

Novel immune checkpoint inhibitors have also been developed over the past several years including antibodies which block cytotoxic T-lymphocyte antigen-4 (CTLA-4) and programmed death receptor-1 (PD-1) resulting in T-lymphocyte activation. A combination of sunitinib and tremelimumab, a monoclonal antibody against CTLA-4, has been evaluated in a phase I study [33]. In this trial, 28 patients with previously treated metastatic RCC were enrolled to receive either sunitinib 37.5 mg daily or 50 mg daily (4 weeks on, 2 weeks off) and tremelimumab (dose escalation in cohorts from 6, 10, or 15 mg/kg) every 12 weeks. There were 21 patients who were evaluable, and of these 9 had a partial response (43 %). An additional seven patients had stable disease as the best response. Dose-limiting toxicities were prominent and included renal failure, colitis, perforated bowel, fatigue, and sudden death. It was recommended that the combination of these two agents not go forward in other clinical studies due to the unacceptable toxicity profile. Another immune checkpoint inhibitor which has shown promising activity in multiple cancer types is nivolumab, an anti-PD-1 antibody. This drug has shown a single-agent response rate of 27 % in metastatic RCC in a phase I clinical trial [34]. A multi-arm dose-finding phase I trial of combination nivolumab with either pazopanib, sunitinib, or ipilimumab (CTLA-4 inhibitor) is planned (NCT01472081).

Combining Molecularly Targeted Agents: Vertical Inhibition

Combining VEGF Pathway Inhibitors

The rationale for utilizing two different VEGF pathway inhibitors which target different points in the pathway is to induce a deeper inhibition of the pathway and hopefully create deeper and more durable responses. It has been shown that the use of VEGF receptor inhibitors increases plasma VEGF ligand levels. Therefore, utilization of an anti-VEGF ligand agent in this setting, such as bevacizumab, could create a superior inhibition of the pathway. The combination of VEGF inhibitors has been evaluated in several early-phase trials with promising clinical activity which has been overshadowed by toxicity (Table 17.2).

Sunitinib Plus Bevacizumab

Efforts to combine sunitinib with bevacizumab have been particularly challenging. In a RCC-specific phase I trial, Feldman et al. evaluated various doses of sunitinib with a fixed dose of bevacizumab (10 mg/kg every 2 weeks) [35]. In this 3+3 design, dose-escalation study, 25 patients received treatment with a primary endpoint of MTD. Although, the MTD was described as sunitinib 50 mg 4 weeks on, 2 weeks off, it was difficult for patients to maintain chronic dosing at these levels because toxicities were common and significant. Common adverse events included fatigue, hypertension, proteinuria, diarrhea, HFS, hemorrhage, and dysgeusia. Additionally, there were multiple cases of microangiopathic hemolytic anemia (MAHA; 20 % of patients) reported and MAHA-like features reported in an additional 12 %. There were also two cases of reversible posterior leukoencephalopathy. Despite the poor tolerability of the combination, a high-activity level was seen with an ORR of 52 % (including 1 CR), and an additional 36 % with stable disease. The median PFS for patients treated in the study was 11 months, and the median OS was not reached at the time of the report. Because of the excessive toxicity of the regimen, it was recommended that this combination not be further pursued.

A phase I trial of bevacizumab plus sunitinib in patients with advanced solid tumors has subsequently been reported that included several RCC patients [36]. This dose-escalation trial evaluated the combination beginning at bevacizumab 5 mg/kg every 2 weeks plus sunitinib 25 mg daily 4 weeks on, 2 weeks off. The trial enrolled 38 patients including 6 patients with metastatic RCC. The MTD as defined by the protocol was found to be sunitinib 50 mg daily 4 weeks on, 2 weeks off plus bevacizumab 10 mg/kg every 2 weeks. Common adverse events included fatigue, hypertension, proteinuria, diarrhea, neutropenia, and thrombocytopenia. One DLT of hypertensive crisis was observed in the trial. Responses were seen across tumor types, including RCC with an ORR of 18.2 %. Interestingly, no cases of MAHA were reported in this study. Although the discrepancy between this trial

Table 17.2 Studies evaluating VEGF inhibitor plus VEGF inhibitor combinations

Combination	Phase	*N*	ORR	PFS (months)	OS (months)	Common adverse events	Reference
Single-arm studies							
Bevacizumab + sorafenib	I	48	52 %	14	NR	Rash, weight loss, proteinuria, hypertension, and HFS	[37, 38]
Bevacizumab + sunitinib	I	25	52 %	11	NR	Fatigue, hypertension, proteinuria, diarrhea, HFS, hemorrhage, and dysgeusia	[35]
Bevacizumab plus sunitinib	I	38	18 %	NR	NR	Fatigue, hypertension, proteinuria, diarrhea, neutropenia, and thrombocytopenia	[36]
Bevacizumab plus sunitinib	Case series, prior sunitinib failure	7	42.80 %	8.5	15.1	Asthenia, anorexia, diarrhea, HFS, and mucositis	[40]
Randomized studies							
BeST trial	II						[39]
Bevacizumab + sorafenib		87	30 %	11.3	Median OS NR	Hypertension, fatigue, HFS, diarrhea, hypophosphatemia, and proteinuria	
Comparator: bevacizumab		86	12 %	8.7	Kaplan-Meier comparison, HR 1.01 (NS)		

N number of patients in trial or arm, *ORR* overall response rate, *PFS* progression-free survival, *OS* overall survival, *HFS* hand-foot syndrome, *NR* not reported, *NS* not significant

and the previous Feldman trial remains unclear, it is possible that the presence of nephrectomy (required in the Feldman trial) predisposed patients to this particular adverse event.

Sorafenib Plus Bevacizumab

The use of sorafenib and bevacizumab has appeared to be more feasible but only with significantly reduced dose levels. Sosman et al. performed a phase I trial of the combination of sorafenib and bevacizumab [37, 38]. In this dose-escalation study, cohorts of six patients were treated with combination doses starting at 200 mg twice daily of sorafenib and 5 mg/kg every 2 weeks of bevacizumab. Forty-eight patients were treated and evaluable on the trial. Common toxicities included rash, weight loss, proteinuria, hypertension, and HFS. The maximum tolerated dose was sorafenib 200 mg daily and bevacizumab 5 mg/kg every 2 weeks. Side effects at higher dose levels required many patients to have dose interruptions and dose reductions in the first few weeks. There were 52 % of the patients who experienced a partial response and an additional 23 patients who had stable disease as best outcome. The median PFS was 14 months which is superior to what has been shown with these drugs when used alone.

The recently reported BeST trial (*Be*vacizumab, *S*orafenib, *T*emsirolimus) further evaluated the combination of sorafenib and bevacizumab [39]. This multicenter, multi-arm randomized phase II trial explored three different combination arms compared to single-agent bevacizumab. These arms included bevacizumab plus temsirolimus, bevacizumab plus sorafenib, and sorafenib plus temsirolimus (the combinations including temsirolimus will be discussed separately in the VEGF and mTOR combinations section below). This trial included patients with metastatic clear cell RCC who had prior nephrectomy (nephrectomy was optional in patients with large metastatic disease burden) and no prior VEGF or mTOR inhibitor treatment. The primary endpoint of the trial was PFS with secondary endpoints including ORR, OS, and safety. The study was powered to detect a 67 % improvement in combination therapy compared to single-agent bevacizumab (15 months vs. 9 months). The study included 340 patients who were randomized, eligible, and treated. The four arms were well balanced based on the MSKCC risk criteria and prior nephrectomy status. The median PFS of the bevacizumab plus sorafenib arm was 11.3 months compared to 8.7 months for bevacizumab. Although this arm had the longest median PFS of any arm in the study, it was not statistically different than the single-agent bevacizumab arm with a HR of 0.84 ($P=0.52$). The ORR was significantly higher in the bevacizumab plus sorafenib arm compared to bevacizumab alone (30 % vs. 12 %, $P=0.02$); however, the combination of bevacizumab had more frequent grade 3 or higher adverse events compared to the bevacizumab arm (81 % vs. 39 %). Dose intensity was well maintained for single-agent bevacizumab; however it was poorly maintained for sorafenib with $\leq$75 % of intended dose administered after the second cycle. This could be a potential explanation for the lack of superiority seen.

Salvage Approach

One interesting approach to the application of combined VEGF pathway inhibitors was evaluated by Medioni et al., in which metastatic RCC patients who progressed on single-agent sunitinib were treated by adding bevacizumab [40]. In this case series, seven patients with metastatic RCC were treated with sunitinib monotherapy until progressive disease. Patients were then initiated on bevacizumab 10 mg/kg every 2 weeks. The dosing of sunitinib was continued at the same dose level which was used as monotherapy (doses varied from 25 to 50 mg 4 weeks on, 2 weeks off). Three patients experienced a partial response with the combination treatment (doses of sunitinib ranged from 25 to 37.5 mg in this group). Four patients experienced stable disease lasting >3 months, and one patient had progressive disease as the best response of the combination. The median PFS for the group was 8.5 months which is an impressive number compared to other second-line agents, albeit this report included a small number of patients. The OS for these patients from the onset of combined therapy treatment was 15.1 months. Common adverse events with the combination included asthenia, anorexia, diarrhea, HFS, and mucositis. Although the numbers in this report are very small, the findings raise the possibility of future pursuit of this combination in a salvage setting.

Combining Molecularly Targeted Agents: Horizontal Inhibition

As opposed to vertical inhibition, horizontal inhibition targets different pathways with the intent of inducing a more prolonged benefit by preemptively inhibiting possible resistance mechanisms created by single drug use. The potential advantages would include selecting agents with non-overlapping toxicities to enhance tolerability. There have been multiple attempts to evaluate this approach with available agents.

EGF Pathway Inhibitor Combinations

Early attempts at horizontal inhibition of molecular pathways in metastatic RCC included the combination of VEGF inhibitors and epidermal growth factor (EGF) pathway inhibitors (Table 17.3). The scientific rationale for this combination has been founded on several observations. Both VEGF and transforming growth factor-α (TGF-α, the EGFR ligand) are hypoxia-inducible genes and increased in clear cell RCC due to VHL loss [41–43]. The EGF receptor (EGFR) is commonly expressed in RCC and has been implicated as a possible prognostic finding and therapeutic target [44–47]. Unfortunately, the single-agent evaluation of EGFR tyrosine kinase inhibitors, erlotinib and gefitinib, as well as anti-EGFR antibody, cetuximab, has shown no activity in RCC [48–50]. However, promising results were seen in one

Table 17.3 Studies evaluating EGF pathway inhibitor plus VEGF pathway inhibitor combinations

Combination	Phase	*N*	ORR	PFS (months)	OS (months)	Common adverse events	Reference
Single arm							
Bevacizumab + erlotinib	II	59	25 %	11	NR	Rash, diarrhea, proteinuria, hypertension, and bleeding	[51]
Bevacizumab + erlotinib + imatinib	I/II	88	17 %	8.9	17.2	Rash, hypertension, diarrhea, and fatigue	[52]
Sunitinib + erlotinib	I	15	29 %	NR	NR	Rash, diarrhea, and fatigue	[54]
Sunitinib + gefitinib	I/II	42	37 %	11	NR	Diarrhea, fatigue, rash, nausea, and stomatitis	[53]
Randomized							
Bevacizumab + erlotinib	II	51	14 %	9.9	20	Hypertension, rash, and diarrhea	[22]
Comparator: bevacizumab		53	13 %	8.5	20		

N number of patients in trial or arm, *ORR* overall response rate, *PFS* progression-free survival, *OS* overall survival, *NR* not reported

phase II trial evaluating the combination of erlotinib and bevacizumab. In this study by Hainsworth et al., 59 evaluable patients with metastatic clear cell RCC were treated with bevacizumab 10 mg/kg every 2 weeks plus erlotinib 150 mg daily [51]. Sixty-eight percent of patients had no prior treatment in the trial, and all patients had prior nephrectomy. The ORR was found to be 25 % with one patient having a complete response. The median PFS was found to be 11 months. The 18-month survival rate was 60 % with the median OS not available at the time of report. Also, subset analysis of patients based on prior therapy showed an ORR of 31 % for untreated patients versus 15 % for previously treated patients. Additionally, patients who were treatment naïve had a longer PFS (12.9 months vs. 8.9 months). The side effects of the combination were deemed tolerable by most patients and commonly included skin rash, diarrhea, proteinuria, hypertension, and bleeding.

In a subsequent placebo-controlled randomized phase II trial, the combination of bevacizumab and erlotinib has been compared to single-agent bevacizumab [22]. This study included patients with clear cell metastatic RCC who had prior nephrectomy and were treatment naïve. The primary endpoints of the trial were PFS and ORR. There were 104 patients enrolled with 53 receiving placebo plus bevacizumab and 51 receiving erlotinib plus bevacizumab. The median PFS was 8.5 months for bevacizumab alone and 9.9 months for the combination (HR 0.86, $P = 0.58$). The overall response rate was also similar at 13 % for bevacizumab and 14 % for bevacizumab and erlotinib ($P = 0.999$). The stable disease rate was identical at 68 % for both arms. Interestingly, there was one CR in the combination arm, and this

patient received treatment for 2 years. There was no difference in OS between the two groups with the median OS for the bevacizumab and erlotinib group being 20 months. Side effects were seen more frequently in the combination group and included hypertension, rash, and diarrhea. These findings led to the recommendation of abandoning this combination.

In an additional attempt to expand on the initial effort by Hainsworth and colleagues, a subsequent phase I/II study evaluated the bevacizumab/erlotinib combination with the addition of the PDGF inhibitor imatinib [52]. The phase I portion of the trial established the MTD of imatinib to be 400 mg daily in combination with erlotinib and bevacizumab. There were 94 patients treated with the combination with 88 evaluable for response. The ORR was found to be 17 % with an additional 61 % having stable disease. The median PFS was 8.9 months, and the median OS was 17.2 months. Common ≥grade 3 side effects included rash, diarrhea, and fatigue. Cumulative toxicity made chronic therapy with this 3-drug regimen challenging, commonly necessitating dose reduction and with ten patients discontinuing due to intolerable side effects. There was also a treatment-related death due to diarrhea, sepsis, and bowel perforation. Given the lack of improved efficacy with the 3-drug regimen and the increased toxicity, this particular combination was not recommended to move forward in further studies.

Other early studies have been performed evaluating EGFR inhibitors with sunitinib. Motzer and colleagues reported a phase I/II trial evaluating the combination of EGFR tyrosine kinase inhibitor gefitinib and VEGF TKI sunitinib [53]. The MTD from the dose-escalation phase I was found to be sunitinib 37.5 mg daily plus gefitinib 250 mg daily. Of the 35 patients who received this dose in the study (including the phase II portion), the ORR was 37 %, and the median PFS was 11 months. In a separate ongoing phase I/II study, Ryan and colleagues described tolerability of the combination of erlotinib and sunitinib [54]. The confirmed ORR from patients evaluable in an early report of the study was 29 %. Although these two small studies have shown tolerability with sunitinib plus EGFR inhibitor therapy, the combinations' effectiveness appears similar to that of single-agent sunitinib. Based on the available clinical studies, the addition of EGF pathway inhibitors to standard VEGF therapy does not appear to improve outcome over single-agent VEGF inhibition alone. This is most likely explained by the lack of single-agent benefit of EGF pathway inhibitors in RCC patients.

Combining VEGF Inhibitors and mTOR Inhibitors

As opposed to the EGF pathway inhibitors, VEGF pathway inhibitors and mTOR inhibitors have both been successfully used as single agents at treating RCC patients. The combination of these two classes therefore would seem a natural way to improve outcomes via horizontal molecular blockade. Molecularly, these two treatment pathways are largely non-overlapping with downstream targets varying. Additionally,

the cells which these drugs are targeting are theoretically separate cells with the VEGF inhibitors blocking signaling in the stromal support cells such as the vascular endothelial cells and the mTOR inhibitors inhibiting the tumor cells themselves in addition to the stromal cells. Based on this rationale and the availability of several active agents, a variety of clinical trials have been conducted in patients with metastatic RCC.

VEGF TKI Plus mTOR

There has been extensive clinical trial experience combining VEGF inhibitors and mTOR inhibitors. The results of these trials are summarized in Table 17.4. Just as with VEGF/VEGF combination therapies, sunitinib has been difficult to combine with mTOR inhibitors. In a phase I study by Patel et al., sunitinib was combined with temsirolimus in previously treated metastatic RCC patients [55]. Even at lower-dosing schedules, two of the first three patients developed dose-limiting toxicities, and the study was terminated. A combination of sunitinib with everolimus has also been evaluated in a phase I trial [56]. In this 3 + 3 dose-escalation trial, 20 patients were treated with varied doses of the combination. The MTD was found to be weekly everolimus at 30 mg per week plus sunitinib 37.5 mg 4 weeks on, 2 weeks off. However, this dose was not able to be used chronically, and a recommended dose was everolimus 20 mg weekly plus sunitinib 37.5 mg 4 weeks on, 2 weeks off. The ORR for the combination on this trial was 25 %. Common adverse events included fatigue, mucositis, diarrhea, hemorrhage, rash, and hypertension. Unfortunately, these efficacy results are not superior to what could be seen with sunitinib alone at full dosing, and given the additive side effect profile, the combination of sunitinib and an mTOR inhibitor does not appear to be appropriate for future studies.

Combinations with sorafenib have also been explored with similar issues of combining at full-strength dosing. A phase I dose-escalation study by Harzstark et al. evaluated 20 metastatic RCC patients with varied dosing of sorafenib and everolimus [57]. The MTD was sorafenib 400 mg twice daily plus everolimus 5 mg daily. Dose-limiting toxicities seen included gout, pancreatitis, and rash. Common adverse events were diarrhea, HFS, hypertension, hypophosphatemia, and rash. The ORR was 25 % which is somewhat higher than expected with these single agents alone. In a more promising phase I study of sorafenib and everolimus, Amato et al. explored the combination in a dose-escalation trial involving 15 clear cell RCC patients who had been previously treated [58]. In this trial the MTD was full dosing of both agents (sorafenib 400 mg twice daily and everolimus 10 mg daily). The ORR for the study was 40 % which included two complete responses. An additional 25 % of patients had stable disease. The median PFS for the group was 5.6 months, and the median OS was 7.9 months. Common adverse events included diarrhea, rash, HFS, alopecia, and mouth sores. The combination of sorafenib and everolimus in this trial appeared to have a higher efficacy rate which was likely explained by a higher MTD which was applicable for these patients.

Table 17.4 Studies evaluating combinations of VEGF pathway inhibitors and mTOR inhibitors

Combination	Phase	*N*	ORR	PFS (month)	OS (months)	Common AE	Reference
Single-arm studies							
Sunitinib + temsirolimus	I	3	NR	NR	NR	Study terminated due to multiple DLTs in first dosing cohort, rash, and TCP	[55]
Sunitinib + everolimus	I	20	25 %	NR	NR	Fatigue, mucositis, diarrhea, hemorrhage, rash, hypertension	[56]
Sorafenib + everolimus	I	20	25 %	TTP = 2–32.4+	NR	Diarrhea, HFS, hypertension, hypophosphatemia, rash	[57]
Sorafenib + everolimus	I	15	40 % (2 CRs)	5.6	7.9	Diarrhea, rash, HFS, alopecia, mouth sores	[58]
Tivozanib + temsirolimus	I	28	28 %	NR	NR	Fatigue, decreased appetite, stomatitis, TCP, diarrhea, nausea, constipation, and dyspnea	[75]
Bevacizumab + temsirolimus	I/II	46 (40 evaluable)	23 %	6-mo PFS rate 40 %; TTP 7.6	20.6	Fatigue, hypertriglyceridemia, stomatitis, proteinuria, abdominal pain, anemia	[61]
Bevacizumab + everolimus	II	80	Treatment naïve 30 % (1 CR); prior treatment 23 %	Treatment naïve 9.1; prior treatment 7.1	Treatment naïve 21.3; prior treatment 14.5	Proteinuria, mucositis, fatigue, and diarrhea	[62]
Randomized studies							
TORAVA	II						[64]
Bevacizumab + temsirolimus		88	27	8.2	NR	Fatigue, skin disorders, proteinuria, and hypertension	
Comparator arms: sunitinib		42	29	8.2	NR		
Bevacizumab + IFN		41	43	16.8	NR		
INTORACT	III						[65]

Bevacizumab + temsirolimus		400	27 %	9.1	25.8	Proteinuria, hypertension, anemia, mucosal inflammation, stomatitis, hypophosphatemia, hyperglycemia, and hyperlipidemia	
Comparator arm: bevacizumab + IFN		*391*	*28 %*	*9.3*	*25.5*		
RECORD-2	II						[63]
Bevacizumab + everolimus		182	23 %	9.3	NR	Stomatitis, proteinuria, diarrhea, hypertension, epistaxis	
Comparator arm: bevacizumab + IFN		*183*	*26 %*	*10*	*NR*		
BeST	II						[39]
Bevacizumab + temsirolimus		81	28 %	7.3	NR	Hypertension, fatigue, diarrhea, proteinuria, hyperglycemia	
Sorafenib + temsirolimus		86	27 %	7.7	NR	Hypertension, fatigue, diarrhea, hypophosphatemia, hyperglycemia	
Comparator arm: bevacizumab		*86*	*12 %*	*8.7*	*NR*		

N number of patients in trial or arm, *ORR* overall response rate, *PFS* progression-free survival, *OS* overall survival, *IFN* interferon-α, *NR* not reported, *DLT* dose-limiting toxicity, *TCP* thrombocytopenia, *HFS* hand-foot syndrome

Bevacizumab Plus mTOR Inhibitor

The evaluation of bevacizumab and mTOR combinations has progressed to later-phase trials given its tolerability in several phase I trials [59, 60]. In a phase I/II trial by Merchan, the combination of bevacizumab and temsirolimus was explored [61]. In this study, 46 patients with previously treated (≤2 prior therapies) clear cell metastatic RCC were enrolled. At the time of the study report, 40 patients were evaluable for response. This study showed an ORR of 23 % and SD rate of 63 %. The 6-month PFS rate was 40 % with a median TTP of 7.6 months. The median OS was 20.6 months. Commonly seen adverse events included fatigue, hypertriglyceridemia, stomatitis, proteinuria, abdominal pain, and anemia. These results compare favorably with the single-agent activity of everolimus in the second-line setting from the pivotal phase III trial [18]. A phase II trial of the combination of bevacizumab and everolimus has also been completed. In this study, 80 patients with clear cell metastatic RCC who had undergone prior nephrectomy were enrolled [62]. Two populations of patients were included: group A, treatment naïve, and group B, previously treated with sunitinib, sorafenib, or both (patients in group B could also have had one other prior systemic therapy). In the group A cohort, the ORR was 30 % (including 1 CR), the median PFS was 9.1 months, and the median OS was 21.3 months. In the group B cohort, the ORR was 23 %, median PFS was 7.1 months, and the median OS was 14.5 months. While the results in the treatment-naïve cohort (group A) were similar to what is seen with single-agent VEGF TKI such as sunitinib or pazopanib, the group B results did compare more favorably with what is seen with single-agent everolimus in this setting. To further answer the question of bevacizumab plus mTOR inhibitor versus mTOR inhibitor in the post-VEGF TKI setting, a large randomized trial is underway. This placebo-controlled phase III trial will evaluate the combination of bevacizumab and everolimus to everolimus alone in patients who have progressed after frontline VEGF TKI therapy and should help to further define VEGF/mTOR combination therapy (CALGB 90802; NCT01198158). A series of phase II and III trials exploring mTOR plus VEGF inhibition in treatment-naïve patients has recently been reported.

RECORD-2

Among these recently reported studies, the RECORD-2 (REnal Cell cancer treatment with Oral RAD001 given Daily-2) trial was an open-labeled randomized phase II trial which explored the combination of bevacizumab and everolimus with the combination of bevacizumab and interferon. This trial was recently reported by Ravaud et al and included 365 treatment-naïve metastatic RCC patients who were randomized 1:1 to receive everolimus plus bevacizumab or interferon plus bevacizumab [63]. The primary endpoint of the trial was PFS with secondary endpoints including ORR and OS. The median PFS for the everolimus plus bevacizumab group was 9.3 months compared to 10 months for the interferon plus bevacizumab

group (HR 0.91, $P=0.485$). The ORR for the two groups was also similar, being 27 % and 28 %, respectively. Of note, the median duration of treatment was similar between the groups (~8 months), and the frequency of discontinuation due to adverse events was also similar (23 % vs. 26 %). The median OS was not reached at the time of this initial report. Side effects of the everolimus plus bevacizumab combination included stomatitis, proteinuria, diarrhea, hypertension, and epistaxis.

TORAVA

The TORAVA trial (*TOR*isel and *AVA*stin) is a randomized 3-arm phase II trial which evaluated the combination of temsirolimus and bevacizumab with the approved therapies of sunitinib and bevacizumab plus interferon [64]. Patients included in the trial were required to have confirmed RCC (all histologies allowed except papillary), treatment naivety, ECOG performance status of ≤2, adequate organ function, and absence of brain metastases. Patients were randomized in a 2:1:1 fashion to receive the combination of bevacizumab 10 mg/kg IV every 2 weeks plus temsirolimus 25 mg IV weekly (BT), or single-agent sunitinib (S) 50 mg orally daily for 4 weeks on then 2 weeks off, or the combination of bevacizumab 10 mg/kg IV every 2 weeks plus interferon 9 MIU three times per week (BI). The primary endpoint was the rate of patients who were progression-free at 48 weeks. This endpoint was chosen over PFS because fewer patients were required to find statistical benefit in the trial. There were 171 patients randomized ($n=88$ BT, $n=42$ S, and $n=41$ BI) over an approximately 14-month period.

Surprisingly, the BT group did not demonstrate a superior efficacy to sunitinib or bevacizumab/interferon-treated patients. The results demonstrated a 48-week PFS rate of 29.5 % for the BT group, 35.7 % for S, and 61 % for BI. The median PFS was 8.2 months, 8.2 months, and 16.8 months, respectively. Additionally, the response rates were 27 %, 29 %, and 43 %, respectively. The clinical benefit rate (CR + PR + SD) was similar between the three groups (77, 77, 76 %). There were 2CRs seen in the BT combination group (2 %), and none seen in the other two arms. There is no mention of durability of these CRs in the publication. Finally, tolerability appeared worse in the BT group with more than 50 % of patients who discontinued therapy doing so because of toxicity rather than disease progression. There was also a much higher rate of dose interruptions in the BT group compared to the others (BT 51 %, S 12 %, and BI 38 %). The most common adverse events for the BT group included fatigue, skin disorders, proteinuria, and hypertension. The efficacy results of the BT combination were surprising in that they failed to show a synergistic or additive benefit for the combination. It appears that the BI group did better than expected likely due to better tolerability (and therefore higher dose intensity) and more low-risk patients in that cohort (39 %). Based on the results of this trial, it is difficult to find reason to continue with the bevacizumab and temsirolimus combination in frontline clinical studies due to its poor long-term tolerability and lack of additive or synergistic efficacy.

BeST

As previously discussed, the randomized phase II BeST trial is a multi-arm trial which not only evaluated the VEGF/VEGF combination of bevacizumab and sorafenib (Arm C) but also evaluated two VEGF/mTOR combinations compared to single-agent bevacizumab (Arm A) [39]. The details of the design of this study were discussed in the VEGF plus VEGF inhibitor section. The two VEGF plus mTOR inhibitor arms were bevacizumab plus temsirolimus (Arm B) and sorafenib plus temsirolimus (Arm D). There was no statistical difference between any of the combination arms compared to single-agent bevacizumab in terms of PFS. The median PFS was 7.3 months for Arm B (HR 0.91, $P=0.62$) and 7.7 months for Arm D (HR 1.11, $P=0.55$). The ORR was significantly higher for both arms compared to bevacizumab (Arm B 28 %, $P=0.03$; Arm D 27 %, $P=0.05$). Toxicities for both Arms B and D were additive between the two agents combinations, and ≥grade 3 toxicities were common (Arm B 75 %, Arm D 82 % compared to Arm A 39 %). Based on these results, it was not recommended that either VEGF/mTOR combination that was evaluated proceed forward with phase III testing.

INTORACT

The multicenter, multinational randomized phase III trial INTORACT (INvestigation of TORisel and Avastin Combination Therapy) was recently presented at the 2012 European Society of Medical Oncology (ESMO) conference which further highlighted the combination of bevacizumab and temsirolimus in a large prospectively treated population [65]. In this study, 791 clear cell RCC patients who were treatment-naïve were randomized 1:1 to receive bevacizumab 10 mg/kg IV every 2 weeks plus temsirolimus 25 mg IV weekly (BT) or bevacizumab 10 mg/kg IV every 2 weeks plus IFN 9 MU (BI) subcutaneously three times weekly. Patients were stratified by MSKCC risk and nephrectomy status. Dose reductions were allowed for temsirolimus and interferon but not for bevacizumab. The primary objective was to compare the PFS between the two treatment arms. The trial was well randomized and balanced for prior nephrectomy status and risk profile. The trial results showed no difference in the median PFS between the two groups with the median PFS for the BT combination group being 9.1 months compared to 9.3 months for the BI group (HR 1.07, $P=0.759$). The ORR for the BT group was 27 % (with 1 CR) and stable disease rate was 55 %. Similarly, the ORR for the BI group was 28 % (with 2 CRs) and stable disease rate was 47 %. Additionally, a lack of overall survival difference was seen. The median duration of response was slightly longer in the BI group at 17 months compared to 11 months for the BT group. However, there was no difference in the median OS. The median OS for the BT cohort was 25.8 months compared to 25.5 months for the BI group (HR 1.04, $P=0.638$). Frequent side effects for the BT combination included proteinuria, hypertension, anemia, mucosal inflammation, stomatitis, hypophosphatemia, hyperglycemia, and hyperlipidemia. Interestingly, the combination of bevacizumab and temsirolimus resulted in fewer

pneumonitis episodes (1 %) than would be expected of temsirolimus alone. Based on these phase III findings of non-superior PFS and OS, there appeared to be no clinical advantage for the use of the combination of bevacizumab and temsirolimus going forward.

Combining Current Targeted Agents with Novel Antiangiogenesis Agents

As compared to VEGF inhibitor-VEGF inhibitor combinations which are vertical in the mechanism of activity, the use of agents which inhibit alternate angiogenesis pathways in combination with VEGF inhibitors is a form of horizontal blockade of angiogenesis. As previously stated, due to the transactivation of hypoxia-inducible genes in most RCC patients via loss of VHL, these tumors are addicted to angiogenesis. The biology of angiogenic escape in the setting of VEGF pathway inhibition of RCC appears to be complex with several mechanisms implicated, including activation of the angiopoietin pathway [66–68] and fibroblast growth factor (FGF) pathway [69–71] and the presence of myeloid-derived suppressor cells and proinflammatory cytokines [72, 73]. Several approaches to combining antiangiogenic agents with VEGF pathway inhibitors are being explored.

Angiopoietin Inhibitor Combinations

AMG-386 is a novel peptibody formed by fusion of peptide and the Fc portion of IgG which interferes with angiopoietin ligand-receptor interaction. It represents a first-in-class inhibitor of both angiopoietin-1 and angiopoietin-2 and has had promising single-agent results in a phase I trial [74]. This agent has been combined with a VEGF TKI in two randomized phase II trials. In the first study by Rini et al., 152 patients with treatment-naïve clear cell RCC were randomized 1:1:1 to receive sorafenib 400 mg twice daily plus either weekly AMG-386 (10 mg/kg), weekly AMG-386 (3 mg/kg), or placebo. The primary endpoint of the trial was PFS. The median PFS for the trial groups were 9.0, 8.5, and 9.0 months, respectively (HR 0.88, $P = 0.523$). There did seem to be an advantage in terms of ORR with the combination groups having a higher response rate (10 mg/kg arm, 38 %; 3 mg/kg arm, 37 %) compared to the sorafenib-alone arm (25 %). Common adverse events for the combination arms included diarrhea, HFS, alopecia, and hypertension.

A second phase II trial has been performed evaluating the combination of AMG-386 with sunitinib in the VEGF inhibitor-naïve metastatic RCC population. This trial was also a randomized trial evaluating two arms: arm A, sunitinib 50 mg daily 4 weeks on, 2 weeks off plus AMG-386 10 mg/kg weekly, and arm B, sunitinib 50 mg daily 4 weeks on, 2 weeks off plus AMG-386 15 mg/kg weekly. The primary endpoints of the trial included the evaluation of adverse events and the frequency of dose interruptions during the first 12 weeks of treatment. Secondary

endpoints included PFS and ORR. The trial was well balanced in terms of MSKCC risk groups between the two arms. Common adverse events included hypertension, HFS, asthenia/fatigue, elevated lipase, diarrhea, mucositis, thrombocytopenia, and neutropenia. These side effects occurred at similar rates between the two arms. The frequency of dose interruptions in the first 12 weeks was 58 % (arm A) and 57 % (arm B). The median PFS for the arm A group was 13.9 months with the median PFS of arm B still being too immature to report. The overall response rate was similarly high in both arms (arm A ORR = 58 % including 1 CR; arm B ORR = 59 %). This study shows impressive response rates and PFS rates compared to that seen with single-agent sunitinib from other frontline studies. Currently, another randomized phase II combination trial is ongoing for pretreated patients with metastatic RCC evaluating the combinations of pazopanib plus AMG-386, bevacizumab plus AMG-386, and sorafenib plus AMG-386 compared to single-agent AMG-386 (NCT01664182).

Vascular-Disrupting Agents

Vascular-disrupting agents (VDA) are drugs which impact the endothelial cell lining of tumor vessels. There are currently two classes of VDAs: agents that disrupt tubulin via the colchicine binding site and flavonoid compounds which induce endothelial cell apoptosis. Either mechanism of VDA activity results in a mechanical disruption of flow in tumoral blood vessels, thus inducing tumoral necrosis. BNC105P is a novel VDA agent which disrupts tumoral blood flow by binding and interfering with tubulin activity. This agent is being evaluated in a phase I/II combination with everolimus in patients previously treated with VEGF TKI (NCT01034631). The phase II portion of this study is ongoing and is randomizing patients to either the combination BNC105P and everolimus or everolimus alone.

Conclusions

For years immune therapies were the main therapeutic option for RCC with a minority of patients treated with cytokine therapy achieving durable responses and no extension of average survival. These clinical outcomes changed with the introduction of VEGF and mTOR targeted therapies. Combination approaches with VEGF inhibitors has been somewhat successful with the FDA approval of bevacizumab and interferon, as well as the tolerability seen with some of the IL-2 and IFN combinations with anti-VEGF agents. However, there still remain many unanswered questions such as the optimal dosing of interferon with agents such as sorafenib. Also, a rigorous evaluation of bevacizumab alone compared to the combination of bevacizumab and interferon is yet to be performed which would prove the necessity of the interferon component of this regimen. From review of the smaller phase I and II studies, at best there appears to be a small additive benefit of cytokine therapy to

VEGF inhibitors with a small increase in response rates, including complete response rates. However, PFS appears not to be any better than with single-agent VEGF-targeted treatment. Therefore, it is unclear if these combination approaches should go forward when active single agents exist with better toxicity profiles. Certainly, more work with newer immune checkpoint inhibitors is required and may be a way forward for combination therapies. The combination of cytokine therapy and mTOR inhibitors does not appear to play any meaningful role based on the available data.

The combination of VEGF pathway targeting agents appears to offer higher response rates at the cost of increased toxicity. The use of sunitinib and bevacizumab together has led to frequent intolerable toxicities and in one study led to an unacceptable degree of MAHA. Other smaller studies have suggested that this combination may be more tolerable in patients with an intact primary kidney as well as in the salvage setting. The combination of sorafenib and bevacizumab appears to be somewhat more tolerable at a significantly dose-modified level but still with a prominent toxicity profile. A phase II randomized trial comparing sunitinib alone to sunitinib plus bevacizumab in treatment-naïve metastatic RCC patients was closed to accrual due to unacceptable toxicity (NCT00491738). Currently, the future of VEGF combinations is unclear in RCC management, although it certainly seems unlikely to be fruitful. Two studies exploring pazopanib and bevacizumab in combination are ongoing with their results anticipated (NCT 01202032 and NCT 01684397).

Certainly, horizontal blockade of molecular pathways with non-overlapping toxicities seems the most reasonable approach; however, identifying an active combination has been challenging. Although combinations of EGF pathway and VEGF pathway inhibitors initially appeared to be promising, subsequent studies have been unable to reproduce these effects, and this approach is no longer felt to be an option. Additionally, based on the data from available trials, the combination of VEGF pathway inhibition and mTOR inhibition does not appear the way forward in improving outcomes for metastatic RCC patients in the frontline setting. Bevacizumab combinations with an mTOR inhibitor appear to be better tolerated than VEGF tyrosine kinase inhibitor combinations. One exception to this appears to be the combination of tivozanib and temsirolimus which in a solitary phase I trial showed tolerability at full doses of both drugs [75]. Unfortunately, frontline clinical activity of VEGF/mTOR combinations is similar to single-agent VEGF TKI or bevacizumab plus interferon, and toxicity appears to be tolerable for some regimens but also additive in nature. The role of VEGF/mTOR combination therapy may be somewhat more promising in the second-line setting. The CALGB 90802 study will shed further light on combination therapy in the post-TKI setting comparing bevacizumab plus everolimus to everolimus.

In summary, we currently find ourselves at an important crossroad in RCC therapeutics. In a very short period of time, the development and approval of new molecularly targeted therapies outpaced our ability to understand mechanisms of resistance and determine who benefits the most from these drugs. Attempts to combine the available agents have demonstrated a lack of additive or synergistic efficacy but do increase the toxicity profile compared to single-agent treatments.

Perhaps as we better understand certain patient tumor subsets in terms of sensitivity to these agents, we can then build individualized combinations where the efficacy *will* be a great leap forward compared to sequential use of single drugs. Finally, there is still hope that new promising targets will be identified and that these drugs will be compatible with our current profile of options in combination treatments. Ironically, the agents which appear to fit this profile currently are immune therapies such as ipilimumab and nivolumab. Although the road behind is littered with negative combination studies, the road forward seems full of hope that we will soon see even greater advancements for this devastating disease.

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Chapter 18
Side Effects of Targeted Therapy

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Immunotherapy

Interferon alfa (IFN-α) and interleukin-2 (IL-2) have been used for years in mRCC treatment. IFN-α provides an overall survival (OS) benefit compared to control arm as demonstrated in phase III trials and a meta-analysis [1–5]. High-dose IL-2 has a higher overall and complete response (CR) rate compared with low-dose cytokines, with a real benefit in the small percentage of patients (5–7 %) that experience a durable CR [6, 7]. The small number of patients who benefit from high-dose IL-2 overall precludes demonstration of a disease-free or OS advantage with high-dose IL-2 for the entire cohort when compared to low-dose cytokine regimens [4, 8].

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Interferon-α Monotherapy

IFN-α became a standard therapy in metastatic RCC, with a 10–15 % objective response rate (ORR) and a median survival of approximately 12 months [5]. The side effects observed in these patients are flu-like syndrome, asthenia, weight loss, anorexia, gastrointestinal disorders, dizziness, confusion, peripheral neuropathy, and hematological toxicity.

In a multicenter, randomized trial, patients with metastatic renal carcinoma were assigned to subcutaneous (SC) interferon-α or oral medroxyprogesterone acetate (MPA). A larger proportion of interferon-α patients than of MPA patients reported moderate or severe symptoms: anorexia, asthenia, nausea, lack of energy, shivering, and dry mouth. At 12 weeks a larger proportion of interferon-α patients than of MPA patients reported moderate or severe lack of appetite, and a larger proportion of MPA patients reported moderate or severe dyspepsia [9].

Interferon-α Combination

The combination of IFN-α and IFN-γ was explored on the basis of in vitro observations indicating a synergism between both agents [10]. Most patients treated with this combination developed fever, anorexia, fatigue, and flu-like symptoms.

The combination of IFNα2a plus vinblastine (VLB) was associated with constitutional symptoms and abnormalities in laboratory parameters, but no toxic deaths were reported [1]. Adverse events noted in patients treated with the combination of IFNα2a plus VLB were characteristic of IFN-α toxicity and similar in type and frequency to toxicities described in previous studies of this combination. The most frequent events were fatigue, fever, and flu-like symptoms.

In a randomized phase III trial conducted by Motzer et al., combination therapy with 13-*cis*-retinoic acid (13-CRA) plus IFNα2a was compared to IFNα2a alone in 284 patients with advanced RCC [11]. There was no difference in grade 2–4 toxicities between treatment arms. Grade 2 toxicities reported in 20–40 % of patients were leukopenia, anemia, fever, and gastrointestinal toxicity. Grade 3 and 4 hematologic toxicity was reported in 21 and 1 % of patients. A total of 32 % patients had grade 3–4 non-hematologic toxicity [11].

Toxicity of IFN in combination with IL-2, bevacizumab, or temsirolimus will be discussed in other sections of this chapter.

Interleukin-2

In most clinical studies, IL-2 was given either as an intravenous bolus (IV) every 8 h or as a 5-day continuous infusion. Because severe side effects are frequent with these protocols, a throughout patient selection has to be done and treatment with high-dose IV IL-2 has to be administered in an intensive care unit or in a monitored standard oncology ward [12–15].

Interleukin-2 Monotherapy

Immunotherapy using subcutaneous IL-2 alone or in combination with SC IFNα2a showed drastically reduced systemic toxicity as compared with IV bolus or continuous infusions. In a single-institution phase II study of IL-2 administered by subcutaneous injection, systemic side effects in the patients were tolerated and accepted and included transient inflammation and local induration at the injection sites, fever and chills, nausea, and some dry desquamation of the skin, especially of the palms and soles. Fever and chills were relatively common and, in most cases, were not controllable with antipyretic treatment [16]. One patient died as a result of myocardial infarction and brain stem ischemia.

A major limitation to the administration of IL-2 has been the occurrence of significant toxicities when given at a high dose. Initially, administration of high-dose IL-2 was associated with mortality rates of up to 4 %. However, the incidence and severity of toxicities have decreased as clinicians have gained experience with this agent and implemented toxicity prevention and management strategies.

The most common major toxicities have been fluid retention, edema, hypotension, and oliguria, which often require support with low-dose dopamine and, in some cases, phenylephrine. In addition, encephalopathy, coma, myocarditis, and cardiac arrhythmias can occur. Many severe IL-2 toxicities are related to capillary leak syndrome (CLS) [15, 17]. While the pathogenesis of CLS is still not completely understood, the clinical spectrum of IL-2 toxicities is well described. These side effects are quite transient and reverse readily after IL-2 therapy is discontinued.

A randomized prospective study performed to compare toxicity of high-dose IV bolus IL-2 and a lower-dose IV bolus regimen for the treatment of metastatic renal cell carcinoma (RCC) demonstrated a greater incidence of grade 3 or 4 thrombocytopenia, malaise, and hypotension in patients who received high-dose IL-2, while patients who received low-dose IL-2 had significantly more infections [18]. Both regimens showed similar frequencies of CNS toxicities, with disorientation and somnolence occurring in 8 and 6 % of high-dose IL-2 courses.

High-dose intravenous IL-2 therapy can be associated with cardiac toxicity. Some authors have reported rates of myocardial infarction in the range of 2–6 % [15, 19]. At the initial experience reported in 1989 by Lee et al., IL-2 was associated with profound cardiovascular changes ranging from sinus tachycardia to myocarditis and respiratory effects ranging from mild shortness of breath to the requirement for intubation [15].

Some authors have described catheter-related bacteremia as possible severe complication of IL-2 therapy [20, 21]. In fact common etiologies of mortality for early study patients included progressive sepsis, besides the abovementioned severe pulmonary compromise, and myocardial dysfunction [22–24].

Since the clinical introduction of intravenous IL-2, much has been learned regarding its therapeutic role and toxicities. Progressive reduction in morbidity and mortality was found with the systemic administration of high-dose IL-2-based therapies over the last 20 years. The improvement in safety most likely reflects the development of strategies to screen eligible patients, optimize therapeutic conditions, and judiciously terminate dosing when significant toxicities are noted.

Interleukin-2 Combination

The main toxicity of IL-2 and IFN-α combination consisted of an alteration of general status, but most of the time this alteration was mild or moderate with fever, fatigue, hypotension, and anorexia [25]. Digestive disorders were commonly observed and usually consisted of nausea or vomiting, rather than diarrhea [26, 27]. Transient elevations of creatininemia were noted. Neurological disturbances include repetitive transient confusion events, mild insomnia, and moderate anxiety.

A prospective multicenter phase II trial of a low-dose subcutaneous regimen of IL-2 and IFN-α in patients with metastatic renal cancer demonstrated that virtually all patients noted a rash and erythema, usually at the site of IL-2 injection. Approximately 50 % of patients developed a generalized erythema, which led to desquamation, most noticeable on the palms [28]. The toxicity of the regimen was modest and consisted primarily of systemic symptoms. The symptom complex of fever, anorexia, fatigue, and weight loss occurred in 85–90 % of patients.

A three-drug combination of IL-2/IFN-α/13-*cis*-retinoic acid was administered in an ambulatory regimen to patients with previously untreated metastatic RCC. Grade 3 or greater toxicities during the first cycle included flu-like symptoms (21 % of patients), fatigue (6 %), and nausea and vomiting (15 %). Significant cumulative toxicities were hyperlipidemia (22 %) and cardiomyopathy (6 %). There was one therapy-related death.

The IL-2 working group reported a randomized phase II trial to determine better the activity of high-dose IL-2 either alone or in combination with IFN-α in patients with metastatic renal cell carcinoma [29]. On the whole, the side effects were similar between the treatment arms and typical of what has been previously reported with either high-dose IL-2 or IL-2/LAK cell therapy [17, 30].

In the study of Negrier et al., 425 patients with metastatic renal cell carcinoma were randomly assigned to receive either a continuous intravenous infusion of IL-2, SC IFN-α, or both. In general patients who received IL-2 had greater toxicity than IFN-α patients. Grade 3 or 4 fever was more common with the combined treatment. Fever and hypertension were the most common adverse events in the two groups receiving interleukin-2 [31].

In an IL-2-based home therapy of SC IL-2 alone, SC IL-2 plus IFN-α, with or without intravenous 5-fluorouracil, grade 3 or 4 toxicity was low. In most patients systemic side effects were limited to grade 1 or 2. Thus, malaise, fever, and chills (grade 1 or 2) were seen in 78, 72, and 57 % of treatment cycles, respectively. Mild anorexia (grade 1 or 2) occurred in 88 % of treatment cycles and was frequently associated with nausea and vomiting (77 % of treatment cycles) and/or diarrhea. Mucositis (grade 1 or 2) occurred in 51 % of treatment cycles but did not require dose modification [32].

Bevacizumab

Vascular endothelial growth factor (VEGF) and its receptor (VEGFR) are overexpressed in the majority of renal cell carcinomas [33, 34]. These characteristics have supported the rationale of targeting VEGF-driven tumor vascularization, especially in clear cell RCC. VEGF-inhibiting strategies include both the use of tyrosine kinase inhibitors and of neutralizing antibodies, which have been evaluated in RCC. Among them, bevacizumab represents an antibody that exclusively targets VEGF, inhibiting the interactions of this ligand with all of the receptors to which it binds [35].

Bevacizumab Profile of Toxicity as a Single Agent

As a single agent, bevacizumab treatment (3 and 10 mg/kg q2w mg) has shown activity across a direct inhibition of VEGF in clear cell metastatic mRCC and an excellent profile of toxicity with the only grade 3 toxicities being hypertension (20 %) and proteinuria (8 %) among 39 patients treated with the highest dose. No life-threatening adverse events potentially related to bevacizumab were observed in this study among 76 patients [36] (Table 18.1).

Bevacizumab Profile of Toxicity in Combination with Interferon-α

Two randomized, multicenter phase III trials, the AVOREN study and the Cancer and Leukemia Group B (CALGB) 90206 intergroup study, have examined the combination of bevacizumab with INF compared with the single-agent IFN in patients with mRCC. The final results of these trials have demonstrated significant clinical benefits with the combination with respect to IFN monotherapy in terms of overall response rate and PFS but not in overall survival [37, 38]. In both studies, patients with previously untreated mRCC were randomized to bevacizumab (10 mg/kg q2w) plus IFN (9 MIU tiw) or IFN (9 MIU tiw). The AVOREN study was placebo

Table 18.1 Adverse events of bevacizumab as monotherapy [36]

	(10 mg/kg q2w) No. patients 39 (%)	(3 mg/kg q2w) No. patients 37 (%)
Epistaxis	8 (20)	5 (13)
Hypertension	14 (36)	1 (3)
Hematuria	5 (13)	1 (3)
Proteinuria	25 (64)	15 (40)

Adapted from J.C. Yang et al. [36]

controlled, and the IFN component was limited to a 1-year duration, with continued use of bevacizumab or placebo permitted beyond 1 year, whereas the CALGB 90206 was an open study. Both trials allowed dose reductions for IFN in case of toxicity [37, 38]. Taking into account these characteristics, the most commonly reported grade 3–4 AEs were those to be associated with IFN such as fatigue and asthenia in the AVOREN study and fatigue and neutropenia in the CALGB trial. In particular, in the AVOREN trial, AEs occurred in 328 (97 %) of patients who received the combination of bevacizumab plus IFN and in 287 (94 %) of those who received only IFN. Serious adverse events were reported in 98 (29 %) patients who received bevacizumab and 50 (16 %) of those who did not. Grade 3–4 AEs in patients who received bevacizumab included arterial hypertension, gastrointestinal perforations, and thromboembolic events. A higher proportion of patients were withdrawn from the bevacizumab treatment due to proteinuria (5 %), hypertension (2 %), and gastrointestinal perforation (2 %). Deaths due to adverse events were reported in 2 % of patients in both arms. Only three (<1 %) deaths of the patients who received bevacizumab (two bleeding events and one gastrointestinal perforation) were related with this drug [37].

In the CALGB 90206 study and among patients evaluable for toxicity (362 for bevacizumab plus IFN and 347 for IFN), 80 % of those receiving bevacizumab plus IFN experienced grade 3 toxicity compared with 63 % of patients receiving only IFN m ($p=0.001$). Bevacizumab plus IFN resulted in significantly more grade 3 hypertension (11 vs. 0 %), anorexia (17 vs. 8 %), fatigue (37 vs. 30 %), and proteinuria (15 vs. 1 %). There were four (1.1 %) treatment-related deaths on the IFN arm and three (<1 %) on the bevacizumab plus IFN arm [39]. Globally, both trials have shown that the addition of bevacizumab to IFN led to higher overall incidences of hypertension, proteinuria, and bleeding with respect to IFN monotherapy. It is worth mentioning that a retrospective analysis of patients in the AVOREN study who received low-dose IFN (3 and 6 MU) indicates that efficacy was maintained while the rates of these AEs grades 3–4 were decreased considerably analyzing the 6-week period after IFN dose reduction compared with the 6 weeks before reduction (44 vs. 18 %) (7) (Table 18.2). However, there has been a suggestion from studies across solid tumors (including RCC) that treatment-induced arterial hypertension (AHTN) may be a biomarker of clinical outcome [40]. In this way, a retrospective analysis carried out in the CALGB study suggested that patients on bevacizumab plus IFN who developed grade 2–3 AHTN had a significantly PFS and OS compared with patients who did not develop AHTN. Even more, on multivariate analysis, the development of AHTN was an independent predictor of OS (HR 0.622; 95 % CI, 0.390–0.992; $p=0.046$) [41].

Bevacizumab Profile of Toxicity in Combination with Other Drugs

There are additional trials analyzing the activity and safety of bevacizumab combined with TK or mTOR inhibitors. In this way, there have been published results of a randomized phase II trial in which patients were allocated to receive bevacizumab

Table 18.2 Bevacizumab treatment-related adverse events [37, 39]

	Bevacizumab + IFN (%)		IFN monotherapy (%)		Bevacizumab + IFN post dose reduction (%)
	All grades	Grades 3–4	All grades	Grades 3–4	Grades 3–4
Fatigue	33–93	12–37	27–90	30	1
Pyrexia	45	2	43	<1	1
Anorexia	36–71	3–17	30–61	3–8	3
Nausea/vomiting	58	7	59	5	3
Influenza-like illness	25	3	25	2	5
Asthenia	32	10	28	7	2
Neutropenia	43	9	36	9	3
Depression	12	3	10	2	1
Dyspnea	13–15	3	9–13	1–2	2
Thrombocytopenia	6–10	2	4–9	<1	1
Diarrhea	20	15	2	<1	8
Headache	23	16	2	1	1
Anemia	10–16	12–20	3–4	4–6	1
Hypertension	26–28	9–11	4–9	<1	15
Bleeding	6–33	1–3	9 to <1	<1	7
Proteinuria	18–71	7–15	3–10	<1	15
Venous thromboembolic	3–4	2	2	<1	1
Arterial thromboembolic	1	<1	1	<1	3
Gastrointestinal perforation	1	0	1	0	0
Wound-healing complications	1	1	1	0	1
Congestive heart failure	<1	0	<1	0	0

Adapted from the AVOREN and CALGB 90206 studies

(10 mg/kg q2w) plus placebo or the combination of bevacizumab (10 mg/kg q2w) and erlotinib (150 mg qd) [41]. Both regimens were similar in terms of efficacy and toxicity, but one death due to gastrointestinal perforation occurred in the bevacizumab-plus-erlotinib arm. AEs associated with TKIs (rash and diarrhea) were found only in the erlotinib arm, and similar rates of hypertension and proteinuria were observed in both arms. More recently, two phase II/III trials have failed to confirm a clinical benefit when bevacizumab is associated with temsirolimus or everolimus over bevacizumab and IFN as first-line therapy for clear cell mRCC [42, 43]. The INTORACT trial, a phase III open-label, multicenter study, compared temsirolimus (25 mg/weekly) plus bevacizumab (10 mg/kg q2w) with interferon (9 MIU tiw) plus bevacizumab (10 mg/kg q2w) in patients with clear cell mRCC. At the data cutoff for final analysis of 489 patients, there were grade ≥3 stomatitis, hypophosphatemia, hyperglycemia, and hypercholesterolemia being more frequently registered in those patients treated with temsirolimus and bevacizumab ($p < 0.001$). On the other hand, the open-label, phase II RECORD-2 trial compared bevacizumab (10 mg/kg q2w) associated with IFN (9 MIU tiw) or with everolimus

(10 mg oral daily). Looking at AEs, there was a tendency to be more frequently registered in the group of patients treated with everolimus and bevacizumab especially in terms of stomatitis (63 %), proteinuria (49 %), diarrhea (39 %), hypertension (38 %), and epistaxis (35 %) with respect of those registered with the combination of bevacizumab and IFN such as decreased appetite (45 %), fatigue (41 %), proteinuria (37 %), and pyrexia (35 %) [43].

Given the multiple treatment options now available in the treatment of mRCC, patient comorbidities and drug-associated toxicities may impact the choice of agents to be used. For example, apart from common AEs related with anti-VEGF TKI such as fatigue and AHTN, bevacizumab is not associated with hand–foot syndrome (HFS) or congestive heart failure. However, it has been associated with other serious AEs that may limit its utility in certain patient subgroups, such as those with nephrotic syndrome, diverticular disease, or a history of arterial thrombotic events. Additionally, the combination of bevacizumab with IFN-a can also give rise to toxicities associated with the latter agent, such as fatigue, flu-like symptoms, and asthenia that may adversely affect quality of life.

Taking into account all these considerations, there are no recommended dose reductions for bevacizumab, but contraindication or discontinuation of this drug must be carried out in patients with the following circumstances:

- Gastrointestinal perforations, diverticular disease, or intra-abdominal abscess.
- Fistula formation involving an internal organ.
- Wound dehiscence and wound-healing complications requiring medical intervention.
- Serious hemorrhage or severe arterial thromboembolic event.
- Severe hypertension not controlled with medical management, hypertensive crisis, or hypertensive encephalopathy.
- Reversible posterior leukoencephalopathy syndrome.
- Nephrotic syndrome. Moderate-to-severe proteinuria.
- Severe infusion reactions.

In addition, temporarily suspension of bevacizumab for at least 4 weeks prior to elective surgery is required.

Sunitinib

Sunitinib, a multi-targeted antiangiogenic tyrosine kinase inhibitor, has been approved in first-line treatment for those patients with good and intermediate prognosis clear cell metastatic renal cell carcinoma, based on the efficacy observed in a clinical comparative trial. With respect to IFN-α, patients treated with sunitinib have a significant improvement in progression-free and overall survival [44]. These long-term outcomes are focusing toward side effects related with sunitinib and how to manage them in order to maintain patients on therapy [45]. On the other hand,

Table 18.3 Adverse events related with sunitinib [44, 48, 108]

AE	Grade 3 (%)	Grade 4 (%)	All grades (%)
Fatigue/asthenia	95 (17.5)	1 (0.2)	397 (73.0)
Diarrhea	38 (7.0)	0 (0.0)	326 (59.9)
Nausea	19 (3.5)	0 (0.0)	290 (53.3)
Taste disturbance	1 (0.2)	0 (0.0)	250 (46.0)
Decreased appetite	9 (1.7)	0 (0.0)	205 (37.7)
Dyspepsia	8 (1.5)	0 (0.0)	189 (34.7)
Stomatitis	13 (2.4)	0 (0.0)	185 (34.0)
Vomiting	17 (3.1)	0 (0.0)	178 (32.7)
Skin discoloration	1 (0.2)	0 (0.0)	144 (26.5)
HFS	46 (8.5)	0 (0.0)	144 (26.5)
Hypertension	55 (12)	0 (0.0)	143 (30)
Mucosal inflammation	8 (1.5)	0 (0.0)	127 (23.3)
Rash	2 (0.4)	1 (0.2)	121 (22.2)
Dry skin	1 (0.2)	0 (0.0)	108 (19.9)
Abdominal pain	10 (1.8)	0 (0.0)	106 (19.5)
Hair color changes	0 (0.0)	0 (0.0)	103 (18.9)
Edema	5 (0.9)	0 (0.0)	99 (18.2)
Pain in extremity	6 (1.1)	0 (0.0)	96 (17.6)
Neutropenia	46 (8.5)	5 (0.9)	89 (16.4)
Thrombocytopenia	37 (6.8)	5 (0.9)	86 (15.8)
Epistaxis	3 (0.6)	0 (0.0)	86 (15.8)
Ejection fraction decrease	16 (2.9)	0 (0.0)	84 (15.4)
Constipation	1 (0.2)	0 (0.0)	83 (15.3)
Headache	3 (0.6)	0 (0.0)	82 (15.1)
Hypothyroidism	6 (1)	0 (0.0)	72 (12 %)

the safety and tolerability profiles are different from those observed with sunitinib and other TKIs. Some similarities, as well as differences, related in part to their mechanisms of action have been observed among the targeted agents. In this sense, sunitinib shows mainly antitumor activity targeting PDGFR, VEGFR, stem cell factor receptor (c-KIT), Flt3, CSF-1R, and RET [46, 47]. The safety profile of sunitinib has been well documented based on several clinical trials and an expanded-access program. Most side effects related with this TKI are mild to moderate in intensity and manageable including mostly fatigue, diarrhea, nausea, hypertension, and hand–foot syndrome (Table 18.3). In the pivotal phase III trial, approximately 50 % of patients in the sunitinib group had a dose reduction, and the safety analysis in the expanded-access program with this drug noted that dose modifications allowed patients to remain on treatment for a long time with no new or unexpected side effects in a group of unselected patients, including a population of 65 or more years of age [44, 48]. Effective management of side effects associated with sunitinib and

other TKIs should involve thorough assessment and appropriate intervention both before and during treatment. This should enable patients to maintain optimal dosing in order achieve the greatest clinical benefit and outcomes.

Fatigue and Asthenia

Fatigue and asthenia represent one of the most frequent AEs related with sunitinib affecting 73 % of patients (18 % grades 3–4). It remains unclear how much fatigue is cancer related and what is sunitinib associated. The origin of this symptom (cancer-related fatigue and sunitinib induced) is unknown [49]. Sunitinib-related fatigue is more frequently presented in men, particularly in young people, with previously treated patients tending to improve during the 2-week off-treatment period. Although a recurrent problem, there did not appear to be an increase with long-term treatment cycles but rather it appears to decrease. Before starting the treatment with sunitinib, it is important to advise patients on the possible occurrence of fatigue; evaluate patients for potential underlying causative factors such as depression, emotional distress, sleep disturbance, hypothyroidism, and anemia; and treat underlying factors according to standard medical practice. During the treatment with sunitinib (specially the first cycles), monitoring of patients regularly for impact of fatigue on quality of life is required as well as looking at signs and symptoms related with anemia, depression, and hypothyroidism, initiating appropriate treatment when necessary. In terms of a specific treatment for fatigue/asthenia, there are very few evidence-based interventions. In example, comparative trials have suggested better response in patients using resistance training for aerobic exercise [50]. On the other hand, additional studies are needed to clarify the role of nutritional supplements such as L-carnitine, melatonin, and American ginseng in fatigue management or the benefit described with psychostimulants [51].

Diarrhea and Other Gastrointestinal Toxicities

Diarrhea is the second more frequent AE related with sunitinib that occurs in 60 % of patients treated with this drug (7 % grade 3). Mechanisms for sunitinib-induced diarrhea are not known, although ischemic colitis that has been reported after treatment with other anti-VEGF TKIs might be one of the reasons [52]. Patient education regarding nutrition before starting treatment is recommended in terms of adequate hydration, temporarily discontinued use of stool softeners, and some fiber supplements as well as magnesium-containing antacids, avoiding spicy foods, fatty foods, as well as caffeine. Dose reductions are rarely necessary for grades 1–2, which can be managed by oral hydration and oral antidiarrheal agents, such as loperamide. Treatment should be interrupted for grade 3–4 diarrhea with dose/schedule

modification in subsequent cycles. Other gastrointestinal side effects related with sunitinib, including indigestion and abdominal pain, occur with varying frequency (46–30 %). In this sense, sporadic cases of acalculous and emphysematous cholecystitis related with sunitinib probably mediated by endothelial ischemic injury in the gallbladder must have special consideration [53].

Emesis

Approximately 50 % of patients treated with sunitinib experience some grade of nausea but less than 5 % is referred as grade 3 [44, 48]. Common antiemetic drugs can be used to relieve or prevent emesis. However, particular care should be used when combining sunitinib with antidopaminergic agents, such as domperidone, or 5HT3 antagonists, such as granisetron, ondansetron, and dolasetron, because they have been associated with QT/QTc interval prolongation [54].

Oral Toxicity (Stomatitis/Mucositis)

Reversible oral changes, including sensitivity and taste changes, dry mouth, and oral mucosal sensitivity, occurred in 50 % of patients treated with sunitinib. Most toxicities are grade 2. Dose adjustments or treatment discontinuation due to grades 3–4 is infrequent (2.4 %). Mechanism and characteristics of this toxicity related with sunitinib appear to be primarily a "functional" irritation of the mucosa and differ from those seen with chemotherapy. Patients report a general sensitivity in the mouth, which feels sore, or they have alterations in taste, but clinical findings are largely normal and patients do not experience the typical physical signs of a mucositis/stomatitis caused by chemotherapy including ulceration.

Before starting treatment with sunitinib, patients must be advised to switch to a pediatric toothpaste and soft toothbrush and avoid alcohols, spirits, and spicy foods. Treatment for oral toxicity developed with sunitinib administration requires using bicarbonate-based mouthwashes containing paracetamol with morphine or codeine. Herbal products containing chamomile, sage, arnica, and zinc may help to reduce discomfort [55].

Skin Toxicity

Generalized erythema and maculopapular or seborrheic dermatitis-like rashes have been reported in approximately 20–35 % of patients treated with sunitinib, being mostly grades 1–2 [56]. These kinds of toxicities rarely require dose reduction, and symptoms tend to decrease over time. Patients should be advised to use

moisturizing skin creams frequently in particular after showers and before bedtime as well as to avoid hot showers and use sun protection. Urea-containing lotions may be helpful, in particular if the skin is very dry. Topical steroid must be reserved only for severe cases. Reversible yellow discoloration of the skin due to the yellow color of the active drug and metabolite as well as hair depigmentation can appear, likely due to signaling block of c-KIT in the melanocyte proliferation/differentiation process [57].

Hand–Foot Syndrome (HFS)

In the sunitinib studies, approximately 35 % of patients developed HFS. Hand–foot changes may present as painful symmetrical erythematous and edematous areas on the palms and soles, commonly preceded or accompanied by paresthesias, tingling, or numbness. Desquamation can occur in severe cases (Fig. 18.1). Painful hyperkeratotic areas on pressure points surrounded by rings of erythematous and edematous lesions as well as painful bullous lesions, blisters, or skin cracking may be noted. These most often occur in areas of flexure or pressure. Preexisting sole hyperkeratosis seems to confer a predisposition for painful sole involvement and functional consequences. HFS induced by sunitinib may clinically resemble the more classic chemotherapy-induced palmar–plantar erythrodysesthesia. The exact mechanism and pathogenesis of this type of HFS are not known. Some publications suggest that dermal vessel alteration and apoptosis might be due to direct anti-VEGFR and/or anti-PDGFR effects of sunitinib on dermal endothelial cells [56]. Histological characteristics include dermal vascular modifications with slight endothelial changes, vascular alterations associated with extensive and linear layers of keratinocyte necrosis with intraepidermal cleavage, and peri-bullous lesions in those cases with severe HFS [58].

Algorithm for the management of sunitinib-related HFS has been published. Prevention measures include frequent contact with clinician to ensure early HFS diagnosis, full-body skin exam, pedicure, thick cotton gloves and/or socks, avoid

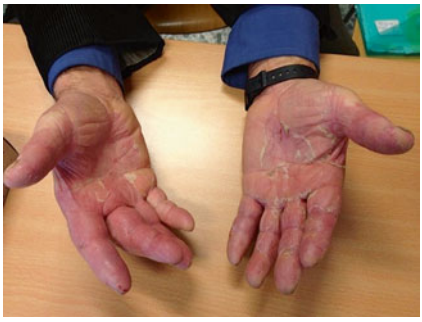

Fig. 18.1 Hand–foot syndrome in a patient receiving sunitinib

hot water and constrictive footwear, and excessive friction. Toxicity grade 1 requires topic moisturizing creams for relief and 20–40 % urea creams without dose reduction of sunitinib. If symptoms worsen after evaluation at 2 weeks or grade 2 is registered at any time, dose reduction of sunitinib to 50 % is required. Treatment includes the above topic creams mentioned as well as clobetasol, lidocaine, codeine, or pregabalin for pain control. Grade 3 HFS requires treatment as with grades 1–2 and dose interruption for at least 7 days until improvement to grade ≤1 with subsequent reduction and/or schedule modifications.

Cardiovascular Toxicity (Hypertension and Cardiac Dysfunction)

Data suggest that the risk of hypertension and other cardiovascular events related with sunitinib and other TKI is dose dependent.

Hypertension (HTN) is the most commonly reported cardiovascular toxicity documented with targeted agents. In a phase III study of sunitinib, all-grade and grade 3–4 hypertension occurred in 30 and 12 % of patients treated with this drug, respectively [44]. In an expanded-access study with sunitinib, the incidence of all-grade hypertension was 22 % and grades 3–4 was 5 % [48]. Inhibition of the VEGF receptor might increase vascular resistance, caused by decreased nitric oxide and prostacyclin production, as well as provoke vascular rarefaction and increased arterial stiffness, resulting in hypertension [59].

Before the initiation of sunitinib, patients should be assessed for prehypertension. Those showing prehypertension should receive antihypertensive treatment for 3–7 days before the commencement of targeted therapy and with regular monitoring during sunitinib treatment. The selection of antihypertensive medication should be based on the general cardiovascular status of the patient, as well as taking into account interactions and contraindications with other drugs. No clear recommendations for specific antihypertensive therapies can be made. Nevertheless, there is some evidence supporting the selection of particular antihypertensive drugs in preference to others based on the patient's status, as described by guidelines published by the European Society of Cardiology [60]. Preference for vasodilatory drugs, such as ACEI, angiotensin II receptor blockers, or calcium channel antagonists, with respect to diuretics or beta-blockers, is based on the vasoconstrictive activity of the anti-VEGFR TKI (Fig. 18.2). Additionally, the potential impact of CYP3A4 induction or inhibition with sunitinib and other drugs should be considered. In this sense, calcium channel blockers, such as verapamil or diltiazem, should be avoided. On the other hand, although elevated blood pressure has been proposed to reflect the clinical efficacy of VEGF signal inhibition with targeted treatment, control of hypertension is essential to avoid serious AEs without detrimental antitumor activity.

Cardiac dysfunction observed with sunitinib includes reduced left ventricular ejection fraction (LVEF), congestive heart failure (CHF), conduction disturbances,

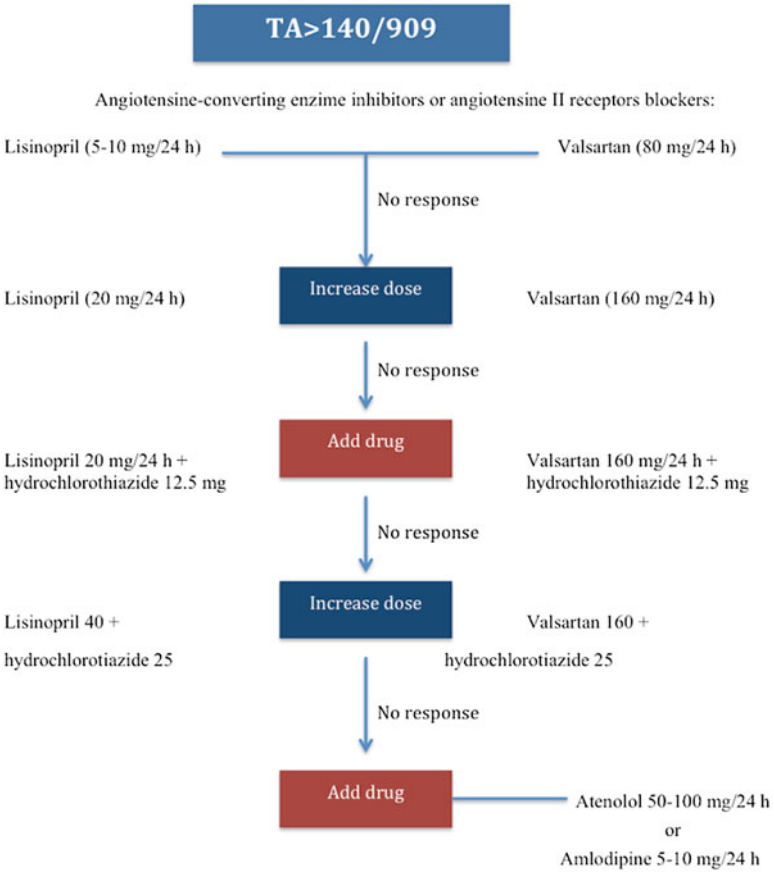

Fig. 18.2 HTA management

and changes in the ST segment or T-wave. A review of treatment-related AEs in phase III trials of targeted therapies in mRCC reported incidences of all-grade and grade 3–4 LVEF decline of 15 and 3 %, respectively, with sunitinib [61]. Pathogenesis of this toxicity could be mediated by cardiomyocyte expression of PDGFR and RAF-1 function. The inhibition of PDGFR might result in the apoptosis of cardiomyocytes and cardiotoxicity. In addition, disruption of RAF-1 function might lead to increased cardiomyocyte apoptosis [62].

Before starting treatment with sunitinib, an ECG and echocardiography of LVEF should be carried out in patients at high cardiovascular risk during every treatment cycle for the first four cycles, and every three cycles subsequently, and every three cycles in patients with no cardiovascular history [62]. If LVEF <50 % or decrease >20 % below baseline, dose interruption should be considered. Treatment of cardiac ischemia and rhythm disturbances can include administration of anti-ischemic and

antiarrhythmic medications to increase blood flow to the heart and promote a regular heartbeat. Patients with signs of ischemia or rhythm disturbances should be referred immediately to the cardiologist and appropriate treatment initiated.

Hematological Toxicity

Sunitinib induces neutropenia and thrombocytopenia in 25 % of patients, although less than 9 % develop grade 3–4 hematological toxicity. Recent data suggest a significantly higher incidence of myelotoxicity in Asian patient populations. In general this AE is not accumulative and blood counts are usually recovered within the 2-week treatment break. Mechanism of sunitinib myelotoxicity is mainly mediated through inhibition of c-KIT.

Complete blood counts should be performed at the beginning of each treatment cycle. In case of myelosupression or thrombocytopenia grade 3-4 sunitinib should be stopped until hematologic recovery. Dose reduction or schedule changes in sunitinib treatment are required in those situations of recurrent grade 3–4 neutropenia or thrombocytopenia persisting for at least 5 days and/or neutropenic fever/bleeding signs. Patients with asymptomatic grade 3–4 neutropenia, at the end of the 4-week treatment period and rapid recovery in the 2-week off-treatment period, may not require a dose reduction, but a schedule change may be considered. Because of the usually rapid recovery of blood counts during the 2-week off-treatment period, hematologic growth factors are rarely indicated.

Hypothyroidism

In clinical trials hypothyroidism has been reported in 12 % of mRCC patients receiving sunitinib, with 1 % of patients experiencing grade 3–4 symptoms [63]. There is a discrepancy between incidence rates reported in prospective trials and retrospective series, most likely due to infrequent testing for hypothyroidism. Thyroid dysfunction while receiving sunitinib can present as TSH elevation only with normal T4 levels (subclinical hypothyroidism) or TSH elevation and low T4.

Underlying mechanism of this toxicity is unknown. Sunitinib effect in the VEGF receptor could produce destructive thiroidiis throguh follicular cell apoptosis. Thyroid hormone replacement clinically benefited only about 40–50 % of the patients treated, suggesting other sunitinib-induced mechanisms for these side effects [64]. Management recommendations include regular surveillance of thyroid function. Patients developing hypothyroidism should be treated with thyroid hormone replacement therapy. Those with asymptomatic subclinical hypothyroidism can be followed without levothyroxine therapy and treated when symptomatic hypothyroidism is present [65]. Treatment interruptions or dose modifications for thyroid dysfunction are generally not necessary (Table 18.4).

Table 18.4 Management of hypothyroidism

Preventive measures	Baseline TSH, T3, T4 testing, repeating every 8–12 weeks for new or existing hypothyroidism
Management strategies	Initiate levothyroxine (25–50 μg/24 h)[a] for patients with elevated TSH level (>10 mU/L) and/or for patients with symptomatic hypothyroidism
Follow-up	Adjust levothyroxine every 6–8 weeks based on lab results, with the objective of achieving a TSH level of 0.5–2.5 mU/L

[a]Patients >50 years old with cardiopathy: initiate levothyroxine at lower doses (12.5–25 μg/24 h)

Pazopanib

Pazopanib is an inhibitor of the vascular endothelial growth factor receptor that has been approved by the FDA and the EMA for patients with metastatic kidney cancer. In the pivotal phase III trial, progression-free survival improved significantly (5 months) compared to placebo. The results of the COMPARZ study have been recently reported, showing that pazopanib is not inferior to sunitinib [66, 67].

The most frequent side effects of pazopanib are diarrhea, hypertension, hair color changes, vomiting, and anorexia. The most frequent grade 3–4 toxicities are hypertension, diarrhea, and increase in hepatic enzymes (Table 18.5).

Asthenia and Anorexia

Asthenia and anorexia are present in 14 and 22 % of cases, respectively. However only 2–3 % of patients presented grade 3–4 toxicity [66]. If anorexia appears, the use of megestrol acetate or cannabinoids should be contemplated. As discussed for sunitinib, potential underlying causative factors for asthenia should be identified and treated.

Gastrointestinal Toxicity

Diarrhea is present in 52 % of patients, but only in 4 % ot them is severe [66]. Fluid replacement is recommended as well as administration of loperamide or racecadotril. In the COMPARZ study, incidence of diarrea (any grade) was 61 %, being severe in 9 % of patients [67]. In case of severe diarrhea, pazopanib should be discontinued, and when patients are recovered, a dose reduction should be considered.

In the COMPARZ study, nausea and vomiting were reported in 26 % of patients, with an incidence of grade 3 emesis in 3 % of them [66]. Particular care should be

Table 18.5 Pazopanib toxicities over 15 % [67]

	All grades (%)	Grades 3–4 (%)
Diarrhea	52	4
Hypertension	40	4
Changes in hair color	38	<1
Nausea/vomiting	26/21	<1/2
Anorexia	22	2
Fatigue	20	2

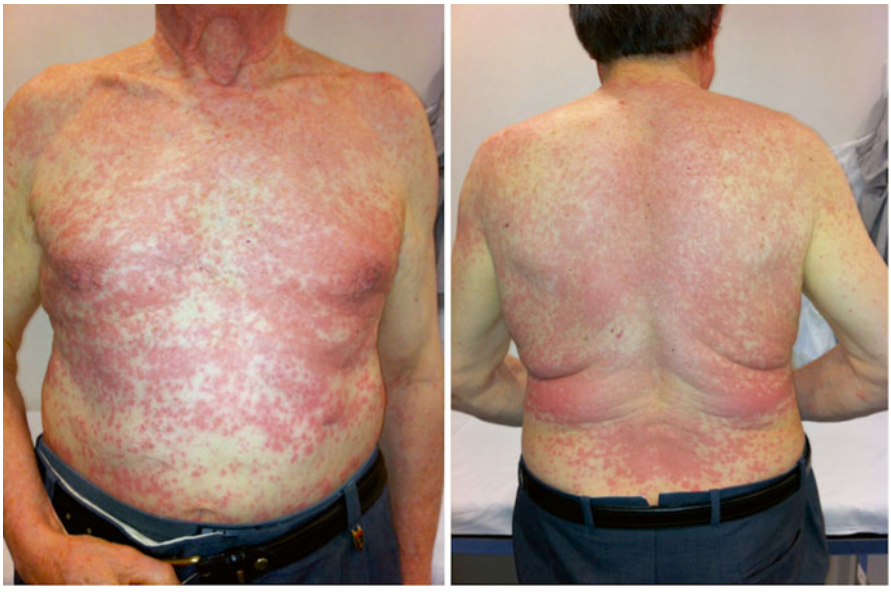

Fig. 18.3 Pazopanib-induced rash. Courtesy of Dr. Enrique Gallardo

taken when combining pazopanib with antidopaminergic agents, such as domperidone, or 5HT3 antagonists, such as granisetron, ondansetron, and dolasetron, because they have been associated with QT/QTc interval prolongation.

Skin Toxicity

Pazopanib causes hair color changes in 38 % of patients. This toxicity is reversible after pazopanib discontinuation [66]. Hand–foot syndrome was observed in 11 % of patients in a phase II trial (grades 3–4 in less than 1 % of them) [68]. The incidence of skin rash in the same phase II trial was 16 %, with an incidence of grade 3–4 rash less than 15 % (Fig. 18.3) [68]. In both pazopanib phase III trials, there was no mention of skin rash.

Hypertension

In a phase I trial with 63 patients, of which 12 had kidney cancer, hypertension was not considered a dose-limiting toxicity (DLT) unless it was not controlled with an antihypertensive medication [69]. With a dose of 800 mg/day, one patient developed severe asymptomatic HTN on day 2. This patient already had a record of controlled HTN; the dose was reduced one level and new antihypertensive drugs were added. However, when increasing again the dose to pazopanib 800 mg/day, the patient developed grade 3 proteinuria.

In the phase II trial by Hutson et al. in 225 patients with kidney cancer, HTN was present in 41 % of the whole group [68]. Only 9 % of patients presented grade 3 HTN and no grade 4 toxicity was recorded. In the pivotal phase III trial compared to placebo, with 435 patients included (randomization 2:1), HTN was present in 40 % of patients, and only 4 % was grade 3 [66]. The COMPARZ study compared pazopanib to sunitinib in first-line setting. In this study the incidence of HTN in any grade was 46 % for pazopanib versus 41 % in the sunitinib arm [67].

Adequate monitoring of arterial pressure is recommended before starting as well as during the treatment. In case of HTN, drugs such as angiotensin-converting inhibitors, angiotensin II receptor blockers, beta-blockers, diuretics, or calcium antagonists (avoiding verapamil or diltiazem) could be used (Fig. 18.2).

Cardiovascular Toxicity

In pazopanib phase II trials, an incidence of QT interval prolongation (≤500 mseg) lower than 2 % was found, with *torsades de pointes* in less than 1 % [69]. In the placebo-controlled phase III study to evaluate efficacy and safety of pazopanib, only three of the 290 patients treated with pazopanib had QT interval values between 500 and 549 mseg [66]. Due to this type of toxicity, it is recommended to use pazopanib cautiously in patients with QT prolongation and in patients taking drugs that could prolong such interval (such as quinidine, procainamide, flecainide, amiodarone, disopyramide, sotalol, macrolides, quinolones, pentamidine, some psychotropes, antidepressants, or 5HT3 antagonists).

Hepatotoxicity

Hurwitz et al. reported an increase of AST and ALT in 38 and 24 % of patients, respectively, with hyperbilirubinemia in 13 % (grade 3 in 2 % of patients) [69]. In phase II and III trials, the incidence of hypertransaminemia was about 50 %, grades 3–4 in 7–12 % of cases. Hepatotoxicity usually occurs early, in the first 18 weeks of treatment [66, 68]. In the same trials, hyperbilirubinemia was present in

28–36 % of patients (grades 3–4 in 1 and 3 %, respectively). In the COMPARZ study, the incidence of hypertransaminemia grades 3–4 was 12 % for AST and 17 % for ALT versus 3 and 5 %, respectively, for sunitinib, with an incidence of severe hyperbilirubinemia similar for both arms [67].

The hepatic function should be monitored before starting the treatment and every 4 weeks during the first 4 months of pazopanib or when indicated. Patients with increase of transaminases eight times the highest normal limit should suspend treatment until they recover to grade 1 or to normal limits. Later on reintroducing treatment at 400 mg/day should be contemplated.

Hematologic Toxicity

Pazopanib induces anemia in 26–32 % of patients, but in only 1–2 % of cases was grades 3–4 [66, 68]. The incidence of thrombocytopenia was 34 %, but it was severe in only 1 % of patients [66].

Patient Preferences

The randomized PISCES trial evaluated the difference in tolerance and safety between pazopanib and sunitinib. One hundred and sixty-eight patients were included in this study, designed not to compare the efficacy of each treatment but the quality of life and patient preference between both drugs. The study showed that most patients, after taking both treatments, preferred pazopanib (70 %) in comparison with sunitinib (22 %). Approximately 8 % of patients did not show any preference for either one [70].

Tivozanib

Tivozanib is a selective VEGFR tyrosine kinase inhibitor that blocks angiogenesis and permeability in tumor tissues. In a randomized phase II trial, tivozanib was administered in second-line renal carcinoma patients [71]. The most frequent side effects were hypertension and dysphonia, with a low incidence of grade 3–4 toxicity.

In the TIVO-1 phase III trial, pazopanib was compared to sorafenib in first line or in second line after progression to immunotherapy [72]. Only 18 % of patients with tivozanib needed dose reductions due to toxicity versus 35 % of patients with sorafenib. Hypertension, diarrhea, and dysphonia were the most frequent side effects (19.6) (Table 18.6).

Table 18.6 Tivozanib toxicities over 15 % [71, 72]

	All grades (%)	Grade 3 (4) (%)
Hypertension	44	24 (2)
Diarrhea	22	2
Dysphonia	21	0
Fatigue	18	5
Weight decreased	17	<1
Asthenia	15	4

Asthenia and Anorexia

Only 10 % of patients presented some degree of anorexia, while the incidence of asthenia was 15–28 % (severe in 4–10 % of cases) [72]. If anorexia appears, the use of megestrol acetate or cannabinoids should be contemplated; in the case of asthenia, it is important to identify and correct potentially treatable causes.

Gastrointestinal Toxicity

The incidence of diarrhea was 22 vs. 34 % for tivozanib and sorafenib, respectively, but grades 3 and 4 diarrhea was 2 and 0 % for tivozanib, respectively. Treatment of diarrhea includes dietary measures and loperamide 2 mg after each deposition, with a maximum of 16 mg daily. Tivozanib provokes nausea in 11 % of patients (1 % grade 3) [72].

Dysphonia

Dysphonia was reported from the start of clinical development of pazopanib. In the phase III trial, Motzer described a 21 % of dysphonia but there was no grade 3–4 toxicity. Up to now, hypotheses regarding the cause of dysphonia have not been formulated.

Skin Toxicity

In the TIVO-1 the incidence of hand–foot syndrome was 13 % for tivozanib (2 % grade 3) and 54 % for sorafenib (17 % grade 3). Therefore, tivozanib seems to present better skin tolerance profile. The incidence of alopecia was only 2 % [72].

Hypertension

In the phase I trial conducted by Eskens, HTN was the most frequent side effect [73]. HTN was described in 45 % of patients in a phase II trial, with grade 3–4 HTN in 12 % of them [71]. The incidence of grade 3–4 hypertension in the randomized trial was greater in patients who were treated with tivozanib (26 %) than those who received sorafenib (17 %) [72]. However, the presence of HTN was an efficiency predictive marker for tivozanib: PFS for patients with diastolic pressure ≥90 mmHg was doubled than those patients with diastolic pressure ≤90 mmHg (18.3 months vs. 9.1 m). The same effect was recorded with systolic pressure.

Hepatotoxicity

Although transaminase increases have been reported in 26–34 % of patients treated with tivozanib, there was a low incidence of grade 3 and 4 elevations of ALT (1 %), AST (1 %), and bilirubin (2 %); no cases met Hy's law for drug-induced hepatotoxicity [71, 72]. Management of hepatotoxicity is similar as described for pazopanib, including drug interruption and dose reduction if needed.

Hypothyroidism

Among patients with normal basal TSH, 24 and 6 % of those treated with tivozanib and sorafenib, respectively, experienced a rise of 10 mIU / L. Only 2–3 % had low levels of free T3 or T4. Recommendations for hypothyroidism management are described in Table 18.4.

Sorafenib

Sorafenib was the first multikinase inhibitor to be approved for use in RCC in USA(2005) and in Europe(2006). Sorafenib inhibits VEGFR and PDGFR tyrosine kinases, as well as tumorigenic Flt-3, c-KIT, and RET receptor tyrosine kinases, and the intracellular serine/threonine kinase RAF-1 [74].

In general, sorafenib is a well-tolerated drug. In the phase III Treatment Approaches in Renal Cancer Global Evaluation Trial (TARGET), sorafenib and placebo had similar rates of treatment discontinuations due to adverse events (10 and 8 %, respectively). Skin toxicities, diarrhea, fatigue, and hypertension were the most frequent side effects of clinical importance (Table 18.7). The majority of

Table 18.7 Sorafenib toxicities over 15 % [76, 77, 79]

	All grades (%)	Grades 3–4 (%)
Diarrhea	48–55	3–7
Rash/desquamation	33–41	1–6
Hand–foot skin reaction	33–56	6–13
Alopecia	31–33	0
Fatigue	29–34	3–7
Nausea	17–19	<1–1
Hypertension	17–19	4–6
Pruritus	11–17	<1
Anorexia	14–22	<1–3

these events were grades 1–2 in severity and occurred early within the first two cycles of treatment [75]. Expanded-access studies of sorafenib in advanced RCC in North America (NA-ARCCS) and Europe (EU-ARCCS) confirmed the safety profile of sorafenib in the clinical setting [76, 77].

Asthenia and Fatigue

The incidence of any constitutional event in the TARGET trial was 51 % with sorafenib and 24 % with placebo treatment. Fatigue, the most frequent constitutional symptom, was reported in 29 and 16 % of patients, respectively, and was predominantly grades 1–2. The incidence of grade 2 fatigue did not differ significantly between the two groups (12 vs. 9 %). There were no differences between treatment groups with respect to grade 3–4 events (≤5 %) [78, 79].

Diarrhea

In the TARGET trial, the events that occurred at a significantly higher rate in sorafenib than in placebo patients were diarrhea (48 vs. 11 %) and oral mucositis (5 vs. 2 %), predominantly grades 1–2 in severity. There were no significant differences in the incidence of grade 3–4 gastrointestinal events [79]. The incidence of diarrhea in the EU-ARCCS study was 55.3 % [77].

Diarrhea continues to be reported at varying frequencies in the later cycles, although at lower rates than initial presentation. The rest of gastrointestinal events tended to develop early during the treatment with sorafenib [80]. Thus, attention to diarrhea is important. Loperamide (2 mg), taken 30 min before the dose of sorafenib, is a preventive measure for diarrhea. For mild diarrhea, dietary changes can reduce it. For mild-to-moderate diarrhea, a dose of loperamide may be taken after each episode. For moderate-to-severe cases, a 5–7-day interruption of sorafenib is necessary. Then, sorafenib may be restarted at a reduced dose and gradually escalated to the full dose [81].

Skin Toxicity

Rash, hand–foot syndrome, alopecia, and pruritus were the most common adverse events affecting skin and cutaneous annexes. Most of patients (81–98 %) had predominantly grade 1–2 adverse effects. Grade 3–4 skin toxicities were reported for HFS (6–12 %), rash (1–5 %), alopecia (<1 %), and pruritus (<1 %) [75–77].

The rates of incidence of HFS and rash significantly reduced after the first cycle, suggesting that the patients who do not experience these adverse events early in the course of the treatment are unlikely to develop them later [80].

Hand–Foot Skin Reaction

HFS is characterized by the formation of thick, often painful, hyperkeratotic lesions in the pressure points that develop within the first 2–4 weeks of treatment. The earliest symptoms are tingling and numbness with slight redness. About 33 % of patients in the TARGET trial and 47 % of patients in the EU-ARCCS study developed HFS [77, 79]. HFS was the most common grade 3 adverse event reported in the TARGET trial and the only skin toxicity to have a significantly greater incident of grade 3–4 events in patients receiving sorafenib as compared to placebo patients (6 and 0 %; $p<0.001$) [78].

In a recent meta-analysis of patients treated with sorafenib, the incidence of HFS was greater in patients with RCC than in those with other tumors (42.0 and 27.6 %, respectively), with no difference for the incidence of grade 3–4 events. The biological basis for this is not known [82].

Before sorafenib is started, some preventive measures can be taken by patients to reduce the risk of HFS: avoidance of exposure to hot water, excessive friction on the skin or pressure on the feet, and vigorous activities placing the hands and feet under stress and wearing cotton socks, soft shoes, or padded insoles. Patients should be suggested to use lanolin or urea-based lotions in the preexisting hyperkeratotic areas and removed feet calluses [58]. Grade 1–2 cases of HFS can be managed with the use of lanolin or urea-based lotions [58]. Grade 3–4 events require interruption of treatment until symptoms return to grade 0–1. Then, sorafenib can be restarted at a reduced dose and gradually escalated to the full dose [81].

Rash

Rash is characterized by erythema of the face and scalp, macular and papular eruptions, desquamation, and sometimes pruritus (Fig. 18.4). The onset is often acute [83]. Erythematous rash is more common in women than in men and especially in Asian women [81]. About 41 % of patients in the TARGET trial and 33 % in the EU-ARCCS study developed rash [77, 79].

For desquamating rash, the use of zinc-oxide-based emollients or lanolin-based creams may help to prevent the development of skin problems and can also treat mild cases of rash. Grade 3–4 cases require interruption of treatment until symp-

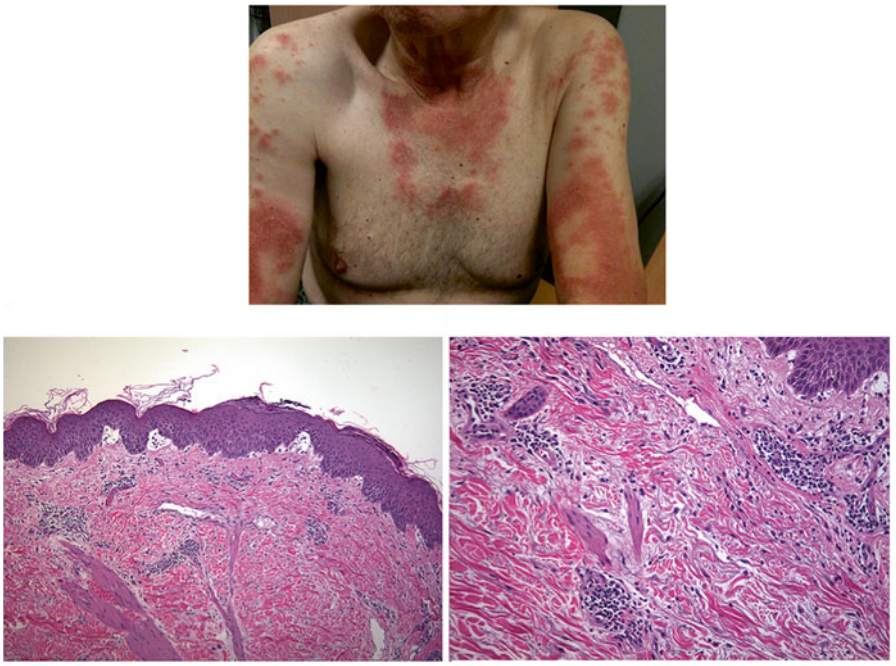

Fig. 18.4 Sorafenib-induced rash. Slight epidermal hyperplasia with spongiosis localized in the deeper side of the epidermis. There is a mixed superficial infiltrate with perivascular lymphocyte presence accompanied by polynuclear neutrophils and occasional eosinophils

toms return to grades 0–1, and then sorafenib can be restarted at a reduced dose and gradually escalated to the full dose [81, 84].

Other Skin Toxicities

Case reports of patients on treatment with sorafenib and developing keratoacanthoma, actinic keratoses, or squamous cell carcinoma (SCC) have been described in the literature. The frequency of keratoacanthoma and SCC is between 0.1 and 1 % of the patients treated with sorafenib [81].

Hypertension

In the TARGET trial and the NA-ARCCS and EU-ARCCS studies, hypertension (all grades) was reported in 17–19.5 % of patients, with grade 3–4 severity in 4–6.1 % of patients [76, 77, 79].

Uncontrolled hypertension can contribute to the onset of cardiovascular events. So, its control is important for the care of these patients. Increases in blood pressure

may arise as early as the first 3 weeks of sorafenib therapy and persist for as many as 18 weeks before stabilizing [85]. Hypertension usually responds to treatment with antihypertensive therapy and very rarely led to treatment discontinuation (<1 %) (Fig. 18.2). In cases of severe or persistent hypertension that do not respond to treatment, sorafenib interruption or discontinuation may be necessary [78].

Cardiovascular Dysfunction

Despite the prevalence of hypertension in these patients, the cardiovascular adverse effects (cardiac ischemia/infarction, left ventricular systolic dysfunction) are rare. Twenty-two patients (4.9 %) randomly assigned to sorafenib reported cardiac ischemic/infarct events, with six related to study drug [79]. In the follow-up of patients from TARGET trial, in 169 patients receiving sorafenib for >1 year, cardiac ischemia/infarction and left ventricular systolic dysfunction had an overall low incidence (2 and 1 %, respectively) and were reported later in the treatment, with no evident particular pattern. TARGET trial, however, was not designed to evaluate cardiotoxicity, and this may underestimate the prevalence of these adverse effects [80].

In the case of cardiac ischemia/infarction or left ventricular systolic dysfunction, temporary or permanent discontinuation of sorafenib therapy may be considered [81].

Axitinib

Axitinib is a potent, highly selective, second-generation inhibitor of VEGFR 1, 2, and 3 that blocks VEGFRs at sub-nanomolar concentrations [86]. Other targets, such as PDGFR, b-RAF, KIT, and FTL-3, blocked by first-generation VEGFR inhibitors, are not inhibited by this agent, which could explain the different toxicity profiles [87].

The most common reported adverse events of axitinib are hypertension, fatigue, decreased appetite, nausea, and dysphonia, the majority grade 1–2 in severity (Table 18.8). In the randomized phase III AXIS trial, comparing effectiveness of axitinib versus sorafenib as second-line therapy in advanced RCC, only 4 % of

Table 18.8 Axitinib toxicities over 15 % [88, 89, 91]

	All grades (%)	Grades 3–4 (%)
Diarrhea	55–61	10–15
Hypertension	40–58	15–16
Fatigue	39–77	8–16
Anorexia	34–48	0–5
Nausea	32–44	0–7
Dysphonia	31–37	0
Hand–foot skin reaction	27–36	5–16
Hypothyroidism	19	<1

patients discontinued axitinib because of treatment-related adverse events (vs. 8 % in the sorafenib arm). The most common adverse events leading to discontinuation of axitinib were fatigue (1 %) and transient ischemic attack (<1 %) [88].

Asthenia and Fatigue

In the AXIS trial, fatigue (all grades) occurred in 39 % of patients treated with axitinib, with 11 % grade ≥3 [88]. Frequency in phase II trials ranges from 48 to 52 % in cytokine-refractory [89, 90] to 77 % in sorafenib-refractory patients [91]. Treatment of fatigue requires assessment and treatment of other exacerbating factors (hypothyroidism, anemia, pain, sleep disturbances), nonpharmacologic interventions (increase of activity, psychosocial interventions, nutritional consultation, sleep therapy), and pharmacotherapy (psychostimulants like methylphenidate and modafinil) [92, 93].

Gastrointestinal Events

In AXIS trial, like in phase II studies, diarrhea is the most frequent adverse event associated with axitinib (55–66 %), with grade ≥3 in 5–15 % of patients. Nausea (25–44 %) and vomiting (16–32 %) are other frequent gastrointestinal adverse events in these trials, predominantly grades 1–2 in severity [88–91].

Patients should be advised to consume frequent small meals, drink fluids in regular small amounts, and avoid foods or drinks that may aggravate diarrhea. Diarrhea requires an early intervention and can be controlled with antidiarrheal agents as loperamide and maximation of fluid intake and dietary changes [92, 93].

Dysphonia

Dysphonia can manifest as a hoarse or weak voice or as an excessively breathy, rough, or harsh voice. In general, some level of phonation is possible. It seems to be intermittent [92]. Frequency in AXIS trial and western phase II studies is 31–37 % of patients [88, 89, 91]. In the Japanese trial, the incidence reached 53 % of patients [90]. There are no evidence-based interventions for the treatment of axitinib-induced dysphonia.

Skin Toxicity

Hand-foot syndrome is the most common cutaneous toxicity of axitinib. In AXIS trial, it was less frequent in axitinib than in sorafenib arm (27 vs. 51 % for all grades and 5 vs. 16 % for grade ≥3). Other skin toxicities, like rash and alopecia, were also

less common for axitinib than sorafenib treatment (13 vs. 32 % and 4 vs. 32 %, for all grades) [88]. Of note, the incidence of HFS in the Japanese phase II study was higher than that reported in the western study in cytokine-refractory patients (75 % vs. NR) [89, 90]. Although severe episodes of HFS may necessitate dose alterations, topical treatments and avoidance of friction, especially in the feet, may provide some relief [93].

Hypertension

Hypertension is the second most common adverse event reported in the published studies (40–58 %), with grade ≥3 in 16 % of patients [88, 89, 91]. In the Japanese phase II trial in cytokine-refractory patients, the incidence reached 84 % of patients, with 70 % grade ≥3 [90]. Hypertension is generally manageable with early and ongoing assessment, using standard antihypertensive medications and treatment interruption in severe cases (Fig. 18.2) [92, 93].

Laboratory Abnormalities

In AXIS trial, laboratory abnormalities were greater in the sorafenib arm than in the axitinib arm for anemia (52 vs. 35 %) and hypophosphatemia (50 vs. 13 %). Axitinib does not appear to cause neutropenia and thrombocytopenia, which have been reported with sunitinib [88].

The incidence of proteinuria in the Japanese study was higher than those reported in the western study in cytokine-refractory patients (58 vs. 8 %). In the Japanese study, 28 % of patients developed proteinuria ≥2 g/24 h and required interruption or reduction of axitinib. Altogether, 11 % of patients discontinued treatment due to proteinuria ≥2 g/24 h with the lowest dose. These results underscore the importance of monitoring patients receiving angiogenesis inhibitors for proteinuria [90]. If proteinuria is ≥2 g/24 h, axitinib treatment must be interrupted until daily protein excretion is <2 g and then restarted at the same dose or reduced by one dose level [93].

Hypothyroidism

The incidence of hypothyroidism in AXIS trial for axitinib reaches 19 % of patients, with grade ≥3 in <1 % of patients (vs. 8 and 0 %, respectively, for the sorafenib arm). In patients who had thyroid-stimulating hormone (TSH) concentrations lower than 5 mU/L before treatment, patients treated with axitinib had more elevations of TSH 10 mU/L or more (32 %) than those treated with sorafenib (11 %) [88]. Hypothyroidism was more frequent in the Japanese cytokine-refractory phase II study (all grades, 48 %), with 88 % of patients experienced increases

and/or transient decreases in TSH levels beyond the normal range during axitinib treatment [90].

Monitoring of thyroid function is recommended during axitinib treatment. Hypothyroidism and associated symptoms may be managed by proactive administration of thyroid hormone replacement therapy (Table 18.4).

mTOR Inhibitors

The main side effects of mTOR inhibitors include skin toxicity, myelosuppression, reversible elevations in liver function test, and asymptomatic hypocalcemia. Other adverse effects, which were generally mild to moderate and reversible, are mucositis, hyperlipidemia, hyperglycemia, hypophosphatemia, anemia, asthenia, nausea, and decreased serum testosterone. The majority of adverse events are dose dependent; however, pneumonitis and mucositis were reported even at lower doses.

Temsirolimus (Torisel®; Pfizer, New York, NY, USA) is a water-soluble prodrug of rapamycin with an added ester at the C43 position. It is rapidly metabolized to sirolimus through de-esterification and both produce suppression of mTORC1 activity.

Based upon the results of a phase II trial, Hudes et al. evaluated temsirolimus in a phase III trial (Global ARCC study) in which 626 previously untreated poor-prognosis patients with metastatic or recurrent RCC were randomly assigned to temsirolimus (25 mg IV/week), the combination of temsirolimus plus IFN-a, or IFN-a as monotherapy [94]. Temsirolimus as a single agent significantly prolonged median progression-free survival and median overall survival compared to IFN-a monotherapy.

Major toxicity findings of the Global ARCC study were asthenia, rash, and anemia; however, the only adverse events that reached grades 3–4 in more than 10 % of the patients were anemia, asthenia, and hyperglycemia (Table 18.9). Further studies have shown the relevance of other toxicities such as drug-related pneumonitis that were underestimated initially [95].

Table 18.9 Temsirolimus toxicities over 15 % [94]

	All grades (%)	Grades 3–4 (%)
Anemia	45	20
Hypercholesterolemia	24	1
Hypertriglyceridemia	27	3
Hyperglycemia	26	11
Diarrhea	27	1
Mucositis	20	1
Dyspnea	28	9
Fatigue	33	3
Nausea	37	2
Vomiting	19	2
Infections	27	5

Everolimus

Everolimus (Afinitor®, Novartis Pharmaceuticals, East Hanover, NJ) is an orally administered mTOR inhibitor, for the treatment of advanced RCC that has progressed on or after treatment with VEGF-targeted therapy. Everolimus also has been approved by the US Food and Drug Administration for treatment of advanced RCC after failure of sorafenib or sunitinib.

The RECORD-1 (Renal Cell Cancer Treatment with Oral RAD001 Given Daily) multicenter, phase III, randomized trial compared everolimus with placebo in patients with clear cell mRCC who had disease progression during or within 6 months of treatment with sorafenib, sunitinib, or both [96]. Patients were randomized 2:1 to receive everolimus 10 mg/day ($n=277$) plus best supportive care or placebo ($n=139$) plus best supportive care. Median PFS for the patients treated with everolimus was 4.9 months (95 % CI, 4.0–5.5 months) versus 1.9 months (95 % CI, 1.8–1.9 months) for the patients who received placebo (HR = 0.33; 95 % CI, 0.25–0.43; $p<0.001$).

The most commonly reported adverse effects in the RECORD-1 trial were stomatitis, asthenia, fatigue, rash, and diarrhea, and most were grade 1/2 in severity (Table 18.10). During the double-blind phase of the study, four deaths were reported in association with AEs: three were attributed to infection and one occurred in a patient with disease progression and everolimus-related grade 3 interstitial lung disease with acute respiratory failure.

Table 18.10 Everolimus toxicities over 15 % [96, 101]

	All grades (%)	Grades 3–4 (%)
Anemia	92	12
Leukopenia	26	1
Lymphopenia	51	18
Thrombocytopenia	23	1
Hypercholesterolemia	77	4
Hypertriglyceridemia	73	<1
Hyperglycemia	57	15
Hypophosphatemia	37	6
Dyspnea	24	7
Pneumonitis	14	4
Infections	37	10
Fatigue	31	5
Anorexia	22	2
Nausea	15	0
Stomatitis	40	3
Diarrhea	17	1
Hypercreatininemia	46	<1
↑ ALT levels	18	<1
↑ AST levels	21	<1
Rash	25	<1

Asthenia and Fatigue

Asthenia has been reported in 51 % of patients treated with temsirolimus in the Global ARCC study (grades 3–4 in 11 % of patients) [94]. The incidence of asthenia with everolimus was inferior in the RECORD-1 trial, 18 % of patients (grade 3 in only 1 % of patients) [96]. In the everolimus expanded-access program, 1,367 patients were treated with everolimus, presenting asthenia grades 3 and 4 in 6.5 and 0.2 % of cases, respectively.

It is important to evaluate and treat all potential causes of asthenia before starting treatment with mTOR inhibitors, such as anemia, depression, or sleep disorders. Patients should be monitored regularly in the first cycles ruling out potentially treatable causes of asthenia. Only in exceptional circumstances a dose reduction is needed.

Oral Toxicity (Mucositis, Stomatitis, and Mouth Sores)

Mucositis, stomatitis, and mouth sores have been reported in almost all the clinical studies with mTOR inhibitors and have been one of the most common adverse events associated with these agents: 20–75 % with temsirolimus and 40 % with everolimus [94, 97, 98]. The incidence appears to be dose related and more common in earlier cycles [99]. The mucositis seen with mTOR inhibitors is distinct from the mucositis typically seen with radiation or chemotherapy (Fig. 18.5). There is no pseudomembrane formation, and the lesions appear as distinct small aphthous lesions confined to the soft mucosa of the mouth, very common on the lips and tongue, and rarely on the hard palate. In most patients mucositis is observed in the first weeks of treatment.

Skin Toxicity

Skin toxicity is commonly reported with mTOR inhibitors and manifests typically as maculopapular of acneiform rash but also as dryness, eczema, skin discoloration, as well as nail dystrophy [97, 99]. The reported incidence of rash for temsirolimus

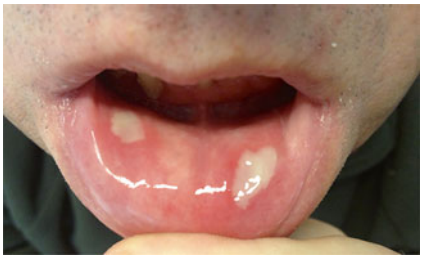

Fig. 18.5 Everolimus-induced mucositis

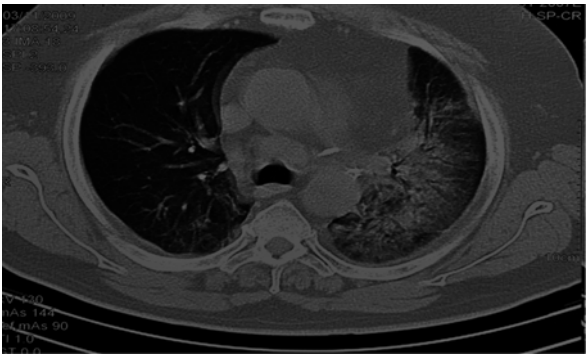

Fig. 18.6 Interstitial pneumonitis in a patient treated with temsirolimus

and everolimus is 47–76 % [94, 97, 98] and 25–50 % [96, 100, 101], respectively, with grade 3 or 4 rash reported in less than 5 % of patients in these trials. Maculopapular rashes (Fig. 18.6) on the face and neck mostly occurred during the first few weeks of treatment and were spontaneously reversible. Grade 1–2 acne-like rash occurred on the face and the upper part of the trunk and was reversible with or without topical steroid cream.

Hyperglycemia

Treatment with mTOR inhibitors may cause hyperglycemia in both diabetic and nondiabetic patients, although patients who present significantly higher glucose levels at baseline are more likely to develop treatment-related hyperglycemia. Close monitoring of glucose levels is recommended in all patients undergoing mTOR inhibitor therapy since the mTOR/S6K1 pathway is involved in glucose metabolism and insulin signaling pathway. In clinical trials, grade 1–4 hyperglycemia occurs in 26 % of patients treated with temsirolimus, with grades 3–4 in 11 % of patients [94]. In the RECORD-1 trial, up to 50 % of the patients treated with everolimus presented any grade of abnormally high glucose but only 12 % of them were grade 3 and none were grade 4 [96]. In the everolimus expanded-access program, 5.5 % of patients presented hyperglycemia grades 3–4.

Before treatment, it is important to educate patients about possible symptoms (polydipsia, polyuria, and weight loss) for early detection. Diabetic patients should obtain adequate glycemic control before starting therapy. The management of mTOR inhibitor-induced hyperglycemia is similar to that of diabetes of other causes, with oral hypoglycemic agents and/or insulin therapy according to standard guidelines and, if possible, under the supervision of an endocrinologist (Table 18.11).

Table 18.11 Management of diabetes, hypercholesterolemia, and hypertriglyceridemia

	Diabetes	Hypertriglyceridemia	Hypercholesterolemia
Initial treatment	Metformin (500–1,500 mg/day) or acarbose (150–300 mg/day)	Lifestyle modifications If triglyceridemia <500 mg/dl, treat hypercholesterolemia	Atorvastatin 10–80 mg/day
Subsequent treatment	Consider add sulfonylureas: glimepiride (1–6 mg/day) or sitagliptin(100 mg/day) If another drug is needed add glitazones (15–45 mg/day)	Use 2,000 mg nicotinic acid and 40 mg laropiprant/day Start gemfibrozil 900–1,200 mg/day (avoid concomitant statins)	Add a bile acid sequestrant (cholestyramine, 4–24 g/day) Use 2,000 mg nicotinic acid and 40 mg laropiprant/day
Prior actions ineffective	Remit to specialist		

If hyperglycemia is diagnosed, it is recommended to follow these instructions [102, 103]:

- Promoting a healthy lifestyle, dietary habits, and exercise if possible.
- Monitor blood glucose levels closely during treatment, and glycosylated hemoglobin levels should be assessed as often as every 12 weeks.
- Conduct health education (early recognition of symptoms) to patients and their families.
- Biguanides such as metformin (500–1,500 mg/day) and glitazones such as pioglitazone (15–45 mg/day) are used to improve insulin sensitivity. Sulfonylureas such as glimepiride (1–6 mg/day) can compensate for insulin deficiency.

Dyslipidemia

mTOR inhibitors have been associated with dyslipidemias in the transplantation and oncologic settings. They have been shown to increase total cholesterol and triglycerides. Impaired clearance of lipids from the bloodstream and inhibition of insulin-stimulated lipoprotein lipase are possible pathophysiologic mechanisms involved in hyperlipidemia [104, 105].

In the two pivotal studies of temsirolimus and everolimus, hypertriglyceridemia was observed in 27 and 71 % of the patients respectively, although less than 4 % of the cases reached grade 3 or greater [94, 96]. For hypercholesterolemia similar results have been reported: 24 % for temsirolimus and 76 % for everolimus, with few cases of grade 3–4 toxicity. Up to 35 % of patients included in these studies had grade 1–2 high total cholesterol/lipid levels at baseline.

Before starting an mTOR inhibitor, it is necessary to perform a lipid profile and assess the patient's cardiovascular risk (smoking, hypertension, family history of coronary artery disease, atherosclerosis, etc.). Most of the guidelines on hyperlipidemia

management recommend to assess cardiovascular risk factors in each patient, to prevent mortality with a long latency period. Total cholesterol levels above 240 mg/dl or LDL-cholesterol >190 mg/dl and/or triglycerides above 500 mg/ml may be sufficient to begin treatment with statins (atorvastatin 10–20 mg/day) without requiring changes in the dose of temsirolimus or everolimus (Table 18.11). For patients who have contraindications in the use of HMGCoA inhibitors, anion exchange resins (colestipol) or inhibitors of cholesterol absorption (ezetimibe) can be used.

As long as the triglyceride level is below 500 mg/dl, controlling LDL is most important; for higher triglyceridemia values, nicotinic acid or fibrates (gemfibrozil 900–1,200 mg/day; avoid concomitant use with statins) should be introduced along with recommendations for a healthy lifestyle.

Hypophosphatemia

Renal tubular abnormalities have been observed among patients treated with rapamycin, leading to hypokalemia and hypophosphatemia [102]. The incidence of hypophosphatemia in phase III trials oscillates from 13 % for temsirolimus to 32 % for everolimus [94, 96]. The mechanism of hypophosphatemia is not known, and symptoms such as generalized fatigue and weakness rarely occur unless the plasma phosphate concentration is quite low (<2 mg/dl, moderate). The most frequent adverse effect from acute hypophosphatemia is compromised muscle and can involve skeletal muscle weakness including diaphragmatic paresis with respiratory failure, decreased myocardial function with congestive heart failure, and even rhabdomyolysis.

In mild-to-moderate cases, hypophosphatemia is corrected with oral phosphate supplements (1,000–2,000 mg per day in 3–4 divided doses) and serum phosphate monitoring, without changes in the dose of mTOR inhibitors. In severe cases intravenous phosphorus is needed, with close monitoring of levels of calcium and magnesium and discontinuation of mTOR inhibitors [106].

Pulmonary Toxicity

Pulmonary toxicity is an often misdiagnosed toxicity of mTOR inhibitors. The incidence of pneumonitis identified in the phase 3 trials of temsirolimus and everolimus was considerably less than that of pneumonitis identified by systematic radiographic analysis. Investigators reported pneumonitis in 2 and 14 % in the pivotal studies of temsirolimus and everolimus, respectively [94, 96]. In posterior retrospective, independent, blinded reviews, 29 % of patients treated with temsirolimus were noted to have radiographic findings consistent with drug-related pneumonitis [95]. In the retrospective analysis of the phase III study of everolimus, the incidence of pneumonitis was 39 % [107].

Table 18.12 Pulmonary toxicity from mTOR inhibitors [109]

Grade	Definition	Treatment	Dose adjustment
1	Asymptomatic Radiographic abnormalities Normal RFT	Not necessary	Not necessary
2	Minimal symptoms Radiographic abnormalities Abnormal RFT	Exclude infectious causes[a] Consider corticosteroids (prednisone 1 mg/kg)	Consider everolimus dose interruption/reduction Restart at reduced dose when grade <2 and consider reescalation
3	Symptomatic, interfering with ADL, oxygen indicated	Exclude infectious causes[a] Consider corticosteroids (prednisone 1 mg/kg)	Hold treatment until recovery to grade 1; may restart within 2 wk at a reduced dose (by 1 level) if there is evidence of clinical benefit
4	Life threatening; ventilation support indicated	Exclude infectious causes Consider corticosteroids (prednisone 1 mg/kg)	Discontinue permanently Supportive measures

ADL: activities of daily living
RFT respiratory function tests (spirometry + CO diffusion test)
[a]Consider: bronchoscopy with bronchoalveolar lavage +/− biopsy, sputum cultures, high-resolution CT, blood galactomannan determination, and pneumococcal and *Legionella* antigen in urine

The exact mechanism for this toxicity is unknown, but studies investigating it have suggested autoimmune mechanisms. Pneumonitis begins in the first weeks of treatment with mTOR inhibitors. Median time to onset of pneumonitis was 8 weeks for temsirolimus and 15 weeks for everolimus, respectively [95, 107]. The most common clinical presentations of pneumonitis are dyspnea, fatigue, dry cough, and fever. In all of these patients, the most common radiographic pulmonary abnormalities were ground-glass opacities and parenchymal consolidation.

The most important aspect in the diagnosis of pneumonitis is the knowledge of clinical–radiological presentation. Asymptomatic pneumonitis (grade 1) should be monitored with CT lung window and PFR with DLCO every two cycles. Infection should be ruled out in case of symptomatic pneumonitis(grade 2–3). Use of corticosteroids should be considered, accompanied by interruption of mTOR inhibitors until resolution of signs and symptoms. Then dose should be reduced by 50 %. In case of grade 4 toxicity, the drug should be discontinued definitively (Table 18.12).

Bone Marrow Suppression

Thrombocytopenia and leukopenia have been reported with mTOR inhibitors with varying incidences. Thrombocytopenia was reported about 14–33 % with temsirolimus and 10 % for everolimus, but less than 1 % were grades 3–4 [94, 96, 97]. Leukopenia

has also been reported up to 6 % for temsirolimus. In the everolimus phase III study, 26 % had leukopenia and 11 % had neutropenia, with no grade 3 or 4 levels in either categories.

Anemia was reported in 91 % of patients in the RECORD trial, grade 3 in 9 % and only 1 patient with grade 4. In the phase III temsirolimus trial, 45 % of patients had anemia, grades 3–4 in 20 % of them.

Conclusion

For several decades, cytokine therapy, using either interleukin-2 or interferon alfa, was the only effective treatment available for patients with mRCC. However, these agents provide only modest increases in survival in a limited subset of patients and are associated with substantial toxicity, particularly at high doses.

In the last years, the introduction of novel targeted agents has changed the algorithm of treatment for patients with metastatic RCC. The different mechanisms of action of TKIs and mTOR inhibitors are also associated with a distinct pattern of adverse events, and until now this toxicity was not frequently observed by medical oncologists. Therefore in order to reduce the frequency, severity, and clinical significance of these side effects, close monitoring and careful management of patients are required.

This chapter summarizes the most important toxicity associated to renal carcinoma therapies and suggests measures to manage adverse events. An appropriate management of side effects ensures adequate treatment compliance to obtaining the best possible benefit from their use.

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Chapter 19
The Role of Targeted Therapy in Special Populations

James M.G. Larkin and Martin E. Gore

Introduction

The treatment of advanced renal cell carcinoma (RCC) has been revolutionised [1] over the last 10 years by the development of drugs that target principally either the vascular endothelial growth factor (VEGF) or mammalian target of rapamycin (mTOR) signalling axes. A number of such drugs are now approved: the multi-targeted kinase inhibitors sorafenib, sunitinib, pazopanib and axitinib and the monoclonal antibody bevacizumab target the VEGF axis, whilst the specific TORC1 (TOR Complex 1) inhibitors everolimus and temsirolimus target the mTOR signalling axis. Registration trials [2–9] of these drugs have been conducted in a variety of clinical contexts, and the approved labels for these agents reflect this; importantly, this has considerable practical bearing on their clinical use. Moreover sorafenib, sunitinib and temsirolimus were the first agents to be approved whilst axitinib has only been relatively recently approved. As a consequence, considerably more clinical experience has accrued with sorafenib, sunitinib and temsirolimus than the other agents. A further issue is that there are relatively small numbers of direct head-to-head comparisons between these drugs [4, 10, 11], and so it can be difficult to say that a particular agent has markedly different efficacy or safety in a particular situation than any other agent. Finally, multi-targeted kinase inhibitors have multiple targets aside from the VEGF receptor 2 (the target probably most relevant for clinical activity in clear cell RCC), but at the current time, it is not possible to say the extent to which activity at other targets, such as the platelet-derived growth factor receptors (PDGFRs), is relevant for efficacy. It is clear however that activity at other targets is important in determining the safety profile of these drugs, and different spectra of toxicity profiles of these drugs reflect differences in potency for these targets.

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What Are 'Special Populations'?

The term 'special populations' can be defined in a variety of ways, but perhaps the most important issue is that patients included in registration trials of new drugs are not broadly representative of patients that are seen in routine clinical practice. This is a consequence of the fact that registration trial patients are, by definition, very fit, often willing to travel long distances to enter a trial, have good organ system function and limited, if any, significant comorbidity. Furthermore, patients with non-clear cell histology, the elderly and patients with aggressive disease biology are underrepresented in registration clinical trials. All of this means that extrapolating trial results into routine clinical practice is not necessarily straightforward, and 'real-life' data sets can be very valuable. In renal cell carcinoma, expanded access programmes have been carried out with a number of drugs, such as sunitinib, sorafenib and everolimus [12–15]; these programmes have tended to have more permissive entry requirements than registration trials and, as a consequence of this, are more representative of routine clinical practice. For example, in the sunitinib expanded access trial, patients with brain metastases, Eastern Cooperative Oncology Group (ECOG) performance status 2 and non-clear cell histology were included. All of these groups were excluded from the sunitinib registration trial.

Taking all of this into consideration, we have chosen to define 'special populations' as follows and will examine the role of targeted therapy in these groups in turn:

1. Organ system dysfunction
2. Metastases in specific areas which may have an impact on choice of targeted therapy, such as the brain or spinal cord
3. Poor performance status and poor-risk disease
4. The elderly population
5. Germ line genetic variation which may predispose to particular toxicities
6. Non-clear cell histology

Organ System Dysfunction

The most important general principle in treating cancer patients with organ system dysfunction is that this should be optimised as much as possible before starting systemic therapy, in conjunction with an appropriate specialist if necessary. Perhaps the commonest example of this in clinical practice in advanced renal cell carcinoma is that of hypertension, which, although common in the general population, is even more common in patients with advanced RCC who on average are over 60, have a solitary kidney and may be obese. Specific examples of the use of targeted therapy in patients with organ system dysfunction will be considered below.

Cardiac Dysfunction

Drugs targeting the VEGF signalling axis are well known to cause cardiac dysfunction. A range of side effects have been described, of which perhaps electrocardiographic (ECG) abnormalities, reduction in left ventricular ejection fraction and hypertension are of greatest clinical relevance.

A range of ECG abnormalities have been described in association with VEGFR kinase inhibitors, but serious abnormalities are rare [16, 17]. Any abnormalities should be managed along standard lines, and a risk/benefit discussion should take place in consultation with the patient regarding the safety of continuing the targeted agent. Practically speaking, we recommend that all patients starting targeted therapy have a baseline electrocardiographic assessment and that further ECGs be carried out in the event of any clinical concern. We would not recommend routine ECG monitoring in the absence of symptoms or signs of cardiac dysfunction.

Reductions in left ventricular ejection fraction (LVEF) are also relatively common with VEGFR kinase inhibitors; we recommend that any patient with a history or coronary artery disease or a suspicion of significant cardiac disease undergo cardiology assessment and echocardiography prior to starting targeted therapy, with regular follow-up during the course of treatment. VEGF receptor kinase inhibitors can be administered safely to patients with mild to moderate impairment in cardiac function, but this should be done with caution and with careful counselling of the patient of the risks and benefits. In the event of a significant reduction in LVEF during treatment, it is mandatory to stop targeted therapy and treat the cardiac dysfunction along standard lines. Anecdotally, recovery in LVEF can occur, but even in this situation, it is only rarely possible to reintroduce anti-VEGF therapy, and if contemplated, this should be done with careful monitoring.

Hypertension is very common in patients treated with VEGF receptor tyrosine kinase inhibitors and may be a surrogate marker of efficacy [18]. Hypertension should in general be managed along standard lines, with the most important principle, as with essential hypertension, that blood pressure is controlled pharmacologically with appropriate medications. The choice of antihypertensive agent will vary from patient to patient but will often include an ACE inhibitor and a calcium channel blocker.

The mTOR inhibitors everolimus and temsirolimus have not been associated with significant cardiac side effects.

Hepatic Dysfunction

There is limited evidence to guide targeted therapy in RCC in those with significant hepatic dysfunction [19–22]. All of the approved agents are metabolised in the liver; toxicity may therefore be unpredictable, and great care is necessary in treating patients with hepatic dysfunction. We recommend that patients with significant

hepatic dysfunction be reviewed on at least a weekly basis until correct dosing is established and that consideration be given to starting therapy at a 25 or 50 % dose reduction. A further issue is that derangement of liver function tests is a class effect of multi-targeted VEGFR kinase inhibitors, with an incidence which varies from drug to drug, but is probably highest for pazopanib [2–4, 9]. Our view would be therefore that in the setting of significant liver dysfunction, of the approved VEGFR kinase inhibitors, sunitinib is preferred over pazopanib in the first-line setting.

Pulmonary Dysfunction

Kinase inhibitors targeted to the VEGFR kinases very rarely cause pulmonary toxicity; indeed, the likeliest mechanism by which pulmonary dysfunction can occur is as a consequence of drug-induced left ventricular dysfunction causing pulmonary oedema.

Inhibitors of mTOR, however, cause interstitial pneumonitis as a class effect, which may occur in up to 10–20 % of patients [23–25]. This is often asymptomatic and is frequently detected only with cross-sectional imaging carried out for response assessment. We recommend that in the setting of asymptomatic pulmonary changes, no specific therapy be initiated, but patients should be counselled that intervention will be necessary if they develop cough or shortness of breath. If symptoms are mild to moderate, management can be on an outpatient basis with oral corticosteroids, but for severe symptoms, mTOR inhibitor therapy should be stopped and the patient treated with high-dose steroids and oxygen as an inpatient. The management of pneumonitis should also include maintaining a high index of suspicion for superimposed infection, and it may therefore be necessary to perform bronchoscopy and bronchoalveolar lavage to rule out infection. Any such infection should be managed along conventional lines.

Renal Dysfunction

Baseline renal dysfunction is relatively common in patients with advanced renal cell carcinoma, given the fact that many have had a prior nephrectomy and many will have concomitant cardiovascular morbidity. Patients with mild to moderate renal impairment simply need close monitoring during targeted therapy, but with significant renal impairment, there is a risk of progression to end-stage renal disease with the need for renal replacement therapy [26]. VEGFR kinase inhibitors do not, per se, increase this risk, but many patients during the course of treatment can become dehydrated, as a consequence of diarrhoea or reduced oral intake; hypovolaemia can worsen renal function, and this should be monitored carefully. There is a risk of nephrotoxicity with mTOR inhibitors, via an unknown mechanism, and again patients

should be monitored very carefully for worsening renal function. A significant deterioration should mandate permanent cessation of therapy and appropriate management of renal dysfunction.

There is a limited evidence base for the use of targeted agents in patients who are undergoing renal replacement therapy, but reports from our group and others [27–29] suggest that both sorafenib and sunitinib can be used safely at standard doses.

Endocrine Dysfunction

The main practical issues in clinical practice regarding the treatment of patients with advanced renal cell carcinoma and endocrine dysfunction relate to diabetes mellitus and thyroid disease.

Diabetes Mellitus

The mTOR inhibitors temsirolimus and everolimus as a class effect can cause hyperglycaemia and impaired blood glucose homeostasis. This needs to be monitored carefully after starting treatment. Interestingly, some multi-targeted kinase inhibitors including sunitinib and sorafenib have been reported to improve diabetic control [30], and again this needs to be monitored carefully during therapy, as does the increased risk of vascular complications and vascular disease in diabetic patients undergoing treatment with anti-VEGF therapy. Hyperlipidaemia and hypercholesterolaemia are also class effects of mTOR inhibitors, and it is worth monitoring blood lipids and cholesterol in patients that have just started therapy and in those that are on therapy for over 6 months. In our experience however, it is rare for blood lipid levels to be sufficiently high to warrant therapy.

Thyroid Disease

Thyroid dysfunction occurs with all VEGF receptor kinase inhibitors [31, 32] and is probably best described with sunitinib: over half of patients of long-term sunitinib therapy may become hypothyroid [33]. We recommend that patients have their thyroid function checked at every other clinic attendance and that replacement thyroxine be given for those with a persistently elevated TSH. Hyperthyroidism has been occasionally reported with VEGF tyrosine kinase inhibitors, and this should be managed along standard lines in consultation with an endocrinologist. Treatment-induced hypothyroidism should not be assumed to be reversible on cessation of VEGFR kinase inhibitor therapy; in those in whom this has occurred, our practice is to monitor thyroid function after targeted therapy has ended and continue thyroid replacement as necessary.

Bone Marrow Dysfunction

Sunitinib in particular would appear to cause more myelosuppression than other VEGFR kinase inhibitors, such as sorafenib or pazopanib, and in patients with poor bone marrow function this may be a reason, for example, to consider pazopanib rather than sunitinib. Although sunitinib causes myelosuppression, predominantly leucopenia and thrombocytopenia, this very rarely causes clinical problems or requires intervention. The mTOR inhibitors can often cause anaemia, which is usually straightforward to manage with blood transfusions.

Metastases in Specific Locations

There are some metastatic sites of renal cell carcinoma for which local therapies are almost always preferred over systemic therapies. Broadly speaking, oligometastatic indolent disease at multiple locations may be treated with local therapies such as surgery or radiotherapy, without the need for systemic therapy. Indeed in some patients with very indolent disease, observation alone can be a valuable strategy [34]. A detailed discussion of these approaches is however beyond the scope of the current review.

The use of local therapies such as surgery or radiotherapy in conjunction with systemic therapy is important in a number of clinical situations such as the management of brain and spinal cord metastases, bone metastases, subcutaneous and thyroid metastases and any metastases causing obstruction of a viscous, such as bowel, ureter or biliary tree. Again the discussion of these individual situations and local therapies is beyond the scope of this review, but there are some important principles for choice of systemic therapy. The first principle, which is particularly relevant to brain metastases, is the question of whether or not there is an optimal drug to use in the setting of brain metastases, given potential concerns about CSF penetration of targeted agents, and this issue will be considered specifically. A second issue is which agent to use in setting where maximum chance of tumour shrinkage is important to prevent severe morbidity. This is potentially relevant in situations where local therapy is unable to control disease that threatens to compress vital structures such as the spinal cord or biliary tract, and this will be considered in detail.

Brain Metastases

Brain metastases are not common in advanced renal cell carcinoma: their incidence is thought to be around 10–15 % [35]. It is unclear if brain metastases are more common in the ‘targeted therapy era’ than they were before the use of targeted agents [36, 37], but our impression is that this may be the case, perhaps because

patients with aggressive disease biology are living longer now than in the past and perhaps also because the brain may be a sanctuary site for metastatic disease in patients treated with targeted agents. Treatment of brain metastases should always take place in a multidisciplinary setting; surgery and localised radiotherapy are generally the first-choice treatments, and drug therapy is often given as an adjunct or only in the setting of disease not suitable for localised therapies.

There are relatively limited data regarding the activity of targeted agents in the setting of brain metastases and certainly no specific prospective clinical trials of which we are aware. However, a number of retrospective series and case reports have reported the efficacy or otherwise for sorafenib and sunitinib in this situation, and the evidence will be reviewed below. Very little, if any, information has been reported for the other targeted agents in renal cell carcinoma.

Sorafenib

The incidence of brain metastases in patients treated with either placebo or sorafenib in the phase III Treatment Approaches in Renal Cancer Global Evaluation Trial (TARGET) at two European centres has been reported [38]. Interestingly, the incidence of brain metastases in patients receiving sorafenib was 3 % (2 of 70) compared with 12 % (8 of 69) in patients receiving placebo ($P<0.05$), suggesting that sorafenib may prevent or delay the development of brain metastases. Furthermore, the safety of sorafenib in the setting of brain metastases has been reported from an expanded access programme in which 2,504 patients from the United States and Canada were treated [14]. Seventy patients (3 %) had brain metastases; the tolerability of sorafenib was not worse in this group than in those without brain metastases, although baseline information concerning the extent of brain disease and prior treatment were limited.

Sunitinib

Data for sunitinib for the treatment of brain metastases have also been reported from an expanded access programme in which 4,564 patients were treated in 52 countries [39]. Of these, 4,371 were included in an intention-to-treat population of which 321 (7 %) had baseline brain metastases. The most common grade 3–4 treatment-related adverse events in this group were fatigue (7 %), thrombocytopenia (6 %) and neutropenia (5 %), the incidence of which were similar to that of the overall population. Of 213 evaluable patients, 26 (12 %) had an objective response. Median progression-free survival and overall survival were 5.6 months (95 % CI, 5.2–6.1) and 9.2 months (95 % CI, 7.8–10.9), respectively.

These large series of patients do not give clear information on the efficacy of sorafenib or sunitinib with respect to controlling disease in the brain in the absence of local therapy, i.e. surgery or radiotherapy. This is an important question and is addressed in a report of six patients with small asymptomatic supratentorial

metastases who safely received sunitinib [40]. All had clear cell histology, none had cerebral haemorrhage or herniation and none had surgical resection or radiation to the brain prior to systemic therapy. All patients had three or more metastatic sites including the brain. Four patients had a solitary brain lesion, one patient had two lesions and one patient had multiple small lesions. Two patients achieved a near-complete response to sunitinib and continued on treatment at the time of the report without recurrence in the brain for 23 and 47 months. Two patients had disease progression in the brain and were salvaged with radiation therapy, and one patient progressed systemically and in the brain after initial improvement in the brain. One patient died of systemic disease without relapse in the brain. Other case reports are consistent with these data [41, 42].

From a practical point of view, these data would lead us to recommend therapy with sunitinib in patients with brain metastases until such time as evidence is reported for the superior safety and efficacy for other targeted agents. We would also stress the need for prospective clinical trials in patients with brain metastases.

Dominant Sites of Metastatic Disease Necessitating Maximum Tumour Regression

The available data show that significant tumour regressions are, in general, more common with VEGFR kinase inhibitors, e.g. sorafenib, sunitinib, pazopanib and axitinib, than with mTOR inhibitors. For example, reported response rates to mTOR inhibitors as single agents are in the range of 0–10 % and even in combination with bevacizumab, in the range of 20–30 % [43–45]. In contrast, response rates with multi-targeted kinase inhibitors in randomised trials range from around 10 to 40 %, with the highest response rates in randomised trials in the first-line setting being reported for sunitinib and pazopanib [3, 9] and in the second-line setting for axitinib [4]. Given that there are no predictive biomarkers suitable for clinical use to rationally select systemic therapy in RCC at the current time [46], in situations where achieving the maximum chance of response is therapeutically important, we recommend that consideration be given to the use of multi-targeted kinase inhibitors.

Poor Performance Status and Poor-Risk Disease

Poor performance status should be distinguished from poor-risk or poor-biology advanced renal cell carcinoma. That is not to say that these categories do not overlap, but importantly, patients with poor performance status are, in general, excluded from registration clinical trials of drugs so the evidence base for the use of targeted agents in advanced RCC is limited for patients in this group. The sunitinib expanded access programme [39] is the largest series of patients reported to date of such patients and included 582 patients (13 %) with an ECOG performance status of 2 or

worse; treatment was tolerable in this group, and there was some evidence of efficacy, with a response rate of 9 %, median PFS of approximately 5 months and an OS of approximately 7 months.

The 3-arm registration trial of temsirolimus versus interferon or the combination [8] included 516 patients (82 %) deemed poor risk partly by virtue of the fact that their Karnofsky performance status was between 60 and 70. In order to be eligible for this trial, patients needed to be systemic treatment naïve and have three of six poor-risk factors (lactate dehydrogenase level of more than 1.5 times the upper limit of normal, haemoglobin level below the lower limit of normal, elevated calcium, time from initial diagnosis of RCC to randomisation of less than 1 year, a Karnofsky performance status of 60 or 70 or metastases in multiple organs). Of note, approximately a third of patients in this study had not had a nephrectomy. Median PFS in the temsirolimus group was 5.5 months and median OS 10.9 months, and as such temsirolimus is a standard of care in this group.

In general terms, patients with poor performance status are unlikely to derive major benefit from targeted therapy for metastatic renal cell carcinoma, which is in line with the fact that most patients with disseminated solid tumours and poor performance status do not benefit from systemic therapy, unless a clear molecular targetable driver is present, such as an activating mutation in a kinase, e.g. BRAF in melanoma [47] or Epidermal Growth Factor Receptor (EGFR) in non-small cell lung carcinoma [48]. Nevertheless, some patients with advanced RCC and an ECOG performance status of 2 in particular may well benefit from targeted treatment, and we think that a trial of treatment is reasonable in this group. Patients with an ECOG performance status of 3 are very unlikely to get significant or durable benefit from treatment, but in treatment-naïve patients, systemic therapy may palliate some symptoms such as malignancy-induced hypercalcaemia or painful bony metastases. Again, a trial of treatment in this situation may be reasonable, but potential benefits should be balanced carefully with the risk of worsened quality of life as a consequence of side effects.

Most patients included in registration clinical trials have had good-risk biology by virtue of the fact that they have had a prior nephrectomy and other prognostic factors have meant that the majority are of either good or intermediate risk, however defined. The only trial that has been done in a substantial number of patients with poor-risk disease is the temsirolimus trial discussed earlier, as a consequence of which temsirolimus is a standard of care in this setting. There has not been a prospective randomised trial of an anti-VEGF drug in this setting. Many oncologists would take the view that a vital overall therapeutic goal is to give patients the maximum chance of prolonged disease control by exposure to as many lines of therapy as possible and that the absence of evidence for anti-VEGF drugs in the group is not evidence for an absence of activity and a trial of a drug such as sunitinib or pazopanib may also be worthwhile. Notably, in a phase 3 trial in the second-line setting in patients who are refractory to sunitinib, sorafenib and temsirolimus were associated with a similar progression-free survival, but overall survival was statistically inferior on temsirolimus [11], based on data presented in abstract form. Further data (not published fully to date) demonstrate that neither the combination of

temsirolimus and bevacizumab nor the combination of everolimus and bevacizumab in the first-line setting has been shown to be superior to the combination of interferon and bevacizumab [44, 45]. Taken together, these data may diminish the use of mTOR inhibitors in advanced renal cell carcinoma in the future unless predictive markers are developed to allow patient selection for treatment with particular drugs. Finally, the treatment of patients with poor performance status and poor-risk disease is a major unmet medical need in renal cell carcinoma, and we would strongly advocate that, where possible, such patients are considered for clinical trial entry.

Elderly Patients

Elderly patients with metastatic renal cell carcinoma have, in general terms, been relatively underrepresented in registration trials, and the median age of patients has been 58–62 [2–8], whereas the median age for diagnosis for renal cell carcinoma is approximately 64 [49]. The elderly in general are likely, by definition, to have greater morbidity than younger patients, and polypharmacy in particular can be a problem in terms of potential interactions between medications. As a consequence of this, it is difficult to extrapolate with confidence from clinical trial data sets, but there have been a number of studies reporting specifically on outcomes for elderly advanced RCC patients treated with targeted agents.

Analyses for elderly patients treated with sorafenib have been carried out on both the North American expanded access programme [50] and the TARGET trial [51]. In the former study, 736/2,504 (29 %) of patients were over 70, and both toxicity and efficacy in the elderly group were similar to the overall population. In TARGET, 115/902 (12 %) of patients were over 70, and again, toxicity and efficacy were similar in both the elderly and the overall populations.

The sunitinib expanded access programme [39] included over 1,000 patients (roughly a third of the overall population) aged 65 and older; response rate in this group (17 %) was the same as in the overall population, as was median PFS (11 months) and OS (18 months). Additionally, an analysis has been reported of pooled data from six trials from just over 1,000 patients who received sunitinib [52], comparing outcomes in those over and under 70; 81 % were of age <70 and 19 % ≥70 years, with median ages of 57 years and 73 years, respectively. Median PFS was similar in both groups as was median OS. Most treatment-emergent AEs occurred at similar rates in both groups; however, some AEs were significantly less common in patients aged <70 years versus ≥70 years, including fatigue (59 % versus 69 %), decreased appetite/weight (29 % versus 53 %), cough (20 % versus 29 %), peripheral oedema (17 % versus 27 %), anaemia (17 % versus 25 %) and thrombocytopenia (16 % versus 25 %; all $P<0.05$). Hand–foot syndrome was more common in younger patients (32 % versus 24 %; $P<0.05$).

For everolimus, an analysis of the RECORD-1 registration trial has been reported according to age [53]. Just over a third of patients were older than 65 and 17.5 % were older than 70. Response rate, PFS and OS were similar in the elderly and the

overall population. The toxicity profile of everolimus was generally similar too although similarly to sunitinib, some adverse events were reported more frequently in the elderly. Specifically, for those over 70 in comparison with the overall population, the all-grade rates were 37 % versus 25 % for peripheral oedema, 40 % versus 30 % for cough and 38 % versus 30 % for diarrhoea.

These data sets have shown that, in principle, age is no barrier to treatment, but close attention needs to be paid to comorbidities and polypharmacy, and in our practice we tend to see elderly patients more frequently than younger patients until optimal dosing of drug is achieved in order to detect and manage toxicity as quickly as possible. Furthermore, age does not impact on choice of targeted agent.

Germ Line Genetic Variation

Most of the work on pharmacogenomics, as it relates to ethnicity, has involved Asian patients from South Korea, China, Taiwan, Singapore and Japan. The expanded access programme of sunitinib which included 4,564 patients from 52 countries has provided one of the main databases for the clinical evaluation of efficacy and toxicity in Asian patients [12]. Lee and colleagues reported on 325 Asian patients from this study and showed that fatigue was slightly greater in Asian compared to non-Asian patients (40 % versus 37 %) [54]. There was a slightly greater difference for stomatitis (39 % versus 26 %) and hand–foot syndrome (39 % versus 23 %). Interestingly, the rate of diarrhoea was very similar between the two populations (42 % versus 45 %) although the frequency of diarrhoea was lower in Asians treated at non-Asian sites (29 %) compared to those treated at Asian sites (49 %). This study found that haematological toxicity was greater in Asian patients and that the median duration of treatment was lower in Asian patients (4.3 months versus 7.8 months) although the rate of dose reduction was very similar between Asians and non-Asians. Progression-free survival was slightly lower for Asians compared to non-Asians (8.7 months versus 10.9 months), but overall survival was very similar (18.9 months versus 18.4 months).

In a detailed analysis of 132 unselected Korean patients, 100 of whom had been entered into the sunitinib expanded access programme, there was a similar finding that myelosuppression was more pronounced in this South Korean population [55]. The most frequent grade 3/4 toxicities encountered were haematological and included thrombocytopenia (37.8 %), neutropenia (29.5 %) and leucopenia (14.4 %). In this study the response rate was as expected, namely, 34 %, with a further 44.7 % of patients achieving stable disease. Progression-free survival was 8.2 months and overall survival 23.1 months, both of which are consistent with the literature. The authors evaluated the pharmacodynamic predictive factors for the toxicity profile seen, and a multivariate logistic regression analysis indicated that low body surface area and previous treatment were significantly associated with grade 3/4 toxicities. They went on to develop a prognostic nomogram for progression-free survival, and body surface area was included in this model.

Zhou has extensively reviewed the reports on sunitinib use in Asian patients and has shown that efficacy in terms of overall survival, progression-free survival and response is consistent with data in the literature [56]. The main difference between Asian and non-Asian populations in relation to sunitinib appears to be the higher rate of myelosuppression, as described.

Naito and colleagues studied 131 Japanese patients treated with sorafenib and demonstrated a 19.4 % partial response with disease control rate of 73.6 % [57]. Median progression-free survival was 7.9 months, and this is consistent with the literature which shows that progression-free survival following sorafenib ranges from 5 to 9 months. However, the overall survival in this study was greater than may be expected at 25.3 months. Most of the subjective grade 3/4 toxicities in this study were similar to the incidence and profile reported in the TARGET trial [2], i.e. below 5 %. However, grade 3/4 hand–foot syndrome occurred in 9.2 % of patients in this study compared to 6 % of patients in the TARGET trial. Rates of myelosuppression were not reported, but there was a difference in the rate of grade 3/4 hypertension which was much higher in this report from Japan (16.8 %) compared to the rate of grade 3/4 hypertension in the TARGET study (4 %). This could explain the good overall survival result reported by Naito and colleagues as it is now well established that hypertension is a good biomarker for efficacy in relation to VEGF-targeted agents in RCC [18]. A study from Yang and colleagues reporting their experience of sorafenib in ethnic Chinese did not confirm the high rate of hypertension seen in the Japanese population, and the rate of grade 3/4 hand–foot syndrome was much higher than either in the TARGET trial or Naito's report (26.7 %) [58]. This was a small trial, but the median progression-free survival rate in the 30 patients reported was impressive at 14 months although the median overall survival was only 16 months.

Rha's group in South Korea compared their Korean patients who entered the expanded access programme for everolimus (REACT) with non-Asian patients entered into the same programme [59]. They analysed 109 of their patients and found that they had a longer exposure to drug (24.1 weeks versus 14.0 weeks), and the overall incidence of grade 3/4 toxicities was slightly higher in their patients (70.6 % versus 61.6 %). The main toxicities that occurred more frequently were anaemia, hypercalcaemia, pneumonia, stomatitis, thrombocytopenia and pneumonitis. Their patients also had a slightly higher rate of partial response (3.7 months versus 1.7 months), and more patients achieved stable disease (67 % versus 51.6 %). A similar subgroup analysis was performed on 24 Japanese patients who entered the pivotal phase III trial of everolimus versus placebo (RECORD 1) [7, 60]. This analysis showed that grade 3/4 toxicities were very similar in the Japanese subpopulation, but some toxicities (grade 1/2) occurred more frequently, namely, stomatitis, infection, rash, dysgeusia, diarrhoea and neutropenia. Pneumonitis was also more common in the Japanese population (27 % versus 14 %—all grades). Progression-free survival was greater in the Japanese subgroup (5.7 months versus 4.9 months), but interestingly the median progression-free survival was also longer in Japanese patients in the placebo arm (3.61 months versus 1.87 months). Median overall survival in the

Japanese subgroup was not reached, whereas it was 14.8 months for the overall population, and the median overall survival for both placebo groups was very similar at 14.9 and 14.4 months.

The explanation why toxicity profiles and severity may be different in Asian populations is not fully understood. As has been described above, it is possible that physical characteristics such as body surface area may play a part, as may pharmacogenomic differences which have not been fully evaluated. It is becoming clear however that a third explanation is a possibility, namely, polymorphisms in ligands, receptors and other molecules along the angiogenic signalling pathway. These polymorphisms have been shown in a number of VEGF ligands and their receptors as well as in specific genes encoding enzymes responsible for metabolism and efflux transporters. These polymorphisms in both pharmacogenomic and pharmacodynamic pathways have been associated with toxicity patterns\severity and outcome for both sunitinib and pazopanib. The studies identifying genomic variations involving the VEGF pathway in individual patients have not been fully validated, and it is uncertain how they relate to different ethnic populations as a whole. This is a highly complex area to study but may have considerable importance in relation to individualising therapy [61–66].

Non-clear Cell

Introduction

This section will deal only with the RCCs, which account for more than 80 % of primary renal neoplasms, with approximately 7 % of malignant tumours of the kidney being due to transitional cell carcinomas and 5 % being termed unclassified [67]. The majority of RCCs are of clear cell type (ccRCC) but approximately 25 % are not, and they are sometimes categorised together as RCC of non-clear type (nccRCC). The 2004 WHO classification of neoplasms of the kidney includes 11 types of non-clear cell histology as well as other tumours of the kidney both benign and malignant such as oncocytoma, adenomas, sarcomas, mixed epithelial and mesenchymal neoplasms, neuroendocrine tumours, haemopoietic and lymphoid neoplasms, germ cell tumours and metastatic cancers [68].

Unclassified RCCs are of high grade, large and more likely to involve the adrenal gland, adjacent organs, nonregional lymph nodes and bones, and they are associated with a poor survival [68–70]. According to the WHO classification, the appearances of unclassifiable types of RCC include mixtures of recognised histological subtypes of RCC, pure sarcomatoid morphology, the presence of mucin, rare mixtures of epithelial and stromal elements and unrecognisable cell types [68, 69]. It is clear that the different histological subtypes of RCC have different molecular features and drivers and indeed aetiologies.

Targeted Agents in ccRCC and nccRCC

In the past some large therapeutic series and studies have included patients with all histological subtypes of RCC even though there was a strong suspicion that some were not particularly responsive to the main therapeutic strategies of the day, namely, immunotherapy. Similarly, in the early days of the introduction of targeted agents, patients were included in some studies irrespective of their histological subtype. Early data suggested that targeted agents such as sunitinib, sorafenib, temsirolimus and everolimus were active both in ccRCC and nccRCC.

An exploratory analysis of the randomised trial of interferon versus temsirolimus versus interferon + temsirolimus in poor-prognosis patients was performed, and temsirolimus appeared to be efficacious in both ccRCC and nccRCC with the median overall survival (OS) being 10.7 months versus 11.6 months, respectively [71]. The same was not true of interferon where the patients with nccRCC did less well on interferon (4.3 months median OS) compared to 8.2 months for those with ccRCC. In the open access study of everolimus, patients who had progressed on other VEGF-targeted agents were enrolled. There were only 75 patients with nccRCC out of a total study population of 1,367. There was only one responder to everolimus amongst those with nccRCC, but this was a similar response rate to the overall population, and likewise the median duration of treatment was similar 12 weeks versus 14 weeks [15, 72]. Thus both those with ccRCC and nccRCC appeared to benefit from everolimus.

In a large open access programme which included over 4,500 patients, sunitinib appeared as active in patients with nccRCC as those with ccRCC [12]. The overall response rate in this study was lower than in previous prospective trials and was 17 % for the study population as a whole and 11 % for those with nccRCC. Progression-free survival (PFS) and OS for patients with nccRCC were only slightly less than for all patients entering the programme; PFS patients for patients with nccRCC was 7.8 months compared to 10.9 months for the overall study population. OS was 13.4 months in comparison with 18.4 months, respectively, in this non-randomised data set.

Sarcomatoid Pathology

All histological subtypes of RCC can exhibit areas of sarcomatoid change. These areas are highly cellular with the cells exhibiting similar morphological features to sarcomas. These cells are often spindle shaped with a high degree of cellular atypia present. The subject has been extensively reviewed by Shuch and colleagues [73]. The authors point out that not all RCCs with sarcomatoid change are high-grade tumours, and this leads to the theoretical consideration that sarcomatoid change may not represent dedifferentiation but maybe the result of a separate de novo process. This review records the large number of chromosomal changes that can take place

within the sarcomatous elements of these tumours. It also notes that the hypoxia-inducible factor (HIF) pathway is highly expressed in the sarcomatoid regions when there is a background of clear cell histology as opposed to those non-clear cell tumours where there is less expression of HIF. Furthermore p53 mutations in the sarcomatoid component appear at a higher frequency than in the non-clear cell component. It has always been felt that patients with sarcomatoid features have a more aggressive phenotype, and there are reports of low median survivals in both patients with metastatic and localised disease with the former having a median overall survival of 3–10 months and the latter having a 5-year survival of under 20 % [74].

Chemotherapy has often been used in these patients, typically with regimens containing doxorubicin combined with either gemcitabine or alkylating agents such as cyclophosphamide, ifosfamide or dacarbazine. From two reviews of the literature [73, 74], it appears that out of 108 patients reported to have been treated with chemotherapy alone, there are 16 responders (15 %), the response rate for patients treated with chemo-immunotherapy, usually 5FU-interferon ± IL2 based, is somewhat higher 36 % (30/83 patients) and for immunotherapy alone mainly IL2 ± interferon, the response rate is 15 % (5/33 patients).

There is one report of chemotherapy being used with sunitinib, and this study showed a response rate of 33 % in the nine patients who were evaluable [75]. Other studies have reported the use of targeted agents alone. The overall response rate to targeted agents is 13.6 % (12/88 patients) [74, 76–78], and median PFS is 4.4–5.3 months with median OS 11.8 months [74, 78]. One study has suggested that median OS might be as long as 18 months and with no difference between those treated with targeted or non-targeted therapies [79]. An interesting sequential study suggested that when patients with sarcomatoid RCC were treated with gemcitabine and doxorubicin, they failed to see any responses and the median time to progression was 6.6 months [80]. However when subsequent sorafenib was given, one patient had an objective response, and the median time to progression was 10.9 months from the start of the second-line targeted therapy.

The majority of patients [62] with sarcomatoid RCC have been treated with sunitinib (reviewed by [74]) with a cumulative response rate of 16 %. There are no responses recorded to everolimus (four patients) or a combination of sunitinib + bevacizumab (two patients), but 1/12 patients have responded to sorafenib and 1/8 patients have responded to bevacizumab [74, 76–78]. These studies report median PFS ranging from approximately 5 to 8 months with median OS ranging from 10 to 17 months.

It is difficult to make definitive recommendations about the treatment of patients who have sarcomatoid components in their tumours because all histological subtypes are involved, there is uncertainty as to whether or not the sarcomatoid component represents the background histological subtype, the percentage of sarcomatoid component is variable and the number of well-conducted prospective therapeutic studies is very limited. In short, the molecular driver of the sarcomatoid component is unknown, but it is likely to differ amongst patients with distinct background histologies.

RCCs associated with a sarcomatous component remain an area of considerable therapeutic uncertainty and clinical need.

Papillary Carcinoma

There are two types of papillary RCC which differ in relation to their phenotype and molecular abnormalities. Type I tumours are more commonly associated with multifocal lesions, and type II tumours are associated with hereditary leiomyomatosis, an autosomal dominant syndrome associated with mutations in the fumarate hydratase enzyme. There are 319 cases recorded in the literature of papillary RCC treated with targeted agents, 2 of these separate patients by subtype [81, 82]. In the preliminary report by Ravaud and colleagues, 1/23 patients with type II papillary RCC responded to sunitinib, whereas there were no response in the 5 patients with type I disease. Lee and co-workers also report responses to sunitinib in patients with type II in 4 out of 17 patients. The other reports record 17 responses out of 264 patients (6.4 %) with a variety of VEGF-targeted agents. Median progression-free survival in these studies has ranged from 1.6 to 11.9 months [14, 76, 77, 83–85].

The EGFR-targeted agent erlotinib has been evaluated in 45 patients with papillary RCC with a response rate of 11 % (5/45 patients) and a median overall survival of 27 months with 31 % of patients not having progressed at 6 months [86].

In a phase 2 study of foretinib, the dual inhibitor of MET/VEGFR2, there was a 13.5 % response rate in 74 patients [85]. Patients were stratified according to MET pathway activation, and the presence of a germ line *MET* mutation was found to be predictive of response with partial responses in this patient group being 50 %, as opposed to 9 % if no such mutation was present. This is a good example of the importance of molecular selection for patients who are being treated with targeted agents.

It is difficult to compare the efficacy of different targeted agents, but response rates from cumulative data calculated from these studies suggest that the response rate to sunitinib is 11 %, 2 % to sorafenib, 6.8 % to everolimus and 13.5 % to the foretinib. PFS is very difficult to compare across the different agents because on the one hand, there appears to be some consistency in the median PFS being about 5–10 months, but on the other, the reported median PFS for those patients treated with sunitinib varies widely from 1.6 to 11.9 months.

Chromophobe Renal Cell Carcinoma

Chromophobe tumours account for about 5 % of renal cell carcinomas and are associated with chromosomal losses and mutations in p53. This type of nccRCC is associated with the Birt–Hogg–Dube syndrome which is an autosomal dominant disease characterised by trichofolliculomas and discomas and lung cysts.

There are 51 patients reported in the literature who have been treated with targeted agents with 11 objective responses (21.5 %) [14, 76, 77, 82, 84, 87, 88]. The cumulative response rate to sunitinib [76, 82, 84] is 27 % (4/15 patients) and to sorafenib [14, 84] 12 % (3/25 patients), and 4/10 patients have responded to temsirolimus or

everolimus (40 %) [77, 87, 88]. Reports on median PFS vary widely from 8.9 to 12.7 months for sunitinib, 5 to 27.5 months for sorafenib and 3.8 to 14 months for the mTOR inhibitors. Our group reported a patient who, although never achieving an objective partial response, gained considerable benefit from sequential therapy with sunitinib followed by everolimus. The disease was controlled initially for 18 months, and then for a further 2 years, there has been ongoing disease stabilisation [89].

Collecting Duct Carcinoma of Bellini (CDC)

CDC accounts for only 1 % of RCCs. CDC is a highly aggressive cancer of the kidney which has in the past been treated by platinum-based chemotherapy.

There are some sporadic case reports on the use of targeted agents in patients with CDC. Nineteen patients in all have been reported [76, 77, 90–93] with 2 patients responding to sunitinib [91, 92]. Procopio and colleagues noted that all but two of their patients had rapidly progressive disease, and these two patients appeared to have long-lasting disease control of 19, 20 and 49 months, respectively, but there were no patients alive at 5 years [90]. Staehler and colleagues treated patients with sunitinib following surgery for relapse, but the disease grew rapidly through this treatment [93].

XP11 Translocation

This type of nccRCC is characterised by translocations involving Xp11.2 resulting in the fusion of the TFE3 gene to a number of genes with the resultant over expression of TFE3 protein. This form of nccRCC affects children and young adults predominantly. The tumour is associated with a poor prognosis, and systemic therapy with chemotherapy or immunotherapy does not seem to improve outcome.

A total of 40 patients are reported in the literature who have received targeted therapies. Single-case reports [94, 95] describe the use of sunitinib or sorafenib in young adults with subsequent responses being seen in both patients; one patient responded for 12 months (sorafenib) and the other (sunitinib) for 3 years. A child had a good but short-lived response to temsirolimus which lasted only 5 months [96].

Two larger series describe the treatment of 15 and 21 patients [97, 98]. Choueiri and colleagues described patients who had received sunitinib (ten patients), sorafenib (three patients) or monoclonal antibodies to VEGFR, bevacizumab or ramucirumab (two patients), and there were three responses, one each for sunitinib, sorafenib and ramucirumab with responses lasting 7, 13 and 27 months [97]. The median PFS was 7.1 months and median OS was 14.3 months. In the report of Malouf and colleagues, 21 patients received targeted agents and 7 achieved an objective response. Targeted agents in first line were either sunitinib (11 patients) or temsirolimus (1 patient), and 9 patients received cytokines. The median PFS was

8.2 months for those treated with sunitinib but only 2 months for those treated with cytokines. There was one complete response in the sunitinib group, and a partial response was observed in another 3; only 1 patient responded in the cytokine group. At the time of reporting, the median OS for patients treated with cytokines was 17 months, but median OS hadn't been reached in the sunitinib group. Eleven patients received second-line treatment, and six patients received third- and fourth-line treatment with targeted agents. Three patients received sunitinib, all of whom responded, and seven of eight patients treated with sorafenib had stable disease with a median PFS of 6 months with one patient continuing to have stable disease at 29 months. Seven patients received mTOR inhibitors and one achieved a partial response. Interestingly this patient had progressed on both cytokine therapy and sunitinib and sorafenib [98].

The data from these studies clearly show that some patients with XP translocations can respond to targeted agents and that a subgroup of patients may get disease control for a prolonged period of time.

Rare Tumours

Mucinous tubular and spindle cell carcinoma is a rare subtype of tumour originating from collecting tubules. Our group has reported the use of sunitinib in a 61-year-old female who presented with metastatic disease who not only showed a partial response to therapy but also derived symptomatic improvement within days of initiating treatment [99].

Renal medullary carcinoma is a very aggressive tumour occurring in patients with sickle-cell haemoglobinopathy. The subject has been well reviewed recently by Ali and colleagues [67]. There is evidence that this tumour type could be a good candidate for targeted agents because HIF1 alpha can be over expressed. In addition, there is evidence that in some of these tumours, anaplastic lymphoma kinase rearrangements can be found, making patients with such tumours potential candidates for crizotinib.

There is a case report of a dramatic complete response to bortezomib, the proteasome inhibitor, in a patient with renal medullary carcinoma. The patient received 7 months of bortezomib and remained disease-free after more than 27 months of follow-up at the time of the report [100].

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Chapter 20
Immunotherapy: The Current Role of Cytokines

Mayer Fishman

Introduction: Cytokines as a Therapeutics Category

Among the molecules of human physiology, *cytokines* encompass hundreds of different proteins released by one cell type and ligating a receptor on the same or other cell types to cause some biological effect. While many have been isolated and studied, only a tiny subset has been synthesized for therapeutic application. For kidney cancer therapy, cytokine therapy is a collective reference to treatment with interferon alpha and with interleukin-2, even though much is known that distinguishes each of their interactions with the tumor, angiogenesis, and different kinds of leukocytes. Another cytokine in widespread contemporary anticancer use and application and testing is GM-CSF, although not a usual component of any standard (non-investigational) kidney cancer treatments.

Some investigational drugs are cytokines or modified cytokines and are in development, including for anticancer application in kidney cancer. There are many other naturally occurring and modified cytokines, including both interferons (such as interferon β 1b, which is indicated in therapy of multiple sclerosis) and interleukins (such as IL-7, IL-15, or IL-21, for which there are contemporary oncology research studies), or modifications of those, or other cytokines that are not structurally related to interleukins or interferons. As discussed briefly below, at least theoretically at this point in time, other cytokine drugs could be of therapeutic utility for kidney cancer treatment.

As therapeutic interventions go, theoretical mechanisms of anticancer treatment with immunotherapy present a striking elegance: A synthetic copy of an endogenous protein ligates specific receptors, physiologically distributed on leukocytes, and the activation of those changes leukocyte behavior, to then trigger a cell-based

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attack upon the cancer. Results can be dramatic—after a limited course of active treatment, there is a durable regression of the cancer and the patient is mainly encumbered only with a surveillance program, for the occasional recurrence.

While *targeted* drugs are, for kidney cancer, mainly directed at the vascular endothelial growth factor [VEGF] and mammalian target of rapamycin [mTOR], or other cell surface proteins, many of which are tyrosine kinases, the crossover of drugs onto targets outside of the tumor that include its associated angiogenic processes or onto elements of the immune system, such as regulatory T cells (Treg) or dendritic cells (DC), has not been ignored [1, 2]. The extent to which there are "class effects" that are homogeneous within the *targeted* or *cytotoxic* drug groups can easily be overstated, and the same is true for *cytokines*. Acknowledging the success of cytokine therapy, but also the limitations which have been partly addressed in the development of targeted drugs, one may be optimistic that as the experience of measuring immune-related parameters on tumors and in patients becomes more sophisticated, the processes of selecting patients and administering therapeutic cytokines will become more often successful.

The revolutionary introduction of the medicines classified *targeted* drugs particularly had an unintended consequence of aggregating together interferon and interleukin-2 and even several molecularly unrelated cytokines into their own category. This potential for overstatement of homogeneity is similar to how the capacity for an anticancer immune response across the incident population of kidney cancer patients could be *assumed* as uniform in a clinical trial where immune parameters are not directly assessed. For now, however, one may commonly see designations as "cytokine refractory" or "cytokine naïve" or "prior cytokine therapy," with little emphasis about which drug or schedule was used, in the targeted drugs' clinical trial literature.

The similarities and differences are particularly of interest for the clinician to distinguish between interferon-α and interleukin-2 treatment. Some research efforts in immune therapies use cytokines including GM-CSF interferon or interleukins as key immunological adjuncts of complex combinations. Some of those combinations may defy categorization into *cytokine* or *targeted* subsets, particularly as we learn of potential for subtle differences among the available and investigational drugs from the targeted group, discussed in detail in other chapters.

For the reader of therapeutic trials of treatments for kidney cancer, the usual usage of the *cytokines* refers to interferon-α and interleukin-2, both in anticancer usages for decades, and contrasted to the newer targeted drugs of the VEGF and mTOR signaling axes. Regardless of the limitations of a nosology derived from idealized mechanisms of action, the chapter here will focus on clinical experiences of interferon-α and interleukin-2, with some attention to other drugs that are, at least structurally, cytokines as well. Interferon-α can be identified as the treatment used as three times a week subcutaneous monotherapy that was the inferior arm in the targeted drug pivotal trial comparisons with sunitinib [3], with temsirolimus [4], and with the combination of bevacizumab and interferon-α [5, 6]. Interleukin-2, on the high-dose bolus schedule, could be summarized as an inpatient, intense treatment associated with complete responses of remarkable unmaintained durability, but only in a low fraction of those treated.

Interferon-α

Historical Role

Over 50 years have elapsed in the study of interferons, which had its roots in the study of viral infections of cultured cells [7]. Therapeutic applications for many disease states have been tried. The earliest kidney cancer regression reported with interferon-α dates to 1983 [8], and some of the early development, encompassing buffy-coat-derived purified protein, and the subsequent introduction of recombinant product expressed in *E. coli* are reviewed briefly in the earlier edition of this book. Practically, the more recent therapeutic testing of interferon-α for kidney cancer treatment used interferon alfa 2a (manufactured by Roche) and interferon-α 2b (manufactured by Merck [MSD]). Modified interferon-α that is pegylated are on the market with indication for treatment of hepatitis, but there was only limited clinical development of that product in formal kidney cancer therapeutic trials (see below). For the most part, no differences between the two are hypothesized, and in the discussion it is referred to as simply interferon-α, INF-α.

Commercially available INF-α as used in kidney cancer therapy is a recombinant copy of the naturally occurring interferon. Interferon-α is a type 1 interferon, one of over 20 human type 1 interferons, which ligates the interferon-α receptor, consisting of two chains (IFNAR1 and IFNAR2) [9, 10]. The distribution of the receptors and the physiologic and pharmacologic effects of exogenous administration are diverse. These include direct effect on receptors on leukocytes, on endothelial cells, on tumor cells, and on other somatic cells. The therapeutic application of interferon-α for kidney cancer treatment is correspondingly complex even on a theoretical basis—effects on the tumor cell, tumor-associated vessels, and global immune function are all plausible. Despite hundreds of clinical trials, encompassing patients with kidney cancer, or other neoplastic diagnoses, or infectious viral diseases such as hepatitis C, the best schedules to use or adjust doses for kidney cancer therapy remain in evolution, with diverse schedules in contemporary research and clinical use.

Older Randomized Trials

Interferon, although never being put through a specific pivotal trial to establish it as an FDA-indicated anticancer treatment for kidney cancer, was nonetheless a de facto reference standard and fundamental medical approach for RCC treatment for over a decade. Many trials were organized with interferon treatment for all patients and then randomization without or with the trial intervention. These include comparisons and combinations with targeted drugs, discussed below, and prior trials from before the advent of VEGF-targeted drugs.

These earlier clinical experiences are notable for what can be called a straightforward patient selection strategy, using almost exclusively clinical factors: site of

origin in the kidney, performance status, and a few organ-function-related blood tests. This inclusive approach did not yield a clear definition for a subset with a high chance of response. Algorithms based on histological features or on other clinical factors have largely not defined a subset of patients for which to reliably predict a high chance for interferon response. The challenge of finding those patient or tumor features that can predict a better chance of anticancer response persists through the present. The dearth of algorithms to select the optimal patient subset for treatment, for interferon, (as well as for interleukin-2), is not unique to cytokines or to kidney cancer, but in general, the targeted drug treatments appear to be less restricted as far as the subset of the incident RCC population for whom there can be a visible impact on the cancer.

The most frequent current use of interferon in kidney cancer treatment is comparable to the schedule of the two largest randomized bevacizumab plus interferon trials [5, 6], but rational bases of other schedules abound. The experience, as described in the subsequent analysis of the European trial [11], in which a better survival was observed in patients with more side effects to the extent that dose reductions were required is consistent with earlier observation that worse cytokine side effects do appear (paradoxically) to be a marker of better anticancer impact [12].

The first randomized trial (using interferon in both arms) accrued 178 patients in Europe, in 1985–1986, and the report was published by Fosså and colleagues in 1992 [13]. The treatment plan was with 18 million IU interferon-α 2a, three times a week, without or with vinblastine, 0.1 mg/kg/dose, IV, every 3 weeks. Among those recruited, 24 were designated ineligible, 9 not evaluable for absent non-bone disease, and 26 withdrew for toxicity, leaving 119 evaluable. The response rate counting one complete response (CR) and 15 partial responses (PR) among 66 patients was 24 % (95 % CI: 15–36 %) in the combination; the monotherapy arm had 1 CR and 5 PR among 53, or 11 % (95 % CI: 4–23 %). These responses were considered to establish interferon as a treatment for RCC, in the absence of any other medical therapy then extant. No differences of overall survival (OS) by treatment arm was observed, but differences by World Health Organization (WHO) performance status (PS) were described with 13 %, 6 %, and zero alive at 5 years for PS 0, 1, or 2, respectively. The effect of pretreatment PS as a dominant determinant of survival was reinforced in many further experiences (e.g., references [14, 15]) and also observed in the randomized bevacizumab and interferon trials, where the between-risk group differences of OS overall survival between-risk groups is much larger than the (nonsignificant) OS overall survival difference [11].

A second randomized trial involving interferon but using vinblastine in both arms was published in 1999; it was comparatively small ($N = 160$), and represented data of a one-country (Finland) three-center trial accrued over 6 years, 1988–1994. In the 1:1 randomization, subjects received treatment either with single-agent vinblastine, once every 3 weeks, or the combination of that with interferon-α 2a, three times a week, the first week at 3 million IU/dose, then after that up to 18 million IU/dose, with reduction to 9 million IU/dose allowed, for a period of 1 year. Assignment to the interferon combination arm was associated with a superior major response rate (7 CR, 6 PR which is 16 % combined, versus 1 CR and 1 PR, 2.4 % on the

vinblastine alone arm) and a superior median time to progression (13 versus 9 weeks) and superior overall survival (OS) (67.6 versus 37.8 weeks). Each of these differences met statistical and clinical significance and some 4- and 5-year survivors as well, noted only in the combination treatment arm [16].

The experience of this randomized clinical trial is discussed by the authors with reference to ten identified interferon + vinblastine treatment combination RCC treatment series, for which there was an aggregate 21.6 % response rate among 416 subjects and five trials reporting median survival over 1 year (and one, 39 weeks). The randomized patients' experience appears comparable. On the other hand, 13 identified interferon-only RCC treatment series reporting altogether 414 subjects' outcomes had an aggregate of 14.5 % overall response rate. Of note the interferon dose was only 2–3 million IU/dose, for some of these regimens, as used for 188 patients.

The issue of an optimal interferon dose (or dose escalation or de-escalation strategy) is discussed again, below, in relation to the bevacizumab plus interferon trials and remains a contemporary challenge to anticancer application of interferon. In this context, as discussed also in the paper, the specific role of vinblastine to augment interferon has remained in doubt. Most subsequent interferon trials used either no conventional cytotoxic therapy or 5-fluorouracil (and related) drugs.

Importantly, for either arm of the latter trial publication, absence of nephrectomy was adverse. This presaged the observation in two randomized trials of interferon therapy as a fixed treatment, but with assignment to nephrectomy or no nephrectomy, in two parallel trials conducted by the Southwest Oncology Group (SWOG) accruing patients from 1991 to 1998 ($N=246$) [17] and the European Organisation for Research and Treatment of Cancer (EORTC) from 1995 to 1998 ($N=85$) [18].

In this next major effort, the RCC randomized trial therapeutic development was anchored on interferon development; both trials used interferon treatment (without randomization) for all the patients. Although this could not accommodate a relative-efficacy assessment against a non-interferon treatment, it represents another larger database of interferon-treated patients. (A similar situation occurs with the bevacizumab plus interferon trials discussed next.) In these two nephrectomy trials, the same result was observed; the EORTC report describes median OS was 17 versus 7 months with a significant hazard ratio of 0.54 (95 % CI = 0.31–0.94), and for the SWOG trial, 11.1 versus 8.1 median overall survival, $P=0.05$ for a two-sided comparison [17, 18]. The aggregated data of the two trials were in agreement, yielding median survival of 13.6 versus 7.8 months, a hazard ratio of 0.69 for the rate of death ($P=0.002$) [19].

This concrete observation of improvement of the median survival as a consequence of nephrectomy, despite metastatic disease, was the basis for a usual consideration of nephrectomy for fit patients with good renal reserve, despite the presence of metastatic disease, in clinical practice. The particular role of interferon, or of cytokine therapy, however was not addressed. Later, with the contemporary usual consideration of VEGF-directed therapy for these patients, the extrapolation of the rationale from the interferon-based experience is now identified as an issue that should be again explicitly addressed.

Some Outpatient Subcutaneous IFN + IL-2 Combinations

Another group of trials has emphasis on combination outpatient low-dose interleukin-2, outpatient interferon as a base treatment. The regimen has a complex schedule that uses different IL-2 doses (5 million or 20 million IU/m^2 per dose) and interferon doses up to 6 million IU/m^2 per dose, on differing schedules of once, three, or five times a week, in an 8-week block.

There were 6 CR and 20 PR, for an overall response frequency of 19.4 % (95 % CI: 13–26 %) observed in an early 134 (evaluable) patient, cooperative group phase II study [20]. In a second multi-institutional series of 152 patients, there were 9 CR and 29 PR recorded, yielding a 25 % (95 % CI: 19–32 %) overall response frequency estimate [21]. Another group observing a similar response frequency in a 38-patient series was reported by Ravaud and colleagues in 1994, with 1 CR and 6 PR, giving a 19 % overall response rate [22]. This response-rate frequency was not duplicated in some later experiences by other groups. A French series with a relatively similar treatment schedule with IL-2, interferon, and 5-FU reported responses of 1 CR and 11 PR and overall response frequency estimate of only 19 % (95 % CI: 10–31 %) and was closed early at 62 patients, for that reason [23]. A Dutch series with 51 found only 6 responders (12 %) and no CR [24]. The combination of IL-2 and interferon, also on a lower-dose, subcutaneous outpatient schedule, is discussed again below as the outpatient dose arm of one of a randomized trial with a high-dose IL-2 arm. There were 91 patients on that arm, 3 CR and 6 PR, or a 9.9 % overall response rate [25], consistent with the lower response frequencies of the latter French and Dutch series.

Additions of third or fourth drug were developed extensively. Examples included in these trials are the chemotherapy drugs, such as 5-fluorouracil (5-FU) and vinblastine and targeted drugs such retinoic acids (directed at retinoic acid receptor) and are used in advanced disease, as maintenance treatment, and on an adjuvant schedule. In an early single-arm trial, reporting on 35 patients treatment with the combination plus 5-FU, there were 4 CR and 13 PR, demonstrating thus a response rate of 48.6 % (95 % CI: 32–66 %) [26]; a report describing addition of oral *cis*-retinoic acid, showed among 24 patients, there were 4 CR and 6 PR, which was a 42 % response rate (95 % CI: 22–63 %) [27]. A randomized trial with a total of 80 patients, comparing the combination with 5-FU versus tamoxifen, showed the latter to be inferior (no responses) but 7 CR and 9 PR, giving overall response rate of 39.1 % (95 % CI: 24.2–55.5 %) [28].

A four-armed, 379 patient study used a regimen with the same subcutaneous interferon and interleukin-2 schedule and additionally 13-*cis*-retinoic acid, with randomization of the lung-metastases-only patients to additional inhaled IL-2, or not, and the other patients to either 5-FU (intravenous) or capecitabine (oral). Overall, there was not a significant difference across the randomizations, but the overall response rate was more in line with that observed in the German cooperative group (DCGIN) studies: in the lung-only patients 29 % and 31 % (+inhaled IL-2)

response rates and in the other 19 % (5-FU) and 26 % (capecitabine). Differences of 3-year PFS were not clinically or statistically significant, with values ranging from 7.8 to 10.8 % [29].

Atzpodien and colleagues, in a trial conducted through DCGIN, describe treatment of 341 patients, for whom the treatment plan was either interferon, interleukin-2, and intravenous 5-FU or the addition of oral *cis*-retinoic acid to that, and these were compared to treatment with interferon and vinblastine, similar to the two-part combination described above. Response rates had a pattern favoring the non-vinblastine arms with a 31 % (with 5-FU), 26 % (with *cis*-retinoic acid), and 20 % (vinblastine), respectively, and the combined cytokines also were statistically superior for the median OS, with a finding of 25, 27, and 16 months, $P = 0.023$ and 0.025 for the two pairwise comparisons of the combined cytokines against the vinblastine containing arms [30].

Trials with low-dose cytokine combinations were developed by several US and European groups. The Cytokine Working Group described 2 CR and 7 PR, among 50 patients treated, for an overall response rate of 18 % [31]. One of the larger reports through the Groupe Français d'Immunothérapie describes treatment of 425 patients allocated to either 120-h continuous infusion interleukin-2 (18 million units/m^2/24 h × 5 days = 120 h) or interferon subcutaneous treatment (9 million units) or both. The response rate statistically favored the combination with observed overall response frequencies of 6.5, 7.5, and 18.6 % [32]. Besides the similarities of response rates, there is a characteristic side-effect profile, consistently with prominent fever, flu-like symptoms, fatigue, nausea, injection site reactions and depression, defining what has become a relatively unappealing choice. Generally, at present, the cumulative experience with multiple injections, and long-term at-home toxicity, and occasional severe impacts on quality of life and adverse events have largely marginalized this use of cytokines, in favor of targeted drug, often oral, monotherapies.

Adjuvant Cytokine Trials

An adjuvant study of the Atzpodien regime, with 203 patients, randomized to the Atzpodien regimen or to observation, was reported with an 8-year follow-up, and was negative, joining the other adjuvant trials to date. Randomization to the active treatment (interferon, interleukin-2, and 5-FU) was associated as actually worse overall survival: for 2-, 5-, and 8-year evaluations, there was 81, 58, and 58 % versus 91, 76, and 66 % in the observation arm ($P = 0.028$); the PFS trend also was adverse, with the treatment arm having 54, 42, and 39 % versus 62, 49, and 49 % but not meeting statistical significance, with log-rank $P = 0.24$ [33]. Several other adjuvant studies incorporated cytokine therapies; although the difference between the treatment and control (generally observation) were smaller, none showed an advantage to define an adjuvant cytokine regimen. Some reviews of these include references [34] and [35]. For this reason, adjuvant use of cytokine therapy is not a usual consideration.

Table 20.1 Comparison of patient inclusion features

Both	CALGB trial [5]	EORTC trial [6]
Kidney cancer	BP < 160/90	Proteinuria <500 mg/24 h
No prior therapy	85 % had prior nephrectomy	Nephrectomy required (or partial nephrectomy with negative margins)
KPS 70 %+	Cardiac comorbidity excluded	RECIST measurable or not measurable allowed
No CNS metastasis	Bleeding or clotting disorders excluded	No recent major surgery

Contemporary Interferon Trials with Bevacizumab

Interferon without or with bevacizumab was studied in two similar trials, conducted through the North American CALGB trial 90206 [5] and European EORTC trial AVOREN [6]. Both were similar in eligibility, treatment plan and outcomes, and sponsorship through Genentech, manufacturer of bevacizumab. In Table 20.1 are a few highlights of similarities and differences of the patient populations and of the treatment plans. All the patients' treatments used interferon, with 1:1 randomization for addition of bevacizumab 10 mg/kg/dose, every 2 weeks. The median PFS were 4.9 and 8.4 months (HR = 0.71, $P < 0.001$, favoring the combination) for the US trial [5] and 5.5 and 10.4 months (HR = 0.571, $P < 0.001$, favoring the combination) for the European one [6]. While this trial design could not address the relative role of interferon, the positive PFS results did establish interferon now as a component of a VEGF-targeted regimen. Although there is not a specific US FDA approval for use of interferon alfa 2a as a treatment for metastatic kidney cancer, the approval of bevacizumab as a treatment for metastatic cancer is with the direct reference to the combination with interferon-α. Practically speaking, recognizing the experiences of the randomized trial of sunitinib versus interferon [3] and temsirolimus versus interferon, in high-risk patients [4], this combination approach—not interferon monotherapy—is the current (2014) usual consideration for interferon treatment of kidney cancer.

The OS results in the two trials however did not meet mathematical significance in terms of an advantage for the combination with 17.4 months in the interferon monotherapy arm and 18.3 months for the combination, but with a *P*-value of 0.069 [5] still not meeting mathematical criteria for significance and similarly in the EORTC trial with 21.3 versus 23.3 months for the combination, the *P*-value of 0.3360 [6] again was not indicative of this being a conclusive difference, even with the apparent bigger difference (2.0 versus 1.1 month median OS) between the differences across trials.

In an unplanned posttrial exploratory analysis among the 233/649 (35.9 %) patients of the EORTC trial who had (nonrandomized) further treatments including treatment with VEGF-receptor tyrosine-kinase inhibitor after the protocol treatment, the overall survival numbers are longer, but comparison did once again not

show a statistically improvement; the difference was longer 33.6 versus 38.6 months in the combination arm. After the application of a (prespecified) Cox regression model that addressed prognostic factors including gender, leukocyte and platelet counts, weight loss, number of sites of disease, and Motzer prognostic score, the advantage of the combination arm versus the interferon monotherapy arm showed *P*-value 0.0219, for the hazard ratio of 0.78 for overall survival, among the overall trial population [36].

Interpretation of these trials puts focus on its separable components. With recognition that the characteristic side-effect profile of interferon, with fever, chills, asthenia, and depression risks has a high quality of life impact, one question is whether the salutary effect on PFS observed in the combination trial arm could be duplicated with a lower interferon dose or even with bevacizumab alone or in another combination. In a hypothesis-generating reanalysis, the observed survival difference in the EORTC trial, among the 131 combination treated patients who required an interferon dose reduction, compared to the overall group of 327 combination treated patients was 26.0 versus 23.3 months (same number as [6]) [37]. This interesting result suggests that the interferon doses, at least among those who required a dose reduction, were sufficient to still put that group at a survival advantage over those receiving full-dose interferon, or interferon (full or reduced dose) without bevacizumab, where the 21.3 months (same number as above) median survival was observed. A further trial does address using lower interferon dosing strategy in the first place, 3 million instead of 9 million IU per dose. In that nonrandomized trial, Melichar and colleagues observed, among 146 patients from a new trial and a comparison group of 283 patients selected from the AVOREN database, that the median PFS, response rates, and median OS were preserved, even with the lower interferon dose. The observed PFS 15.3 months (95 % CI: 11.7–18.0) compared favorably to that seen in AVOREN 10.5 months (95 % CI: 10.1–12.9); the major response rates were 29 and 36 %, and the median OS of 30.7 was not worse than the 25.8 months for those patients in the AVOREN database [38]. As a bottom line, it is safe to say that optimal dosing strategies of interferon continue to evolve, particularly with an eye to starting interferon at a lower dose than 9 million units, on this schedule.

Another question relates to the use of OS as a trial endpoint—should one expect that a large PFS increment produce an OS change as well? The issue is not unique to kidney cancer nor to targeted drug treatments. A significant, observable median OS increment was absent in the AVOREN and CALGB 90206 bevacizumab plus interferon studies. As recently as a decade ago, with no approved targeted therapies, the preponderant majority of patients did not receive second-line therapies with likely substantial OS impact. However, now, with several VEGF-axis and also mTOR-axis drugs available, differences of PFS in a trial population may be significant, but the control arm population (with earlier termination of the on-trial treatment) will have disproportionately more exposure to other off-trial and posttrial treatments, necessarily narrowing the observable differences between treatment arms; this is discussed also by Delea [39].

On the other hand, in a meta-analysis encompassing over 10,000 RCC patients across 31 therapeutic trials, there was correlation, with every 1 month PFS increment being a basis to expect averaged 1.17 month (95 % CI: 0.59–1.76 months) OS improvement [39]. Although the correlation coefficient was relatively low (0.28) for this computation, it does underscore experience of the large, interferon-based trials as ones for which PFS differences on first-line treatment were seen, but still smaller nonsignificant OS differences were seen.

Trials of Bevacizumab with Another Drug Besides Interferon

Bevacizumab is used in the first VEGF-targeted monotherapy trial in RCC, conducted at the NIH [40]. The question converse to that of the interferon-based bevacizumab trials was addressed in a bevacizumab-based trials using bevacizumab as the base treatment and either interferon or another drug (temsirolimus) as the partner. The results from the 791 patient INTORACT trial suggest that the contribution of interferon could be substituted by another drug. In this randomized trial, the bevacizumab and temsirolimus combination median PFS and OS were seen at 9.1 and 25.8 months, not different from those observed in the bevacizumab and interferon arm, 9.3 and 25.5 months [41]. This contrasts with the earlier report from the French TORAVA trial, in which 171 patients were assigned (2:1:1 randomization) to bevacizumab with temsirolimus, sunitinib, or bevacizumab with interferon. In that report the median PFS results showed 8.2, 8.2, and 16.8 (95 % CI: 6–26 months), nonsignificantly in favor of the conventional schedule with the bevacizumab and interferon combination as on the AVOREN trial [42].

Trials of Interferon with Other VEGF-Axis Drugs

The combination with sunitinib and interferon was found to be feasible, in a phase I study, with 25 patients treated with either sunitinib 50 mg or sunitinib 37.5 mg and interferon at three times a week dose of 3 or 6 or 9 million IU per dose. While major responses were seen, dose reductions were frequent; the aggregate overall response rate of 12 % (with another 80 % with SD as a best response) does not compare favorably to the other sunitinib monotherapy experiences, where over 30 % response rates were observed. The summative assessment was that the treatment was generally not sustainable, thus favoring sunitinib monotherapy over an interferon combination [43].

Jonasch and colleagues reported on a phase 2 randomized trial in which 80 patients were treated with either sorafenib 400 mg po BID or with the addition of interferon at a dose of 9 million IU three times a week. The monotherapy arm appeared to be as good, not suggesting additional benefit attributable to interferon. The observed ORR were 30 and 25 % (combination), PFS 7.4 and 7.6 (combination)

months; the OS median was not attained at the point of publication (sorafenib arm, lower 95 % CI: 22.3 months) versus 27 months (combination) [44].

The combination of sorafenib and interferon was addressed also in a European trial (RAPSODY) with a similar phase 2 format trial but using sorafenib 400 mg po BID and combination either the same 9 million IU three times a week schedule, as above, or a lower-dose schedule with 3 million IU five times a week. The latter arm appeared more active, with borderline significance of the overall response rate (17.6 % versus 34.0 %; $P = 0.058$) and little difference of the media PFS (7.9 versus 8.6 months) but a clinically significant difference of response durability, median durations of 8.2 versus 19.2 months ($P = 0.0013$). Fatigue and asthenia (added together; 28 % versus 16 %, $P = $NS) were more frequent in the 9 million units schedule [45]. Gollob and colleagues reported on a 40-patient, single-arm trial using interferon at 10 million IU, three times a week, in combination with sorafenib at 400 mg twice a day, with an 8-weeks-on/2-weeks-off overall schedule. They describe 2 CR and 11 PRs, for an overall 33 % response rate, 10-month median PFS, and 12-month median response duration, with the OS not reported [46]. This appears to be a potential improvement.

The biggest question for these interferon combinations is whether interferon should be (skeptically) assigned to most of the side effects but unknown specific incremental therapeutic impact in the bevacizumab or sorafenib combinations. While one should acknowledge that bevacizumab monotherapy can impact progression-free survival meaningfully and with less of a burden of side effects compared to the combination with interferon, at least to this point, some clinical data would still favor assigning some of the medical impact to the interferon itself.

Patient Characteristics

Some of the general patient characteristics can be compared across these generations of interferon trials. To look at a couple examples, in the Fosså et al. series [13], the median patient age was 57, there were 71 % men, and lung (61 %) and lymph node (18 %) were the main identified metastatic sites; in the Pyrhönen randomized trial versus vinblastine [16], median age was 61, there were 64 % men, and lung (70 %) and lymph nodes (42 %) were the dominant pattern of spread. In the 246 patient CALGB study of nephrectomy, the median age was about 59, 69 % were men, and about 69 % had *only* lung metastasis [18]. The DCGIN trial of interferon and interleukin-2 together had 379 patients, with median age 60, 76 % men, and lung metastases in 71 % and lymph node metastases in 34 % [29]. Comparably among the 732 patients in the report of bevacizumab plus interferon versus interferon, the median age was 61, 73 % were men, and there were lung (60 %) and lymph node (about 35 %) as most frequent site of spread [5]. Thus, the patient populations' general characteristics appear generally consistent for some major clinical characteristics.

Interleukin-2

Historical Development

The original isolation of interleukin-2 was as a soluble factor in the supernatant of cultured T cells that had the physiologic function of triggering T-cell proliferation. In further study since that point, the 15.5 kDa protein had been cloned and developed as a medical treatment that is commercially available as aldesleukin (trade name Proleukin™), with FDA indications for therapy of metastatic melanoma and of metastatic kidney cancer. While the clinical protocols for organized, safe, high-dose bolus IL-2 administration have remained relatively static, the philosophical, biological, and medical basis for selection of it as a therapeutic choice are in active evolution. These issues are discussed in detail below.

As described on the Proleukin™ package insert, there are biochemical differences of the aldesleukin product versus native interleukin-2: aldesleukin is not glycosylated like the naturally occurring eukaryotic cytokine, because it is produced in *E. coli*. Two sequence differences are that the N-terminal alanine is absent and there is serine instead of cysteine at position 125. These or other factors mean that the aggregation state is likely to differ from that of native interleukin-2; in the product, there are non-covalently bound microaggregates with an average size of 27 molecules.

In a contrast with the biological distribution of receptors for interferon-α upon many different cell types in the body, the expression of the cell surface receptors for IL-2 is limited to leukocytes. The IL-2 receptor (IL-2R) has three separate proteins, which have been characterized in detail, including 3-D models of specific amino acids' binding and crystallized structure [47]. The initial interaction is the IL-2R β chain. Then (apparently after a conformational change induced by the binding) the IL-2R γ chain associates; this three-part (IL-2, IL-2R β, IL-2 R-γ) complex is a low-affinity IL-2receptor/IL-2 complex. The IL-2R α chain (CD25) when present is a high-affinity component. The four-part (IL-2, IL-2Rα, IL-2R β, IL-2 R-γ) complex is formed with the interaction of IL-2 with the high-affinity receptor.

The γ chain is also a component of receptors for other cytokines, including IL-4, IL-7, IL-9, IL-15, and IL-21 [48]. Genetic defects of the IL-2R γ chain are associated with X-linked severe combined immunodeficiency disease (X-SCID [49]). The IL-15 binding is also to the same β and γ chains (IL-2R β, IL-2R γ) chains on lymphocytes, but a different α chain (IL-15 Rα) forms the fourth component of that high-affinity complex. The relative binding constant of IL-15 to the IL-15 Rα/IL-2 R β/IL-2Rγ complex is stronger than that of IL-2 to the IL-2R α/IL-2 R β/IL-2 R γ complex. Although there are not clinical data to compare between IL-2 and IL-15, the receptors have key similarities [50]. This is of interest, to understand how some of these other cytokines may have potential for treatment of kidney cancer; ongoing studies with IL-15 (NCT01021059) and IL-21 [51] in therapy of kidney cancer are examples.

Pharmacodynamic Effects of IL-2 on Leukocytes

While it would be straightforward to say that lymphocytes kill cancer and IL-2 induces lymphocyte activation and proliferation, that clearly would gloss over important details. That basic model falls short in the current understanding of different types of lymphocytes and their potential contributions to the interaction of the immune system with the tumor. Differential activation versus proliferative responses of lymphocytes may have important implications for both side effects of the cytokine when administered as a drug and the anticancer potential. For example, proliferation of regulatory T cells could be counterproductive. The fact that the anticancer response varies so widely among patients, with most not having a useful response, underscores the need for a better understanding, both of the response of the immune system and the mechanisms by which the cancer cells resist attack.

Three types of lymphocytes with IL-2 receptors bear particular attention, natural killer (NK) cells, cytotoxic T lymphocytes (CTL), and regulatory T cells (Treg). The NK cells bear low-affinity IL-2 receptors (without IL-2Rα) and may mediate direct antitumor activity; however, they may also mediate toxicity of the high-dose IL-2 treatment mediated by secondary cytokine release. Cytotoxic T lymphocytes, include T cells derived from tumor material (TIL, tumor-infiltrating lymphocytes). The CTL may mediate anticancer effects and particularly durability of the response when the memory phenotype is acquired.

On the other hand, regulatory T cells, characterized by CD4+, CD25+ [IL-2R α], FoxP3+ phenotype, may attenuate and decrease responses, including potential anticancer CTL reactions, and respond to IL-2 [52]. A specific anticancer-therapy blocking effect of Treg, which may increase in number during IL-2 therapy but then decrease again in responders, has not been specifically demonstrated [53]. Overall, the relative importance of these cellular sub-compartments or their interactions remains a difficult theoretical issue in understanding when IL-2 therapy could or could not work for a particular patient.

Clinical Experience of IL-2 Responses

In the report of the first cumulated response database observed among 255 patients with metastatic RCC across seven phase 2 trials [54], responses of CR were seen in 12 (5 %) and PR in additional 24 (9 %) or an overall response rate of 14 % with a 95 % CI of 10–19 %. Eleven of the 12 CR were patients designated as performance status 0. Across those seven trials, two trials had a dose schedule of 720,000 IU/kg/dose, and five were at 600,000 IU/kg/dose. The key distinctive feature of the CR population is the remarkably durability, including a later report emphasizing that the median progression-free survival had not been reached, even at 80+ months [55].

The median overall survival duration for the entire group of ever-treated patients was 16.3 months. The median age and time from diagnosis to treatment were 52

years and 8.5 months, respectively. These features are in contrast to more contemporary kidney cancer initial-therapy studies, including [3, 5, 6] and others uniformly reporting median age about 60 and median survivals over 20 months.

Other contrasts of the older IL-2 data include less stringent criteria about histological subtype. The enrichment of the VEGFR-TKI and other trials with older patients also may reflect a relative depletion of some of the histological subtypes of kidney cancer that have more aggressive clinical courses and for which there is a relative concentration of younger patients. With these and other well-recognized issues of bias that could be a consequence of comparing trials conducted with differing entrance criteria, at different times and on different continents, it is clear that for the physician and patient faced with a choice among kidney cancer treatments, a simple comparison of the "official" prescribing information data gives little guidance on whether to choose initial therapy with interleukin-2, a combination of interferon and bevacizumab, or on oral VEGF-receptor tyrosine-kinase inhibitor.

There is a relatively stable experience with interleukin-2 across many years, in the experiences several multi-institutional series of kidney cancer patients treated in single-arm or randomized series, as well as on the Proleukin™ prescribing information package insert. It is instructive to review these, from an efficacy standpoint and then from a side-effect perspective. This serves a few developmental and application goals: One is in setting reasonable expectations for patients and physicians to consider during the patient selection process and another is as a reference point for development of new strategies incorporating IL-2.

The overall pattern for high-dose bolus is with a 15-min infusion of the IL-2, at a weight-specified dose of 600,000 IU/kg/dose, at 8-h intervals, for a series of up to 14 doses. Higher doses, up to 720,000 IU/kg/dose, are considered still on label, in recognition of what was later recognized as systematic dosing difference that occurred in patients treated at the NCI. There has been formal testing of many regimens with lower per dose and lower total-dose-target and slower-dose administration (including subcutaneous administration and continuous infusion), and combination treatments with interferon are discussed below.

A survey of lower-dose IL-2 regimens includes both single-arm trials and the nominally inferior arms of the randomized experiences described above. A unifying concept of lower-dose IL-2 development is that at a lower dose, some of the side effects might be attenuated as far as severity and frequency, while the population for whom the treatment could be considered would be a wider piece of the incident population, and the immune effects would be similar or even enhanced by the longer durations of treatment. For those lower cumulative dose-target regimens that still are in the "high-dose IL-2" category, the ligation of the low-affinity (IL-2R β/IL2 R γ) dimer receptor is still considered as a key part of the mechanism of action.

On the other hand, with the lower-dose regimens, where the IL-2 concentration does not reach high levels, such as intravenous 72,000 IU/kg/dose, or 250,000 IU/kg/dose SQ followed by 125,000 IU/kg/dose, the high-affinity receptor (Il-2R α/IL2 R β, IL-2 R γ) is ligated at about the same level as when the IL-2 dose is at the high level. Since the extent of the pharmacodynamic impact of different dose levels of IL2 on different leukocytes (such as NK cells, T-helper cells, or regulatory T cells)

is not actually well defined, the mechanistic connection mapping of a dose regimen to the mechanism of clinical response is not one that has been discerned in these studies.

Randomized Comparisons, in Advanced Disease

High-dose bolus IL-2 has been compared with lower-dose cytokine treatment in two larger-format, multicenter randomized studies for therapy of advanced kidney cancer [25, 56] and in a study to address potential relevance of tumor staining for carbonic anhydrase IX as a marker for a better chance of anticancer response [57]. Another randomized study addressed use of high-dose bolus interleukin-2, given for one course, as an adjuvant therapy immediately following curative-intent nephrectomy or metastasectomy [58]. Another randomized study looked at a continuous infusion schedule, compared to subcutaneous interferon combination, or both together (discussed above) [32].

The first study accrued patients over a decade, from 1991 to 2001; from 1991 to 1993 there was a 1:1 randomization between 720,000 and 72,000 IU/kg/dose, with either on an every-8-h intravenous schedule. Starting in 1993, the regimen described by Sleijfer [59] was included, with 1:1:1 randomization between that and the two previous arms. Only patients with clear cell type cancer were included. The 2003 report describes the results for the comparison of the two intravenous regimens ($N=156$ and 149 evaluable) or for the three-arm comparison ($N=96$, 93, or 94, respectively) [56].

In the two-arm comparison of 720,000 IU/kg/dose versus 72,000 IU/kg/dose, there was a higher response rate, with 11 CR and 22 PR, giving 21 % overall response rate, compared to 6 CR and 13 PR, an overall response rate of 13 %. This favored the high-dose arm, with borderline statistical significance, reported as $P=0.048$ by chi-squared test but $P=0.067$ by Fisher's exact test. In the three-arm comparison, the responses again favored the high-dose arm with 6 CR and 14 PR but for the lower-dose intravenous arm 1 CR and 9 PR and for the low-dose subcutaneous arm 2 CR and 7 PR. Statistical analysis of the overall response frequency (21 % versus 11 % versus 10 %) again favors the high-dose arm, with $P=0.033$ by χ^2 test and $P=0.043$ by Fisher's exact test [56].

In the second randomized study using high-dose bolus IL-2, the IL-2 dose was at the lower dose on the label (600,000 IU/kg/dose), on days 1 through 5 and days 15 through 19, with a maximum of 28 doses, separated into cycles at 12 week intervals. The low-dose comparator was combination schedule using both IL-2 (5 million IU/m^2 subcutaneously every 8 h for three doses on day 1, then daily 5 days/week for 4 weeks) and IFN (5 million IU/m^2 subcutaneously three times per week for 4 weeks) and then 2 weeks off, with the cycle repeating every 6 weeks. (To compare the doses, the on-label dose of 600,000 IU/kg in a 70 kg, 1.7 m^2 human would work out to 42 million IU versus on high dose but 8.5 million on the 5 million IU/m^2 dosing.) The lower-dose IL-2 schedule had been developed by Sleijfer and colleagues [59].

For the efficacy comparison, there were 8 CR and 14 PR among 95 high-dose arm patients, but only 3 CR and 6 PR among the 91 in the combination arm. The statistical analysis of the 23.2 % versus 9.9 % overall response rates favors the high-dose arm, $P=0.018$, by Fisher's exact test [25].

In summary, the initial approval and schedule for IL-2, still in use today, were based on 255 patients from nonrandomized trials. The results of the prospective, randomized, multicenter comparisons to low-dose cytokine treatment favor the continued use of the high-dose bolus schedule, in appropriately selected patients; this is consistent with the package insert Proleukin™ prescribing information, which identifies lower response rates in two nonrandomized, smaller kidney cancer treatment reports.

Selection of Patients for IL-2 Treatment

In a third but more contemporary nonrandomized, single-arm, multicenter phase 2 study of high-dose bolus IL-2 treatment of metastatic RCC accruing patients from November 2007 to July 2009, the SELECT trial addressed the issue of utility of staining for carbonic anhydrase IX, a gene product which is upregulated during VHL dysfunction [60], as a predictor for IL-2 response. The results of the trial were presented at the June 2010 ASCO meeting, in which there were seven investigator-assessed CR and 28 PR (29 % overall response rate). The study hypothesis that response would be more frequent in the subset of patients with high CA-IX staining was not supported, with a nonsignificant trend favoring the response frequency in those who did *not* have that high CA-IX staining feature [57].

The database of these experiences reinforce about the same general impression about IL-2 responses—the patients with major responses are a minority; the overall response rate in these series ranges from about 15 to 29 %, with the latter experiences being higher. The durability of the response, among those responding patients, is generally excellent. Some isolated experiences, such as Shablak and colleagues in the UK [61], describe higher response rates, contingent on particular and heuristic patient selection patterns. The favorable features include those defining good-risk (performance status, nephrectomy status, hemoglobin above lower limit, and LDH and calcium not elevated) [14] and histological features such as clear cell type, alveolar pattern, low proportion of granular cells, and absent papillary pattern. In this way, 28 of 57 patients meeting the criteria (49 %) had major response, including 25 % with CR.

The central conflicts of low-frequency but durable responses and high up-front but low long-term toxicity continue to present a challenge for incorporation of IL-2 into the setting of other treatment choices for metastatic kidney cancer. Clinical factors dominate the patient selection strategy: good performance status, absence of prohibitive cardiovascular or respiratory comorbidities, clear cell subtype, and nominal "good-risk" features, such as absence of anemia, hypercalcemia, and high LDH. Longer time from diagnosis to evident metastasis suggests a more indolent cancer growth pattern. Of note, most patients who have been treated had debulking resection of the

cancer first. The subset is different than the subset who would be treated with VEGFR-TKI or mTOR inhibitors, particularly with reference to the high-risk features that defined the study population of the temsirolimus pivotal trial [4].

Retrospective data in a series of 23 with progression through prior VEGFR-directed treatment found that for patients treated with inpatient high-dose IL-2, there appeared to be low response rate and excess toxicity. Resistance to therapy was consistent with the observation only 1 of the 23 even continuing to get a second cycle of IL-2 and no major responses. Forty percent had cardiac toxicity (95 % CI: 16.3–67.7 %) [62]. While not prospective or mechanistic in nature, the report of this experience can be considered as a basis for not using a criterion such as immediately preceding progression through a VEGFR-type therapy as a central consideration in a decision for recommending or not recommending IL-2 treatment. Whether the underlying reason for such a conclusion is that there are left over, albeit potentially subtle, cardiovascular alterations to the body from VEGF inhibition that increase the risks from IL-2 treatment or whether the biological features of the progressing cancers themselves or of the host leukocytes are not compatible with IL-2 response is moot, from the perspective of clinical decision making. This criterion can be summarized as "IL-2 is either first-or-never." In a later, also retrospective, series of 40 patients getting high dose IL-2 therapy of kidney cancer following prior VEGF treatment, 2 CR, 3 PR and 13 SD were observed, with a much for feasible toxicity experience [63].

Retrospective analysis of dendritic cell (DC) phenotype in a series of 16 patients receiving therapy for kidney cancer or melanoma found that 5 responding patients had the highest (more normal) ratio of mature DC to myeloid-derived suppressor cells (MDSC). This single-institution series used an IL-2 schedule with four courses of 5 planned doses, at weekly intervals, instead of two courses of 14 planned doses [64].

Overall, both inclusive features, such as patient general features, leukocyte features, time to recurrence, and histology and exclusionary features, such as comorbidities or recent progression through VEGF-axis treatments, represent a patient selection process for IL-2, albeit one for which grey areas remain.

Toxicity of IL-2 Treatment

Treatment with interleukin-2 is appropriately associated with a distinct safety profile, or one could say, a distinct toxicity experience. On a theoretical basis, the underlying basis is the storm of cytokines synthesized and released by leukocytes with ligated IL-2 receptors and further secondary events, particularly fever, chills, and capillary leak syndrome (CLS) and interstitial fluid leak and hypotension. While these are predictable in the general sense, from patient to patient there may be a wide variation of timing and intensity. Anticipation and mitigation of these events is key. The potential for an IL-2-mediated toxicity of extreme or life-threatening intensity warrants the careful patient selection criteria and the organized and anticipated approach to mitigate those toxicities. As annotated in the boxed warning, capillary leak syndrome encompasses potential for toxicity in many organ

systems. The “loss of vascular tone and extravasation of plasma proteins and fluid into the extravascular space” can result “in hypotension and reduced organ perfusion which may be severe and can result in death.” Particular events can include “cardiac arrhythmias (supraventricular and ventricular), angina, myocardial infarction, respiratory insufficiency requiring intubation, gastrointestinal bleeding or infarction, renal insufficiency, edema, and mental status changes.”

For a meaningful anticancer response and to avoid an untenable risk element of the therapeutic risk to benefit ratio, both tumor features and patient features must be considered. Considering the patient features, with respect to safety, these are outlined specifically in the manufacturer’s prescribing information. The practical application of pretreatment testing and thresholds of organ function tests including cardiac, pulmonary, hepatic, renal, and central nervous system function, for example, remain for individualized clinical judgment.

Patient attributes for relatively safe drug administration are outlined in the package insert information (Proleukin™ prescribing information, www.proleukin.com) and echoed across many clinical trials’ formalized inclusion criteria. Cardiac features that are contraindications (referring to the prescribing information) do include abnormal stress thallium test, prior IL-2-induced sustained ventricular tachycardia, other uncontrolled arrhythmias, chest pains with associated EKG changes that would be consistent with angina or myocardial infarction, and cardiac tamponade. Requirements for lung function and reserve could include no prior intubation for over 72 h and normal pulmonary function testing. Of note, in some cases some organ function testing may have been already addressed during evaluations for recent surgical procedures.

Infection risk issues are certainly different for cytokines than for conventional cytotoxic drugs that cause predictable severe neutropenia. However, IL-2 treatment may induce impaired neutrophil function, and the package insert identifies increased risk of “disseminated infection, including sepsis and bacterial endocarditis,” and attention to risks from preexisting bacterial infections or indwelling central lines is prone to “infection with gram-positive microorganisms.” The package insert describes that incidences of staphylococcal infections can be attenuated through antibiotic prophylaxis.

Other more straightforward toxicity management included in the prescribing information includes features of the CNS, such as the boxed warning to withhold further infusions in case of “in patients developing moderate to severe lethargy or somnolence; continued administration may result in coma.” The brain should be evaluated and CNS disease controlled before treatment. This in addition to recognition of the potential for the occurrence of new neurologic signs or events, encompassing anatomically identifiable new lesions, “changes in mental status, [or] speech difficulties, cortical blindness, limb or gait ataxia, hallucinations, agitation, obtundation, and coma” including in patients without overt metastasis. A useful review of strategies for safe administration of IL-2 was written by Schwartzentruber [65], but the key issue of using a center with a volume of patients and specific physician, pharmacist, and nursing experience remains invaluable.

Evolutions of Cytokine Therapy

Drugs that are modifications of conventional cytokines have been developed, but with none advancing to pivotal trial testing in kidney cancer patients. Both have been modified in different ways. The most trials were done with pegylated interferon, which was also concurrently tested in therapy of metastatic melanoma and in therapy of hepatitis C. Contemporary usage for these indications has developed, in contrast to their use in kidney cancer treatment, and the safety and tolerability experiences appear better, for those patients, than with conventional interferon-α.

Two pegylated (polyethylene glycol modified) interferon drugs were tested in kidney cancer-directed trials, PEG-Intron™ (40 kDa pegylated interferon alfa2a) and Pegasys™ (pegylated interferon alfa2b). The pegylation increases the molecular weight and markedly increases the half-life for clearance and the area under the curve for the overall exposure. Among 27 patients in five dose levels in the phase 1 study of Pegasys™, at Memorial Sloan Kettering Cancer Center, 5 had PR (19 %), and the dose of 450 mg, three times a week, was the one recommended for potential further treatment [66]. Another 32 evaluable patients were treated in a phase II trial, with a more promising response experience, one CR and 9 PR (31 %); another 15 had SD. Median OS was at 31 months, but the median PFS was 5 months [67], not very different from many conventional interferon monotherapy experience.

In a phase I/II study of Peg-Intron™, enrolling patients with metastatic melanoma and with kidney cancer at Cleveland Clinic, 44 kidney cancer patients were treated. The response rate among the kidney cancer patients was 14 % [68]. A phase II study with 22 patients at the Netherlands Cancer Institute described 3 patients with PRs, one of whom later had a CR and another 10 patients with SD. Similarly, as with the pegylated interferon alfa 2, there was not a clear advantage over the unmodified cytokine in these four studies [69].

In combination treatments, a schedule of Pegasys™ and subcutaneous IL-2, comparable to combination of outpatient interferon and low-dose interleukin-2, was tested at Cleveland Clinic; 34 patients were treated, at 6 dose levels. Five PR (15 %) were observed; the median progression-free survival (9 months) and overall survival (31.9 months) were encouraging [70]. Further clinical testing with the Cytokine Working Group in a phase I/II included 54 patients, 33 of whom were in the phase II portion. Clark and colleagues reported that the phase I portion was expanded for concerns about cardiac and neurologic toxicity, and then the phase 2 portion was stopped at 33 patients because of 11 observed serious events. The overall frequency of PRs was 30.2 %, somewhat higher than the monotherapy trials mentioned above, but with a different, worse, perspective about relative safety [71].

While these practical early-phase trial experiences with the modified interferon were acceptable in terms of response frequency, and generally for toxicity, they did not reach a tipping point of pivotal trial development for kidney cancer and commercial development directed at use of this pegylated cytokine for kidney cancer. Biological activity of other modified cytokines, which ligate the same receptors in different ways, has been of interest through the present. Several others of these

cytokines with modifications or additions have been brought to clinical testing. Among these are IL-2 modifications which include changes of isolated amino acids or conjugation with targeting proteins.

The issue of modification of IL-2 so that the relative extent of impact on NK cells versus on T cells will be different was a motivation in design of BAY 50-4798, "an IL-2 analogue featuring a single amino acid substitution (arginine for asparagine at position 88) that alters its binding to the high-affinity IL-2 receptor on T cells and the lower-affinity receptor on NK cells [which] results in a preferential activation of T cells over NK cells" [72]. This derives from the rationale that the former mediate most of the aldesleukin antitumor activity but that NK-derived inflammatory cytokines mediate toxicities [72]. This durability of anticancer response may be particularly an outcome of specific T-cell activation. An alternative view is that NK cells mediate a significant part of the antitumor response and are not therapeutically dispensable. The clinical testing of BAY 50-4798 is reported for a trial of 45 patients, 33 of whom had metastatic RCC. For the 20 RCC patients in the dose expansion cohort, 1 had confirmed PR of 4 months, and 13 had SD for at least 2 months; one of those was durable over 5 years. Side effects, overall, had a general pattern similar to that for conventional high-dose IL-2 administration.

Selectkine (EMD 521873 or NHS-IL2LT) "is a fusion protein consisting of modified human IL-2 which binds specifically to the high-affinity IL-2 receptor, and an antibody specific for both single- and double-stranded DNA, designed to facilitate the enrichment of IL-2 in tumor tissue" [72]. In a phase 1 trial with 39 patients, advanced, refractory cancers (histological sites of origin not reported), no major tumor responses were observed. They report on a first-in-man pharmacodynamics study addressing immune response in 39 patients treated with increasing doses. These showed Treg cells (CD4+ CD25+ FoxP3+) increased over tenfold. The authors comment that an increase of inhibitory function may not occur because of IL-10 increases. The CD4+ and CD8+ circulating T-cell numbers also were increased, including an observation of more CD8+ T-cell activation. Neutrophils, monocytes, and NK cells had little change. No major clinical responses were observed. Some PFS curves are presented, but without clinical details of the subjects [70].

A few IL-2/antibody fusion drugs have been developed in early-phase trials. GD2 is a disialoganglioside present in melanoma. The product EMD 273063 (hu14.18-IL2) is a humanized anti-GD2 monoclonal antibody fused to interleukin-2 (IL2) and was reported in a trial with 33 melanoma patients. Across all dose levels, eight (24 %) patients had SD, with no major regressions observed, in this phase 1 study that was not designed to estimate a clinical response rate [74]. The pharmacodynamic assessments showed an increase in lymphocyte count, an increase in the percentage of CD16+ and CD56+ PBMCs, an increase in NK lysis, and an increase in ADCC [74]. A further report on pharmacodynamics in nine additional subjects with metastatic melanoma concluded that there was not NK activation apparent but that there were "intratumoral changes in some patients consistent with increased intratumoral infiltration by CD8+ T cells" [75].

The KSA protein is a 40 kDa epithelial membrane antigen, expressed in the distal nephron of normal renal tissue and at higher frequency in collecting duct type

and chromophobe-type kidney cancer. It was expressed in >50 % of cells in only 8 % of 318 clear cell kidney cancer specimens [76]. The fusion protein HuKS-IL-2 has component that is an antibody to KSA and two IL-2 molecules. A trial in prostate cancer patients defined a tolerable dose [77]. The drug was not developed further; no kidney cancer trials were conducted.

Fusion of IL-2 with a T-cell receptor protein, with specificity for the 264–272 peptide of p53 when presented in the context of HLA-A*0201, is the investigational drug ALT-801 [78]. A more recent poster presentation at the February 2013 ASCO GU meeting addressed the proposed mechanism of action. It described that the presence of HLA-A*0201 may be not required for immune activation and that intratumoral macrophages may be repolarized, and T cells bearing NGK2T antigen are increased by the drug [79]. A mixed diagnosis phase 1 trial of single-agent treatment included nine patients with tumors of kidney origin, including one with collecting duct cancer. Some patients had apparent disease stabilization, and one melanoma patient had a durable CR (demonstrated after resection of residual nodule showing no viable tumor) [78]. Current development is in melanoma, urothelial cancer, and myeloma but does not include kidney cancer.

Conclusions

The cytokines, as a clinical application in kidney cancer, are the two drugs with clinical experiences decades longer than those of the VEGF-axis and mTOR drugs. For whom are they a good choice? For whom are they not? That mature experience defines limitations as well as strengths. As is increasingly recognized, the biological, anatomic, and other heterogeneities of metastatic kidney cancer are significant. Some patients will not need medical therapy, having no immediate potential for net benefit. For others it is a key step to get control of the cancer, if only for a time. If the question is a straightforward one of clinical practice, there will be some patients for whom cytokines are good fit. They will be a minority, but an important one. Bevacizumab combination with interferon-α appears to offer a progression-free survival increment over single-agent interferon-α. Side effects are prohibitive for some patients, and for others use of an oral medication has a huge appeal over anything infused or injected.

For the well-selected patient, and that selection process may indeed be poised to become better defined, interleukin-2 can be a key choice. The implications of a durable CR can include years of cancer-free, treatment-free life with a relatively short-term commitment to an intense treatment plan. For some patients, the disease features, such as histology or clinical risk factors, can define that the chance of IL-2 response is very low. For them, the non-IL-2 treatments can give a treatment approach that, at the least, offers less toxicity. For others, among those with favorable disease features, the psychological focus on a curative-intent approach is an important part of confronting the illness. Obviously, those who do get durable CRs benefit enormously.

Those who do not (within the group of patients with the histological, clinical, and comorbid features that define a good IL-2 candidate) may still be encouraged that the treatment plan could prioritize that issue ahead of other treatments, that is to say targeted drug therapies, where the impact on the disease is of a different character.

Although the chapter here focuses on interferon-α and interleukin-2, the field of immunotherapy of cancer and of kidney cancer in particular is growing in a few directions. Some examples of late-phase trials include anti-PD1 antibody nivolumab (BMS-936558, pivotal trial NCT01668784); Immatic's product IMA-901 is a nine-peptide cocktail administered with cyclophosphamide and a cytokine not discussed here, GM-CSF (NCT01265901); the ADAPT study uses Argos' product, which is an autologous tumor-derived mRNA to pulse dendritic cells, which are used as a vaccine (NCT01582672). Whether interferon-α, IL-2, or other cytokines, used as adjuncts to these or other immune manipulations can further impact on kidney cancer remains for future study. However, as long as the immune system is involved, the natural question about changing the host leukocytes or amplifying a marginal response into a more effective one will remain. Although at present the one or two drug approaches with cytokines are the ones validated for clinical practice, as immune therapy develops and cytotoxic T lymphocytes, natural killer cells, dendritic cells, macrophages, and other cell types are activated in more defined ways, it seems certain that cytokines will remain a part of the picture, perhaps in doses and schedules radically different from those described in many clinical trials from the past.

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Chapter 21
Immune Checkpoint Inhibitors (Anti-CTLA4, Anti-PD-1) in RCC

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Overview of Checkpoint Inhibition

The normal immune response requires two signals for T-cell activation and proliferation. The first signal consists of the major histability complex (MHC)/antigen-presenting cell (APC) interaction with the T-cell. A second costimulatory signal is then required for further immune system activation, and several costimulatory molecules can provide this signal. A critical costimulatory family is the CD28 receptor on the T-cell which binds to B7 ligand subtypes CD80 and CD86 on the APC. Checkpoint inhibitors such as CTLA4 and PD-1 share these ligand-binding sites to inhibit costimulation but use different pathways to achieve this goal. CTLA-4 competes with CD28 and binds to its ligands with higher affinity, thus downregulating or blunting T-cell proliferation and cytokine production. The PD-1 receptor on the T-cell is upregulated in response to inflammation and is thus another checkpoint which shuts off the immune system response to prevent overstimulation [1–3] (see Fig. 21.1). Both the PD-1 molecule and its ligand (PDL-1) have been studied as potential targets for checkpoint inhibition.

Research in the field of immunology has led to a greater understanding of mechanisms the human body employs to recognize self from foreign and how it often fails to contain malignancies. Immunoncology is a newly evolving field of medicine, focused on therapies that improve the body's potential to generate an immune response against cancer. The human immune system is adaptable and has developed

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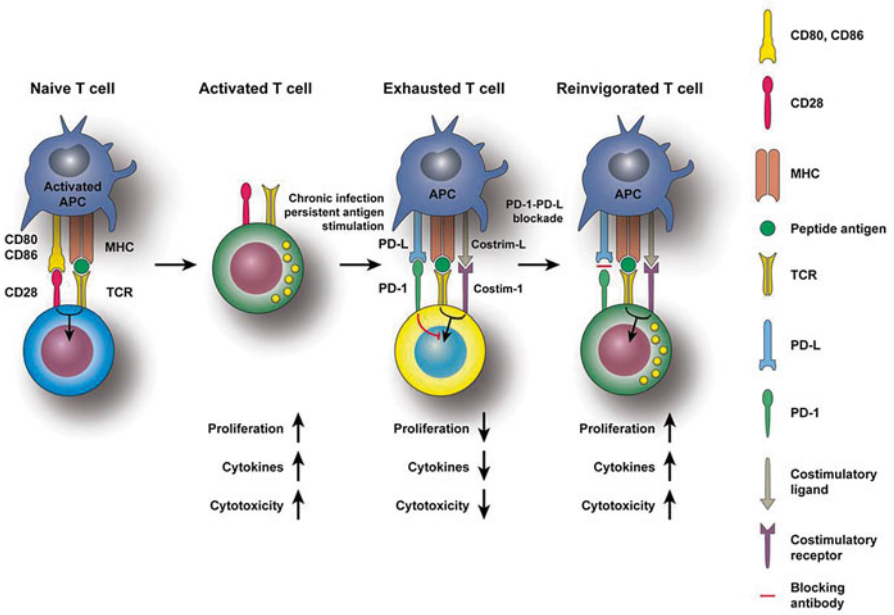

Fig. 21.1 PD-1 in T cell activation, exhaustion, and reinvigoration. T cells are activated via (1) binding of MHC plus peptide on an APC to the TCR and then (2) binding of CD80/86 on an APC to CD28 on the T cell. In patients with cancer, tumor cells can also serve as APCs. Upon T-cell activation, expression of PD-1 is induced. In situations of chronic infection or persistent stimulation, PDL-1 signals through T-cell PD-1 to "turn off" T cells in order to minimize damage to healthy tissue. Tumor cells can upregulate PDL-1 in order to "turn off" T cells that would potentially destroy them. Blocking the PD-1/PDL-1 signaling pathway allows T cells to become "reinvigorated" and recover their effector functions. In patients with cancer, reinvigorated tumor-specific T cells can kill tumor cells and secrete cytokines that activate/recruit other immune cells to participate in the anti-tumor response/fight the tumor. Reproduced with permission from David McDermott, MD

multiple checkpoints to prevent inappropriate activation. These checkpoints are in place to prevent initiation of inappropriately directed responses and to limit the size, duration, or premature focusing of the immune response once it is activated. While largely in place to control the immune system in response to infection rather than malignancy, alteration of these checkpoints can be employed to combat malignancy.

Tumors can overwhelm the immune system by multiple strategies such as altering antigen expression and interfering with T-cell activation. Immune editing is a technique in which tumors create and manipulate a microenvironment to escape detection and eradication by the immune system [4, 5]. Thus targeting the immune system with monoclonal antibody checkpoint inhibition has the potential to make a substantial impact on tumor growth and proliferation. These treatments shift the microenvironment from pro-tumor to antitumor to facilitate the death of the malignant cells [6, 7]. Investigation in these pathways have led to promising new developments in agents that block the regulation of T-cells by inhibiting CTLA-4, PD-1, or PDL-1.

CTLA-4 Description

One of the most critical immune checkpoints is the CTLA-4 pathway, which has been employed to target cancer cells in several types of malignancies, including RCC and metastatic melanoma. CTLA-4 is a close homologue of CD28 and binds both the B7.1 and B7.2 ligand pair to function as a negative regulator of immune responses. CTLA-4 has significantly higher affinities for both B7 ligands than does CD28. The interaction of CTLA-4 with B7.1 is stronger than that with B7.2, whereas CD28 is predicted to bind to B7.2 more effectively than B7.1 [8–10]. The inhibition of CTLA-4 has been shown to suppress autoreactive T-cell immunity, preventing unwanted autoimmunity and creating a tolerance to self-antigens. Early studies have shown that CTLA-4 knockout mice develop a rapidly lethal polyclonal CD4-dependent lymphoproliferative disorder within 3–4 weeks of birth [11–13]. The clinical benefit of targeting CTLA-4 is becoming recognized in several fields including transplant medicine, rheumatology, and oncology. Two CTLA-4 inhibitors (ipilimumab and tremelimumab) have been studied in several cancer types.

CTLA-4 Inhibition: Clinical Activity

The CTLA-4 inhibitory agents ipilimumab (YERVOY) and tremelimumab have been studied the most in metastatic melanoma and renal cell carcinoma. Ipilimumab is a fully human monoclonal antibody which inhibits CTLA-4. This drug was FDA approved for melanoma in March 2010, after a phase III study showed an improvement overall from 6.4 months to 10.0 months when compared to the vaccine GP100 [14, 15]. Its use in renal cell carcinoma is still being explored. Ipilimumab was tested in a phase II single-center trial of 61 patients with metastatic renal cell carcinoma. Twenty-one patients in cohort A were treated with a loading dose of 3 mg/kg followed by subsequent doses at 1 mg/kg. Forty patients in cohort 2 were given 3 mg/kg for all doses. All patients in cohort A had received prior IL-2 treatment, and 26/40 in cohort B had received prior IL-2. Response rates were 5 % in cohort A and 12.5 % in cohort B (5/40 patients). Three patients with longer duration responses (12, 17, and 21 months) had not received IL2 therapy previously. Forty percent of patients (17/40) in cohort B developed significant immune-related toxicities, including enteritis and hypophysitis. Three patients suffered colonic perforation, and two patients died as a consequence of the perforation. In this trial, there was a significant association between toxicity and tumor regression. The response rate was 30 % among the patients with significant autoimmune toxicity, but was 0 % in the patients free of toxicity. No complete or durable regression of renal cancer was documented in this study. Treatment of autoimmune toxicities with high-dose corticosteroids did not seem to affect antitumor response [16]. While results of this study are encouraging, further investigation of CTLA-4 blockade in RCC will need to be done to assess efficacy. Ipilimumab is also being studied in non-small cell lung cancer, small cell lung cancer, and advanced prostate cancer.

Tremelimumab is a second CTLA-4 antibody which has been studied in renal cell carcinoma. In a phase I trial of tremelimumab combined with sunitinib, 25 patients with metastatic renal cell carcinoma were enrolled. Dose-limiting toxicity was reached at the 6 mg/kg dose of tremelimumab with sudden onset of acute renal failure which was unexpected. The maximum tolerated dose was determined to be 6 mg/kg of tremelimumab combined with 37.5 mg of sunitinib. Of the 21 patients evaluated at this dose, 43 % (9/21 patients) achieved a partial response, and four of these responses were ongoing when the study was published [17]. Tremelimumab has also been studied as a single agent in melanoma with a durable response of 35.8 months vs. 13.7 months in the standard chemotherapy arm. While durable response was prolonged, the study failed to demonstrate a statistically significant survival advantage of tremelimumab over standard of care chemotherapy [18]. Tremelimumab is currently being studied in mesothelioma, prostate cancer, and hepatocellular carcinoma. Based on these data, use of CTLA-4-blocking antibodies in RCC may be worth further study.

Combination therapy with IL-2 and ipilimumab has been studied in animal models as well as clinical trials in humans. Because Il-2 has been shown to stimulate regulatory T-cells which express CTLA-4, it has been theorized that combination therapy could potentiate the antitumor effects of both agents and provide a synergistic effect. A phase I/II clinical trial tested this hypothesis in 36 patients. Objective response rates were 22 % (8/36) as compared to historical 15 % response rate with IL2 alone. Six of the eight responders had continued tumor regression at 11–19 months, demonstrating a durable clinical benefit. Grade III/IV autoimmune effects occurred in 14 % of patients, as compared to 12 % of patients treated with ipilimumab alone. Correlative serum studies demonstrated increased levels of activated T-cells (CD4+ HLA-DR and CD45RO) in patients after treatment [19]. While the response rate in this small series did not suggest synergy between these two agents, this study showed that they could be administered together safely, and durable benefit was achieved in a subset of patients. Larger, randomized studies will be necessary to confirm the clinical benefit of these two immune agents. Although CTLA-4 inhibition has been pursued, newer agents that block PD-1 and PDL-1 have shown more promise in efficacy against RCC. Several of these agents are currently in clinical development.

PD1/PDL1 Description

In addition to CTLA-4, PD-1 inhibition with the use of PD-1-/PDL-1-blocking antibodies has been recognized as a second and perhaps even more potent target of checkpoint inhibition. PD-1 is expressed on activated, but not resting, CD4+ and CD8+ T-cells, B-cells, and monocytes. Its role in the periphery is to decrease T-cell response to inflammation and limit autoimmunity. PD-1 has two known ligands, PDL-1 (B7-H1) and PDL-2 (B7-DC), which are upregulated in response to inflammation. Proinflammatory cytokines such as interferon gamma upregulate PDL-1 on

many types of cells. The inflammatory cytokine IL4 induces upregulation of PDL-2 on dendritic cells and macrophages. In addition to expression on normal antigen-presenting cells, PDL-1 is upregulated on many tumor types, including melanoma and renal cell carcinoma. The engagement of PD-1 with PDL-1 inhibits TCR-mediated proliferation and cytokine production [20–28].

PDL-1 expression on kidney cancer tumor cells is thought to be a poor prognostic marker as shown in an analysis of RCC tissue from 196 patients at the Mayo Clinic. After adjustment for TNM stage, tumor size, nuclear grade, and tumor necrosis, patients with ≥10 % PDL-1 expression were three times more likely to die from RCC with a relative risk of 2.91 (95 % CI 1.39–6.13; $P=0.005$). The combination of tumor and tumor-infiltrating lymphocyte PDL-1 expression was an even stronger predictor of poor outcome [21, 22]. Investigators from the Mayo Clinic have also developed a model called a BioScore which combines several clinicopathologic factors to determine prognosis. Immunohistochemical staining of tumor B7-H1/PDL-1, survivin, and Ki-67 expression was performed on paraffin-embedded tissue of 634 patients, and a multivariate analysis was used to create a BioScore. High levels of these markers, when combined and integrated into the BioScore scoring system, correlated with increased RCC-related mortality. Patients with high BioScores were five times more likely to die from RCC compared to patients with low BioScore (hazard ratio, 5.03; 95 % confidence interval, 3.82–6.61; $P<0.001$) [23]. In addition to RCC, PDL-1 expression has been shown in other cancers such as breast, pancreas, stomach, some melanoma, bladder, lung, liver, and ovary to correlate with poor prognosis [24–28]. Because of the implications of these studies and potential impact of disease aggressiveness, PDL-1 has pursued as a target in RCC.

Tumors themselves are known to contain tumor antigen-specific T-cells, called tumor-infiltrating T lymphocytes (TIL) which have been shown to express PD-1, while T-cells in normal tissue and peripheral blood T lymphocytes (PBL) do not. Studies of PD-1 expression on TIL in metastatic melanoma have shown that PD-1-positive TIL exhibit impaired function when compared to PD-1-negative TIL and PBL in the same patient. A small fraction of PD-1-positive T-cells produced cytokines such as IFN-γ at markedly reduced levels, which was used as a measure of T-cell function. Blockade of the PD-1/PDL-1 pathway increased IFN-γ secretion in response to dendritic cell stimulation. Thus, inhibition of this pathway may restore T-cell function and shift the microenvironment to target tumor cells, justifying this target for immune therapy [25].

PD1/PDL1 Clinical Activity

There are currently several PD1/PDL1 inhibitors being investigated (see Table 21.1), and clinical trials in humans are showing promising early results (see Table 21.2). Nivolumab (BMS 936558, MDX-1106) is a fully human IgG4 monoclonal antibody directed against PD-1. It has a high affinity for PD-1 and blocks binding of both PDL-1 and PDL-2. A recent phase I/II trial of 296 patients demonstrated clinical

Table 21.1 PD-1/PDL-1 agents in development

Name	Generic name	Sponsoring company	Description	Target	Phase
BMS-936558	Nivolumab	Bristol-Myers Squibb	Fully human IgG4 monoclonal antibody	PD-1	III
CT-011	–	CureTech	Humanized IgG1 monoclonal antibody	PD-1	II
MK-3475	–	Merck	Humanized IgG4 monoclonal antibody	PD-1	I
BMS-936559	–	Bristol-Myers Squibb	Fully human IgG4 monoclonal antibody	PDL-1	I
RG7446, MPDL3280A	–	Roche, Genentech	Monoclonal Antibody	PDL-1	I
AMP-224	–	GlaxoSmithKline and Amplimmune	B7-DC/IgG1 fusion protein	PDL-2	I

benefit with the use of this agent. The trial was composed of 104 patients with melanoma, 122 with NSCLC, 34 with RCC, 17 with castrate-resistant prostate cancer, and 19 with colorectal cancer. This was a dose-escalation study of doses from 0.3, 1, 3, and 10 mg/kg given every 2 weeks of an 8-week cycle. The maximum tolerated dose was not reached. The most common treatment-related adverse effects were fatigue, rash, diarrhea, pruritus, decreased appetite, and nausea. 14 % of patients had drug-related grade 3 or 4 AEs, and there was a serious adverse event rate of 11 %. Autoimmune-related adverse events included pneumonitis, vitiligo, colitis, hepatitis, hypophysitis, and thyroiditis. The rate of pneumonitis was 3 % (9/296 patients) with three treatment-related deaths due to pulmonary toxicity. In the RCC cohort, 10/34 patients had major tumor responses (complete response or partial response), and 9 other patients had stable disease for longer than 24 weeks. In addition, two patients had a persistent reduction in target lesion tumor burden in the presence of new lesions and were not categorized as responders. Among those treated at the 10 mg/kg dose level, 70 % were progression-free at 6 months, and four patients have yet to progress despite stopping treatment at 96 weeks [26, 27]. Based on the promising results of this phase I trial, a phase III trial is currently underway randomizing patients with advanced or metastatic RCC who have previously been treated with antiangiogenic therapy to receive nivolumab vs. everolimus (NCT01668784).

One challenge in the evolving field of immunotherapy is to identify predictive biomarkers to confer responses to treatment and help select patients for appropriate therapies. The phase I study of nivolumab described previously evaluated PDL-1 expression in 61 pretreatment tumor specimens from 42 patients (18 with melanoma, 10 with non-small cell lung cancer, 7 with colorectal cancer, 5 with RCC, and 2 with prostate cancer). Twenty-five of the 42 patients had biopsies positive for PDL-1 expression by immunohistochemical staining. 9/25 (36 %) of these patients

Table 21.2 Ongoing clinical trials

Trial number	Phase	Agent(s)	Status	Title
NCT01668784	III	Nivolumab	Recruiting	A Randomized, Open-Label, Phase 3 Study of BMS-936558 vs. Everolimus in Subjects With Advanced or Metastatic Clear-Cell Renal Cell Carcinoma Who Have Received Prior Anti-Angiogenic Therapy
NCT01358721	I	Nivolumab	Recruiting	An Exploratory Study to Investigate the Immunomodulatory Activity of Various Dose Levels of Anti Programmed-Death-1 (PD-1) Antibody (BMS-936558) in Subjects With Metastatic Clear Cell Renal Cell Carcinoma (RCC)
NCT01441765	II	CT-011	Recruiting	Phase II Study of PD-1 Blockade Alone or In Conjunction With the Dendritic Cell (DC)/Renal Cell Carcinoma (RCC) Fusion Cell Vaccination
NCT01067287	II	CT-011	Recruiting	Blockade of PD-1 in Conjunction With the Dendritic Cell/Myeloma Vaccines Following Stem Cell Transplantation
NCT01096602	II	CT-011	Recruiting	Blockade of PD-1 in Conjunction With the Dendritic Cell/AML Vaccine Following Chemotherapy Induced Remission
NCT01295827	I	MK-3475	Recruiting	Phase I Study of Single Agent MK-3475 in Patients With Progressive Locally Advanced or Metastatic Carcinoma, Melanoma, and Non-Small Cell Lung Carcinoma
NCT01704287	II	MK-3475	Recruiting	Randomized, Phase II Study of MK-3475 Versus Chemotherapy in Patients With Advanced Melanoma
NCT01352884	I	AMP-224	Active, not recruiting	Study to Assess the Safety, Tolerability, and Pharmacokinetics of AMP-224 in Patients With Advanced Cancer
NCT01375842	I	MPDL3280A	Recruiting	A Phase I, Open Label, Dose Escalation Study of the Safety and Pharmacokinetics of MPDL3280A Administered Intravenously As a Single Agent to Patients With Locally Advanced or Metastatic Solid Tumors
NCT01472081	I	Nivolumab + sunitinib, pazopanib, or ipilimumab	Recruiting	A Phase 1 Study of Nivolumab (BMS-936558) Plus Sunitinib, Pazopanib, or Ipilimumab in Subjects With Metastatic Renal Cell Carcinoma
NCT01629758	I	Nivolumab + IL-21	Recruiting	A Phase 1 Dose Escalation Study of BMS-982470 (Recombinant Interleukin-21, rIL-21) in Combination With BMS-936558 (Anti-PD-1) in Subjects With Advanced or Metastatic Solid Tumors
NCT01024231	I	Nivolumab + ipilimumab	Recruiting	A Phase 1b, Open-label, Multicenter, Multidose, Dose-escalation Study of BMS-936558 (MDX-1106) in Combination With Ipilimumab in Subjects With Unresectable Stage III or Stage IV Malignant Melanoma

had an objective response, while none of the 17 patients who had PDL-1-negative tumors had an objective response. The number of patients evaluated for PDL-1 expression in this study is small, and results must be interpreted with caution. It is still unknown whether PDL-1 expression will predict for response to therapy. Ongoing studies are attempting to determine whether IHC staining for PDL-1 can select patient who are likely to benefit from these inhibitory agents in future trials.

The question remains as to whether PDL-1 expression is associated with responses to other therapies. Currently a phase I biomarker trial with nivolumab is underway to try to answer these questions. Patients on this study will have a pre-treatment biopsy as well as an on-treatment biopsy of their tumor to assess for potential markers such as PDL-1. Serum markers are also being collected. This trial may provide some insight into PDL-1 expression and correlation with clinical response as a potential biomarker (NCT01358721).

CT-011 (CureTech) is a humanized IgG1 monoclonal antibody that inhibits PD-1 and has been tested in a phase I clinical trial in patients with acute myelogenous leukemia (AML), non-Hodgkin's lymphoma (NHL), chronic lymphocytic leukemia (CLL), myelodysplastic syndrome (MDS), and multiple myeloma (MM). Patients were enrolled in a dose-escalation fashion at five levels of doses: 0.2, 0.6, 1.5, 3, and 6 mg/kg. A total of 17 patients were enrolled at varying dose levels. No dose-limiting toxicity was reached. Clinical benefit was seen in 33 % of patients. CT-011 is now being studied in the phase II setting for other tumor types [28]. CT-011 is also currently being tested in combination with a dendritic cell (DC) vaccine using patient-derived tumor cells fused with dendritic cells in patients with metastatic RCC. This vaccine has previously been tested in RCC and shown to induce antitumor immunity and disease regression in 22 % of patients [29]. In this study patients receive CT-011 alone vs. CT-011 in combination with the DC vaccine. The primary end points are toxicity as well as response rate. This study is currently actively recruiting patients (NCT01441765). This combination of CT-011 and a DC vaccine is also being tested in patients with multiple myeloma after autologous stem cell transplantation (NCT01067287) and in patients with acute myelogenous leukemia after chemotherapy-induced remission (NCT01096602).

MK-3475 (Merck) is a PD-1 antibody in development in multiple tumor types. A phase I study in locally advanced or metastatic carcinoma is underway to assess for safety and toxicity (NCT01295827). The trial was recently expanded to include greater numbers of patients with NSCLC and melanoma. Early interim data was presented at the 2012 Society for Melanoma. Preliminary data showed 51 % objective response rate in patients with melanoma (43/85 patients) with 9 % (8 patients) sustaining a complete response at 12 weeks. Seven patients experienced grade III/IV immune-related adverse events. A phase II study of MK-3475 vs. standard chemotherapy in patients with metastatic melanoma is now being conducted (NCT01704287).

AMP-224 (Applimmune and GSK) is a PDL-2/IgG1 fusion protein that blocks PD-1 signaling. This molecule is currently in phase I testing to assess safety and tolerability. (NCT01352884). PDL-1 has also been developed as a target for inhibitor molecules, many of which are currently in the early stages of trial development. BMS-935559 (MDX1105-01) is a fully human PDL-1-specific IgG4 monoclonal

antibody which inhibits binding of PDL-1 to PD-1 and CD80. In a phase I study of patients with NSCLC, melanoma, renal cell carcinoma, ovarian cancer, gastric cancer, and breast cancer, MDX1105 was given by IV infusion every 2 weeks with dose escalation from 0.3 to 1 mg/kg to 3–10 mg/kg. The 207 patients had the following tumor types: 75 had non-small cell lung cancer, 55 had melanoma, 17 had RCC, 17 had ovarian cancer, 14 had pancreatic cancer, 7 had gastric cancer, and 4 had breast cancer. The maximum tolerated dose was not reached. Most common drug-related adverse events included fatigue, infusion reactions, diarrhea, arthralgias, rash, nausea, pruritus, and headache. 9 % of patients had grade III or IV event. Of the 17 patients with renal cell carcinoma, 2 had an objective response (ORR 12 %), both at the 10 mg/kg dose. 7 patients (41 %) had stable disease lasting 24 weeks. Responses in other tumor types were also seen [30].

MPDL3280A (Genentech) is a PDL-1 antibody currently in phase I testing in multiple solid tumor types (NCT01375842). It is also being tested in combination with bevacizumab in several tumor types, including RCC, and it is being tested in combination with vemurafenib in patients with metastatic melanoma.

Combination Therapy

In part due to its favorable toxicity profile, PD-1 blockade may be combined more easily with other strategies in the hopes of creating a synergistic effect. Several preclinical studies have shown that increased levels of VEGF may have immunosuppressive effects [31]. VEGF receptor tyrosine kinase inhibitors (e.g., pazopanib, sunitinib) may reduce the numbers of myeloid-derived suppressor cells (MDSC) and limit the negative effect of VEGF, thereby reversing tumor-induced immunosuppression [32, 33]. It is clear that tumors develop resistance to single-agent treatments, making opportunities for combination therapy more appealing. Combination therapy with VEGF agents and checkpoint inhibitors is currently under investigation. Currently, nivolumab in combination with sunitinib, pazopanib, or ipilimumab is being assessed for safety and tolerability in patients with mRCC (NCT01472081). As mentioned previously, the Genentech PDL-1 antibody MPDL3280A is currently being studied in combination with bevacizumab.

Combination studies with immune agents have the potential for augmenting benefit based on preclinical studies showing synergism. Gamma-chain cytokines IL-2, IL-7, IL-15, and IL-21 are shown to upregulate PD-1 on purified T-cells in vitro. Thus, these cytokines can be used as adjuncts to enhance T-cell responses and augment the inhibition by PD-1 immunotherapy [34]. Based on this rationale, nivolumab is being studied in combination with IL-21 in a phase I study of patients with advanced solid tumors (NCT01629758).

In melanoma, ipilimumab and bevacizumab have been studied in combination in the phase I setting. Preliminary results show this combination to be tolerable, and clinical activity suggests a synergistic effect. CTLA-4 inhibitors have been shown to induce an immune-mediated tumor vasculopathy, and VEGF has been shown to suppress antigen-presenting cells (APCs). This phase I study showed clinical

benefit in 14/21 patients treated, and all responses were durable (>6 months) [35]. Based on this data, the combination of ipilimumab and bevacizumab may be worth studying in RCC.

Lastly, combination therapy with ipilimumab and PD-1/PDL-1 inhibitors may allow for a more robust immune response without the immune-related toxicities. The two agents affect T-cell activation in different ways—ipilimumab by removing the inhibitory break on T-cell proliferation and anti-PD-1 removing a break on T-cell effector function. Combining these two immune strategies may prevent resistance to either therapy given alone. A phase I trial of the combination of nivolumab with ipilimumab in patients with melanoma is currently ongoing (NCT01024231). The recent explosion of trials and new immune agents being developed for metastatic renal cell carcinoma will change the face of cancer treatments for the future.

Conclusion

Checkpoint inhibition is a new and evolving field in the treatment of solid tumors. Early trials with CTLA-4 and PD-1/PDL-1 blockade have revealed durable clinical benefit in a subset of patients with less toxicity than had been seen with older agents (e.g., IL-2). Further development in this field is rapidly expanding with several clinical trials open to accrual more in planning stages. Ipilimumab is currently FDA approved for melanoma, but its utility in RCC is still not fully understood. Other CTLA-4 antibodies such as tremelimumab also show promise in RCC and are entering larger trials. The PD-1 antibody nivolumab is currently undergoing confirmatory testing in the phase III trial. Other PD-1 and PDL-1 antibodies show significant promise in several tumor types. Patient selection will be an expanding area of research as we look toward biomarker development to predict response to these therapies. Combination therapy with immune treatments targeting different immune mechanisms as well as combinations with traditional antiangiogenic therapies is being further explored. Due to the low toxicity profile of these newer immune therapies, the use of checkpoint inhibitors as adjuvant treatment should be considered in future development. Checkpoint inhibition has opened many doorways in the exploration of immune system manipulations to treat RCC. By minimizing toxicity and offering patients the potential for durable remissions, it is hoped that these immunotherapies will be the future of cancer treatment in RCC and could set the stage for development in other solid tumors.

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Chapter 22
Vaccines in RCC: Clinical and Biological Relevance

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RCC as an "Immunogenic Cancer"

Since the 1960s, rare but reproducible reports of the spontaneous regression of RCC metastases (typically pulmonary or hepatic) post-nephrectomy have been reported [2–13], with implications for the critical involvement of inflammatory cell infiltrates and/or enhanced peripheral T-cell function in patients with resolving disease [2, 3, 7, 14, 15]. Additional research suggested that RCC tumors exhibiting defects in their ability to be recognized by the adaptive immune system are "ineligible" to undergo spontaneous regression in vivo [16]. Such studies suggested that RCC tumor cells can be both recognized and regulated under certain conditions by RCC-specific T effector cells that may mediate (at least transient) resolution of disseminated disease [2–15].

In the decades following such initial observations, we have gained tremendous insights in the immunobiology of T cells and their requirements for effective recognition of specific target cell populations, including tumor cells. This knowledge has fueled our capacity to isolate, clone, expand, characterize, and even adoptively transfer antitumor T effector cell populations into autologous patients as a

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therapeutic modality that has yielded objective clinical responses (OCR, based on Response Evaluation Criteria In Solid Tumors (RECIST)) in patients with RCC [16–23].

Anti-RCC T-cell populations have also served as effective probes for the molecular identification of RCC-associated antigens (RCCAA; Table 22.1) [24–98]. Ultimately, both the specific T-cell receptor (TCR) employed by anti-RCC T cells to recognize a given RCCAA and the RCCAA itself have proven to have significant clinical utility. A cloned RCCAA-specific TCR can be used to engineer (via recombinant viral infection) an army of transgenic, tumoricidal T cells for use in adoptive cell transfer (ACT)-based immunotherapy. Indeed, ACT-based therapies have proven capable of eradicating large tumor burdens in patients, particularly when combined with lymphodepletion regimens [99]. On the other hand, RCCAA gene products (mRNA, protein, and peptides) can be used in vaccine formulations and for the monitoring of specific T-cell responses in RCC patients receiving any form of treatment believed to result in the modulation of a patient's adaptive antitumor immune system.

When immunotherapies have been applied in the cancer setting, RCC (and melanoma) has tended to be among the most clinically responsive solid cancer types based on OCR as an endpoint [99–106]. For instance, the treatment of RCC patients with high doses of rIL-2 (see also Chap. 20) or with humanized antibodies against cytotoxic T-lymphocyte antigen-4 (CTLA-4; see also Chap. 21) leads to the activation and improved Type 1 effector cell function of patient T cells, in association with OCR rates in the range of 25–50 % [99, 101, 103–111]. Of arguably equal or even greater importance, many patients responding to treatment with high-dose rIL-2 or CTLA-4 blockade exhibit durable antitumor benefits as a consequence of these interventional strategies [99, 101, 105–107, 110, 111].

Active-Specific Vaccination Against RCC

From the available clinical and translational literature, one may conclude that barring RCC patient treatment with ACT (which is costly, subject to off-target effects including autoimmune pathology and/or insertional mutagenesis/leukemia [112] and can only be performed in highly specialized clinical centers) as a preferred immunotherapy, clinically beneficial antitumor T-cell-mediated immunity may be best developed via active, specific vaccination. To achieve this goal, the vaccine should be injected into tissue sites that are minimally impacted by the suppressive tumor microenvironment in order to access the patient's antigen-presenting cells (such as $CD141^{hi+}/XCR1^{+}$ dendritic cells (DC) [113–115]) that can subsequently promote robust, RCCAA antigen-specific T-cell responses in vivo. The vaccine should contain RCCAA mRNA, cDNA, protein, or peptides (cross presented in MHC complexes expressed by DC to the TCR expressed on responding T cells) and an adjuvant capable of maturing host DC into transport-competent $CCR7^{+}$ APC that migrate to vaccine site-draining lymph nodes where they may preferentially activate Type 1-polarized, anti-RCCAA T effector cell responses [116].

Table 22.1 Molecularly defined RCC

Antigen	Frequency of expression among RCC tumors (%)	CD8+ T-cell recognition: patients with HLA class I allele(s)	CD4+ T-cell recognition: patients with HLA class II allele(s)	Hypoxia-responsive gene product?	Increased gene product under hypomethylation conditions?	HSP90 client protein?	References
Survivin	100	Multiple	Multiple	+	+	+	[24]
OFA-iLR	100	A2	NR	NR	NR	NR	[25, 26]
VEGF (cryptic)	100	B27	NR	+	+	NR	[27]
IGFBP3	97	B7	Multiple	+	+	NR	[28–30]
EphA2	>90	A2	DR4	+	NR	+	[31–33]
RU2AS transcript		B7	NR	NR	NR	NR	[34]
G250, CA-IX	90	A2, A24	Multiple	+	+	NR	[35–37]
EGFR	85	A2	NR	+	+	+	[38, 39]
HIFPH3	85	A24	NR	+	NR	NR	[40]
RGS1	81	A2, A3	NR	+	NR	NR	[41]
APOL1	81	A2	NR	NR	NR	NR	[30]
c-Met	>80	A2	NR	+	NR	+	[30, 42]
WT-1	80	A2, A24	NR	+	NR	+	[43–46]
MUC1	76	A2	DR3	+	+	+	[47–49]
5T4	75–95	A2, Cw7	DR4	NR	NR	NR	[50–54]
iCE	75	B7	NR	NR	NR	NR	[55]
MMP7	75	A3	Multiple	+	NR	NR	[30, 57]
Cyclin D1	75	A2	Multiple	+	NR	+	[30, 58, 59]
HAGE	75	A2	DR4	NR	+	NR	[60]
hTERT	>70	Multiple	Multiple	+	+	+	[61–63]
RGS5	63	A2, A3	NR	+	NR	NR	[64]
FGF-5	>60	A3	NR	NR	NR	NR	[65]

(continued)

Table 22.1 (continued)

Antigen	Frequency of expression among RCC tumors (%)	CD8+ T-cell recognition: patients with HLA class I allele(s)	CD4+ T-cell recognition: patients with HLA class II allele(s)	Hypoxia-responsive gene product?	Increased gene product under hypomethylation conditions?	HSP90 client protein?	References
mutVHL	>60	NR	NR	+	+	+	[66, 67]
MAGE-A3	60	Multiple	Multiple	NR	+	NR	[68, 69]
SART-3	57	Multiple	NR	NR	NR	NR	[70–73]
SART-2	56	A24	NR	NR	NR	NR	[74]
PRAME	40	Multiple	NR	NR	+	NR	[75–78]
p53	ML	Multiple	Multiple	+	+	+	[79, 80]
CYP1B1	33	A2	NR	+	+	NR	[30]
MAGE-A9	>30	A2	NR	NR	+	NR	[81]
MAGE-A6	30	Multiple	DR4	NR	+	NR	[82, 83]
MAGE-D4	30	A25	NR	NR	+	NR	[84]
Her2/neu	10–30	Multiple	Multiple	+	+	+	[85–90]
HLA-G	27	A24	NR	+	+	NR	[91, 92]
SART-1	25	Multiple	NR	+	NR	NR	[93–95]
RAGE-1	21	Multiple	Multiple	NR	+	NR	[75, 81, 96, 97]
ACSL4	9	A3, B7	NR	NR	NR	NR	[30]
Hsp70-2	Orphan mutant	A2	NR	NR	NR	NR	[98]
GUCY1A3	NR	A2	NR	NR	NR	NR	[64]
PLIN2/ADRP	NR	A2	NR	NR	NR	NR	[64]
PRUNE2	NR	A2	NR	NR	NR	NR	[64]

A summary is provided for RCCAA that have been defined at the molecular level. RCCAA are characterized with regard to their prevalence of (over)expression among total RCC specimens evaluated, whether RCCAA expression is modulated by hypoxia or tumor DNA methylation status, whether the RCCAA is a known HSP90 client protein, and whether HLA class I and class II alleles have been reported to serve as presenting molecules for T-cell recognition of peptides derived from a given RCCAA

Abbreviations: *ACLS4* acyl-CoA synthetase long-chain family member 4; *ADRP* adipose differentiation-related protein (also known as PLIN2); *APOL1* apolipoprotein L1; *c-MET* MET oncoprotein (also known as hepatocyte growth factor receptor; HGFR); *CYP1B1* cytochrome P450 family 1, subfamily B, polypeptide 1; *G250, CA-IX* (carbonic anhydrase-IX); *EGFR* epidermal growth factor receptor; *EphA2* EPH receptor A2; *FGF* fibroblast growth factor; *GUCY1A3* guanylate cyclase 1-soluble subunit alpha-3; *HAGE* helicase antigen; *Her2* human EGFR2 (also known as Neu); *HLA* human leukocyte antigen; *HIFPH3* hypoxia-inducible factor prolyl hydroxylase 3; *HSP* heat shock protein; *hTERT* human telomerase reverse transcriptase; *iCE* intestinal carboxyl esterase; *IGFBP3* insulin-like growth factor binding protein 3; *MAGE* melanoma-associated antigen; *MMP7* matrix metalloproteinase 7; *MUC1* mucin 1; *NR* not reported; *OFA-iLR* oncofetal antigen-immature laminin receptor; *PRAME* preferentially expressed antigen in melanoma; *PRUNE2* protein prune homologue 2; *RAGE* renal cell carcinoma-associated antigen; *RGS1* regulator of G-signaling protein 1; *RGS5* regulator of G-signaling protein 5; *SART* squamous antigen rejecting tumor protein; *VEGF* vascular endothelial growth factor; *VHL* von Hippel–Lindau tumor suppressor protein; *WT-1* Wilms' tumor 1 protein; *5T4* (also known as trophoblast glycoprotein, TPBG)

Since tumor-induced immunosuppression can limit the operational immunogenicity of vaccines in the cancer-bearing host, corrective therapeutic agents may need to be coadministered in order to optimize the magnitude, efficacy, and durability of vaccine-induced immunity in vivo (as will be discussed later in this chapter).

Vaccines for RCC: Whole or "Subunit" Tumor Cell-Based Vaccines

Driven by the existing technology (or lack thereof) and an only rudimentary understanding of complex cancer immunobiology in the late 1980s and early 1990s, preliminary forms of RCC vaccines were based on irradiated, autologous/allogeneic tumor cells administered with or without bacterial adjuvants (such as Bacille Calmette–Guérin; BCG) or recombinant cytokines (such as interleukin (IL)-2, interferons (IFN-α, IFN-β, or IFN-γ), or granulocyte–macrophage colony-stimulating factor (GM-CSF)) known to enhance the function of DC and/or T cells in vivo (Table 22.2) [117–134]. In some cases, patients were also coadministered alkylating drugs (e.g., low-dose cyclophosphamide; CY) shown to enhance immune reactivity to specific vaccination based on the: (1) elimination of Treg cell populations and/or (2) "normalization" of previously dysfunctional DC and T-cell populations in vivo, thereby allowing for the improved activation and expansion of vaccine-induced T cells [135–139]. Using delayed-type hypersensitivity (DTH) responses as a cutaneous (in vivo) measure of Type 1 T-cell responsiveness to vaccination, phase I/II clinical trials have reported treatment-associated activation of antitumor immunity in approximately 1/3–2/3 of vaccinated RCC patients, with evidence of OCR in minority of such cases (Table 22.2). Enthusiasm for the continued use, and refinement, of vaccines in the RCC setting has been sustained by reports that virtually all patients that develop OCR postvaccination also displayed coordinate evidence of treatment-associated improvement in their anti-RCC immune responsiveness [119, 121].

Since irradiated RCC tumor cells used in early vaccine formulations would likely retain their immunosuppressive qualities (i.e., production of suppressive mediators (such as IL-10, TGF-β, gangliosides, and prostanoids, among others) and/or plasma membrane expression of T-cell co-inhibitory molecules such as Fas-L, PD-L1, PD-L2, among others; see also Chap. 21) [140–143], a range of corollary approaches have since been developed. These include the use of autologous or allogeneic RCC lysates [122–128], heat shock protein (HSP)-peptide complexes from lysed RCC cells [129, 130], or RCC cells fused with allogeneic/autologous lymphocytes or DC (to form heterokaryons that present RCCAA-derived peptides in patient-matched MHC complexes to T cells in a manner that benefits from the potent stimulatory characteristics of allogeneic cells and/or DC, while presumably minimizing the immunosuppressive aspects of RCC based on genetic complementation in the

Table 22.2 Clinical Vaccines for RCC Incorporating Whole RCC or RCC Lysates

Vaccine format	Co-therapy	Vaccine-targeted antigen	Trial phase	Number of RCC patients treated	Vaccine immunogenicity	Clinical outcomes	References
Irradiated AutoRCC + BCG	None	Natural RCCAA	II	60	38/54 evaluable patients with positive DTH responses postvaccination	No changes in DFS or OS	[117]
Irradiated NDV-infected AutoRCC	IL-2, IFN-α	Natural RCCAA	II	40	DTH response after 3 vaccinations predictive of survival advantage	5 CR, 6 PR, 12 SD	[118]
Irradiated AutoRCC + BCG	IFN-α, IFN-γ	Natural RCCAA	II	14	No DTH responses to tumor; possible CTL development in clinical responders	3 MR, 5 SD	[119]
Irradiated AutoRCC + BCG	IFN-β, GM-CSF, CY	Natural RCCAA	II	25	6/18 evaluable patients with positive DTH response postvaccination	No OCR	[120]
Irradiated AutoRCC	GM-CSF	Natural RCCAA	I/II	22	7/19 evaluable patients with positive DTH response and 10/17 evaluable patients with increased CD4+ and CD8+ T-cell responses postvaccination	1 CR, 7 SD; improved TTP and OS if patients developed immune response postvaccination	[121]
AutoRCC lysate	None	Natural RCCAA	III	276	Not evaluated	Increased 5-year PFS	[122]
AutoRCC lysate	None	Natural RCCAA	CUP	692	Not reported	Extended OS if vaccinated	[123]
AutoRCC plasma membrane coated on silica beads	CY, IL-2	Natural RCCAA	III	31	Not reported	1 PR, 12 SD; no difference in OS or PFS	[124]
AutoDC + AutoRCC lysate + KLH	None	Natural RCCAA	I	2	1/2 patients with positive DTH response postvaccination	1 SD correlated with positive DTH response postvaccination	[125]
AutoDC + AutoRCC lysate + KLH	IL-2	Natural RCCAA	I/II	6	2/6 patients with increased Type 1 T-cell responses postvaccination	1 SD associated with Type 1 T-cell response and reduced Treg	[126]

(continued)

Table 22.2 (continued)

Vaccine format	Co-therapy	Vaccine-targeted antigen	Trial phase	Number of RCC patients treated	Vaccine immunogenicity	Clinical outcomes	References
AutoDC + AutoRCC lysate + KLH	IL-2	Natural RCCAA	I/II	12	Not observed	Extension of SD	[127]
AlloDC + AutoRCC lysate + KLH	CY	Natural RCCAA	I/II	20	No T-cell response against lysate RCCAA; 1 MR patient with enhanced IFN-γ against OFA postvaccination	2 MR, 3 SD; strongest anti-KLH responses postvaccination in 2 MR patients	[128]
AutoRCC HSP(gp96)-peptide complexes (vitespen)	IL-2	Natural RCCAA	III	84 (60 evaluable)	Not reported	2 CR, 2 PR, 7 SD	[129]
AutoRCC HSP(gp96)-peptide complexes (vitespen)	None	Natural RCCAA	III	361	Not reported	Trend toward better recurrence-free survival in patients with earlier stage disease	[130]
AlloRCC-AlloLymph fusion cells	None	Natural RCCAA	I/II	11	Not reported	2 CR, 2 PR	[131]
AutoDC-AlloRCC fusion cells	None	Natural RCCAA	I	10	7/10 patients with positive DTH response postvaccination	1 PR, 6 SD correlated with positive DTH response to vaccine	[132]
AlloDC-AutoRCC fusion cells	None	Natural RCCAA	I/II	22	Not reported	3 OCR; 11 SD	[133]
AlloDC-AutoRCC fusion cells	None	Natural RCCAA	I/II	24	10/21 patients with Type 1 CD4+ and/or CD8+ T-cell responses postvaccination	2 PR, 8 SD	[134]

Abbreviations: Allo, Allogeneic; Auto, Autologous; BCG, Bacillus Calmette–Guérin; CTL, Cytotoxic T Lymphocyte; CUP, Compassionate Use Program; CY, Cyclophosphamide; DFS, Disease-Free Survival; DTH, Delayed-Type Hypersensitivity; GM-CSF, Granulocyte-Macrophage Colony Stimulating Factor; IFN, Interferon; MR, Mixed Response; IL, Interleukin; KLH, Keyhole Limpet Hemocyanin; OCR, Objective Clinical Response; OS, Overall Survival; PFS, Progression-Free Survival; RCC, Renal Cell Carcinoma; SD, Stabilization of Disease; RCCAA, Tumor-Associated Antigen(s); TTP, Time To Progression.

hybrid cells) as vaccine-incorporated immunogens [131–134]. As in the case of whole cell-based vaccines, these "subunit" (i.e., cellular lysates and purified HSP complexes) and hybrid cell-based vaccines appeared to be well tolerated by patients and capable of imparting clinical benefit to immunized patients. In the largest trials involving the injection of autologous RCC lysates, patients receiving the vaccine displayed increased 5-year progression-free survival (PFS; $p = 0.0204$) [122] or extended overall survival (OS; $p = 0.030$) [123]. Among RCC patients treated with vaccines composed of autologous DC pulsed with RCC lysates alone or in combination with IL-2 or CY co-therapy, disease stabilization was the most common OCR, with the clinical responders tending to exhibit the strongest levels of enhanced T-cell-mediated immunity post- versus pretreatment [125–128]. Furthermore, the results of a small phase I/II study by Baek et al. [126] using a lysate-based vaccine in six RCC patients suggested that OCR might be most commonly observed in patients with treatment-associated increases in Type 1 immune reactivity and coordinate reductions in Treg cell numbers in peripheral blood.

"Immunogenic" components within tumor lysates include cytosolic HSP-peptide complexes [144]. HSPs serve as molecular chaperones in classical MHC–peptide complex formation [144–148], with some HSPs (i.e., including HSP90/gp96) being able to directly bind peptide species that ultimately serve as MHC-presented epitopes on target cells, including tumor cells, that are directly recognized by $CD8^+$ T lymphocytes [144–148]. When purified and injected into mice as a vaccine, tumor cell-derived HSP90 (i.e., gp96)-peptide complexes are taken up by professional APC, including DC, that subsequently cross present these epitopes in MHC complexes to antigen-specific T cells [148–151]. Autologous RCC gp96-peptide vaccines (i.e., vitespen, aka HSPPC-96 or Oncophage from Agenus) have recently been investigated in phase II and III clinical trials where OCR were observed in a minority of patients [128, 129], particularly among a cohort of patients with early stage disease [129]. Unfortunately, the monitoring of vaccine-induced T-cell responses against RCCAA has not been systematically integrated into clinical trials evaluating gp96-based vaccines, making it difficult to draw firm conclusions regarding the comparative immunogenicity of this approach versus alternate vaccine strategies in treated RCC patients.

RCC hybrid cell-based vaccines are conceptually attractive given their intent to engineer and implement a superior tumor-specific APC cell via the use of simple cell–cell fusion technologies to produce immunogenic heterokaryons. However, the logistics of isolating viable primary RCC cells and/or DC as components for hybrid construction and low recovery rates for injectable stable fusion products have served to limit the use of this approach to small pilot clinical trials performed over the past 15 years [131–134]. Nevertheless, when patients have been treated with RCC cell fusion vaccines, approximately 1/2–2/3 of them have developed specific Type 1 T-cell-mediated immunity (based on DTH or T-cell proliferation/IFN-γ production in response to vaccine-associated proteins) posttreatment, often with coordinate evidence of OCR [132, 134].

Clinical Vaccines: Genetically Modified RCC or Dendritic Cell-Based Protocols

Beyond cell–cell fusion technology, another way to make tumor cells intrinsically more "immunogenic" involves the use of molecular engineering to transfect a patient's autologous RCC cells or an "off-the-shelf" allogeneic RCC cell line with cDNAs encoding stimulatory cytokines or co-stimulatory molecules that enhanced the ability of these laboratory-modified APC to activate antigen-specific T cells in vivo. Landmark studies in murine tumor models by Golumbek et al. [152], Dranoff et al. [153], and Dorsch et al. [154] supported the ability of irradiated, cytokine gene-modified tumor cells to activate both innate (i.e., DC, natural killer cells (NK), eosinophils, polymorphonuclear leukocytes (PMN)) and adaptive immunity in association with the establishment of protective/therapeutic T-cell-mediated immunity in vivo. These studies, and many others, fostered the translation of this approach into the clinic (Table 22.3) [155–163]. Early trials implemented vaccines consisting of irradiated RCC cells transfected to produce granulocyte–macrophage colony-stimulating factor (GM-CSF), based on the superior antitumor efficacy noted for this strategy in head-to-head comparative studies [153]. In virtually all cases (and independent of RCC source, cytokine/co-stimulatory molecule cDNA engineered into RCC or presence of coadministered rIL-2), these vaccine approaches elicited positive DTH and/or Type 1 T-cell responses in the majority of treated patients [155–163]. OCR were observed in cases involving treatment with autologous RCC-based vaccines, with extended time to progression (TTP) or OS noted among vaccinated patients (Table 22.3).

Clinical vaccines have also been developed using autologous RCC (AutoRCC) or AutoDC transfected with cDNA- or mRNA-encoding RCCAA (Table 22.3) [164, 165]. In excess of 75 % of patients vaccinated using these approaches developed specific T-cell-mediated immunity posttreatment, with stabilization of disease noted as best OCR in small phase I/II studies [164, 165].

Clinical Vaccines: Vaccines Based on RCCAA Protein/Peptides

In the early 1990s, it became possible to identify RCCAA at the molecular level using gene cloning- and mass spectrometry-based approaches [166]. The implementation of in vitro-stimulated (IVS) populations of RCC-specific T cells as probes and cDNA-transfected APC as target cells allowed for immune-targeted RCCAA cDNAs to be isolated and cloned [166]. Alternatively, RCCAA-derived peptides could be affinity purified and then extracted from tumor MHC class I complexes, allowing for their subsequent amino acid sequencing using combined liquid chromatography and mass spectrometry [167–169]. Using these complementary approaches, as well as, molecular cloning technologies based on RCC patient

Table 22.3 Clinical vaccines for RCC incorporating molecularly engineered tumor cells or dendritic cells to elicit T-cell responses against endogenous, natural TAA

Vaccine format	Co-therapy	Vaccine-targeted antigen	Trial phase	Number of RCC patients treated	Vaccine immunogenicity	Clinical outcomes	References
Irradiated AutoRCC.GM-CSF	None	Natural TAA	I	16	14/16 evaluable patients with positive DTH response to tumor; strongest DTH responses in patient with OCR	1 PR	[155]
Irradiated AutoRCC.GM-CSF	None	Natural TAA	I	4	4/4 patients with positive DTH response and 3/4 patients with specific CTL responses postvaccination	2/4 with extended OS	[156]
Irradiated AutoRCC.GM-CSF/IL-7	None	Natural TAA	I/II	10	Increased CTL postvaccination; strongest Type 1 T-cell response in patient with CR	1 CR, 1 PR, 2 SD	[157]
Irradiated AutoRCC.CD80	IL-2	Natural TAA	I	15	5/13 evaluable patients with positive DTH response postvaccination; positive DTH responses in 3 of 4 patients with OCR	2 PR, 2 SD	[158]
Irradiated AutoRCC.CD80	IL-2	Natural TAA	II	39	22/27 evaluable patients with positive DTH responses (lymphocytic infiltrate) correlating with increased median survival	1 CR, 1 PR, 24 SD	[159]
Irradiated AutoRCC.IL-2	None	Natural TAA	II	30	Not detailed; some evidence of Type 1 T-cell responses in patients postvaccination	1 CR, 4 PR, 9 SD; improved OS if vaccinated	[160]
Irradiated AlloRCC26.CD80.IL-2	None	Natural TAA	I	15	11/12 evaluable patients with positive DTH responses to vaccine and 9/12 with TAA-specific T-cell responses in vitro	No OCR; extended median TTP/OS if vaccinated; evidence of ES and reduced Treg postvaccination	[161, 162]

(continued)

Table 22.3 (continued)

Vaccine format	Co-therapy	Vaccine-targeted antigen	Trial phase	Number of RCC patients treated	Vaccine immunogenicity	Clinical outcomes	References
Irradiated AlloRCC26.IL-7.CD80	None	Natural TAA	I	10	No Type 1 T-cell responses observed	No OCR	[163]
Irradiated AutoRCC.PGRP-S	None	Natural TAA	I/II	4	3/4 patients with positive DTH responses postvaccination	1 SD	[164]
AutoRCC mRNA transfected AutoDC	None	Natural TAA	I	10	6/7 evaluable patients with Type 1 T-cell responses against TAA postvaccination	1 SD	[165]

Abbreviations: *CR* complete response, *CTL* cytotoxic T lymphocyte, *DC* dendritic cell, *DTH* delayed-type hypersensitivity, *GM-CSF* granulocyte–macrophage colony-stimulating factor, *IL* interleukin, *MR* mixed response, *OCR* objective clinical response, *OS* overall survival, *PGRP-S* peptidoglycan recognition protein short form (also known as tag7), *PR* partial response, *RCC* renal cell carcinoma, *SD* stabilization of disease, *TAA* tumor-associated antigen(s), *TTP* time to progression

serologic (i.e., antibody-based; serological expression (SEREX) cloning) reactivity [170], an ever-growing list of RCCAA has been defined (Table 22.1).

Several RCCAA have now been investigated in phase I–III clinical trials of peptide- or RNA-/DNA-based vaccines (Table 22.4) [67, 171–184]. Given the diversity of vaccine formulations and variation in targeted RCC patient subpopulations, as well as the use of nonstandardized laboratory testing for immune correlates, it is virtually impossible to compare and contrast these trials for their comparative immunogenicity and clinical efficacy. When taken as a whole, RCCAA-specific vaccinations have been reported to be safe and well tolerated by RCC patients, with minimal treatment-associated toxicities and no diminishment in quality of life [67, 171–185]. Where evaluated, vaccine antigen-specific Type 1 T-cell responses have been detected in 50–100 % of patients receiving peptide-based vaccines and in 20–80 % of RCC patients receiving genetic vaccination (Table 22.4). Low frequencies of OCR have been observed in most trials, with patients who develop strong immunity postvaccination tending to have a better clinical prognosis (Table 22.4).

Vaccines as a Single-Modality Immunotherapy: Lessons Learned

From the results of RCC vaccine trials summarized in Tables 22.2, 22.3, and 22.4, one is led to conclude that each vaccine approach (whole cell, lysate, gene modified, and peptide) is capable of eliciting specific immunity in a fraction of patients and that it is within this subset of patients that objective clinical benefits are most likely to be observed. Furthermore, among the immune responders, those patients that develop the most robust responses (based on (1) polyfunctional/poly-specific T-cell responses, (2) diversification in the antitumor T-cell repertoire (manifest in the form of "epitope spreading" or vaccine-associated development of Type 1 T cells reactive against disease-relevant specificities not intrinsic to the vaccine), and (3) the reduced preponderance of Treg cells) tend to be the individuals exhibiting OCR [64, 126, 162, 172, 179]. Further implications from the results of these trials are that vaccines appear to provide superior clinical benefits when administered to patients of early stage [130] (which may relate to stage-dependent expression of vaccine-targeted RCCAA [186, 187] or enhanced immunosuppression/anergy [187]) or limited tumor burden [184].

In a systematic review and meta-analysis of 12 clinical trials performed using DC-based vaccines in 172 RCC patients, Draube et al. [188] reported that an enhancement in specific cellular immunity postvaccination was associated with an improved clinical benefit rate (CBR = OCR + SD; odds ratio (OR), 8.4, 95 %, CI 1.3–53.0) and that the magnitude of the vaccine-induced immune response was directly influenced by the immunizing dose of antigen (i.e., the dose of DC applied in the vaccine formulation OR, 7.0, 95 %, CI, 1.9–25.0). These data are analogous to those reported for vaccines applied to patients with melanoma [187] or prostate carcinoma [188]. For instance, Chi and Dudek [189] performed a similar meta-analysis

Table 22.4 Clinical vaccines for RCC incorporating molecularly defined antigens

Vaccine format	Co-therapy	Vaccine-targeted antigen	Trial phase	Number of RCC patients treated	Vaccine immunogenicity	Clinical outcomes	References
Polypeptide vaccine	IL-2 or IFN-α	Selection of 4 defined TAA peptides from panel based on patient in vitro responses pretreatment	I	10	8/10 patients with positive DTH response to vaccine; 2/10 patients with increased Type 1 T-cell response to peptides postvaccination	6 SD	[171]
AutoDC + MUC1 peptide + PADRE peptide + IL-2	IL-2	MUC1	I	20	10/20 patients with Type 1 T-cell response to peptides; T-cell responses correlated to OCR	1 CR, 2 PR, 2 MR; 5 SD	[172]
MKC1106-PP gene prime/peptide boost	None	PRAME, PSMA	I	2	2/2 patients with Type 1 T-cell response to peptides	2/2 with SD >11 months	[173]
CA-IX (G250) peptide + IFA	None	CA-IX	I	23	5/10 evaluable patients with Type 1 CD8+ T-cell response to peptide	3 PR; 6 SD	[174]
Mature AutoDC + CA-IX (G250) peptides	None	CA-IX	I	6	No specific immunity to CA-IX	No OCR	[175]
Tumor antigen RNA + GM-CSF	GM-CSF	MUC1, CEA, HER2/neu, telomerase, survivin, MAGE-A1	I/II	30	8/10 evaluable patients with Type 1 T-cell response to vaccine peptides	16 SD; 1 PR	[176]
Telomerase peptide + Montanide adjuvant	None	Telomerase	I/II	4	Not described, progressor tumor in 1 patient was an MHC-I loss variant	No OCR	[177]

AutoDC + telomerase peptide	None	Telomerase	I/II	10	2/8 evaluable patients with DTH response; CTL (IFN-γ) responses only in 3 patients with OCR	2 MR; 1 SD	[178]
Mutant VHL peptide + Montanide adjuvant	None	Mutant VHL	I/II	6	4/5 evaluable patients with CD8+ T-cell responses to antigen	No OCR, but extended OS if patients responded to vaccination	[67]
IM-901	GM-CSF ± CY	9 natural MHC-I-presented TAA (PLIN2, APOL1 (2), CCND1, GUCY1A3, PRUNE2, MET, MUC1, RGS5) + 1 natural MHC-II-presented TAA (MMP7) peptides	I + II	96	64–74 % of evaluable patients displayed CD8+ T-cell responses to 1 or more vaccine peptides	Better OS observed if patients made immune response to multiple peptides; serum biomarkers of response/OS = APOA1 and CCL17	[64]
TG4010 (vaccinia virus)	IL-2, IFN-α	MUC1	II	37	6/28 and 6/23 evaluable patients made CD4+ and CD8+ T-cell responses to vaccine, respectively; immune responders with longer OS	6/20 patients with SD for more than 6 months	[179]
MVA-5T4 (TroVax; modified vaccinia Ankara)	Sunitinib, IL-2, IFN-α	5T4	III	288	56 % of evaluable patients developed anti-5T4 antibody responses postvaccination; T-cell responses not evaluated	Magnitude of antibody response was associated with longer OS, greatest impact for smaller tumors and patients with normal mean corpuscular Hb concentration (MCHC) levels	[180–184]

(continued)

Table 22.4 (continued)

Vaccine format	Co-therapy	Vaccine-targeted antigen	Trial phase	Number of RCC patients treated	Vaccine immunogenicity	Clinical outcomes	References
IMA901	Sunitinib	9 Natural MHC-I presented TAA (PLIN2, APOL1 (2), CCND1, GUCY1A3, PRUNE2, MET, MUC1, RGS5) + 1 natural MHC-II presented TAA (MMP7) peptides	III	330	In progress, results expected in 2014	In progress, results expected in 2014; http://clinicaltrials.gov/ct2/show/NCT01265901	In progress, results expected in 2014

Abbreviations: *AlloDC* allogeneic dendritic cells, *APOL1* apolipoprotein L1 antigen, *AutoDC* autologous dendritic cells, *CA-IX* carbonic anhydrase-IX antigen (also known as G250), *CCND1* cyclin D1 antigen, *CEA* carcinoembryonic antigen, *CR* complete response, *CTL* cytotoxic T lymphocyte, *CY* cyclophosphamide, *DTH* delayed-type hypersensitivity, *GUCY1A3* guanylate cyclase 1-soluble subunit alpha-3 antigen, *HER2* human epidermal growth factor receptor 2 (also known as neu), *IFA* incomplete Freund's adjuvant, *IFN* interferon, *IL* interleukin, *MAGE-A1* melanoma-associated antigen 1, *MET* c-MET proto-oncogene antigen (also known as hepatocyte growth factor receptor), *MHC* major histocompatibility complex, *MMP7* matrix metalloproteinase 7 antigen, *MR* mixed response, *MUC1* mucin 1 antigen, *OCR* objective clinical response, *OS* overall survival, *PADRE* Pan-DR epitope, *PR* partial response, *PRAME* preferentially expressed antigen in melanoma, *PRUNE2* protein prune homologue 2 antigen, *PSMA* prostate-specific membrane antigen, *RGS5* regulator of G-protein signaling 5 antigen, *SD* stabilization of disease, *TAA* tumor-associated antigen, *VHL* von Hippel–Lindau protein, *5T4* trophoblast glycoprotein antigen (also known as TPBG)

of 56 phase II and III clinical vaccines involving the treatment of 4,375 melanoma patients and found that more than ¼ of patients received clinical benefit from vaccination, that vaccination improved disease control compared to rIL-2 therapy alone (OR, 2.79, 95 %, CI, 1.62–4.80), and that patients that developed tumor-specific immune responses postvaccination exhibited improved OS (OR, 2.15; 95 %, CI, 1.88–2.44).

Potential pretreatment serum/blood biomarkers (see also Chap. 23) in patients that are most likely to receive greatest clinical benefit from RCCAA-based vaccines have been suggested to include: (1) normal mean corpuscular hemoglobin concentration (MCHC; an indicator of underlying inflammatory state and tumor burden) [184], (2) elevated serum concentration of APOA1 (a major protein component of high-density lipoprotein, with low APOA1 levels associated with chronic inflammation and suppressed adaptive immunity) [64], and (3) elevated serum concentration of CCL17 (a chemokine made by myeloid DC that promotes T-cell infiltration into tumors) [64]. The latter two indices were reported to be most predictive in a combined vaccination protocol that included patient preconditioning with CY [64]. It has also suggested that CXCL10, a chemokine responsible for recruiting Type 1 $CXCR3^+$ TIL, may be detected at elevated levels in the pretreatment serum of patients with RCC who are more likely to develop OCR in response to DC-based vaccination, although this association did not reach statistical significance based on the small number of patients evaluated in this trial ($n=8$; $p=0.07$) [190]. These results could suggest that patients exhibiting elevation in their baseline serum levels of TIL-recruiting chemokines may be most receptive to the subsequent trafficking of therapeutic T cell-induced postvaccination into the RCC tumor microenvironment [190, 191].

Immune Deviation in RCC: MDSC, Suppressive DC, and Treg

Multiple mechanisms appear to be involved in downregulating T-cell responses against RCCAA, with suppressor cell populations such as myeloid-derived suppressor cells (MDSC) and Treg playing significant roles in this process.

MDSC are a heterogeneous population of immature myeloid cells, containing subpopulations bearing a range of phenotypes associated with granulocytes (neutrophils, PMN-MDSC), monocytes (M-MDSC), as well as dendritic cells [192]. MDSC suppress T-cell immunity via a variety of mechanisms including their expression of arginase-1 (an enzyme that reduces levels of arginine, an amino acid that is critical for TCR-ζ chain expression and T-cell activation and proliferation) [193]. MDSC can similarly consume and sequester L-cysteine leading to T-cell hyporesponsiveness to antigenic (i.e., RCCAA) stimulation [194]. Reactive oxygen species (ROS) produced by MDSC can be generated as a consequence of either L-arginine metabolism or by the activation of NADH oxidases, such as NOX2 in MDSC [195, 196], resulting in significant immune suppression. In murine tumor models, NADPH oxidase is overexpressed in a STAT3-dependent manner which,

upon specific knockdown using siRNA-based approaches, resulted in the negation of the suppressive activity of MDSC [197]. Likewise, increased ROS production was observed in MDSC isolated from melanoma patients in association with increased levels of oxidative stress and activated STAT3 [198]. Inducible nitric oxide synthase (iNOS; NOS2) produced by MDSC converts L-arginine into citrulline and generates NO as a by-product, which in the presence of ROS is converted to the (immmuno)suppressive radical peroxynitrate [199]. The nitration or nitrosylation of the TCR promotes T-cell anergy in tumor-bearing mice [199] and fosters the loss of cytotoxic activity, proliferation, and IFN-γ production in T cells from patients with RCC [197, 200–203]. Additionally, peroxynitrite produced by MDSC can modify the chemokine CCL2 which hinders the migration of $CD8^+$ T effector cells into the tumor [201]. MDSC may also alter the trafficking of T cells by reducing the expression of the selectin CD62L on the surface of $CD4^+$ and $CD8^+$ T lymphocytes thereby hindering T-cell recirculation to lymph nodes which may limit their ability to be primed and boosted in vaccine site-draining lymph nodes [204]. Furthermore, MDSC are known to promote the expansion of antigen-specific Treg cells and to promote the direct conversion of naïve T cells into Treg cells [205, 206], in addition to promoting angiogenesis in the TME in a STAT3-dependent manner [207, 208].

Aberrant expansion of MDSC in the tumor-bearing host is supported by multiple cytokines including granulocyte colony-stimulating factor (G-CSF), granulocyte–macrophage colony-stimulating factor (GM-CSF), interleukin 6 (IL-6), vascular endothelial growth factor (VEGF), S100 proteins, interleukin 3 (IL-3), and stem cell factor (SCF) [192]. GM-CSF alone, or in combination with G-CSF or IL-6, induces MDSC development from bone marrow precursors in vitro [209–211]. Interestingly, while GM-CSF is required as an adjuvant in many vaccine studies and can promote T-cell responses based on its positive influence on immunostimulatory DC, under certain conditions, GM-CSF can also result in increased MDSC numbers after vaccination in melanoma patients and to associated immunologic unresponsiveness [212]. Others have suggested that while low doses of GM-CSF promote specific T-cell-mediated immunity, higher doses of this cytokine impair vaccine efficacy [213]. While GM-CSF and other cytokines promote the expansion of MDSC in the TME, select products appear to enhance the functional activation of MDSC, including IFN-γ, COX2, IL-4, IL-13, and TGF-β [211]. In fact, these products of activated T cells may actually be necessary for MDSC to proliferate and retain their immunosuppressive function (in an apparent immune-regulatory feedback loop) [211]. Their production also blocks myeloid cell maturation leading to an accumulation of immature myeloid cells including dysfunctional DC [214]. Under the influence of hypoxia in the TME, DC can become suppressive as they may acquire expression of the adenosine receptor (A2B) [192, 215] which mediates polarized Type 2 versus protective Type 1 T-cell responses. Excess adenosine produced in tumors can also stimulate DC to produce proangiogenic proteins (i.e., IL-6, VEGF, IL-8) and to boost production of suppressive mediators (i.e., IDO, COX2, and IL-10) [216]. The TME can also condition DC to express arginase and/or IDO (indoleamine 2,3-dioxygenase), leading to the catalysis of L-arginine and L-tryptophan, respectively, and to T-cell hyporesponsivness [217, 218].

In cancer patients, several subsets of MDSC have been discriminated based on immunostaining and FACS analysis [219] including granulocytic (PMN; $CD33^{low+}HLADR^{neg}CD15^{+}CD14^{neg}$), monocytic ($CD33^{low+}HLADR^{neg}CD15^{+}CD14^{neg}$), and lineage negative ($CD33^{low+}HLADR^{neg}CD15^{neg}CD14^{neg}$). Interestingly, in RCC patients [202, 203, 220, 221] and certain other tumors (brain, lung, head and neck, and pancreatic cancer) [64, 222–224], PMN-MDSC are the dominant population. In RCC patients, PMN-MDSC appear to be composed of activated neutrophils with arginase-promoting suppressive activity [202, 203, 225] as well as immature neutrophils ($CD33^{low+}HLA\text{-}DR^{neg}CD66b^{+}$) that minimally express CD16 found on mature neutrophils [226]. The importance of MDSC in promoting tumor growth is suggested by the observation that increased baseline levels of M-MDSC and PMN-MDSC in advanced-stage RCC patients correlate with poor overall survival [64].

Treg are typically defined as $CD4^{+}$ T cells that express high levels of the IL-2Rα chain (CD25) and the transcription factor forkhead box p3 (FoxP3) which appears essential for their development and maintenance of immunosuppressive activity [226]. Mutation or deletion of FoxP3 impairs Treg development, which can result in severe autoimmune disease [227]. The two main types of Treg include natural Tregs (nTreg) that develop in the thymus and which can suppress the proliferation of T effector cells in a contact-dependent fashion as a foundation for peripheral tolerance mechanisms [226, 228]. Treg cells can also evolve in the periphery in response to antigenic stimulation [226, 229]. Additional proteins expressed by Treg cells have been associated with their suppressive activity, including GITR (glucocorticoid-induced TNF receptor) [230–232], CTLA-4 (a co-stimulatory molecule with negative impact on T-cell activation) [230–233], IL-10 and TGF-β [229], and granzyme-B [234, 235]. Treg cells can also promote T-cell suppression based on the expression and activity of CD39 (i.e., ectonucleoside triphosphate diphosphohydrolase 1) and CD73 (i.e., ecto-5′-nucleotidase), which degrade ATP and cause elevated levels of extracellular adenosine that ultimately inhibits the function of T effector cells expressing the A2A receptor [236, 237].

In addition to classic $FoxP3^{+}$ Treg cells, alternate immune-suppressive $FoxP3^{neg}$ T cells exist, including regulatory Type 1 (Tr1) and T helper 3 (Th3) cells. Tr1 are $CD4^{+}$ T cells found in the peripheral blood that expand in response to self-antigens in the presence of IL-10 and promote T effector cell suppression via a mechanism involving IL-10 and TGF-β [238]. Th3 cells also produce TGF-β which can inhibit DC maturation and Type 1 immune responses in the solid cancer setting [239].

Treg cells appear to play an important role in promoting T-cell immune suppression resulting in RCC progression. Frequencies of Treg are elevated in the peripheral blood of patients with RCC versus age-matched normal healthy donors [240–247], with many but not all studies demonstrating a correlation between increased numbers of peripheral blood Tregs and reduced disease-free survival [242, 244, 246]. Elevated levels of Treg in the TME (versus peripheral blood of the same patient) has also been observed in many RCC studies [242, 244, 246, 248], and in one study, Treg numbers in the tumor were predictive of shorter overall survival [248]. Other studies provide support for a positive correlation between the levels of $FoxP3^{neg}CD4^{+}$ Treg cells (i.e., Tr1 or Th3) and poor clinical outcome in patients with localized RCC [247].

Potential Agents for Use in Combination Vaccines for RCC

Given the expanding nucleus of clinical observations in support of the immunogenicity for most vaccine formulations applied to patients with RCC, albeit with only modest antitumor benefits being noted, one is left to consider how best to improve the efficacy of such approaches. Simple consideration of including increasingly stimulatory adjuvants in vaccine compositions may enhance the frequency of circulating anti-RCC T cells in patients, as well as the overall immunologic response rates monitored in clinical trials. However, one must also consider the possibility that current vaccines are limited in their capacity to promote the effective recruitment of vaccine-induced T cells into tumors and to bolster the survival and antitumor function of T effector cells once they have arrived in the RCC microenvironment. In this context, combinational vaccine approaches must be developed and empirically evaluated in randomized phase I/II clinical trials. Co-therapies should include agents (Table 22.5) [220, 221, 241, 249–325] that do not negatively impact the activation and function of therapeutic T cells but which (1) remove or antagonize the immunosuppressive influence of Treg and MDSC, (2) promote the improved recruitment of vaccine-induced T cells into tumor sites, (3) improve the ability of vaccine-induced T cells to differentially recognize and react against RCC tumor cells versus normal tissue (kidney) cells, (4) protect vaccine-induced T cells from premature death (i.e., apoptosis) or anergy (i.e., hyporesponsiveness), and/or (5) reduce tumor burden (and hence the total number of tumor cells that must be targeted/eradicated by limiting levels of vaccine-induced T effector cells).

The removal of Treg and MDSC suppression at the level of cellular deletion or functional antagonism (Table 22.5) has been intensively investigated in the setting of cancer therapy over the past decade [249, 262, 326, 327]. Preclinical modeling in which Treg can be specifically ablated from tumor-bearing animals based on the genetic susceptibility of this regulatory cell subset to systemic administration of diphtheria toxin (DT) clearly supports the effectiveness of this strategy in promoting the recruitment of antitumor $CD8^+$ TIL and superior therapeutic efficacy, particularly when combined with active vaccination against tumor-associated antigens [326]. Since Treg cells express high levels of the IL-25Rα (CD25), a range of targeted approaches using CD25-depletional strategies have also been extensively studied, including control and toxin-conjugated (DT; denileukin diftitox; ONTAK) anti-CD25 Abs [249–252]. These agents have shown variable antitumor impact, with some agents promoting strong antitumor effects in vivo and others actually blunting protective immunity based on their negative effects on $CD25^{dim+}$ T effector cells [328, 329]. Importantly, Dannull et al. report the enhancement of vaccine-mediated antitumor immunity in patients with metastatic RCC after depletion of regulatory T cells using ONTAK [249].

Treg cells also preferentially express high levels of the cell surface molecule cytotoxic T-lymphocyte antigen-4 (CTLA-4; aka CD152) that mediates a negative signal into antitumor T effector cells. While early development of CTLA-4 targeting strategies was based on the supposition that these agents would reduce Treg numbers or their functional capacity to suppress protective immunity [330], instead,

Table 22.5 Co-therapeutic agents for use in combination vaccines against RCC based on complementary mechanism(s) of action

Co-therapy agent	Deletion or antagonism of Treg/MDSC	Enhanced TIL recruitment	Enhanced T-cell recognition of target cells	Protection of T cell vitality and/or function	Reduction in tumor burden (target number)	References
ONTAK	●					[249]
Anti-CD25	●					[250–252]
TKI (sunitinib, axitinib, others)	●	●		●	●	[220, 221, 241, 253–261]
Gemcitabine, 5-FU	●				●	[262–266]
ATRA, vitamin D	●			●		[262, 267–269]
Cox-2i, PDE-5i	●	●			●	[262, 270–274]
STAT3i	●			●	●	[220, 258–260, 262, 275, 276]
Chemokines (CXCR3 ligands + others)	●	●			●	[271, 277–280]
IDOi (1-MT)	●					[262]
HDACi[a], 5-aza-2′-deoxycytidine		●	●		●	[281–288]
mTORi[a]		●		●	●	[289–291]
Interferons (IFN)		●	●	●	●	[190, 292–295]
Bortezomib[a]/PS-341			●		●	[296–301]
HSP90i	●	●	●		●	[302–304]
Anti-CTLA-4				●		[305–316]
Anti-PD-1/Anti-PD-L1				●		[305, 309, 317–319]
Anti-Tim3				●		[318–320]
Anti-BTLA				●		[320]
Radiotherapy		●	●		●	[321–325]

[a]May increase frequencies of MDSC and/or Treg in treated patients

Abbreviations: *5-FU* 5-fluorouracil, *ATRA* all-*trans* retinoic acid, *BTLA* B- and T-lymphocyte attenuator, *CTL4* cytotoxic T-lymphocyte antigen-4, *HSP* heat shock protein, *PD-1* programmed cell death-1, *STAT3* signal transducer and activator of transcription-3, *Tim3* T-cell immunoglobulin mucin-3, *TKI* tyrosine kinase inhibitor

it is now believed that such agents limit the ability of T effector cells to be counter-regulated by CTLA-4-mediated signals leading to a robust (uncurbed) inflammatory response in the cancer setting [305–316]. Indeed, the greatest degree of antitumor efficacy for CTLA-4 blockade strategies occurs in patients with significant autoimmune sequelae including enterocolitis, hepatitis, dermatitis, hypophysitis, and even immune-related renal failure [354–357]. For instance, RCC patients treated with CTLA-4 blockade with ipilimumab (MDX-010) exhibit an overall OCR of 14 %, but among those patients that develop enterocolitis, the OCR rate is 35 %, versus only 2 % in patients that fail to exhibit autoimmune phenomenon [305]. Based on substantial preclinical and clinical modeling, CTLA-4 blockade would be anticipated to lead to the superior immunogenicity and antitumor efficacy of vaccines in the RCC setting [309–315].

MDSC or non-Type 1 polarized populations of tumor-associated macrophages (TAM) also act as potent suppressors of antitumor T effector cells and may be antagonized by therapeutic strategies that directly target these cell subsets or that alter the tumor microenvironment in a manner that indirectly leads to their conditional removal. In many cases, such interventions may lead to the differentiation of these immature myeloid cell populations into cells that fail to exert immunosuppressive qualities and may even serve as stimulatory antigen-presenting cells in vivo [262] (Table 22.5). For instance, the deletion of TAM via administration of neutralizing antibodies reactive against colony-stimulating factor-1 (CSF1) or its receptor (CSF1R) removes immunosuppression and promotes improved antitumor $CD8^+$ T-cell responses [327]. Furthermore, antagonism of the chemokine receptor CCR2 expressed by TAM/MDSC limits their recruitment and suppressive influence within the tumor microenvironment [327]. Functional inhibitors of TAM/MDSC suppressor function include IDO inhibitors (i.e., 1-methylytryptophan; 1-MT [262]) and STAT3 inhibitors (such as sunitinib among others) [221, 253, 258, 260, 262] (also see Chaps. 8–10). TKI such as sunitinib (Chap. 8) and axitinib (Chap. 10) have demonstrated superior adjuvanticity when combined with cancer vaccines in preclinical modeling [253, 258, 331], with sunitinib currently being assessed in a phase III clinical trial in RCC patients receiving a combination vaccine formulation (i.e., IM-901; Table 22.4).

Once activated by specific vaccination, anti-RCC T cells have been readily detected in the peripheral blood circulation of many treated patients (Tables 22.1, 22.2, 22.3, and 22.4). However, these potentially therapeutic T cells are clinically meaningless unless they can be recruited in sizeable numbers into the RCC tumor microenvironment. Such T-lymphocyte recruitment into RCC tissue is driven by chemokine receptors (i.e., CXCR3, CXCR6, CCR5, and CCR6) expressed by vaccine-induced T cells that respond chemotactically to locoregional production of their corresponding chemokine ligands (i.e., CXCL9–CXCL11, CXCL16, CCL3–5, and CCL20, respectively) within the TME [332]. Although sparingly generated in the untreated TME, when chemokines such as CXCL10/IP-10 are detected at high levels in serum, this may be predictive of beneficial outcome in RCC patients receiving therapeutic vaccination [190]. Furthermore, production of many T-cell-recruiting chemokines may be conditionally upregulated within tumors as a consequence of

cytokine or chemokine gene-based therapy [333, 334] or via pharmacologic agents such as the TKI sunitinib or axitinib [258, 331], HDAC inhibitors [282], and mTOR kinase inhibitors [290], in addition to chemotherapy or localized radiotherapy [321, 325, 335]. As such, specific vaccination in advance of activating the conditional recruitment of antitumor T effector cells into the TME would be expected to provide greater clinical efficacy than vaccination alone.

Once vaccine-induced T effector cells are recruited into the TME, they must recognize their cognate targets (i.e., tumor cells or tumor-associated stromal cells) in order to mediate therapeutic benefits. Therefore, combination therapies that allow T cells to better recognize cognate MHC–peptide complexes expressed by target cells might represent an attractive strategy to improve RCC patient outcomes. Such modulation in tumor antigenicity can be envisioned under pharmacologic conditions that promote upregulation in MHC class I or class II expression (i.e., treatment with Type I or Type II interferons [190, 292–295]) or that facilitate the preferential processing and loading of MHC complexes with target antigen-derived peptides (i.e., treatment with proteasome inhibitors such as bortezomib/PS-341 [296–298], HSP90 inhibitors [303, 304], histone modulators [281–286], or radiotherapy [322–325]). Given known co-therapeutic agent toxicities, such approaches would be arguably best applied on a metronomic schedule after vaccination to first expand circulating levels of anti-RCC T cells in patients receiving combination vaccines.

Finally, once having recognized a clinically relevant target cell in the TME, effector T cells must recycle and sustain their functionality over as long a period as is possible to provide optimal protection. Means by which to protect vaccine-induced T cells from premature death (i.e., activation-induced cell death) or anergy (i.e., hyporesponsiveness) would be expected to reinforce the therapeutic benefits associated with combination vaccine strategies. In this regard, antagonists of co-inhibitory/death receptors such as programmed cell death-1 (PD-1; see also Chap. 23), T-cell immunoglobulin mucin-3 (Tim-3), and B- and T-lymphocyte attenuator (BTLA) have become highly competitive areas of translational research over the past several years [305, 309, 317–320]. Of these, clinical use of PD-1-blockade therapies (i.e., anti-PD-L1) has resulted in OCR in multiple disease indications, including RCC, where sustained occupancy of T-cell-expressed PD-1 molecules could be demonstrated for ≥2 months in association with antitumor efficacy (i.e., durable OCR of 27 % (9/33) for RCC patients) [317, 336]. Anti-PD-L1-based therapy yielded durable OCR of 6–17 % and prolonged stabilization of disease rates of 12–41 % in patients with advanced cancers, including RCC [317]. Very recent translational studies in colon and ovarian carcinoma models suggest that dual blockade of PD-1 and CTLA-4 enhances the therapeutic efficacy of specific vaccination by coordinately sustaining antitumor T effector function [309].

In addition to optimizing the activation, delivery, and sustained function of vaccine-induced T cells as a means to improve the clinical efficacy of combination approaches, the shear bulk of disease should be reduced in order to limit the absolute number of targets that the adaptive immune response must address. The debulking regimen chosen for inclusion in combination immunotherapy designs must simultaneously promote reductions in tumor size and either have a neutral impact

on the immune system or preferably, immunostimulatory effects. In addition to certain chemotherapeutic agents and localized radiotherapy, a broad range of antiangiogenic agents (see also Chaps. 8–11), including anti-VEGF antibodies (i.e., bevacizumab/Avastin), VEGF trap (i.e., aflibercept), and TKIs, conform to these requirements [337–339] (Table 22.5).

Furthermore since each of these combination agents impacts one or more, but not all, of the biologic endpoints thought to be required to optimize anti-RCC vaccine efficacy (Table 22.5), additional combinations of these "conditioning" regimens should be considered in order to provide synergistic benefits (without a concomitant increase in toxicity).

Summary and Future Perspectives

Over the past several decades, we have gained a better understanding of how adaptive immunity is generated and sustained in the setting of chronic diseases, such as cancer. Vaccine formulations have been translationally developed in the laboratory and empirically evaluated in exploratory phase I trials, with a number of strategies advancing to phase II and III trials. Regardless of the antigenic format employed in the vaccines, a consensus can be reached that these approaches have proven to be safe and well tolerated by patients and that they frequently activate specific T-cell-mediated immunity, with a minor subset of these cases exhibiting durable OCR. It is also clear that RCC vaccines as a single modality cannot provide optimal antitumor benefit in the clinic and that combinational vaccine strategies must be pursued to overcome existing immune dysfunction in RCC patients and to further optimize antitumor T-cell functionality, directionality (i.e., effective recruitment into the TME), and sustainability. In achieving these goals, there is every confidence that the frequency of patients developing long-term OCR can be improved, in association with the maintenance of quality of life and extended overall survival.

Combinational approaches integrating agents that challenge the boundaries of autoimmune pathology (i.e., anti-CTLA-4, anti-PD-1) appear to hold great promise in the face of moderate safety concerns. Application of vaccines commensurate or after a range of "conditioning" regimens that antagonize immune suppression networks (Treg, MDSC) while enhancing the recruitment (into the TME) and antitumor function of vaccine-induced T cells is arguably the most conceptually attractive protocols for prospective investigation.

Therapeutic vaccines for the treatment of RCC are expected to continue to evolve over the next decades based on a more comprehensive view of the cellular constituents of the TME, each of which may be targeted via vaccine strategies. We and others have recently developed vaccines capable of eliciting therapeutic $CD8^+$ (cytotoxic) T cells reactive against antigens expressed by cells that compose tumor blood vessels (i.e., vascular endothelial cells and pericytes) [340–342]. Of note, such vaccines have proven curative in preclinical tumor modeling in cases where tumor cells cannot serve as direct targets for vaccine-induced T cells [341, 342].

One can also readily consider the design of therapeutic vaccines targeting additional stromal cell populations within the TME that exhibit unique (tumor-conditioned) antigen profiles, including fibroblasts, myofibroblasts, tumor-associated macrophages, and a range of "stem cell"-like populations (i.e., tumor stem cells or mesenchymal stem cells) [343–352]. Such non-tumor, host stromal cell populations lack the inherent genetic instability of cancer cells that is associated with immune evasion/escape, which should make them less of a "moving target" for therapeutic regulation by vaccine-induced T effector cells.

Furthermore, while still somewhat anecdotal, where it has been evaluated in preclinical tumor models and clinical trials, the therapeutic broadening of the antitumor T-cell repertoire over time postvaccination appears predictive of superior treatment benefit [162, 172, 353–356]. Such evolving diversification of T-cell responses is a classic paradigm in the setting of pathologic autoimmune diseases where it is referred to as "epitope/determinant spreading" [357, 358]; however, in the cancer setting, this "manifestation of autoimmunity" may be a clinically preferred endpoint. Since such immunity develops over extended periods of time (during which reiterated cycles of immune attack, tumor cell death, and corollary cross priming of "second-set" antitumor T-cell repertoires are generated), the efficacy of clinical vaccines may be improved with the adoption of chronic administration schedules (versus the typical abbreviated regimens in phase I/II trial designs). For instance, in clinical trials of NY-ESO-1 peptide-based vaccines, the majority of immunologically responsive melanoma patients developed late onset but durable tumor antigen-specific $CD8^+$ T-cell responses after 4 months of repeated immunization [359]. Furthermore, chronic booster vaccination in tumor models has been reported to longitudinally develop antitumor $CD8^+$ T cells of increasing functional avidity and antitumor efficacy [360]. Hence, while it is anticipated a cohort of RCC patients will acutely respond to specific (combination) vaccination, there may also be a substantial cohort of patients that can ultimately be coerced to respond to such intervention only after a protracted period of time, leading to higher overall rates of durable OCR postvaccination.

In closing, it should be appreciated that we have tremendously expanded our understanding of how tumors and the immune system coevolve in cancer patients (i.e., RCC) and how conditional restraints placed on the antitumor immune response may be removed via specific stimulation (via vaccination) and strategic alteration of the TME to recruit, enhance, and sustain the therapeutic efficacy of T effector cells (via coadministered agents such as those described in Table 22.5). There is great promise for improving rates of durable OCR in RCC patients treated with combination vaccine approaches, although patience must be exercised given the vast number of possible combinations that can already be envisioned for evaluation in randomized protocol designs. Given advances in defining biomarkers associated with patient responsiveness to combined vaccination approaches (i.e., basal levels of T cell-recruiting chemokines in serum, presence or absence of PD-L1 expression by RCC cells pretreatment, etc.), there is great expectation that these evolving treatments may be optimized for safety and efficacy in the most biologically relevant patient cohorts.

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Chapter 23
Development and Incorporation of Biomarkers in RCC Therapeutics

Sumanta K. Pal and Robert A. Figlin

Introduction

In 1992, interleukin-2 (IL-2) was approved for the treatment of metastatic renal cell carcinoma [1]. While this therapy represented a much needed option for patients, unfortunately, only a small proportion of individuals derived benefit from the therapy [2, 3]. Durable rates of response to IL-2 range from 5 to 10 %, implying that the vast majority of patients who receive the treatment will progress [2]. Furthermore, cardiopulmonary toxicity from IL-2 (which can be fatal) further compromises the risk-benefit profile of the agent. Several retrospective reports in the literature suggested that certain clinicopathologic features (i.e., sites of metastasis, MSKCC score, etc.) and biomarkers (i.e., carbonic anhydrase IX [CAIX]) might predict response to IL-2 [4, 5]. Such a study would have been transformative—understanding which patients had optimal outcomes with IL-2 would spare other patients the toxicities of the agent. To validate several hypotheses related to predictive factors for IL-2 response, the SELECT study was launched [6]. This trial included a total of 126 patients with newly diagnosed mRCC—ultimately, neither clinical features (MSKCC score, UCLA risk group) nor pathologic features (clear cell versus non-clear cell histology, CAIX score) were associated with IL-2 response.

Without predictive factors derived from SELECT, the application of IL-2 remains somewhat nonuniform—there are no firmly established criteria to decipher appropriate candidates for this therapy. This clinical dilemma has only been magnified in

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recent years with the advent of multiple-targeted therapies. Over the past decade, a total of seven targeted agents have gained FDA approval for use in mRCC [7]. These agents all have antiangiogenic properties and function through either (1) inhibition of vascular endothelial growth factor (VEGF)-mediating signaling (bevacizumab, sunitinib, sorafenib, pazopanib, and axitinib) or (2) mammalian target of rapamycin (mTOR) signaling (temsirolimus and everolimus) [8–14]. Current clinical guidelines proposed by groups such as the National Comprehensive Cancer Network (NCCN) suggest that any number of agents (either pazopanib, sunitinib, or bevacizumab with interferon-α [IFN-α]) can be used for patients with previously untreated clear cell mRCC with good- or intermediate-risk disease (by MSKCC criteria) [15]. In the second-line setting (after failure of VEGF-directed therapy), either axitinib or everolimus are recommended. Finally, beyond second-line treatment, patients may be offered what is colloquially termed a "dealer's choice" of treatment. The NCCN and similar guideline panels offer no preference for specific therapeutic options, resulting in equipoise in virtually every therapeutic scenario. Thus, much like the dilemma surrounding IL-2, clinicians must utilize their own subjective assessment of each agent when counseling patients.

Also mirroring the development of IL-2, there are multiple retrospective experiences that report putative biomarkers that may predict response or clinical outcome with specific-targeted therapies. In the current review, the most promising biomarkers amongst these are reviewed. Furthermore, a framework for developing these biomarkers for routine clinical use is discussed (Table 23.1).

Table 23.1 Relevant biomarkers in RCC and status of clinical evaluation

Biomarker	Agent	Assessment
CAF profile	Pazopanib Sorafenib	• Low osteopontin or VEGF predict superior PFS with sorafenib + IFN-α versus sorafenib alone • Low HGF, IL-6, and IL-8 predict increased tumor shrinkage with pazopanib • A 6 CAF panel (including IL-6, IL-8, osteopontin, VEGF, TIMP-1, and HGF) correlates with PFS and OS
SNPs in VEGF and related mediators	Bevacizumab Pazopanib	• SNPs in *VEGFR1* predicted PFS with bevacizumab • SNPS in *VEGFA* predict response with pazopanib • SNPs in *HIF1A*, *IL8*, and *NR1I2* also predict clinical outcome with pazopanib
Expression of VEGF and related mediators	Axitinib Sunitinib Sorafenib	• Decrease in sVEGFR2 associated with higher response rate with axitinib • Increase in $CD45^{dim}VEGFR2^{+}$-expressing cells in the circulation associated with improved PFS and OS with sorafenib and sunitinib
BRAF mutation	Sorafenib	• Mutations in BRAF (V600E, K601E) associated with inferior PFS with sorafenib
PD-L1	Nivolumab	• Response to nivolumab limited to patients with PD-L1 expression
LDH	Temsirolimus	• Temsirolimus (as compared to IFN-α) significantly prolong survival in patients with elevated LDH
Cholesterol	Temsirolimus	• Increases in cholesterol during temsirolimus associated with improved OS

Cytokine and Angiogenic Factor (CAF) Signatures

It is now well-documented that beyond their antiangiogenic effect, VEGF-directed therapies may have complex effects on the immune milieu that augment their anticancer activity. These effects may be distinct amongst VEGF-directed therapies—for instance, Xin et al. examined Renca tumor-bearing mice treated with sunitinib [16]. In this model, sunitinib was shown to decrease accumulation of myeloid-derived suppressor cells (MDSCs) and regulatory T cells (Tregs) in tumor tissue via STAT3-dependent mechanisms. STAT3 inhibition by sunitinib was shown to occur before any effects were seen on tumor vasculature. In a separate study, sorafenib was found to have a very different effect on immune function. Hipp et al. assessed C57BL/6 mice treated with both sorafenib and sunitinib [17]. In comparison to sunitinib, sorafenib treatment reduced the ability of antigen-presenting cells (APCs) to stimulate lymphocyte production. Furthermore, sorafenib treatment was found to inhibit $CD8^+$ T-cell responses. These studies suggest that the immune effect of each VEGF-directed agent may be distinct.

Zurita et al. performed a CAF profiling analysis in 69 patients with mRCC enrolled in a randomized study of sorafenib alone or sorafenib with IFN-α [18]. CAFs were assessed at baseline, at 28 days, and at 56 days. The CAF profile assessed was broad—54 moieties were assessed using the Bio-Plex 200 system, including 4 moieties assessed through a customized panel (matrix metalloproteinase-9 [MMP-9], E-selectin, epidermal growth factor [EGF], and transforming growth factor-α [TGF-α]). Four additional moieties (osteopontin, CAIX, VEGF-A, and soluble VEGFR2) were assessed using enzyme-linked immunosorbent assay (ELISA). On univariate analyses, 14 of these elements demonstrated an association with progression-free survival (PFS). However, on multivariate analysis, only three showed independent prognostic value (IL-5, M-CSF, and EGF).

Notably, the parent study had previously shown no difference in PFS with either treatment arm (7.39 months with sorafenib versus 7.56 months with sorafenib plus IFN-α; $P = \text{NS}$) [19]. The investigators thus explored CAFs that might differentiate the clinical benefit derived from either treatment [18]. In this effort, an association between osteopontin and VEGF was uncovered—low expression of either biomarker predicted superior PFS with sorafenib plus IFN-α as compared to sorafenib alone. Hierarchical clustering analyses were subsequently used to define a more comprehensive predictive model—this model included osteopontin, soluble CAIX, VEGF, TRAIL, collagen V, and soluble VEGFR2.

A similar approach has been used to classify a CAF profile associated with response and clinical outcome with pazopanib, both in the setting of non-small cell lung cancer (NSCLC) and mRCC. In NSCLC, 33 patients with early-stage disease received 6 weeks of pazopanib preceding surgery. The primary endpoint of the study was volumetric tumor reduction (assessed by high-resolution computerized tomography [HRCT]). CAFs were assessed at baseline and at 6 weeks of therapy. Ultimately, tumor shrinkage was found to be associated with either increases in soluble VEGFR2 or decreases in IL-4.

The largest evaluation of CAF profile (in the context of antiangiogenic therapy) was performed in association with the phase II and III studies of pazopanib in mRCC [8, 20, 21]. The phase II experience, initially designed as a randomized discontinuation trial (RDT) in either treatment-naïve patients or patients with prior bevacizumab or cytokine exposure, served as a testing cohort. Plasma samples were collected at baseline from 129 patients, of whom 64 had substantial tumor shrinkage and 65 had tumor growth or limited response. Of 17 CAFs assessed in this group, low levels of HGF, IL-6, and IL-8 correlated with the greatest degree of tumor shrinkage ($P < 0.05$ for each).

A total of 344 baseline plasma specimens were available from the phase III evaluation of pazopanib. In this larger experience, 7 CAFs were explored for associations with PFS and response, including IL-6, IL-8, osteopontin, VEGF, HGF, TIMP-1, and E-selectin. IL-6 was found to independently predict PFS benefit; patients with higher baseline IL-6 levels appeared to derive greater benefit from pazopanib compared with placebo (as compared to patients with low baseline IL-6). A hierarchical clustering analysis revealed a panel of six CAFs (including IL-6, IL-8, osteopontin, VEGF, TIMP-1, and HGF) that correlated with PFS and OS. Patients with high levels of these CAFs had a poorer prognosis based on these two endpoints but appeared to derive greater benefit from pazopanib therapy. Currently, the utility of this biomarker panel is somewhat limited, as it cannot be applied to differentiate the relative benefit of selected TKIs. Further prospective assessment may be necessary to utilize this as a decision-making tool.

VEGF and Related Mediators

Presumably, subtle variations in the VEGF pathway could result in distinct activity of VEGF-directed therapies. Several studies have explored single-nucleotide polymorphisms of moieties along the VEGF pathway. In one such effort, Lambrechts et al. assessed serum DNA collected from patients in two phase III studies assessing bevacizumab [22]. The first study, the AViTA trial, compared gemcitabine/erlotinib/bevacizumab to gemcitabine/erlotinib/placebo [23]. The second study, AVOREN, compared bevacizumab/IFN-α to bevacizumab/placebo [24]. Exploration of a total of 138 SNPs revealed variants in *VEGFR1* that were predictive of PFS and OS benefit with bevacizumab in the AViTA. Specifically, an SNP at rs7993418 (located in the tyrosine kinase domain of VEGFR1) was implicated. This SNP was also predictive of PFS in the AVOREN study (HR 1.81, 95 % CI 1.08–3.05, $P = 0.033$). Notably, outside of its role in angiogenesis, preclinical studies have implicated the role of VEGFR1 in formation of the "pre-metastatic niche," a feature of the benign tissue microenvironment that may serve as a harbinger for metastasis [25, 26]. It is possible that this alternative role of VEGFR1 may be associated with its predictive value.

SNPs in VEGF and related mediators have also been explored in the context of pazopanib therapy. A panel of 27 SNPs housed within 13 genes was assessed in a total of 397 patients treated with pazopanib across three clinical trials [27]. In this

study, response rate was reduced in patients with the *VEGFA* 1498CC genotype as compared to the 1498TT genotype (33 % versus 51 %). Along the VEGF signaling axis, several other key SNPs were recognized. In particular, both PFS and response rate were inferior in patients with the *HIF1A* 1790AG genotype as compared to wild type. Other SNPs predictive of clinical outcome were found in *IL-8* and *NR1I2*. Importantly, IL-8 has been previously implicated as a potential mediator of resistance to sunitinib [28].

As opposed to genetic aberrations in VEGF, levels of VEGF and downstream moieties also have a purported prognostic and predictive role. In a phase II study, 64 patients with cytokine-refractory mRCC were treated with axitinib 5 mg oral twice daily [29]. Decreases in soluble VEGFR2 (sVEGFR2) were associated with a higher response rate (64.75 % versus 37.5 %, $P=0.045$) and longer PFS (12.9 months versus 9.2 months, $P=0.01$). Surface expression of VEGFR2 on bone marrow progenitor cells has been characterized in a separate study. In this effort, 55 patients with mRCC receiving either sunitinib or sorafenib were assessed for $CD45^{dim}VEGFR2^{+}$ cells at baseline and at day 14 [30]. Elevated levels of this subset at baseline was associated with an increased risk of progression (HR 2.5, $P=0.001$). Surprisingly, increases in this subset were associated with both improved PFS and OS.

BRAF

Initially, the development of sorafenib was based (at least in part) on abrogation of RAF kinase activity [31]. With a broader examination of the agent in preclinical models of RCC, it is thought that the anticancer effect is mediated mainly through VEGF signaling [32]. A dose escalation study by Manusco et al. included patients with mRCC that had progression on a prior VEGF-TKI [33]. Patients were started at the standard dose of 400 mg twice daily but escalated to 600 mg twice daily in the absence of toxicity at 12 weeks. Interestingly, 3 of 19 patients (15.7 %) demonstrated BRAF mutation at V600E, and 1 patient demonstrated BRAF mutation at K601E. PFS was significantly shorter amongst those patients bearing mutation in BRAF (2.5 months with mutations versus 9.1 months with wild-type BRAF; $P<0.05$). These data may spark renewed interest in further examining the prognostic role of BRAF mutation in the setting of sorafenib therapy. Furthermore, there may be rationale for examining BRAF-directed therapies (i.e., vemurafenib) in selected patients with mRCC [34, 35].

Programmed Death-Ligand 1 (PD-L1)

The interaction of programmed death-1 (PD-1) on the activated T cell with PD-L1 induces T-cell anergy [36]. As such, efforts have been made to abrogate this interaction, and thereby preserve the antitumor immune response. Although many PD-1

inhibitors are in various phases of clinical testing, there is a great deal of enthusiasm surrounding the monoclonal antibody BMS-936558. In a phase I study including 296 patients with a wide array of tumor types, the responses were seen in patients with melanoma, NSCLC, and mRCC [37]. Of 33 patients with mRCC, 9 patients (27 %) demonstrated objective responses. Responses encountered to therapy were largely durable, and toxicities were generally mild. Ongoing studies in mRCC seek to characterize synergy between BMS-936558 and approved VEGF-directed therapies (specifically, sunitinib and pazopanib) [38]. Correlative studies accompanying the phase I experience included assessment of formalin-fixed, paraffin-embedded (FFPE) tissue with anti-PD-L1 antibody. Using a threshold of 5 % to define positivity, clinical outcome was compared in cohorts with and without PD-L1 expression. Adequate pretreatment tissue was available from 42 patients (including 5 patients with mRCC). Of the 25 patients with PD-L1 expression, 9 patients (36 %) had a response. In contrast, no responses were seen amongst the 17 patients lacking PD-L1 expression. PD-L1 has been explored independently as a prognostic marker in RCC. Specifically, Krambeck et al. performed immunohistochemical (IHC) analyses in 298 patients with localized RCC for both PD-L1 and survivin (a regulator of apoptosis) [39]. In a multivariate analysis including numerous clinicopathologic features, high PD-L1 and survivin expression were associated with lower disease-specific survival (DSS). To the authors' knowledge, no predictive role for PD-L1 has been established in the context of VEGF- or mTOR-directed therapies.

Lactate Dehydrogenase (LDH)

LDH has previously been incorporated in several prognostic schema for mRCC, including the original MSKCC criteria, the modified MSKCC criteria, and the Mekhail criteria [40–42]. However, the predictive value of this moiety has not been resolved until recently. Armstrong et al. examined pretreatment LDH values in patients treated on the pivotal phase III study comparing temsirolimus, temsirolimus with IFN-α, and IFN-α in patients with poor-risk mRCC. The study included a total of 404 patients—ultimately, on multivariate analysis, it was found that an elevated LDH was independently associated with an increased risk of death (HR 2.81; 95 % CI 2.01–3.94, $P<0.001$). Temsirolimus (as compared to IFN-α) was found to significantly prolong survival in those patients with an elevated LDH (6.9 months versus 4.2 months; $P<0.002$). In contrast, temsirolimus did not prolong OS compared to IFN-α in those patients with a normal LDH ($P=0.514$). In the subset of patients with an elevated LDH and poor-risk disease, this study clarifies the choice of temsirolimus. However, a clinical question that remains is whether patients with poor-risk disease may derive benefit from VEGF-directed therapies—at this point, LDH cannot discern the relative merit of this approach.

Serum Cholesterol

Akin to the evaluation of LDH, the predictive value of serum cholesterol has been assessed in the context of temsirolimus therapy. With specimens derived from the aforementioned phase III experience, associations were sought between increases in serum cholesterol, glucose and triglycerides, and clinical benefit [43]. Notably, all three of these moieties were noted to increase to a greater extent with temsirolimus therapy as compared to therapy with IFN-α. Increases in cholesterol during protocol-based treatment were noted to be associated with improved OS (HR 0.77, $P < 0.0001$) and PFS (HR 0.81, $P < 0.0001$). No predictive value was ascertained for changes in glucose or triglycerides. Relative to LDH, one challenge in further implementation of this biomarker is that it is collected post hoc, i.e., a change in serum cholesterol can only be ascertained after treatment with temsirolimus is started. In contrast, for serum LDH, baseline values alone are predictive.

CAIX

As noted previously, CAIX has been assessed extensively in the context of immunotherapy. However, there have been recent efforts to determine the predictive value of this moiety in the context of targeted therapies. Initially, Choueiri et al. had assessed tissue from a total of 118 patients who had received initial therapy with VEGF-directed agents [44]. In this preliminary study, it was suggested that patients who had received sorafenib therapy with high CAIX values might have a greater degree of tumor shrinkage. Similar findings were not seen amongst patients who had received prior sunitinib therapy. The same investigators then assessed paraffin-embedded tissue obtained from 133 patients who participated in the phase III TARGET study, comparing sorafenib and placebo in a largely cytokine-refractory population [45]. CAIX showed limited prognostic value—PFS was similar amongst patients with low and high CAIX on both the sorafenib arm (5.4 and 5.5 months, respectively; $P = 0.97$) and on the placebo arm (1.5 and 1.7 months, respectively; $P = 0.76$). Comparisons of tumor shrinkage on both arms similarly showed that CAIX had limited predictive value. Imaging modalities based on CAIX have recently been developed for RCC—most recently, the REDECT study assessed the role of 124I-girentuximab in patients with localized clear cell RCC, suggesting both a high specificity and sensitivity [46]. Unfortunately, the collective data from Choueiri et al. suggest that these novel approaches may ultimately yield limited predictive value in patients receiving targeted agents.

Imaging

Since the advent of antiangiogenic strategies, it has been well recognized that traditional radiographic criteria to define progression (i.e., Response Evaluation Criteria in Solid Tumors [RECIST]) may be somewhat misleading. Targeted agents abrogating VEGF and mTOR signaling may ultimately lead to a decrease in tumor size, but this effect is generally preceded by tumor necrosis and cavitation. Kayani et al. have reported the use of ^{18}FDG-PET as a biomarker of response in mRCC [47]. A total of 44 patients with previously untreated mRCC received standard doses of sunitinib, with ^{18}FDG PET-CT obtained at baseline, 4 weeks, and 16 weeks. Amongst 24 patients (54 %) who responded at 4 weeks, no significant improvement in PFS or OS was seen. However, at 16 weeks, disease progression assessed by ^{18}FDG PET-CT (occurring in 12 patients, or 28 %) was associated with both decreased PFS (HR 12.13, 95 % CI 3.72–46.45) and OS (HR 5.96, 95 % CI 2.43–19.02).

Conclusions

As described herein, there are a number of biomarkers being explored in mRCC, ranging from traditional analytes (i.e., LDH and cholesterol) to novel cytokine arrays. Biomarkers have already found their way into prognostic models for mRCC—for instance, the Heng criteria (initially established through a retrospective cohort of 645 patients who received first-line VEGF-directed therapy) has now been externally validated in a cohort of 1,028 patients [48]. This prognostic model omits LDH (an original component of the MSKCC prognostic model) but does include baseline neutrophil and platelet count. Beyond a prognostic role, however, there are multiple challenges that lie ahead for the implementation of biomarkers as a decision tool to guide therapeutic selection. The most recently unveiled issue is tumor heterogeneity. Gerlinger et al. performed an elegant study in four consecutive patients with mRCC treated with everolimus therapy [49]. Multiple samplings were performed of both primary tumor and metastatic sites, and three distinct methods (IHC, mutation functional analysis, and mRNA profiling) were performed to characterize differences amongst the sampled tissues. Notably, both favorable and unfavorable gene expression signatures were identified in specimens derived from the same patient. Although this may be perceived as a hazard to only tissue-based biomarkers, tumor heterogeneity may mar the feasibility of blood-based assays as well. If intra-patient variation is indeed as diverse as this study implies, cytokine profiles and other blood-based assays may reflect changes induced by a subset of tumors.

A second challenge to implementation of biomarkers is related to study design considerations. All of the efforts to characterize biomarkers described herein are retrospective. In order for a biomarker to be useful in clinical decision-making, the marker must be prospectively assessed in a trial with

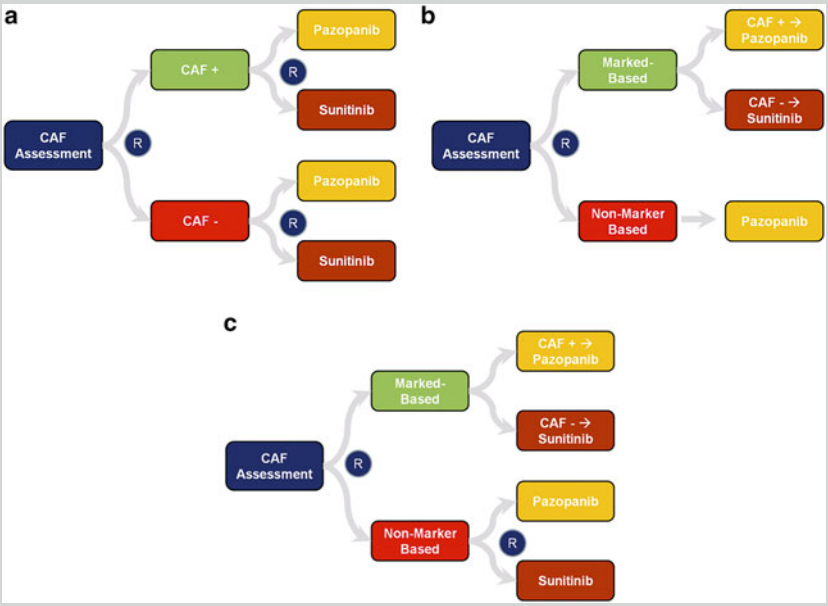

Fig. 23.1 (**a**–**c**) Three theoretical studies aimed at prospectively validating the role of the CAF signatures in predicting clinical benefit with pazopanib as compared to sunitinib

treatment interventions based on biomarker status. Sargent et al. have assembled a widely referenced review that outlines such trial designs. In the first strategy, patients with biomarker negative or positive disease are independently randomized to two distinct treatments (Fig. 23.1a). As an example, we have proposed an evaluation of the CAF signature associated with pazopanib, comparing this agent to another comparable choice, the first-line setting such as sunitinib. A second option involves randomization to either a non-marker-based or marker-based strategy (Fig. 23.1b). In this study design, patients randomized to a marker-based approach could, for example, receive pazopanib if they possess the appropriate CAF signature or could receive sunitinib if they do not. Patients randomized to the non-marker-based strategy could simply receive pazopanib. A flaw in this study design is the allocation of all patients in the non-marker-based strategy arm to one therapy, leading to some deficiencies in this control population. A third possibility delineated in Fig. 23.1c closely resembles the previous strategy, but patients randomized to non-marker-based treatment would be further randomized to sunitinib or pazopanib. Although this third trial design might be construed as preferable to the second, it would entail a significantly higher number of patients to facilitate the second randomization. In truth, as eloquently articulated by Sargent et al., any of these trial designs would likely require thousands of patients, far

beyond the scope of any trial in mRCC performed to date. It is doubtful that the financial incentive associated with biomarker development will facilitate the completion of such trials. Pushing aside the financial barriers for a moment, the investigative community must also decide whether trials to prospectively validate existing biomarkers should take priority over trials investigating novel therapeutic approaches—these two categories of studies are mutually exclusive.

Given the logistical challenges associated with clinical validation and implementation, biomarkers face an unclear path toward use as a clinical decision-making tool. Despite this, further biomarker-related research is critical for progress in mRCC therapy. Nearly two decades ago, the first studies emerged documenting overexpression of VEGF in mRCC, triggering the current plethora of VEGF-directed strategies [50–52]. In a similar fashion, one can envision newly identified moieties identified through gene expression studies of RCC patients (i.e., SETD2, JARID1C, or UTX) as representing next-generation therapeutic targets [53]. Most recently, Kapur et al. have assessed a pool of 145 patients with clear cell RCC and mutations in PBRM1 or BAP1 (two mutually exclusive mutations identified in prior gene profiling studies) [54]. Patients with BAP1 mutations had an OS that was significantly lower than patients with PBRM1 mutations (4.6 years versus 10.6 years, HR 2.7, $P=0.044$). These results were further validated in a cohort derived from The Cancer Genome Atlas (TCGA)—in this cohort, a nearly identical hazard ratio for survival was observed (2.8 favoring PBRM1; $P=0.004$). Although targetable entities have yet to be identified, a treatment paradigm built around such targets may be necessary to break through the apparent plateau in clinical outcome with VEGF- and mTOR-directed therapies.

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Chapter 24
Molecularly Targeted Staging Strategies in Renal Cell Carcinoma

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Introduction

In the United States, it is estimated that in 2013, approximately 65,150 new cases of renal malignancies will be diagnosed and more than 13,680 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 renal cell carcinoma (RCC) can vary widely. Metastatic or recurrent RCC carries a poor prognosis and long-term survival is rare. Historically, the 3-year survival rate 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. Thus, accurate diagnosis, staging, and determination of prognosis are useful for patient counseling, selecting treatment, and considering enrollment for clinical trials.

Ideally, diagnosing and staging of any malignancy is performed without invasive procedures and with minimal risk of morbidity. Cancer staging involves determining the extent cancer has progressed by spreading. In routine clinical practice, cancer is staged using imaging studies such as x-ray, high-resolution computerized tomography (CT) scans, and magnetic resonance imaging (MRI). However, the resolution of current imaging studies limits the accuracy of staging. Therefore, an important goal of modern imaging research is to assess tumor tissue at the molecular

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level to improve staging accuracy. The term molecular imaging refers to a wide variety of imaging strategies designed to visualize tumors at the cellular level or provide information about cellular function, including proliferative state and metabolic state. In the era of targeted therapies, molecular imaging has the potential to identify molecular signatures to help direct treatment and monitor response, without need for invasive biopsies. In this chapter, we review current molecular imaging techniques that have been proposed for RCC.

Molecular Imaging Techniques

Imaging modalities can be classified by the information they provide. Imaging can provide information about anatomy, physiology, cellular function, or molecular state. Standard imaging (e.g., CT, MRI, and ultrasound) provides information on anatomy and physiology. Molecular imaging provides information on cellular function or molecular state. We will concentrate on the molecular imaging strategies currently being used in RCC staging, which can be broadly categorized as positron emission tomography (PET) and optical imaging.

PET Imaging

PET is a nuclear medicine imaging technique which produces images that reflect functional processes. To produce these images, a biologically active molecule is tagged with a positron-emitting radionuclide (tracer) and introduced into the body. The active molecule is taken up by the target tissue and concentrated. After imaging the tissue for gamma rays and obtaining a CT scan, computer analysis is performed to obtain three-dimensional images that localize the tracer in the body. PET tracers are commonly produced using isotopes of elements often occurring in natural biological compounds such as fluorine and iodine. The characteristic molecular defect in clear cell RCC (ccRCC) is the inactivation of the von Hippel-Lindau (pVHL) protein. This defect contributes to upregulation of hypoxia-inducible factor (HIF) and subsequent transcription of hypoxia-regulated genes, including angiogenic factors such as vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), glucose transporter (Glut), and carbonic anhydrase IX (CAIX). Several drugs that target angiogenesis have recently been approved for RCC. One of the first targeted therapies approved for RCC is sunitinib, which inhibits the receptors for VEGF and PDGF. Since the uptake of certain PET radiotracers (e.g., ^{18}F-fluorodeoxyglucose and ^{124}I-girentuximab) is dependent on HIF signaling, PET has a potential role in RCC imaging and may even report the modulation of the HIF pathway by targeted drugs such as sunitinib [5–7]. In the following sections, we review currently available PET radiotracers being used for RCC (Table 24.1).

Table 24.1 Diagnostic PET imaging for RCC

Imaging type	Study	Tumor site	Year	*n*	Sensitivity	Specificity
FDG-PET	Wang et al. [10]	Renal	2012	Meta-analysis (14 studies)	62 %	88 %
		Extrarenal			79 %	90 %
		Extrarenal[a]			91 %	88 %
FDG-PET	Kang et al. [8]	Renal	2004	66	60 %	100 %
		Extrarenal	2004		75 %	100 %
FDG-PET	Nakatani et al. [9]	Recurrence	2009	23	81 %	71 %
FDG-PET/CT	Kayani et al. [11]	Renal/ extrarenal[b]	2011	44	87 %	95 %
^{124}I-cG250-PET/CT	REDECT trial [17]	Renal	2013	195	86.2 %	85.9 %
^{111}In-Bevacizumab PET	Desar et al. [22]	Renal	2010	14	Accumulated in all patients with RCC	

[a]FDG-PET/CT
[b]PET-predicted response to sunitinib

^{18}F-Fluorodeoxyglucose (FDG) PET

FDG is an analog of glucose, and FDG uptake indicates glucose uptake and metabolic activity. The sensitivity and specificity of FDG-PET are somewhat limited for primary tumors because the normal kidney has high background uptake of the radiotracer (Figs. 24.1 and 24.2). However, FDG-PET may be more useful for imaging metastatic lesions (Fig. 24.3). Kang et al. [8] reported their experience with FDG-PET in 66 patients with either primary, metastatic RCC or local recurrence of RCC. Rapid progression to death due to RCC, growth on follow-up imaging, or histopathologic confirmation served as the "gold standard" for assessing the imaging results. The results of PET were compared with conventional imaging. In primary RCC, PET was 60 % sensitive and 100 % specific. For nodal metastases in the retroperitoneum and/or local recurrence, PET was 75 % sensitive and 100 % specific. PET had a sensitivity of 77 and 100 % specificity for bone metastases. However, 6 out of 52 patients had metastatic disease detected on conventional imaging that was missed on PET.

In a study by Nakatani et al. [9], 23 postsurgical patients were evaluated for recurrent disease using FDG-PET. Diagnostic accuracy was evaluated by comparison with the final diagnosis determined histologically or by growth on follow-up imaging. The recurrent disease was seen in 21 of 28 cases. The sensitivity of PET imaging for detecting recurrent disease was 81 % and the specificity was 71 %. It was noted that PET detected all intra-abdominal and bone recurrences.

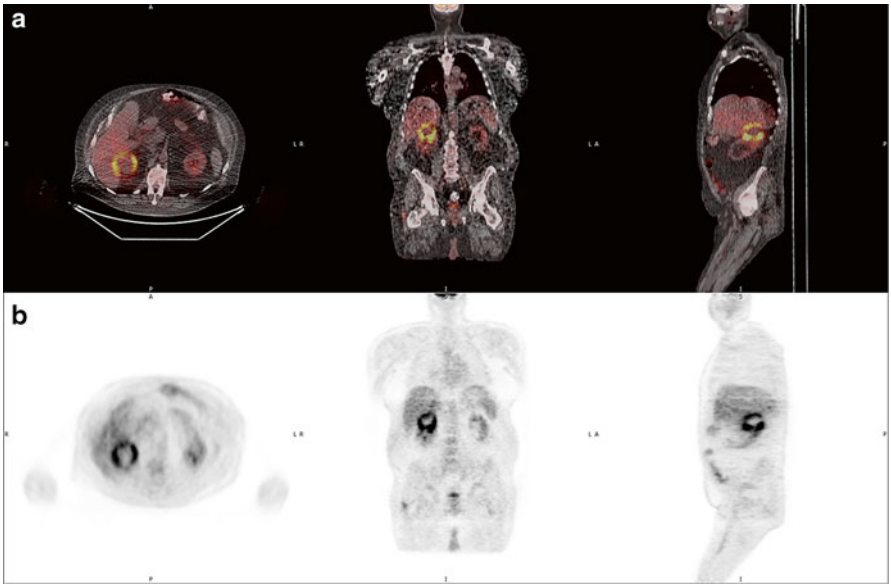

Fig. 24.1 ^{18}F-Fluorodeoxyglucose (FDG) PET/CT scan of a clear cell renal tumor located on the right kidney (**a**), FDG-PET scan that was fused to the CT scan (**b**). The tumor is metabolically active at the periphery and has a necrotic center

Wang et al. [10] performed a meta-analysis of 14 eligible studies. The sensitivity and specificity of FDG-PET for detection of primary RCC lesions were 62 % and 88 %, respectively. However, both sensitivity and specificity were higher for detecting extrarenal lesions, 79 % and 90 %, respectively, and they were further increased to 91 % and 88 %, respectively, with the use of hybrid FDG-PET/CT.

FDG-PET/CT was evaluated by Kayani et al. [11] as an imaging tool to assess the response to sunitinib in 44 patients with metastatic RCC. In a phase II prospective multicenter trial of sunitinib, patients were evaluated with FDG-PET/CT prior to treatment and after 4 and 16 weeks of treatment. FDG-PET/CT had a sensitivity of 87 % and specificity of 95 % for detecting tumor site pretreatment, suggesting a potential role for FDG-PET/CT in initial staging. For each patient, the lesion with the most intense standard uptake value (SUV) was used as the index lesion. Metabolic response was defined as a decrease in SUV by >20 %, and disease progression was defined as an increase in SUV by ≥20 % or development of a new metastatic site. After 4 weeks of treatment, 24 (57 %) patients had a metabolic response, but this did not correlate with progression-free survival (PFS) or overall survival (OS). After 16 weeks of treatment, 12 (24 %) patients had metabolic progression after 16 weeks of treatment. This progression correlated with a decreased OS and PFS. Patients with FDG-PET/CT progression at 16 weeks had more metabolically active disease at baseline and the majority had an initial metabolic response to sunitinib (10 of 12 patients). The traditional radiological methods of monitoring disease response to systemic treatment, such as response evaluation criteria in solid tumor (RECIST),

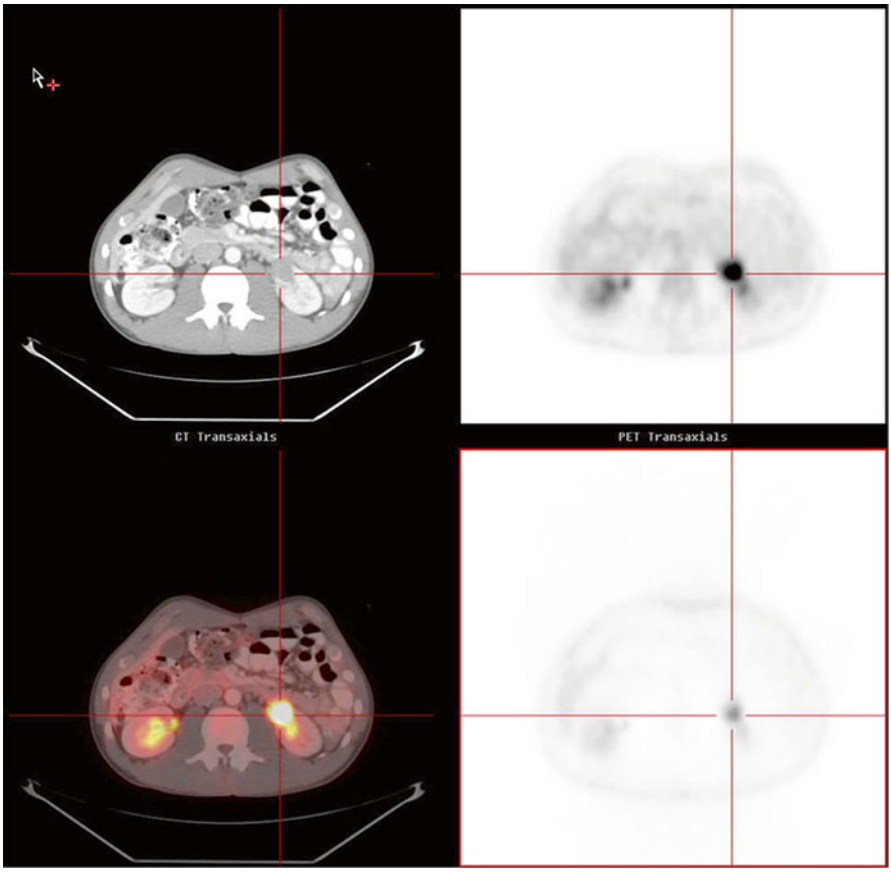

Fig. 24.2 ^{18}F-Fluorodeoxyglucose (FDG) PET/CT scan of a clear cell renal tumor located on the left kidney

only measure tumor size and have limitations in renal cancer. For example, tumors may respond to treatment and become highly necrotic; however, if the tumor size is unchanged, then RECIST fails to capture the response. Data from this study suggest that FDG-PET/CT provides a more effective means to monitor response [12].

Iodine-124-Girentuximab (cG250) PET/CT

cG250 (girentuximab, Redectane®) is a monoclonal antibody (Mab) that binds specifically to a functional epitope of CAIX. Since the transcription of CAIX is induced by HIF, it is almost universally expressed in ccRCC [13–15]. In a retrospective analysis, Choueiri et al. [16] investigated the predictive role of CAIX expression in primary tumors from patients with metastatic ccRCC. A total of 94 patients treated between August 2001 and November 2007 were included in the study. All patients

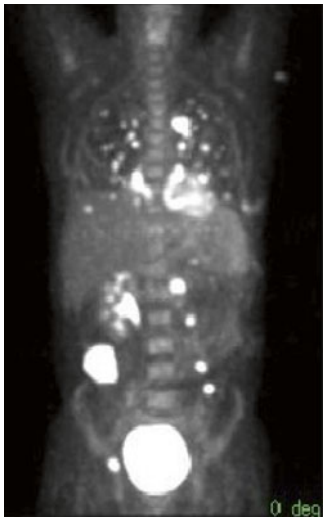

Fig. 24.3
^{18}F-Fluorodeoxyglucose (FDG) PET scan showing multiple metastatic lesions in the chest and abdomen

were treated with VEGF-targeting agents with the majority receiving sunitinib (41 %) or sorafenib (43 %). There was heterogeneity in tumor responsiveness to sunitinib or sorafenib according to CAIX status determined by immunohistochemistry. With sunitinib treatment, the average tumor shrinkage was 17 % vs. 25 % for high CAIX-expressing vs. low CAIX-expressing tumors, respectively. With sorafenib treatment, the average tumor size decreased 13 % if CAIX was high, while the average tumor size increased 9 % if CAIX was low ($p = 0.05$ for interaction). Therefore, the authors suggest that cG250 status may be a predictive biomarker for response to sorafenib treatment.

cG250 has been labeled with ^{124}I for PET imaging. cG250-PET/CT was evaluated in a phase III study that enrolled 226 presurgical patients with renal masses in 14 centers in the United States [17]. All patients had a cG250-PET/CT prior to surgery. The scans were interpreted and compared with contrast-enhanced CT by three readers who were blinded to patient information. The final tumor histology was interpreted by a single, central pathologist who was also blinded to patient information. One hundred ninety-five patients were included in the analysis. The average sensitivity was 86.2 % and 75.5 % for PET/CT and CT, respectively, for diagnosis of ccRCC. The average specificity was 85.9 % and 46.8 % for PET/CT and CT, respectively. This study concluded that the accuracy for diagnosis of ccRCC using PET/CT was comparable to biopsy. However, the results do not permit conclusions regarding other subtypes of RCC.

^{18}F-Fluoro-3′-deoxy-3′-l-fluorothymidine (FLT) PET

Shields et al. [18] first described FLT as a tracer for PET imaging. FLT is an analog of thymidine, which is normally incorporated into DNA during cellular proliferation. FLT is actively taken up by cells and monophosphorylated by thymidine kinase

1, which traps the tracer inside the cell. Intracellular FLT is resistant to degradation and directly reflects thymidine kinase 1 activity and thus cellular proliferation.

Wong et al. evaluated FLT-PET for assessing cellular proliferation in RCC. They evaluated 27 patients with newly diagnosed RCC. All patients had preoperative FLT scans. Immunohistochemical (IHC) staining using Ki-67, which correlates with cellular proliferation, was performed on all resected tumors. The degree of Ki-67 IHC staining was compared with preoperative FLT-PET imaging. FLT signal strongly correlated with Ki-67 expression [19, 20].

Lui et al. [21] used FLT-PET/CT to detect proliferative changes in RCC and other solid cancers during sunitinib treatment and withdrawal. Sixteen patients with metastatic lesions visualized on FLT-PET/CT who had no prior exposure to anti-VEGF therapy were enrolled in this study. All patients received FLT-PET/CT imaging at baseline, during sunitinib exposure, and following sunitinib withdrawal on a 4/2 or 2/1 schedule. The authors found an increase in cellular proliferation during sunitinib withdrawal in patients with RCC, which was consistent with an expected flare in angiogenesis when sunitinib is held. Interestingly, in an exploratory analysis, patients with a larger flare were less likely to benefit clinically from sunitinib therapy. Taken together, the studies of FLT-PET/CT suggest that this imaging modality may be useful in assessing tumor proliferation and directing therapy.

^{111}In-Bevacizumab (In-Bevacizumab) PET

Another promising strategy is to radiolabel active drugs for PET imaging. Bevacizumab is a humanized Mab that binds and inhibits VEGF. Bevacizumab is approved for the treatment of a variety of malignancies including RCC. Desar et al. [22] evaluated In-bevacizumab PET imaging in 14 patients with RCC who were scheduled to undergo a nephrectomy. In nine patients, neoadjuvant sorafenib was administered and In-bevacizumab PET was performed before and after sorafenib treatment. Scans were performed in five control patients who did not receive neoadjuvant therapy. In all 14 patients, In-bevacizumab preferentially accumulated in the renal tumor. After treatment with sorafenib, there was a 60.5 % reduction in the mean uptake in the primary tumor in patients with ccRCC, which correlated with decreased vascularity seen by IHC staining of the tumor. Interesting, there was no change in PET imaging or tumor vascularity in one patient with papillary RCC. This study suggests that this imaging modality may be useful for staging RCC and monitoring response to anti-angiogenic therapy.

Molecular Optical Imaging

Optical imaging is another technique for molecular imaging being applied to RCC. Optical imaging can be used noninvasively or at the time of surgery to detect molecular markers. Molecular optical imaging involves the detection of light signals emitted by various probes used to infer biologic or chemical properties of the

target tissue. Initial optical imaging research in urology was applied to the diagnosis of bladder cancer [23]. More recently, the focus has been broadened to evaluate RCC. Both fluorescence and non-fluorescence-based optical imaging techniques are being utilized.

Non-fluorescent: Tissue Spectroscopy

Optical spectroscopy (OS) is a noninvasive technique used to identify relative changes in the way light interacts with tissue. In OS, light is scattered by cell analytes or absorbed by chromophores in the tissues. Malignant cells are structurally different from normal cells, resulting in changes in optical properties. These differences are detected with spectroscopy after illumination with light [24]. OS can be used to differentiate tissue types by providing information on structure. This approach has been used to detect pancreatic tumor [25].

Parekh et al. [26] published the first ex vivo study in RCC using OS. The authors looked at kidney samples from ten patients after radical nephrectomy using OS and compared the findings to the pathology. Six patients had ccRCC, three had papillary RCC, and one had a cystic nephroma. Malignant tissue had higher reflectance intensities, ranging between 600 and 800 nm, compared to normal renal tissue.

Bensalah et al. [27] also assessed OS to reliably differentiate tumor and normal tissue in renal specimens obtained from tumors and normal parenchyma. The optical reflectance spectroscopy slopes were assessed. A total of 21 (13 radical and 8 partial nephrectomies) specimens was analyzed, and based on OS, 15 were determined to be malignant (14 ccRCC and 1 papillary), and 6 were determined to be benign (oncocytoma). There was a significant difference between the average OS spectral slopes and intensities between tumor and normal parenchyma ($p = 0.03$). The data indicate that OS may be helpful in detecting positive margins during partial nephrectomy. These studies suggest a potential role for OS in differentiating malignant and normal renal parenchyma.

Non-fluorescent: Optical Coherence Tomography (OCT)

Optical coherence tomography, first developed for ophthalmological applications, provides in situ imaging of tissue morphology with resolution approaching the micron scale (1–2 μm) with a depth of penetration of 2–4 mm. The images show the microscopic tissue structures observed in histology [28, 29].

The technique involves directing a beam of near-infrared light (1,300 nm) from a low-coherent fiber-coupled light source (e.g., a superluminescent diode) at the target tissue and detecting light that is backscattered from the targeted tissue. This phenomenon is similar to ultrasound imaging, but differs in using light rather than sound to produce cross-sectional images of tissue. The imaging depth is limited by the attenuate of light as it penetrates deeper into the tissue. The attenuation is quantified by measuring the decay of signal intensity per unit depth. The attenuation

coefficient can be derived and used to characterize tissue [30]. For example, malignant tissues have irregular nuclei resulting in a higher refractive index; the attenuation coefficient is higher compared to normal tissue.

In 2011, Barwari et al. [31] reported results from an ex vivo pilot study using OCT to differentiate malignant from normal renal tissue. The study demonstrated that the abundant cellular structures in malignant tissue resulted in a higher degree of scattering and a higher attenuation coefficient than normal renal parenchyma. A follow-up, phase I in vivo human study was performed. During surgery, OCT images were obtained from 16 renal tumors and the surrounding normal renal parenchyma. Ex vivo OCT images were also obtained. The pathology was then compared with the images. Using attenuation coefficients, there was a significant difference between normal renal parenchyma and malignant tumors. However, the difference in attenuation coefficients between malignant and benign tumors was not significant. The authors postulated that with a larger sample size, a clear difference might be found [32].

Linehan et al. [33] used OCT imaging to assess histologic subtypes of RCC. After radical or partial nephrectomy in 20 subjects, both the normal renal parenchyma and the tumor were evaluated with light microscopy and a benchtop OCT system. OCT images were compared with histological slides. OCT was most successful in distinguishing angiomyolipoma and urothelial carcinoma from normal parenchyma. OCT is less useful for identifying oncocytoma. ccRCC and other subtypes of RCC had a heterogeneous appearance, precluding reliable identification. The authors speculated that higher-resolution OCT, such as optical coherence microscopy (OCM), may be more useful for subtyping RCC.

OCM combines OCT with confocal microscopy to improve imaging depth compared to that of standard confocal microscopy [34]. Lee et al. [35] examined the use of OCT and OCM to assess human renal tissues. A total of 35 renal specimens from 19 patients, consisting of 12 normal tissues and 23 tumors (16 ccRCC, 5 papillary RCC, and 2 oncocytomas), was imaged ex vivo after surgical resection. OCT and OCM images were compared with standard histology. Three pathologists blinded to histology evaluated sensitivity and specificity of the images for differentiating normal from neoplastic renal tissues. Each imaging method was useful for assessing morphology. OCT and OCM matched well with the corresponding histology. Three observers achieved 88 %, 100 %, and 100 % sensitivity and 100 %, 88 %, and 100 % specificity, respectively, when evaluating normal vs. neoplastic specimens.

These results indicate OCT and OCM can be used to identify distinctive morphological patterns and achieve diagnostic accuracy. While the technology is limited by depth of penetration, it has the potential to serve as an adjunct during surgery since it offers the advantage of real-time pathologic information.

Fluorescence Optical Imaging

Fluorescence results when a photon is absorbed by a molecule at one wavelength and emitted at a longer wavelength. A variety of fluorescent reporters, known as fluorophores, have been described and they can be proteins, dyes, or nanoparticles.

Fluorescein is an example of a fluorophore. Fluorescein has limited use in oncology because fluorescent signals are absorbed and scattered by both normal and malignant tissues. Additionally, normal tissue produces background fluorescence in a similar wavelength spectrum as malignant cells. One strategy to overcome this limitation is to use fluorophores that emit in the near-infrared spectrum where there is minimal background fluorescence and light signals penetrate deeper into tissue. To differentiate between healthy and diseased tissue, fluorophores can be used to tag antibodies and other molecules that specifically bind disease-specific markers.

Cyanine Dyes

Indocyanine green (ICG) is a fluorescent dye that absorbs near-infrared (NIR) light and emits light at a slightly longer wavelength, which can be detected by a NIR camera. ICG binds to plasma proteins when injected intravenously. The vasculature can then be visualized with a NIR camera. Additionally, when compared to normal kidney, renal tumors have lower expression of bilitranslocase, which is the carrier protein for ICG [36]. Thus, cortical tumors are less efficient at taking up the dye and appear hypofluorescent when viewed with a NIR camera.

This technology can be applied during partial nephrectomies to achieve complete resection of the tumor. Tobis et al. reported their initial clinical experience with 11 robotic-assisted partial nephrectomies using ICG [37]. Of the malignant tumors, seven were hypofluorescent and three were isofluorescent compared to surrounding renal parenchyma. All surgical margins were negative on final pathology. They concluded that the technology aided in differentiating the tumor mass from normal parenchyma and the renal vessels.

Krane et al. [38] recently performed a prospective study using ICG in 47 consecutive patients with renal masses suspicious for malignancy who underwent robotic partial nephrectomy (RPN). This cohort was compared to 47 consecutive patients who had undergone RPN without near-infrared fluorescence. The group found no significant difference in positive margin rate or Clavien complications. There was a small decrease in warm ischemia time in the ICG group. The authors felt ICG helped identify vasculature, but it did not help with the dissection of the mass, even in cases of endophytic tumors. These early studies provide mixed results on the usefulness of intraoperative ICG, but they demonstrate a technology that has the potential to improve oncologic and functional outcomes of partial nephrectomy.

Fluorescence: 5-Aminolevulinic Acid (5-ALA)

5-ALA is metabolized to protoporphyrin IX, a fluorescent agent. Protoporphyrin IX accumulates in rapidly proliferating cells, thus allowing differentiation of malignant versus normal parenchyma [39]. 5-ALA was first studied in a murine model and showed promise for identification of malignant tumors [40]. Hoda et al. [41] applied this technology to RCC in a prospective, non-randomized single-center study.

The authors described the use of protoporphyrin IX imaging for detection of malignant renal masses and to evaluate surgical margin status during laparoscopic partial nephrectomy. This imaging modality had a sensitivity of 95 % and specificity of 94 % in identification of a renal tumor as RCC. Furthermore, they reported a sensitivity of 100 % for detection of positive margins.

Conclusions

PET and optical imaging techniques are being actively evaluated in the management of RCC. PET has the potential to provide noninvasive means of determining histology and malignant potential of a renal mass. Patients with localized disease can be risk-stratified for observation, nephron-sparing surgery, or radical nephrectomy. Patients with metastatic disease may be imaged to determine the best therapy, and treatment response can be monitored with serial imaging.

Preliminary studies in optical imaging offer promise in differentiation of normal from malignant renal tissue. Optical imaging techniques have potential to provide histological information without the need to remove the tissue from the patient. This information may be used to diagnose RCC or used intraoperatively to assess surgical margins during partial nephrectomy.

Many of these techniques remain investigational and validation of early results is necessary, but the potential advantages are clear. Further advances in our understanding of the molecular basis of RCC are expected to produce parallel advances in strategies for molecularly targeted imaging.

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Chapter 25
Clinical Prognostic Factors in Metastatic Renal Cell Carcinoma

Nimira Alimohamed, Toni K. Choueiri, and Daniel Y.C. Heng

Introduction

Prior to the development of molecularly targeted agents, the mainstay of therapy for patients with mRCC were cytokine immunotherapy agents such as interferon-α and interleukin-2 (IL-2). However, the use of these agents resulted in significant systemic toxicity with limited responses [1]. Agents targeting the VEGF and the mTOR pathways have now become the standard of care and have improved the clinical outcome of patients with mRCC [2–10]. As the treatment armamentarium for mRCC rapidly increases, the need to continue to improve on prognostication is paramount.

Clinical prognostic factors in mRCC include situations, baseline conditions, or characteristics of patients which are used to predict outcome following systemic therapy: tumor response, progression-free survival (PFS) and most commonly overall survival (OS) [11]. These factors, collated from clinical trials and retrospective population-based analyses, have also been combined into prognostic models. Risk stratification by prognostic strata is important for patient counseling, guiding therapeutic decisions, and current and future clinical trial design and interpretation.

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Patient Factors

Patient symptoms and performance status have consistently been one of the most important prognostic factors in mRCC, as well as several other advanced malignancies. In the immunotherapy era, a large review of patients treated with IL-2 after nephrectomy found constitutional symptoms including weight loss and decreased appetite to adversely impact survival [1]. Another large retrospective study found performance status to be one of five consistent prognostic factors of survival across three independent series evaluating outcomes of patients treated with interferon-α or IL-2 [2]. A Karnofsky performance score of less than 80 % was similarly determined to be an adverse prognostic factor in another large series of patients treated with interferon-α [3]. In the era of targeted therapy, Eastern Cooperative Oncology Group (ECOG) performance status greater than zero was one of five independent adverse prognostic factors for Progression Free Survival (PFS) on multivariate analysis from a retrospective analysis of patients enrolled in clinical trials treated with VEGF-targeted agents at the Cleveland Clinic [4]. ECOG performance status was 1 of 11 pretreatment predictor variables for predicting the probability of PFS for patients treated with sunitinib [5]. In two other larger analyses of patients with mRCC treated with targeted therapy, performance status, regardless of the scale used, is a common prognostic factor [6, 7].

Disease-Related Factors

Tumor Burden

Primary tumor characteristics such as tumor size, grade, and extent of invasion to adjacent structures have important prognostic impact in localized disease but not in metastatic RCC [8]. Factors indicative of tumor burden in mRCC include sites of metastases, the number of metastatic lesions, and the resectability of these lesions. One study of patients treated in the immunotherapy era found that complete resection of pulmonary metastases led to a 5-year survival of 41.5 % compared with 22.1 % in those patients who were incompletely resected. On multivariate analysis, predictors of survival after complete resection were the number of pulmonary metastases, involvement of lymph nodes, and length of disease-free interval [9]. Another series found the size of the metastatic pulmonary nodule, less than 4 cm conferring a better prognosis, and the completeness of pulmonary resection (negative margins) as important prognostic factors [10]. With brain metastases, an important adverse prognostic factor for CNS recurrence is having multiple brain lesions, and survival after resection remains poor at 12 % at 5 years [11]. The presence of bone metastases has recently been determined to be predictive of poor survival in patients treated with sunitinib [7]. However, if patients have resectable bone metastases, bone-only lesions and solitary lesions do not lead to a worse survival as demonstrated in a retrospective review of 295 patients with mRCC treated surgically for

skeletal metastases [12]. In the era of targeted therapy, metastatectomy is feasible in selected patients with limited morbidity and oligometastases [13]; however metastasis resectability its use as a prognostic factor remains to be elucidated in prospective clinical trials.

Serum Markers

Serum markers indicative of tumor burden or a proinflammatory state are valuable prognostic factors. In the immunotherapy era, elevated lactate dehydrogenase (LDH), low hemoglobin, and elevated corrected calcium level indicative of high tumor burden were consistently identified as negative prognostic factors [3, 14]. Elevated alkaline phosphatase level has also been demonstrated on multivariate analysis to portend a poorer prognosis [2]. Biologic signs of inflammation including erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) were also validated as independent predictors of worse survival in the immunotherapy era [2]. However, their significance with recent treatments has yet to be proven. Anemia and hypercalcemia have remained consistent predictors of poor outcome in patients with mRCC treated with targeted therapy [4, 6]. Neutrophilia was first identified as an important prognostic factor in patients treated with immunotherapy or chemotherapy [15], and its prognostic value has been confirmed in patients treated with targeted therapies [4, 6]. Thrombocytosis has also been validated as a poor prognostic factor in patients treated with antiangiogenic therapies, as platelets contain VEGF and may play an important role in angiogenesis and tumor progression [4, 6].

Recent studies have evaluated the prognostic role of serum sodium level in mRCC. Hyponatremia (serum sodium less than the lower limit of normal) was validated as independent prognostic factor for worse survival and also predicted for lack of response to therapy in series of 240 patients treated with immunotherapy [16]. In a retrospective analysis of 855 patients treated with VEGF-targeted agents, hyponatremia (serum sodium less than 135 mmol/L) was associated with shorter overall survival and time to treatment failure on multivariate analysis after adjusting for poor risk criteria [17]. The mechanism by which hyponatremia leads to a worse outcome is not entirely clear, but it may be other indicator of an elevated tumor burden, thus leading to the syndrome of inappropriate anti-diuretic hormone (SIADH) in patients with more extensive pulmonary or central nervous system disease [16].

Treatment-Related Factors

Nephrectomy Status

Cytoreductive nephrectomy has been well established as a prognostic factor in mRCC patients with acceptable PS and limited disease burden. The EORTC randomized study found that radical nephrectomy before IFN-based immunotherapy

demonstrated improved time to progression and overall survival [18]. Similarly, a randomized study conducted by Southwest Oncology Group (SWOG) found a significant improvement in survival in patients treated with nephrectomy followed by IFN therapy and then with IFN therapy alone, a benefit that was independent of performance status, metastatic sites, and the presence or absence of measurable metastatic lesions [19]. Randomized trials are underway to determine the impact of cytoreductive nephrectomy in the era of targeted therapy, including the CARMENA trial (NCT 00930033) comparing nephrectomy followed by sunitinib therapy versus sunitinib alone in mRCC and the SURTIME trial (NCT 01099423) evaluating immediate versus delayed nephrectomy in patients with synchronous mRCC. However, data from retrospective analyses confirms the importance of nephrectomy with current treatment options. A recent retrospective study of 314 patients with mRCC, of which 201 underwent cytoreductive nephrectomy, found overall survival of 19.8 months for those who received nephrectomy versus 9.4 months without (HR 0.44, 0.32–0.59, $p < 0.01$) [20]. On multivariable analysis when adjusting for established prognostic risk factors, this survival benefit persisted. Subgroup analyses revealed marginal benefit for patients with a low KPS and brain metastases or those in the poor-risk category, thus illustrating how prognostication may be useful to guide therapeutic decision-making.

Disease-Free Interval

The disease-free interval in mRCC has been defined as the time from diagnosis or nephrectomy to the treatment of metastatic disease with a shorter interval indicating more aggressive disease. A time from diagnosis to treatment of less than 1 year (by consensus) has been an independent predictor of worse outcome on several multivariate prognostic models including those from the immunotherapy era and with recent VEGF-targeted therapy [3, 6, 7]. A disease-free interval of less than 2 years has also been demonstrated to be an independent adverse prognostic factor for PFS [4].

Multivariable Prognostic Models

The large number of independent clinical prognostic factors in mRCC has led to the development of several multivariable prognostic models. These models have been used to increase the accuracy of predicting outcomes both in clinical practice for patient counseling and in clinical trials. Patients are classified into poor-, intermediate-, and favorable-risk categories. The concordance index (*c*-index) is the ability of the prognostic model to accurately discriminate patients into these different prognostic categories with a *c*-index value of 1 corresponding to the ideal prediction.

Memorial Sloan-Kettering Cancer Center (MSKCC) Model

In the immunotherapy era, Motzer and colleagues developed the largest and most widely used prognostic model at the MSKCC [3, 14]. Initially a retrospective analysis of 670 patients enrolled in consecutive clinical trials of immunotherapy and chemotherapy for mRCC between 1975 and 1996 was undertaken. Inclusion criteria were stage IV disease, measurable metastatic lesions, adequate KPS, adequate organ function, and nonsignificant comorbidities. Median survival of the entire cohort was 10 months. Five factors were found on multivariate analysis to independently predict for poor survival: low KPS (<80 %), elevated LDH (>1.5 upper limit of normal), low hemoglobin (<lower limit of normal), elevated corrected serum calcium (>10 mg/dL), and absence of prior nephrectomy. These factors were used to categorize patients into three risk groups: favorable risk (0 risk factor) with a median survival of 19.9 months, intermediate risk (1–2 risk factors) with a median survival of 10.3 months, and poor risk (3–5 risk factors) with a median survival of 3.9 months. This model was updated in 2002 with an analysis of 463 patients with advanced RCC who were treated with first-line IFN-α. Median overall survival was 13 months. Multivariate analysis revealed low KPS, elevated LDH, low serum hemoglobin, elevated corrected serum calcium, and the time from initial diagnosis to the start of IFN-α treatment of less than 1 year as being independent predictors of short survival. Cytoreductive nephrectomy was replaced by time to IFN-α therapy in this updated model as during the time interval between the two analyses, the benefit of cytoreductive nephrectomy had been demonstrated in two randomized trials [18, 19], and thus time to treatment became a more appropriate risk factor. Median survivals for favorable-, intermediate-, and poor-risk patients were 29.6 months, 13.8 months, and 4.9 months, respectively. The Cleveland Clinic group externally validated this model and found prior radiotherapy and having greater than one site of metastases to be additional adverse prognostic factors [21]. The updated MSKCC model became widely utilized in clinical trial design for patients with advanced RCC.

Groupe Français d'Immunothérapie Model

Negrier and colleagues also assessed prognostic factors in the immunotherapy era by evaluating clinical variables in 782 patients treated in successive multicenter trials with IFN-α or IL-2 or both [2]. Median survival in this cohort was 12.8 months. Nine adverse clinical prognostic factors for survival were found. Results from this analysis were integrated with previous studies, yielding five validated prognostic factors predictive for overall survival: biologic signs of inflammation (ESR ≥100 or CRP ≥50), less than 1 year from renal tumor to occurrence metastases, low hemoglobin level (<115 g/L females, <130 g/L males), the number of metastatic sites (>1), and poor ECOG performance status (≥1). One proposed risk model discriminated patients into three risk groups: 0–1 risk factor, 2–3 risk factors, and 4–5 risk factors. Median survivals for these patient groups were 42 months, 15 months, and

6 months, respectively. In addition, four prognostic factors predictive of rapid progression with cytokine therapy were identified: presence of liver metastases, less than 1 year from renal tumor to occurrence of metastases, elevated neutrophil count ($>7.5 \times 10^3$/L), and the number of metastatic sites (>1).

International Kidney Cancer Working Group Model

A collaborative group of researchers from Europe and North America assembled a large database of 3,748 patients treated in clinical trials by 11 different groups from 1975 to 2002 for prognostic factor evaluation in mRCC [22]. The majority of these patients were treated with immunotherapy. Multivariate analysis revealed nine independent prognostic factors for survival: treatment, performance status, number of metastatic sites, interval from diagnosis to treatment, pretreatment hemoglobin, white blood cell count, LDH, alkaline phosphatase, and serum calcium. These factors were integrated into three risk groups (favorable, intermediate, and poor) with median survivals of 26.9 months, 11.5 months, and 4.2 months, respectively. This model was then tested and validated among patients treated with VEGF-targeted agents from an external data set [6] and showed good concordance (*c*-index 0.741), suggesting its applicability to patients treated with targeted therapies.

Cleveland Clinic Model

Choueiri and colleagues were the first to evaluate clinical prognostic factors in patients with mRCC treated exclusively with VEGF-targeted therapy [4]. One hundred and twenty patients with metastatic clear-cell RCC treated on clinical trials at the Cleveland Clinic were evaluated. Overall median survival for this patient population was 13.8 months with an objective response rate of 34 %. Multivariate analysis revealed five independent adverse prognostic factors for PFS: time from diagnosis to current treatment less than 2 years, baseline platelet count >300K/microL, baseline neutrophil count >4.5K/microL, baseline corrected serum calcium <8.5 mg/dL or >10 mg/dL, and initial ECOG performance status >0. Patients with 0–1 adverse prognostic factor had a median PFS of 20.1 months, those with two adverse factors had a median PFS of 13 months, and those with >2 adverse prognostic factors had a median PFS of 3.9 months.

Prognostic Factors from the Sunitinib Phase III Trial

Motzer and colleagues used individual patient data from the sunitinib arm of the phase III randomized clinical trial of sunitinib versus IFN-α to investigate the correlation between pretreatment clinical features and PFS [5]. Eleven pretreatment

variables were incorporated into a prognostic nomogram to predict 12-month PFS with sunitinib treatment: corrected serum calcium, the number of metastatic sites, hemoglobin level (≥LLN or <LLN), prior nephrectomy, the presence of lung metastases, the presence of liver metastases, ECOG performance status (0 or 1), thrombocytosis, time from diagnosis to treatment, alkaline phosphatase level, and lactate dehydrogenase level. The concordance index for the nomogram was 0.633. On multivariate analysis, only 6 of these factors were independently associated with PFS; however all 11 factors were included in the nomogram because of the deleterious effect on its predictive accuracy. Patil and colleagues similarly used patient data from the randomized phase III trial of first-line sunitinib in an attempt to apply the MSKCC multivariable prognostic model to the era of targeted therapy [7]. For sunitinib therapy, five independent predictors of PFS were identified: ECOG performance status >0, absence of nephrectomy, LDH level, platelet count, and ≥2 metastatic sites. Six independent predictors for overall survival with sunitinib therapy were also identified: ECOG performance status >0, time from diagnosis to treatment <1year, LDH level, corrected calcium level, hemoglobin level, and bone metastases. The presence of bone metastases was the only new prognostic factor for overall survival identified compared with the original MSKCC model [3], suggesting that the MSKCC model remains applicable in the era of targeted therapy with this addition. The prognostic nomogram and the prognostic model described from the sunitinib phase III trial are unique among multivariable prognostic models as both were derived from prospectively followed patients, however may not be generalizable to patients not taking sunitinib.

Prognostic Models in Patients Treated with mTOR Inhibitors

Both the Cleveland Clinic and International mRCC Database Consortium model were developed in patients treated with anti-VEGF therapy, and minimal data exists on the applicability of these models to patients treated with mTOR inhibitors. Hudes and colleagues evaluated the mTOR inhibitor temsirolimus in a phase III trial of poor prognosis patients with mRCC. Poor prognosis in this study was defined as having three of the following six predictors of short survival: LDH > 1.5 times the upper limit of normal, hemoglobin < lower limit of normal, corrected serum calcium > 10 mg/dL, time from initial diagnosis to randomization of less than 1 year, KPS of 60 or 70, and metastases in multiple organs. Thus patients who would have been defined as intermediate risk by the MSKCC model may have still been eligible for this study. Similarly in the phase III RECORD-1 trial evaluating the mTOR inhibitor everolimus versus best supportive care after progression with VEGF-targeted therapy, pretreatment prognostic profiles were assessed. The MSKCC risk categories were confirmed to predict for outcome in this patient population as the 12-month probability of survival was 70 % for the favorable-risk group, 56 % for the intermediate-risk group, and 26 % for the poor-risk group. Additional variables predictive for shortened PFS and OS in this study were the presence of liver or bone metastases, elevated neutrophils, and prior treatment with sunitinib.

Table 25.1 International mRCC Database Consortium prognostic model

Adverse factors	Parameter
Low Karnofsky performance score	<80 %
Short interval between RCC diagnosis and treatment	<1 year
Low hemoglobin level	<LLN
Elevated corrected serum calcium level	>ULN
Elevated neutrophil count	>ULN
Elevated platelet count	>ULN
Risk categories	Median survival
Good prognosis (0 factor)	Not reached
Intermediate prognosis (1–2 factors)	27 months
Poor prognosis (>2 factors)	8.8 months

RCC renal cell carcinoma, *LLN* lower limit of normal, *ULN* upper limit of normal

International mRCC Database Consortium Model

To further explore prognostic factors in the era of targeted therapy, Heng and colleagues performed a large multicenter, retrospective analysis of 645 patients with mRCC treated with sunitinib, sorafenib, or bevacizumab [6]. Six independent predictors of worse overall survival were identified: low hemoglobin (<lower limit of normal), elevated neutrophils (>upper limit of normal), elevated platelets (>upper limit of normal), elevated corrected serum calcium (>upper limit of normal), low Karnofsky performance status (<80 %), and a short disease-free interval (<1 year from diagnosis to treatment). Three risk categories were assembled: favorable risk (0 risk factor), intermediate risk (1–2 risk factors), and poor risk (3–6 risk factors) (Table 25.1). Median overall survival, not reached in the favorable-risk group, was 27 months and 8.8 months in the intermediate- and poor-risk groups, respectively (Fig. 25.1). In comparison to the MSKCC model, the Heng et al criteria found prognostic benefit to neutrophilia and thrombocytosis, while elevated LDH was not a significant independent prognostic factor. This model's discriminatory ability was good with a *c*-index of 0.73 and also proved to be generalizable with the inclusion of patients treated with a variety of VEGF-targeted agents. This model has recently been externally validated with an additional 1,028 patients [23]. All six risk factors were deemed independent predictors of poor survival in the external validation set with a concordance index for the model based on the three risk categories of 0.663 (compared to 0.640–0.668 for the other commonly used prognostic models). When the six prognostic factors are not collapsed into three risk groups, the *c*-index is 0.71. Overall survival of patients in the validation set was 43.2 months, 22.5 months, and 7.8 months for favorable-, intermediate-, and poor-risk groups, respectively.

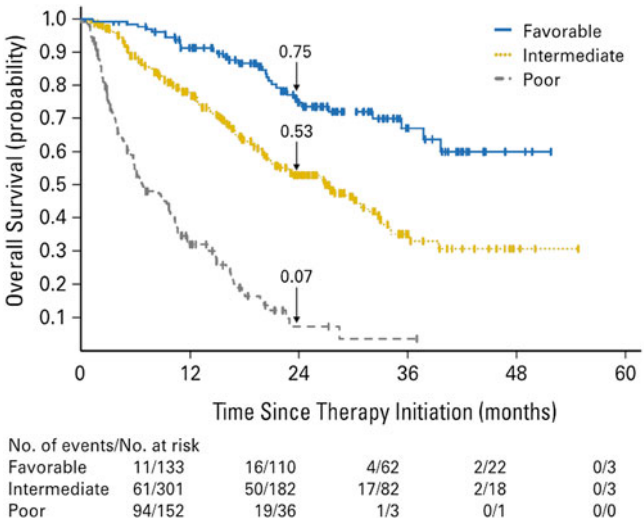

Fig. 25.1 Overall survival for the International mRCC Database Consortium model [17]

Comparison of Prognostic Models

Although there have been a multitude of prognostic models developed to predict outcomes in patients with advanced RCC, with several similarities among them (Table 25.2), one standard model has not been employed by the entire academic community. The MSKCC model is widely accepted for its application to patients treated with immunotherapy and was applied in clinical trials for current standard treatments. The International mRCC Database Consortium model has been externally validated and now can be applied to clinical trials as new benchmarks in overall survival have been set in the era of targeted therapy. When compared to other prognostic models, the International mRCC Database Consortium model outperformed the others in areas of model fit, including having the most similar numbers of reported versus predicted deaths at 2 years [23]. When compared to the MSKCC and French models, the Database Consortium model had better model fit and reclassified patients into the more correct risk group when measured against 2-year overall survival. This supports using the Database Consortium model to stratify patients in clinical trials and for counseling patients about prognosis.

Future Directions

There are several limitations to the clinical prognostic factors and models developed for use in patients with mRCC. Analyses conducted in the era of immunotherapy require further validation in the era of targeted therapy, affecting the generalizability

Table 25.2 Comparison of prognostic models for metastatic renal cell carcinoma

	MSKCC model	Groupe Française d'Immunothérapie model	International Kidney Cancer Working Group model	Cleveland Clinic model	RCC Database Consortium model	Sunitinib phase III trial
	Motzer et al. (2002)	Negrier et al. (2002)	Manola et al. (2011)	Choueiri et al. (2007)	Heng et al. (2009)	Patil et al. (2011)
Patient population	463 patients treated with IFN-α	782 patients treated with IFN-α or IL-2	3,748 patients, majority treated with immunotherapy	120 patients treated with VEGF-targeted agents	645 patients treated with VEGF-targeted agents	375 patients treated with sunitinib
Adverse prognostic factors included						
Performance status						
Karnofsky performance score	<80 %				<80 %	
ECOG score		≥1	≥1	>0		>0
Disease-free interval	<1 year	<1 year	<134 days	<2 years	<1 year	<1 year
Number of metastatic sites		>1	0–5 or more			
Bone metastases						Present
Prior nephrectomy						
Hemoglobin level	<LLN	<115 g/L women, <130 g/L men	≤12.5 g/dL		<LLN	<LLN
Platelet count				>300K/μL	>ULN	
Neutrophil count				>4.5K/μL	>ULN	
White blood count			>7.6K/μL			
LDH level	>1.5× ULN		>193 U/L		>ULN	>ULN
ESR or CRP elevation		ESR ≥ 100 or CRP ≤ 50				
Corrected serum calcium level	>10 mg/dL		>9.5 mg/dL	<8.5 mg/dL or >10 mg/dL	>ULN	>ULN
ALP level			>99 U/L			
Treatment			Varied			

ECOG Eastern Cooperative Oncology Group, *LDH* lactate dehydrogenase, *ESR* erythrocyte sedimentation rate, *CRP* c-reactive protein, *ALP* alkaline phosphatase, *LLN* lower limit of normal, *ULN* upper limit of normal

of common models to current clinical practice. The majority of models were derived from data of patients in clinical trials, again limiting the generalizability to the subset of patients who are eligible. In addition, the different prognostic models have not been directly compared to each other or validated in prospective clinical trials. Retrospective analysis may have caused selection bias of certain types of information, and many other data points of importance may not have been routinely collected or assessed. The accuracy of the current prognostic models for mRCC is fair; however, this is also an area for further improvement.

With the rapid evolution of therapy to treat mRCC and the growing list of targeted therapies in development, the need to offer personalized medicine is needed more than ever. Molecular biomarkers to guide therapy have been investigated in mRCC from several aspects of the von Hippel-Lindau (VHL) pathway, including VHL gene mutations and serum VEGF levels [24]. These biomarkers are not ready for clinical practice and at this time do not add to the available clinical prognostic factors. Future prognostic scoring models for mRCC are likely to include clinical and molecular factors in hopes of attaining a higher discriminatory ability.

Conclusions

The treatment of mRCC has evolved from immunotherapy to therapies targeting the VEGF and mTOR pathways. Along with this evolution have come improvements in survival. However, mRCC remains a heterogeneous disease and the ability to prognosticate for patients remains important. Clinical prognostic factors include several patient-, disease-, and treatment-related factors that have been combined into multivariable prognostic models. The most commonly used models are currently the MSKCC model and the RCC Database Consortium model, both stratify patients into favorable-, intermediate-, and poor-risk groups. These models have been applied to clinical trial design and interpretation, as well as patient selection for certain therapies and patient counseling. Moving forward, the role of molecular biomarkers will continue to be elucidated, and incorporation of validated clinical and molecular biomarkers into prognostic models will hopefully improve prognostication in patients with mRCC.

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Chapter 26
The Role of Advocacy in Renal Cell Carcinoma

William P. Bro and Paul Larson

Introduction

Since the founding of the Kidney Cancer Association (KCA) in 1990, the medical community has made enormous strides in developing new strategies to improve the health outcomes of patients diagnosed with kidney cancer—and ultimately, find a cure.

These advancements have been particularly impressive in the last decade; a time during which the Food and Drug Administration (FDA) approved seven new therapeutic options to slow the progression of kidney cancer.

When the FDA approved Nexavar® (sorafenib tosylate) and Sutent® (sunitinib malate) in 2005 and 2006, these additions provided the first new medications to treat kidney cancer in a decade. Over the next several years, Nexavar and Sutent were quickly followed by Votrient® (pazopanib), Avastin® (bevacizumab), Torisel® (temsirolimus), Afinitor® (everolimus), and, most recently, Inlyta® (axitinib), approved in 2012.

Over this time, surgical techniques and options also advanced, including remarkable progress in noninvasive, laparascopic techniques as well as refinements in ablative therapies.

All of this occurred against the backdrop of the completion of the Human Genome Project in 2003, which opened a new era of scientific inquiry and facilitated significant new discoveries in genetics that will almost certainly change our understanding of RCC and our approaches to treating it.

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The dizzying pace of these scientific advances has created one of the most extraordinary periods ever for kidney cancer clinicians, patients, and families—and has given the RCC community great optimism for the future.

The National Cancer Institute estimates that there are between 100,000 and 200,000 kidney cancer survivors living in the United States right now, and with the recent advances in treatment, it is expected that even more patients will live with kidney cancer in the future, continuing to maintain their normal lifestyles [1].

But for all of this hope and optimism, we face serious challenges that threaten the arc of our progress.

Though spiraling health-care costs have grown more slowly in recent years, they continue to pose a very real threat to the goal of bringing our new treatments and innovations to patients. The introduction of the most extensive changes to the health-care system in generations—via the Affordable Care Act—promises to create a period of great uncertainty and potentially negative consequences for sectors of the cancer research community [2].

The rise of chronic disease, combined with an aging population and new pressures on entitlement programs such as Medicare and Medicaid, creates a more complex health-system picture that affects all clinical activity—including cancer research. Health-care disparities in the United States, measured by race and income, continue to impact large sectors of the population and affect RCC outcomes [3].

Significant cuts to federal research-funding threaten innovation, while federal regulatory issues pose obstacles to the development of and access to new drug therapies. The pool of participants for clinical trials continues to lag, along with research funding, at a time when the need for more and better clinical data is growing [4].

The fast-changing and volatile environment for pharmaceutical, biologic, and medical device development impacts the way new drugs come to market—and poses serious new questions for our research infrastructure [5]. New international issues related to drug and medical device regulation, combined with an increasingly global health-care marketplace, can negatively impact supply and demand, leading to issues such as drug shortages [6].

In this environment, strong advocacy for the RCC community is a must: The hope of patients and families, the dedication of physicians, and the perseverance of researchers and innovators grow from shared commitment, strength of purpose, and unity of voice.

Advocacy paves the way for progress by helping accelerate policy development, by raising awareness of key issues, and by encouraging synergy between potential partners across sectors in health care. It operates as a catalyst for research and discovery, helping the RCC community identify priorities, fend off threats to those priorities, and mobilize resources to accomplish its goals. It joins with research and education as the vital third element in the triangle of progress upon which the RCC community's recent accomplishments have been built.

Advocacy occurs on many levels and in many permutations within the RCC community, ranging from individuals organizing backyard fund-raisers to 501c3 organizations lobbying Congress and celebrity spokespersons endorsing special initiatives. In addition to traditional activities, such as letter-writing campaigns,

op/eds, media outreach, and testimony before policy makers, a major manifestation of RCC advocacy emerges via educational symposia and the development of informational materials for patients and caregivers. These key activities help sharpen the focus of the RCC community, defining and reinforcing the issues that matter and must be addressed to continue progress toward cures.

The growth and diverse range of advocacy activity associated with RCC—and its measurable impact over the last decade—is substantial. It can be argued that the rise of organized advocacy over the last two decades has played a strong role in the RCC community's recent spectacular progress in developing new therapies and treatments. It has unquestionably been integral in improving the quality of life for the growing community of kidney cancer survivors.

Defining RCC "Advocacy"

What do we mean by "advocacy" in the RCC community—and who are its "advocates"? What role do they play and what should their role be in the future?

Broadly speaking, RCC advocacy encompasses:

- Improving the care and treatment of cancer patients by influencing state and federal policy through direct outreach to legislators, regulators, and other policy makers.
- Mobilizing physicians, patients, and caregivers in support of specific policy positions.
- Educating physicians, patients and caregivers, and the public about kidney cancer.
- Sponsoring and encouraging research.
- Fund-raising to support the goals of the RCC community.
- Building a stronger RCC community by encouraging partnerships and synergy among diverse organizations and individuals.
- Fostering the free and open exchange of ideas and support and strengthening relationships between patients and physicians.

A broad cross section of the RCC community is actively engaged and contributing to these efforts.

Patients

The so-called empowered patient is a well-documented phenomenon that is radically changing the dynamics of health-care advocacy [7]. With high-deductible health plans and out-of-pocket expenses on the increase, patients are becoming more consumer oriented, insisting on better understanding their treatment options. They have also become more demanding of evidence-based information, largely as

a result of resources such as the Internet, and are less likely to passively accept authoritarian directives from a health-care provider. Consequently, patients are poised to play an ever-increasing role in impacting the way health care is delivered.

Those diagnosed with cancer often become the most committed of these new health-care consumers, gradually becoming vocal advocates as a result of their experience. Advocacy provides a means to turn a negative experience into something positive—and a way to help others. The empowerment that comes with taking an active role as an advocate serves as a counterweight to the feelings of fear and vulnerability that often come with an initial diagnosis. Speaking about one's own experience can play an important part in the healing process following surgery and treatment.

The rise of organized patient advocates within the RCC community has been one of the most important developments of the last two decades, spurred largely by the Internet and the growth of social media.

RCC patients are more engaged than ever before in virtually all levels of advocacy today, organizing fund-raisers and public events, assisting organizations such as KCA on special projects, writing blogs, contacting legislators and policy makers, and speaking at conferences and symposia.

The effectiveness of first-person testimonials, especially in terms of sharing stories of navigating the health-care system, has added to the popularity of publications such as the KCA's *We Have Kidney Cancer* and *Survivors Stories* and books such as *Smile for Your Child: A Parent's Guide to Finding Positive Energy During Diagnosis, Treatment, & Life After* by Tracy Gray. These provide strong channels for RCC advocacy on issues of concern to patients.

Chat rooms and online forums have become a particularly powerful mechanism for RCC patients, enabling information exchange and personal support that has accelerated the transmission of news about clinical developments, treatment options, and other resources.

To provide just one example of the strong reach and impact of online communications, participation in KCA's various electronic forums has grown explosively in the last eight years, increasing from 4,000 users in 2005 to more than 65,000 today. The ability to convey information quickly and widely via electronic communication has led to a better-educated grassroots community that is more accurately informed today on key issues such as clinical trials, federal funding for research, and drug development and delivery than in any previous patient era.

Families and Caregivers

The role and impact of those who serve as caregivers for cancer patients has been extensively studied in recent years [8]. The physical, social, emotional, psychological, and financial burdens of caregiving can be extreme, profoundly impacting patients and families and influencing RCC treatment.

Well over a million new cases of cancer are diagnosed each year in the United States [9], many of them eventually requiring support from a family caregiver. With cancer increasingly becoming a chronic condition, and with the number of kidney cancer survivors on the upswing, these impacts are expected to increase. Additionally, new treatment models, including the Patient Centered Medical Home and Accountable Care Organizations, place a new emphasis on the inclusion of families and caregivers as central components of care teams [10].

As a result, caregivers and families are considered a growing part of the equation for success in RCC treatment. As in many cancer communities, the participation of caregivers and families in shaping and advancing RCC advocacy is on the rise. They are among the most active supporters of efforts such as fund-raisers and public awareness campaigns. As such, they become passionate and effective advocates in the search for cures.

At the same time, caregivers and families can become important advocates for their *own* needs, which form a distinct subset within the overarching priorities of RCC advocacy. Individuals and groups that provide support to other caregivers and families, while raising awareness and visibility of issues related to caregiving, play a key role in the spectrum of RCC advocacy.

Physicians, Nurses, and Other Health-Care Professionals

Health-care professionals who specialize in cancers of the kidney represent a core advocacy group. As physicians and other health professionals have become more specialized in recent decades, the power, reach, and influence of their representative organizations have grown.

Membership in specialty organizations such as the American Society of Clinical Oncology (ASCO) and the American Society for Radiology Oncology (ASTRO) has expanded as membership in generalist organizations such as the American Medical Association (AMA) has retracted. In 2012, AMA membership slipped to 217,490 [11]—a relatively small percentage of the nation's physicians.

At the same time, oncology-targeted organizations for other health professionals, such as the Oncology Nursing Society (ONS), with more than 35,000 nurse members, have also expanded their scope and impact [12].

US medical specialty societies have introduced a much more focused, sophisticated, and issue-specific form of public advocacy as they have grown in stature. Accompanying this new organizational advocacy has been a growing sense that "advocating for patients" should be considered a part of a physician's professional responsibility. In 2002, for example, the American Board of Internal Medicine (ABIM), in its charter on medical professionalism, called for a "commitment to the promotion of public health and preventive medicine, as well as public advocacy on the part of each physician" [13].

Similarly, the AMA formally states that physicians must "advocate for the social, economic, educational, and political changes that ameliorate suffering and contribute to human well-being" [14]. Today "advocacy" is part of everyday life in medical specialty organizations: Advocacy "resource centers" are common—often prominent—features of their websites, as are links to Political Action Committees (PACs) and grassroots lobbying networks, all part of the expectation that physicians, nurses, and others should help shape the health-care environment as a part of their professional commitment.

Recent studies show that while large numbers of physicians, nurses, and other health professionals *endorse* the new role of advocacy in organized medicine, fewer of them actively *engage* in it [15]. Still, those that do engage form an important force for change and progress. This is certainly the case in the RCC community, where physician and nurse leaders play prominent roles in the governance and strategic positioning of organizations such as the KCA [16]. They also figure prominently as spokespersons in the media and as key members of lobbying teams interacting with policy makers, research funders, and others—where they are able to raise public awareness of issues such as cancer detection, screening, and the importance of clinical trials.

Another important avenue for physician, nurse, and physician assistant advocacy in the RCC community is found in educational colloquia and symposia, which provide the opportunity for clinicians to raise the visibility of emerging trends, opportunities, and challenges in clinical treatment and discovery.

Not-For-Profit Organizations

A variety of not-for-profit organizations and private foundations provide wide-reaching advocacy services that benefit the RCC community, ranging from the American Cancer Society and the National Cancer Institute to the National Coalition for Cancer Survivorship and the Kidney Cancer Association.

Smaller organizations, such as the VHL Alliance for patients with von Hippel-Lindau disease, the Cancer Leadership Council, National Comprehensive Cancer Network, Patient Advocate Foundation, the National Coalition for Cancer Research, and Friends of Cancer Research, also add to the tapestry of advocacy organizations that include a focus on RCC in their efforts.

Similarly, international organizations such as the International Alliance of Patients' Organizations and the World Cancer Congress also exist, in many cases closely integrated with the activities of US groups.

The breadth of advocacy activity engaged in by professional staff of these organizations includes patient and physician education, special events, research development, clinical trial placement services, peer-to-peer counseling and advice, formal lobbying, publishing, and fund-raising.

Of these, fund-raising to support research toward cures is a prime activity of many of the not-for-profits, with advocates planning fund-raising activities ranging from cancer walks and the sale of products to annual direct mail campaigns.

Corporations

The support of pharmaceutical, biologic, and medical device manufacturers is an important part of the advocacy network that weaves together the RCC community. The discovery and development of new therapies and treatment options in the last decade have been critically important in creating a new era of hope and possibility for kidney cancer patients.

With federal research funding on the decline, the support of corporate foundations and charitable initiatives continues to play a role in advancing RCC advocacy. Corporate funding of activities such as scientific meetings, web-based patient resources, educational publications, and research projects helps advance many of the key goals of the RCC community.

The Kidney Cancer Association and the Impact of Organizational Advocacy on RCC

As an unusual form of cancer, RCC was for many years simply one of many cancers that fell within the general advocacy efforts of organizations like the American Cancer Society and the American Society of Clinical Oncology.

In 1990, a small group of patients, spearheaded by Eugene P. Schonfeld, PhD, and physicians in Chicago, founded the Kidney Cancer Association—the first patient-physician-based organization dedicated solely to supporting the needs of kidney cancer patients.

From the beginning, advocacy was a core KCA activity. In 1994, Dr. Schonfeld raised the organization's profile considerably when he began an intense personal lobbying effort in Washington, D.C., traveling to the capital on almost a weekly basis. He testified before the Food and Drug Administration in favor of approval of interleukin 2 as an agent for treating RCC—a successful effort that had a huge impact on patients and families. He also advocated forcefully with policy makers for reforms to the nation's patent processing systems and pushed for new approaches to the structure and purpose of clinical trials.

In its early years, the Kidney Cancer Association brought together physicians specializing in RCC to make presentations and provide clinical updates at meetings for patients. But it quickly learned that the exchange of information from physician to physician was as just as important as the effort to educate patients. In the early 1990s it began putting a strong focus on providing physician education and the exchange of clinical information in addition to patient education and updates, in this way helping to facilitate and advance new clinical discoveries. Its first physician symposia were extremely successful, providing funding for expansion of other services and activities.

In the early 2000s, the KCA made a fundamental operational shift that has profoundly impacted its work as an advocacy organization, changing from a membership model—with "members" paying annual dues and voting to elect officers—to a

donor-supported, staff-and-board-governed model, with no yearly dues. The switch saved the enormous postal costs of processing dues and ballots and freed significant administrative time and resources.

Over the course of two decades, the KCA also shifted its fund-raising model: In the early years, it hosted gala events to raise visibility and recognize donors—but these events were often revenue neutral or money losers. Starting in 2003, the association turned its focus to direct funding-solicitations and smaller, de-centralized events hosted by kidney cancer patients and families in their own localities. By the mid- to late 2000s, the focus of fund-raising had moved increasingly outward, using the Internet as an organizing tool.

Similarly, the KCA has moved to a new lobbying and advocacy model: In its early years it relied on traditional centralized "Day on the Hill"-style lobbying efforts, lining up one-on-one sessions to educate legislators in Congress about RCC at significant volunteer travel and staff cost. In the mid-2000s, it began shifting to a more nimble and coalition-based advocacy model, in which it works in partnership with a broad group of cancer advocates who have similar overarching goals. The united voices of the KCA's cancer coalition have a much greater impact on broad cancer-related issues than they would if each group advocated on behalf of its own unique cancer constituents.

KCA has had enormous success as an advocacy organization, especially for an agency of its size. The most important milestone activities were KCA's successful efforts in helping convince regulators to approve the use of interleukin 2 for RCC patients in the 1990s and, more recently, its work in helping support the development of seven new drugs for kidney cancer patients in less than a decade.

Also of significance is the KCA's role in developing the RCC physician network in the United States and internationally and its work in creating a cohesiveness between US and international scientific efforts. The KCA's international and European symposia have connected RCC physicians in ways that have significantly altered the research environment.

One of the most important components of this new international network was the development, over the course of 10 years, of a database that compiled US and European RCC data. The compilation effort was the largest undertaking of its kind ever, pulling together RCC patient data from as far back as the 1970s. Formally called the International Kidney Cancer Working Group, the physician-led task force that compiled the database began its work in 2002. Among the tools developed as a part of this work is the KCA's online Kidney Cancer Risk Score Calculator, which provides an individual risk assessment for RCC, based on the extensive data compiled in the international database [17].

Today, the KCA reaches more than 70,000 people in 102 countries. Approximately 60 % of its constituents are located outside the United States, with a fast-growing presence in Southeast Asia. It is the largest organization of its kind dedicated to the eradication of RCC in the world and its publications have been translated into 13 languages.

The KCA of today represents a new not-for-profit advocacy model, one which is lean in personnel and relies largely on outsourced and online services, connecting

with its audiences primarily online through the use of social media. It operates with a small staff of two full-time employees and one part-time employee, relying heavily on outsourced services for day-to-day operations. In the new advocacy model, the KCA works across sectors, actively engaging industry, government, and academia as essential partners in its effort to develop new scientific discoveries to benefit patients.

The Role of RCC Advocacy in Health Policy

Sustaining the tremendous clinical progress that has occurred in the RCC community in recent years will require constantly renewed and focused advocacy on a variety of health policy issues that affect the search for cures.

The past two decades have seen the rise of a wide variety of resources for RCC patients and health-care providers alike. They range from effective new drugs from the pharmaceutical industry and the development of enhanced surgical techniques by physicians to the staging of international RCC educational symposia, the creation of nurse/physician advisory councils, and the extensive growth of support resources such as books and online forums for patients and expanded continuing medical education for health-care providers. The Kidney Cancer Association and other RCC-oriented organizations, such as the VHL Alliance, remain at the forefront of encouraging and supporting these resources.

Standing, as we are, on the cusp of significant change in our health-care delivery system, continued economic uncertainty, and new public health issues that complicate cancer care—such as the rise of chronic disease—it is more important than ever for the communities that developed the resources of the last two decades to coalesce now as advocates to protect and sustain them.

Each of the major advocacy groups—patients, families and caregivers, health-care providers, not-for-profit organizations, and corporations—can contribute to this effort, focusing their efforts collectively on the key policy issues that pose significant challenges to RCC progress in coming years. These challenges include the following.

Preserving Federal Funding and Support for RCC Research

Since President Nixon launched a national effort to eradicate cancer with the National Cancer Act of 1971, the United States government has committed substantial funding to the effort, with significant portions of its funding channeled through the National Cancer Institute (NCI), located within the National Institutes of Health (NIH). The NCI has spent approximately $90 billion on research and treatment initiatives since the Act was signed [18].

Between the years of 1998 and 2003, spending on cancer research accelerated sharply. Federal funding of the NIH doubled, from $13.6 billion to $27.5 billion [19]. After 2003, however, funding increases flat lined, did not keep pace with inflation, and included a 1.5 % spending cut in 2011. Despite a one-time-only increase of $10.4 billion in 2009 as a part of the federal stimulus plan [19], the lack of NIH funding growth over the last decade has essentially eroded the buying power for medical research by roughly 20 % [20].

In the wake of the nation's economic collapse and recession, lawmakers have now proposed even deeper spending cuts to the NIH and NCI—as much as 10 % across the board [21].

Budget analysts point out that the NIH budget, at roughly $31 billion, is less than 1 % of the total US budget of $3.7 trillion. A 10 % cut to the NIH budget would have minimal impact on the overall US budget (saving roughly 0.08 %), but it would have significant impact on the cancer research community—enough so that former American Association for Cancer Research President Judy Garber, MD, has declared that for researchers, "funding is in crisis" 40 years after the start of the war on cancer [22].

The specter of significant cuts to federal research funding cannot be underestimated. NIH funding has historically accounted for nearly 80 % of all funding for not-for-profit medical research in the United States [23]. Most biomedical researchers and major medical centers rely heavily on NIH funding. RCC research has been particularly dependent on NIH funding.

Hundreds of RCC clinical trials that are currently under way, and much-needed trials under consideration, are vulnerable in this cost-cutting environment.

A potentially new threat to the goal of stabilizing RCC research funding at the federal level is implementation of comparative effectiveness (CE) studies, authorized under the American Recovery and Reinvestment Act, signed into law by President Obama in February 2009 [24]. The Act provided $1.1 billion for CE research, intended to "conduct, support or synthesize research that compares the clinical outcomes, effectiveness, and appropriateness of items, services, and procedures that are used to prevent, diagnose, or treat diseases, disorders, and other health conditions" [25]. A strong emphasis was put on oncology as a focus of CE research.

A growing concern with the government's approach is that it is intended to determine effectiveness of current treatment options via randomized clinical trials—which are already stretched thin in the United States and have difficulty enrolling participants. In this environment, the challenge for the RCC community will be balancing participation in CE studies in a way that does not inhibit the continued development of novel therapies: Oncologists will have to choose whether difficult-to-fill clinical trials should be devoted to CE-oriented studies of well-characterized, currently available therapeutic agents or studies of purely investigational agents [26]. Both have long-term value to the RCC community, but it is vitally important that the pipeline of investigational research remains active and constantly moves forward. RCC advocates will need to monitor the discussion of CE implementation to ensure it does not jeopardize clinical efforts.

Cutbacks in federal funding could also impact the work of the nation's not-for-profit community dedicated to supporting RCC patients, caregivers, and health-care providers. Today, a wide range of not-for-profit cancer-related organizations are actively raising funds in this effort [27]. Access to federal grants and other resources is an important component of most cancer-related not-for-profits' funding profiles. Awareness-building efforts of the needs of RCC patients are a key component that have helped pave the way for support of RCC-oriented research and discovery; these efforts have been the principle domain of organizations such as KCA. Adequate funding for these patient-driven organizations is of central importance.

Supporting a Robust Pipeline of Drug and Medical Device Discovery

At a time of flux in federal funding, many pharmaceutical companies have also reduced their research spending as a result of mergers, market forces, and budget cutting [19]. The RCC community must build strong relationships with industry and engage in active advocacy and partnerships with it to ensure the industry-supported pipeline of new therapeutic agents and medical devices is robust.

The journey from drug discovery to approval is arduous: Only one of every 10,000 potential medicines investigated by US pharmaceutical companies makes it through the research and development pipeline to gain approval for patient use by the FDA. Gaining approval of a new drug takes, on average, 15 years and is estimated to cost more than $1 billion [28]. New medicines must pass through a variety of stages and tests before they are approved; once formally introduced, the documentation for approval is extremely complex. Typically, tens of thousands of pages of paperwork go into the development of an FDA New Drug Application (NDA) [29].

A new issue impacting the pipeline of drug therapies for RCC patients is the research community's changing approach to cancer treatment. For decades, RCC has been a difficult cancer to treat, with surgery being the cornerstone of clinical options. Systemic therapies were limited to toxic agents such as interferon and interleukin 2. But with advances in the understanding of two major RCC pathways—mammalian target of rapamycin and VEGF/VEGFR—and the rapid development of seven new drug therapies for RCC over the last decade, the orientation of the research community has changed.

The RCC community now faces the challenge of taking its revolutionary discoveries to a new level of impact for patients, rather than focusing on areas that are unlikely to result in major improvements or advances over existing therapies. New drugs are needed in key areas outside the two major pathways that have been identified and for categories of kidney cancer that lack effective therapies—non-clear-cell RCC, for example.

This requires advocacy and partnership with the pharmaceutical industry to prevent duplication of effort and the introduction of "me too" trials and therapies. Only about one-third of the drugs approved annually in the United States are new compounds; the rest represent modified forms of—or new uses for—existing drugs [29]. The RCC community should strive, collectively, to articulate and formalize a vision for its research future, prioritizing goals and targets—and putting an emphasis on research in new, understudied targets.

Our updated understanding of the genomic and molecular nature of cancer has placed a new importance on the use of biomarkers in helping identify novel treatment targets, identify patients who are eligible for clinical trials, and to better monitor responses to therapies under study [30]. As a part of its vision for new drug development, the RCC community must advocate strongly for increased study and focus on the use of biomarkers in research.

Most of the important new drugs introduced by the pharmaceutical industry over the past 40 years were developed with some contribution from public-sector research [29]. Advocacy from the RCC community should be directed at ensuring that the critical connection between private-sector innovation and public-sector funding in drug development is maintained and strengthened.

Encouraging Enrollment in Clinical Trials

Successful development of new therapies for cancer patients is heavily dependent on our ability to translate scientific discoveries into practice via clinical trials. In 2013 there were well over 600 RCC trials under way worldwide [31], and a major focus for the RCC community should be ensuring that our clinical trials are successfully completed and new ones started.

Randomized clinical trials (RCTs) are of great importance in this effort, but enrollment in RCTs faces many challenges in the United States. It is estimated that only between 2 and 7 % of cancer patients participate in trials [32]. According to pharmaceutical industry analysis, an estimated 80 % of trials fail to meet their enrollment timelines, with many research trial sites enrolling one or no patients [33]. Survey data shows that awareness of clinical trials among both cancer patients and the general public is very low. One survey of cancer patients showed that 80 % had not considered the possibility of participating in a clinical trial because they were not aware of the option [32].

Among the reasons for under-enrollment are a lack of insurance coverage for trials, the logistics of participation—which can be challenging for patients—and strict eligibility criteria. Some studies indicate the problem may be exacerbated by negative beliefs and misconceptions about clinical trials, on the part of both patients and physicians [32]. Physicians are critical gatekeepers to the clinical trial process, but many are reluctant to enroll their own patients, for a variety of reasons [4].

Studies indicate that another major issue in clinical research is financial pressure, including understated infrastructure needs and reimbursement costs associated with recruitment and retention of patients. Substantial cost outlays are necessary to organize a clinical trial, but the nation's current focus on containing costs through value-based and "accountable" health-care models creates potential new hurdles on the expansion of trials [4]. In addition, achieving racial, ethnic, and gender balance in clinical trials continues to be a challenge [4].

Additional factors impeding new trial development are administrative requirements and regulatory burdens, including institutional policies governing clinical investigation that may actually discourage, rather than encourage, participation among physicians and by institutions in community settings vs. academic centers.

Beyond the serious issue of under-enrollment, clinical trials face other challenges; these include concerns about bias in data analysis—which have come to light in newer studies. According to a recent literature review published in the *Annals of Oncology*, for example, a third of RCTs in breast cancer published results that showed bias in the reporting of endpoints, with two-thirds showing bias in reporting toxicity [34]. According to researchers, authors of the published results "used spin in an attempt to conceal bias." "Bias in the reporting of efficacy and toxicity remains prevalent," said lead researcher Ian F. Tannock, M.D., of the University of Toronto. "Clinicians, reviewers, journal editors, and regulators should apply a critical eye to trial reports and be wary of the possibility of biased reporting" [34].

Clinical trial advocacy for the RCC community begins with heightened awareness-building activities—including outreach to physician audiences to promote the competitive advantages of helping connect their patients with cutting-edge therapies. The KCA strongly supports collaborative efforts—such as the Clinical Trials Transformation Initiative (CTTI) [35]—to raise awareness. Success will most likely be achieved through strong partnerships that should include the federal government, patient and physician organizations, academia, and the pharmaceutical industry. Emphasis needs to be placed on building awareness among minorities, women, and underserved populations in order to create more representative clinical trial populations. The RCC community should also focus efforts on encouraging development of clinical trials in community settings, which would address many issues that impact patient enrollment (e.g., travel time and cost).

Updated national criteria and guidelines are needed for clinical trial development, aimed at creating new administrative constructs and processes that will reduce enrollment and participation barriers for both patients and physicians.

As clinical research moves more aggressively toward molecular and genetic strategies, the RCC community should take the lead in helping educate patients and clinicians about the emerging importance of genomics and biomarkers in the testing of targeted therapies. As a part of this effort, new approaches to molecular screening of patients in clinical research studies are needed, along with insurance reimbursement for this key step in the discovery process.

Food and Drug Administration Effectiveness

On July 12, 2012, President Obama signed into law the Food and Drug Administration (FDA) Safety and Innovation Act, which introduced a number of sweeping reforms that impact the future of RCC research and discovery [36].

The law's new provisions require the FDA to meet performance goals related to timely review of drug applications, increase interaction with drug sponsors during the review process, and expand interactions with patients, including those with rare diseases. In addition, it mandates accelerated approval tracks for certain categories of substances, requires the FDA to work with other regulatory agencies to reduce duplication of studies needed for premarket approval, and makes other structural changes intended to create a more efficient drug approval and oversight process [36].

The new law addresses many of the issues that have been a focus for RCC advocacy in recent years, including expanding access to new drugs to patients in need, improving quality controls over drug approval process, and increasing transparency required in the manufacturing of drugs in a complex global marketplace. In addition, the new law makes the drug approval process more predictable for industry [36].

The RCC community must continue to monitor progress at the FDA and advocate for adequate funding and structural enhancements that help expedite the delivery of new therapies to RCC patients.

Physician Payment Reform, Including Repeal of the SGR

The rapidly changing professional environment for physicians has created a number of issues that potentially affect care for kidney cancer patients.

Rising health-care costs and the implementation of the US Affordable Care Act have contributed to the acceleration of concepts such as Affordable Care Organizations (ACOs) and the Patient Centered Medical Home, which seek to deliver more efficient, outcomes-based health care [37].

Structural changes to the health delivery system introducing these and other new concepts must be made in a way that protects the patient-physician relationship, preserves physician autonomy, and ensures the physician voice is included in the governance and clinical decision-making of the new systems. It is important that new payment models are structured and introduced in a way that doesn't impact access, especially to underserved populations.

An ongoing issue for the medical community is the Sustainable Growth Rate (SGR), which continues to play havoc with physician reimbursement rates and threaten access to care for kidney cancer patients.

A key focus of RCC community advocacy should be creating a stable practice environment for physicians. An unstable SGR puts a strain on practices.

The SGR, which impacts reimbursement for physicians who care for Medicare beneficiaries and thus potentially impacts millions of Americans, is long overdue for reform in order to ensure stable access to health care. The RCC community should support a move away from the current payment formula, which connects Medicare reimbursement rates to the growth of the Gross Domestic Product (GDP) [38]. Because it bases reimbursements on the GDP and not on actual health practice costs incurred by physicians, it consistently recommends unrealistic cuts to Medicare reimbursement rates that must be overridden by Congress [38].

Congress has long recognized issues with the SGR, but has yet to address them on a permanent basis. New payment methods are needed in order to maintain access to care among RCC patients on Medicare; an effort that begins with a plan to phase out SGR.

CE Research in the Affordable Care Act

The spiraling cost of health care in recent years has led to a pendulum swing of cost containment that is well placed, but not without risks. Pressure is increasing from a variety of health-care system stakeholders—including insurers—to justify the cost of cancer therapies along with safety and effectiveness. In a recent PricewaterhouseCoopers PwC poll, 80 % of insurers who responded said they now require evidence of cost savings or a distinct clinical benefit in order for cancer therapies to be added to lists of covered drugs [39].

The rise of ACOs and other new care delivery models will add to this pendulum swing, as will provisions of the Affordable Care Act. The Act includes several features aimed at improving the value of health care by paying hospitals for quality rather than quantity.

Among these features is comparative effectiveness (CE) research, which, as noted earlier, is the study of which medical tests and treatments deliver the best results for various patients under different circumstances. While CE is intended as a way of encouraging more efficient and less costly health care by giving patients and health-care providers data to help them make better-informed treatment decisions based on demonstrated outcomes, there has been increasing discussion that CE data could lead to a system in which patients are denied potentially lifesaving treatments using cost as a justification [40].

Minority and disability groups have expressed concerns that CE research, if not conducted properly and taking into account broad enough population samples, could lead to flawed decision-making [40].

As CE continues to be discussed and developed, the RCC community should advocate for CE study designs that take into account patient individuality and the new focus on the genomic and molecular nature of cancer. The goal of RCC community advocacy should be the prevention of misuse of CE data—for example, by insurance providers, who could make one-size-fits-all coverage decisions using CE studies as a basis [40].

In the new era of molecular, genetic-based approaches to cancer, therapies will be much more targeted than in years past and the cost of developing them is expensive. The specter of researchers retreating from potentially effective novel therapies because of cost issues is very real and should be a focus of RCC community vigilance and advocacy.

Safeguarding Against Drug Shortages

Drug shortages have dramatically increased in recent years, forcing physicians to alter their treatment plans and putting patients at risk. According to the FDA, the number of drugs in short supply tripled between 2005 and 2011 [41].

The oncology community has been particularly hard hit by drug shortages. The National Coalition for Cancer Research noted that of the 22 cancer agents on the drug shortages list in 2011, 15 are urgently needed for clinical research [42]. According to the Coalition of Cancer Cooperative Groups, approximately half of all active cooperative group cancer clinical trials in 2011 had at least one drug on the government's shortages list [42].

The impact of such shortages is potentially devastating, introducing disruption and delay to the development of the hundreds of new cancer therapeutics that are in active clinical trials. In some cases, for example, shortages of basic chemotherapy agents have delayed or even halted enrollment in clinical trials sponsored by the NCI [43].

Cancer research is inherently more prone to damage from drug shortages because placebos are rarely used in its clinical trials and are never used alone if an effective treatment is available to trial participants. Cancer trials test the safety and efficacy of the standard of care against, or in combination with, new treatments that are being investigated [42].

Beyond clinical trials, the impact of drug shortages on patient outcomes is clear and well documented. In an analysis of children treated for Hodgkin's lymphoma in 2012, for example, results showed that switching to cyclophosphamide (Cytoxan) when mechlorethamine (Mustargen) supplies ran short lowered the rate of 2-year event-free survival in patients from 88 to 75 % [44]. Previous literature review had suggested that the two drugs were interchangeable.

While to date the RCC community has not been impacted at the level of other cancer communities by recent drug shortages, the potential for damage is present. Moreover, the structural issues that affect drug supply impact the entire cancer community and require unified advocacy in response. As the globalization of medicine continues, and international economic and policy issues increasingly impinge upon drug supply and demand, the potential for drug shortages relating to the RCC community will increase.

In response, the FDA should develop a more robust policy aimed at reducing our vulnerability to drug shortages, while ensuring appropriate contingency plans are in place for shortages of critically important drugs.

Recommended action steps include improving communication between the industry and health-care providers to anticipate shortages in a more timely manner, increasing the extent of supply responsiveness in the market, and creating better criteria and distribution options for drugs considered vulnerable to a shortage.

Health Equity/Health Disparities

The US government has long included addressing disparities in health-care access and outcomes—including disparities among racial and ethnic groups—as a national priority. The goal of reducing health disparities has been a part of the national "Healthy People" initiative for many years, but little progress has been made in reducing these disparities [45]. New research shows that health inequities continue and that they are impacting the RCC community; a recent study showed that black patients with kidney cancer have poorer survival rates than white patients [46]. In a study of National Cancer Institute data on nearly 40,000 patients with RCC, researchers found that 72.6 % of white patients survived at least five years, compared with 68 % of black patients.

The higher survival rates in white patients occurred in all subgroups of patients, ranging from gender and age to tumor stage and size. This, despite the fact that a higher percentage of black patients were diagnosed with localized cancer and smaller tumors—which normally should point to a better chance of survival. Researchers also found that blacks were less likely to receive surgical treatment, which is associated with a less favorable prognosis [46].

Health inequities have been shown to exist at many other levels of the US health-care system, ranging from urban vs. rural settings to income levels to genders. All Americans should have access to the care they need, and RCC community advocacy should be focused on overcoming this problem. Among the solutions are improving the delivery of health services in the United States—including more strategic use of preventive measures—as well as improving access to health insurance and increasing health literacy among targeted populations.

Strengthening Patient Education

In an era of increased cancer survivorship, the education of patients and their caregivers is increasingly important. Aligning the patient-physician-caregiver relationship to ensure effective patient education opens the pathway to the information and treatment strategies that can extend survivorship and add to the quality of life.

At a time when health practitioners' jobs are becoming more complex and the number of kidney cancer survivors is growing, however, finding the time and resources to devote to better patient education can be challenging. The focus of clinicians can be heavily skewed toward surgical and therapeutic outcomes, rather

than comprehensive, long-term recovery plans geared toward helping survivors cope with life after diagnosis and treatment.

A recent Mayo Clinic study, for example, demonstrated that a surprisingly low number of physicians recommend basic, well-established strategies for dealing with debilitating fatigue after cancer treatment—despite the existence of formal guidelines for patients. Only one tenth of patients studied reported that their care teams had instructed them about the full regimen of fatigue strategies available—often opting instead to recommend medication as the primary response [47].

The RCC community can address this issue by unifying around a new survivorship-oriented paradigm of training for health-care professionals, which places greater emphasis on services to patients and their families after initial diagnosis and treatment.

Key features of this new paradigm would include the standard use of long-term cancer-care plans and a new emphasis on enhancing communication with patients and families, more consistent use of treatment summaries that offer clearly defined and detailed follow-up care for survivors, expanded and updated approaches to palliative care, and increased funding for studies of long-term cancer survivorship.

A key to success in promoting this new paradigm is in strengthening connections between patients and their health-care teams. RCC advocates can do this by creating more opportunities for interaction between patients, caregivers, and health-care professionals outside of clinical settings—at education and support meetings and conferences and in online forums, for example—and by creating materials that keep patients and caregivers well informed of advances in treatment and research. KCA annual conferences are structured to include representation by patients and caregivers, as well as physicians, nurses, and others, in the spirit of open exchange of information and dialogue. Patient advocates typically prepare summaries of medical presentations at such conferences, which can be easily understood by patients and their families.

Medical Workforce Issues

ASCO predicts that by 2020 there will be a shortage of 4,000 oncologists in the United States [48]. The RCC community should take steps to bolster the pipeline of kidney cancer specialists in the oncology workforce by advocating for stabilized residency funding in key physician specialties. Working with partners such as ASCO, it should raise the visibility of recent advances in RCC research and the need for RCC specialists in a dynamic research and practice environment.

As survivorship grows, the RCC community may need more specialists with expertise in supportive care for kidney cancer patients, particularly for issues such as fatigue and depression. The RCC community should advocate for stabilized residency funding in key physician specialties and for efforts to bolster the workforce pipeline of critically important members of cancer-care teams—including oncology nurses. Workforce projections indicate that the nation will face a severe shortage of

registered nurses by 2020 [49], and some nursing positions, such as advanced practice nurses and oncology clinical nurse specialists, will be especially important to the oncology community [50].

To promote the continued proficiency of RCC physicians, nurse specialists, and others in this new lean workforce environment, professional development and targeted continuing medical education will be crucial. The RCC community should put an emphasis on continued efforts to expand conferences, symposia, and online learning opportunities for health-care professionals.

The KCA has expanded its medical education activities in recent years, providing new opportunities for physicians, nurses, and others to exchange cutting-edge information at special clinical summit meetings and symposia. In 2013, the KCA hosted its 8th European Kidney Cancer Symposium, which was offered in addition to its annual International Kidney Cancer Symposium, held in Chicago.

Raising Public Awareness of RCC

A variety of trends have begun coalescing in recent years to create challenges for advocacy-based organizations seeking to raise visibility for their causes. The most impactful of them is the sheer volume of cause-related communications that now bombard consumers. Billions of pieces of cause-related mail are delivered in the United States every year [51], along with a growing stream of Internet updates, alerts, and fund-raising appeals.

According to the National Center for Charitable Statistics (NCCS), more than 1.5 million nonprofit organizations are registered in the United States [52]—all competing for the hearts and minds of their targeted audiences in an environment characterized by "information overload." In an era of government funding cutbacks and economic hardship among consumers, the competition for financial support has also become more challenging.

In this environment, the ability to present a compelling case for one's cause is paramount, and studies show that the best way to do so is by effectively telling the stories of the people who are most impacted by the cause. In the case of the RCC community, our advocacy can be amplified and strengthened through the stories of kidney cancer patients and their families.

A recent study published in the *American Sociological Review* found that diseases tied to strong patient-advocacy organizations, which tell their public stories through a strong lens of patient experience, received millions of dollars more in research funding between 1989 and 2007 than organizations whose patient orientation was not as strong [53].

In the United States, the KCA and other kidney cancer groups have focused on raising visibility of RCC by community-building—bringing groups of people impacted by kidney cancer together to share their experiences and ideas and highlighting their inspirational and compelling stories.

Publications such as *We Have Kidney Cancer* and *Survivors Stories*, published by the KCA, provide first-person accounts and advice from actual kidney cancer patients, with a strong emphasis on hope. As a service to international audiences, *We Have Kidney Cancer* has been translated into 13 languages and distributed worldwide.

KCA offers KidneyCancer.me, a peer-to-peer collaboration website for patients, survivors, and caregivers, and it hosts weekly informal Facebook group chats that encourage information sharing and personal stories. It also hosts dozens of local education and support meetings nationally that bring small groups of patients and caregivers together to exchange ideas and provide support for each other.

The use of celebrity spokespersons has become an effective visibility-raising tactic for a wide variety of health-oriented organizations. In recent years, the KCA has built a strong relationship with actress Denise Richards, whose mother died from kidney cancer in 2007. It recently launched a joint project with Richards and online apparel company ShoeDazzle to raise funds for kidney cancer research, education, and advocacy through sales of two custom shoe designs (one by Richards and one by a teen who died of RCC). The project has been supported by a partnership between KCA, the Urology Care Foundation, and the Conquer Cancer Foundation [54].

Richards has participated in other fund-raising activities on behalf of the KCA—ranging from video contests to run/walks—and has recorded public service announcements on its behalf. As a part of its visibility-building efforts, KCA offers a variety of branded awareness-building products, prominently featuring the color orange as a branding device—including posters and commitment bracelets.

Combined, these activities form the base of an awareness-building enterprise that has raised the public profile of RCC significantly over the last decade. The creation of a strong community of people whose lives have been impacted by RCC provides a national fund-raising network that has helped raise millions of dollars for kidney cancer research [55].

Special Advocacy

Two health conditions related to RCC are the subject of special advocacy within the RCC community. These include:

VHL

Von Hippel-Lindau disease (VHL) is a rare, genetic disease that causes tumors and cysts to grow in the body. Clear cell RCC is the cell type associated with the VHL gene mutation in hereditary kidney cancer. Because of its genetic basis, VHL is frequently misdiagnosed or not diagnosed at all. Because there is no cure for VHL, early detection is of critical importance. VHL tumors are most effectively treated when found early. The primary advocacy organization for VHL, the VHL Alliance and Cancer Research Fund, has grown significantly since its founding in 1993.

Wilms' Tumor

Wilms' tumor, or nephroblastoma, is the cancer of the kidneys that typically occurs in children, rarely in adults. It is named for Dr. Max Wilms, the German surgeon who first described this type of tumor. Approximately 500 cases are diagnosed in the United States annually. The majority occur in children. A variety of small support groups and online resources exists nationally, supporting Wilms' tumor advocacy. Its strongest public advocate is Tracy Gray, the mother of a Wilms' tumor child, who wrote a book about her experiences titled *Smile for Your Child: A Parent's Guide to Finding Positive Energy During Diagnosis, Treatment, & Life After.*

The Role of RCC Advocacy in Research and Discovery

Research discoveries over the last decade have yielded three key areas of progress for the RCC community:

- Significant progress in surgical techniques and surgical management.
- Better understanding of the histological basis of RCC and its underlying biology.
- Advances in medical management and the integration of surgery into the medical management model.

The impressive pace of scientific advance in the RCC community has given us great optimism for the future; but it has also created an environment in which researchers find themselves contemplating just as many new questions as new answers. With the addition of so many options for treatment in the last decade, along with rich new data sets for study, the clinical research environment is more complex and fluid than ever.

With solid new drug therapies established, researchers have now begun to focus on early detection strategies, creation of less invasive techniques for treatment, and new strategies aimed at advanced-stage RCC.

The research community is also now seeking to have a better understanding of how to individualize treatment using the new RCC therapies and why tumors may become resistant to them.

Just as it does in health policy issues, advocacy can play a critical role in RCC research and discovery by helping focus priorities and to identify and respond to threats and challenges to our clinical progress.

The Kidney Cancer Association has played an important role, both as a fundraising organization and a catalyst for discussion and consensus building, to help focus the RCC community's energies on establishing research priorities. It has raised millions of dollars to support RCC research, including approximately $1 million invested in research-related program activities in its two most recent fiscal years [55]. Through its annual Young Investigator Awards, it recognizes and rewards

promising young researchers in RCC. It also advocates aggressively on a wide range of research-related issues with the federal government and other stakeholders in health care. In 2011, it hosted a major symposium for young researchers, titled "Kidney Cancer Research: Developing a New Vision for the Future."

Organizations such as KCA, along with individual physician and nurse leaders, can be instrumental in helping focus attention on the research needs of the RCC community. A variety of key issues in research and discovery will require the attention of the RCC advocates in the future. Among them:

Expansion of Drug Categories

While a large number of drugs are available to patients, they are grouped in only three categories: cytokine, VEGF, and mTOR. In order to get more leverage from recent discoveries and move RCC treatment to the next level, drug categories should be expanded, with novel targets identified.

A better understanding and definition of the mechanisms of resistance to pathways such as VEGF and mTOR are also needed. In seeking new drug categories, an emphasis should be placed on addressing non-clear-cell RCC, which has lagged behind clear-cell RCC in terms of research attention [56].

Clinical Trials

Robust clinical trials are essential for RCC's research future, but, as noted earlier, there are serious issues impeding clinical trial growth. Beyond the problems of low enrollment and funding cutbacks, there is a lack of collaboration across research centers [56]. Specific trials are lacking for non-clear-cell RCC, a key area of future research focus.

The RCC community could magnify the impact of its clinical trial activity by working collectively to address consistency in the structural details of trials, including a new effort to incorporate the use of predictive biomarkers and other standard features across multiple trials.

Advocacy organizations such as KCA can help move a new vision of clinical research forward by raising visibility of the need for new structural innovations at forums and through publications and other communications with the RCC community. The goal is to generate a culture that fosters truly informative and original clinical research and builds upon the existing RCC knowledge base. Central to this effort should be encouragement of collaborative research between academic and community research centers.

Research Consortia

The RCC community lags behind other disease states (e.g., prostate cancer and myeloma) in organizing translational consortia to help advance research needs not met by industry and cooperative groups. RCC community advocacy should be focused on encouraging development of consortia and seeking new ways to address the funding and liability issues that can inhibit the launch of new investigator-initiated projects at academic medical centers. Advocacy should emphasize the need for stronger partnerships among government, industry, and academia.

Biopsies

The usefulness of needle biopsy continues to be debated in the RCC community, with discussion centering on the quality of tissue, the prohibitive costs of biopsy as a research tool, the lack of patient support and buy-in, and whether other methods of tissue-access could be developed [56].

More study is needed on how to advance percutaneous biopsy in a way that minimizes its complications. Better standards for the use of biopsy in guiding therapeutic decisions are also needed.

Tumor Registries

A notable weakness in the RCC community is the lack of a robust tumor registry or a comprehensive system of tissue collection—a resource that has helped other cancer communities accelerate research. Organizations such as KCA can play a valuable role, utilizing its convening and communicating capabilities, by advocating across institutions for the development of a more comprehensive, multicenter approach to research that would include the development of tumor registries.

Multisite cooperation and collaboration are essential in order to leverage the RCC community's recent research momentum. Other medical communities—thoracic medicine, for example—have been successful in creating minimum data sets and core data elements that are collected from research conducted discipline wide [56]. This serves as a good model for RCC.

A new collaborative, multicenter data collection model could be focused on several key objectives, including the study of the role of renal-mass biopsy. A unified process would conceivably yield much more impactful data.

Surgical Techniques

The last decade has been an exciting period of progress in the RCC surgical community. Surgeons have a much better understanding of chronic renal insufficiency and its implications for long-term survival in patients without kidney cancer and extrapolating that information to those with kidney cancer [56]. This understanding has informed new thinking on the benefits of partial versus radical nephrectomy.

Closely related are advances in the development of energy ablative techniques of cryosurgery and radio-frequency ablation. These advances have significantly enhanced patient outcomes and improved quality of life.

But the surgical community faces future challenges. For example, in recent years there has been strong support among surgeons for the use of partial nephrectomy as a strategy for patients with small tumors, in order to spare them from long-term health consequences, such as chronic kidney disease. But long-term studies confirming this reasoning are lacking, and a recent randomized trial actually suggested that overall survival may be better for some patients who have radical nephrectomy rather than partial nephrectomy [57].

A key focus of RCC advocacy in the future will be encouraging, as a research priority, fundamental examination of all the factors related to partial versus radical nephrectomy. A related question to be explored will be: How can surgeons reliably predict those for whom *any* kind of treatment will be beneficial? While the kidney cancer community has learned much about the biology of tumors and has refined its treatment options, it must do a better job of determining more accurately who will benefit from surgery and why. For some patients, treatment may be worse than doing nothing.

The RCC surgical community also lags in its understanding of the optimal integration of surgery and systemic therapy. Among the key questions: What is the potential role of new systemic therapy for patients with larger tumors and existing metastatic disease? Conversely, what is the best role of systemic therapy as an adjuvant for surgical therapy in patients with intermediate disease?

Risk Factors for Kidney Cancer

The RCC community has a poor understanding, from an epidemiological perspective, of RCC risk factors. As a research priority, it should explore opportunities for early detection, such as developing urine tests, for example, and other screening measures aimed at conditions such as neoplasia.

How can we best leverage our new-found data wealth? How can we share this data faster and more broadly in order to maximize its impact? In view of all we have learned over the last several years, what should our research priorities be? And perhaps most important of all—how can we encourage the development of a new generation of researchers, inspired to move our clinical knowledge base to the next level?

Connecting Patients: The Role of Social Media in Advocacy

The number of Americans using the Internet as a basis for health information continues to grow. Almost 75 % of all US adults use the Internet, and more than 60 % of them have looked for medical information online [58].

Within the community of Internet users, the growth of social media continues to accelerate and impact virtually every sector of the economy. An estimated 96 % of all 18–35-year-olds are actively engaged with at least one social network, and 69 % of all adults say they use social media [59]. Facebook usage grew by 40 % between 2011 and 2012 [60], and the company reports that 2.7 billion "likes" are registered and 300 million photos uploaded daily at the site [61]. One fifth of the global Internet population now uses Twitter, and the service continues to grow rapidly [62].

These statistics have radically transformed the face of not-for-profit advocacy efforts, which now rely heavily on social media as a core tool for everything from communications to delivering member services to fund-raising.

Fund-raising, in particular, has moved rapidly online and into social media in recent years. A growing percentage of not-for-profit funding now originates online annually, with an estimated 87 % of not-for-profits having at least one online gift of $1,000 or more during the previous year [63].

The Kidney Cancer Association has used online access and social media as a key component of its RCC advocacy in recent years. In addition to raising the visibility of kidney cancer, KCA social media resources help patients, families, and caregivers raise funds for medical expenses and organize support networks.

Among the social media platforms used regularly by the KCA are Facebook, Twitter, YouTube, and Vimeo. KCA staff regularly post items of interest to the RCC community on the KCA Facebook page, which in early 2013 had been "liked" by more than 65,000 people worldwide [64]. The association's first page was launched in 2007 and modified in 2009. Posting is frequent—often as many as a half dozen items per day—ranging from links to emerging research and news reports to inspiring stories of cancer survivors. Facebook has become a prime "gathering place" for the RCC community and is one of the most important channels in the KCA's communications network.

Kidney Cancer Connect (www.kidneycancer.me) is the KCA's dedicated social media site for cancer patients, families, and caregivers—with special features that allow participants to share stories and photos and engage in group discussions. The password-protected site features an interactive map that helps participants find other kidney cancer patients in their area. More than 8,000 people have registered as Kidney Cancer Connect participants.

In 2009, the KCA expanded its media capabilities by creating an in-house multimedia studio that allows low-cost recording of podcasts and webinars. KCA podcasts are available through iTunes, and its YouTube and Vimeo video channels feature a wide range of educational materials for both physicians and patients. Research presentations from major conferences and symposia are regularly posted.

In recent years, the KCA has begun developing a variety of iPhone/iPad and Android apps that help users find resources and connect with others. A special clinical trials app, helping patients connect with clinical trial information, has also been developed for the iPhone and iPad.

In addition to connecting the RCC community through social media, the KCA also uses it as tool for advancing advocacy and fund-raising. Using the online service Capwiz, the KCA sends advocacy messages and alerts and helps connect its audiences with their representatives in Congress and state government. The service also connects advocates from the RCC community with local media.

Online fund-raising is growing as a percentage of the KCA's annual fund-raising total. Total revenues from fund-raisers organized by kidney cancer survivors and families, facilitated mostly through social media and online communication, have more than doubled between 2008 and 2013. Patient fund-raisers now account for approximately one-third of the KCA's noncorporate support.

The Future of RCC Advocacy: Where Do We Go from Here?

Clearly, the RCC community faces future challenges, but it is in a remarkably strong position—still benefiting from one of the most productive periods in RCC research history. The progress in the fight against kidney cancer is part of an encouraging trend line for cancer overall.

The good news for Americans is that cancer death rates for both men and women, and for all major racial and ethnic groups, continue to decline in the United States. The American Cancer Society has reported that the 5-year relative survival rate for all cancers diagnosed between 1999 and 2006 is 68 %, up from 50 % in 1975–1977 [65]. According to the Annual Report to the Nation on the Status of Cancer, 1975–2009, published in early 2013, cancer death rates between 2000 and 2009 decreased by 1.8 % a year among men and 1.4 % a year among women [66]. The National Cancer Institute estimates that well over 11 million Americans with a history of cancer are alive today [65].

During that time period, according to the Annual Report to the Nation on the Status of Cancer, death rates from kidney cancer, for both men and women, decreased. However, the *incidence* of kidney cancer *increased* for both men and women during the same time period [66].

The annual report, published by the American Cancer Society, the Centers for Disease Control and Prevention, the National Cancer Institute, and the North American Association of Central Cancer Registries, confirms something we've known within the RCC community for some time: As kidney cancer becomes an increasingly chronic condition and, at the same time, more people are diagnosed with it, we must step up our efforts at providing health strategies, tools, and resources focused on improving the quality of life for survivors.

In this new era of increased survivability, helping patients cope with a wide range of social, emotional, physical, and financial issues that arise after diagnosis will be

important. Cancer survivors have many challenges to overcome beyond the initial burdens of surgery and/or therapy. They deal with a wide variety of side effects, ranging from pain and nausea to fatigue and depression. Often, they receive no treatment plan at the beginning of their care or a treatment summary or follow-up plan at the end of their treatment. Their care may require visits to multiple practitioners and facilities. A lack of clear coding guidelines and reimbursement policies for the treatment of survivors often leads to unfair and inadequate reimbursement for practitioners.

The advocacy of the RCC community can help move the nation toward new approaches to cancer survivorship, including better coordination of care among patients, caregivers, and health-provider teams.

The KCA strongly supports the provisions and framework of the Comprehensive Cancer Care Improvement Act, legislation that was introduced in Congress in 2011 and was intended to enhance survivorship by creating new comprehensive cancer-care planning services, funding grant programs to increase provider education of palliative care and symptom management, and promoting survivorship-related research. The measure also included provisions to ensure that physician reimbursement for survivorship is fair and adequate [67].

More research on the topic of survivorship is needed to examine the efficacy of various survivorship care models as we continue to extend lives through enhanced surgical and therapeutic techniques.

As a part of its advocacy for a new paradigm of treatment and services for kidney cancer survivors, the RCC community should promote changes in medical school curricula to reflect the new era of survivorship. Enhanced education for cancer specialists should include more expansive training in survivorship, long-term complications of treatment, pain management, palliative care, and other aspects of care that are becoming more commonly encountered in clinical settings. New training should be provided at multiple levels, including academic medical centers, nursing schools, hospitals, and physician practices.

Evidence is mounting that our cancer survivorship efforts are being complicated by bad diets, a lack of physical activity, and the rise of chronic conditions such as obesity. Beyond the clinical complications that arise from these conditions, health problems such as obesity can impact patients' eligibility for participation in clinical trials. The RCC community, therefore, must be part of the national effort aimed at the prevention of chronic disease, as outlined in the US Surgeon General's new National Prevention Strategy [68].

We should link more aggressively with public health efforts aimed at health behaviors such as smoking, a well-known risk factor for kidney cancer. Among persons with active cancer or a past history of cancer, 57 % have a history of smoking, compared with 44 % of adults with no history of cancer [69].

All of these efforts should be leveraged globally to help the thousands of kidney cancer patients around the world who can benefit from advances in the United States. The Kidney Cancer Association and other RCC organizations can help by continuing their efforts to build international online communities and to provide translated clinical and patient-support materials.

One of the highest advocacy priorities for the RCC community in the future in order to make this new survivorship paradigm a reality will be stabilizing funding for research—which will include new categories for investigation beyond drug development and will encompass topics such as symptom management, population health, and palliative care.

Conclusion

The RCC community has begun the second decade of the twenty-first century in a strong position, with three overarching advantages that will help it succeed in its efforts to develop new therapies and treatments for patients:

A strong not-for-profit organization and mobilized community of advocates. Comparisons of fund-raising totals by disease type show that the heaviest funding for research in recent years has been for types of cancer that are supported by well-recognized not-for-profit organizations and extensive patient networks [70]. The RCC community is anchored by the Kidney Cancer Association, with strong support from other RCC-oriented not-for-profit organizations. Our patient networks and committed volunteers and fund-raisers are well established and growing.

Demonstrated scientific progress toward a cure. The extraordinary drug-development successes of the mid-2000s have established momentum that benefits the RCC community's future fund-raising and advocacy efforts. Our demonstrated success raises the visibility of RCC researchers and innovators, attracting new resources and potential collaborators.

Potential for translational impact. RCC research has the potential to provide insights that can be leveraged by the wider cancer community. The RCC community's remarkable breakthroughs in the understanding of VHL syndrome and the mTOR pathway, for example, have led directly to radically new targeted therapies and provided important markers for cancer research in general.

With these advantages as a springboard, the RCC community is poised for continued success. Strong advocacy can help pave the way forward by encouraging wise policy on key issues, raising public awareness, building synergies, and galvanizing funding support.

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