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Review

State of the Science: An Update on Renal Cell Carcinoma

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Abstract

Renal cell carcinomas (RCC) are emerging as a complex set of diseases that are having a major socioeconomic impact and showing a continued rise in incidence throughout the world. As the field of urologic oncology faces these trends, several major genomic and mechanistic discoveries are altering our core understanding of this multitude of cancers, including several new rare subtypes of renal cancers. In this review, these new findings are examined and placed in the context of the well-established association of clear cell RCC (ccRCC) with mutations in the von Hippel-Lindau (*VHL*) gene and resultant aberrant hypoxia inducible factor (HIF) signaling. The impact of novel ccRCC-associated genetic lesions on chromatin remodeling and epigenetic regulation is explored. The effects of *VHL* mutation on primary ciliary function, extracellular matrix homeostasis, and tumor metabolism are discussed. Studies of VHL proteostasis, with the goal of harnessing the proteostatic machinery to refunctionalize mutant VHL, are reviewed. Translational efforts using molecular tools to elucidate discriminating features of ccRCC tumors and develop improved prognostic and predictive algorithms are presented, and new therapeutics arising from the earliest molecular discoveries in ccRCC are summarized. By creating an integrated review of the key genomic and molecular biological disease characteristics of ccRCC and placing these data in the context of the evolving therapeutic landscape, we intend to facilitate interaction among basic, translational, and clinical researchers involved in the treatment of this devastating disease, and accelerate progress toward its ultimate eradication. *Mol Cancer Res; 10(7); 859–80. ©2012 AACR.*

Introduction

A rapid series of discoveries in clear cell renal cell carcinoma (ccRCC), bolstered by advances in genomic biology and the embrace of targeted therapy, have ushered in a new era of biological investigation and therapeutic opportunity for this challenging disease. ccRCC is unresponsive to traditional chemotherapies, highly resistant to radiation, and lacks the hallmark genetic features of solid tumors, such as *KRAS* and *TP53* mutations. The unique tight association between ccRCC and mutations in the *VHL* gene, and the resulting constitutive stabilization of hypoxia-inducible factor (HIF)-1α and HIF-2α have been the subject of intense study for almost 2 decades now. Stemming directly from studies of *VHL* insufficiency is an enhanced understanding of the intricate relationship between this tumor type and the tumor endothelial vascular network, and the result has been the development of therapies that can not only reduce the tumor burden but also extend the natural life expectancy of patients with metastatic disease.

In this review, we examine recent developments that are poised yet again to produce a paradigm shift in our understanding of the biology of ccRCC and other tumors, as well as to generate a landscape that is ripe for the development of new therapeutics. Experts from around the world provide a concise description of the most relevant developments in their field regarding ccRCC. Andy Futreal summarizes the discoveries that have arisen from deep-sequencing studies conducted over the last few years, and Ian Davis and Cheryl Walker describe the impact of recently described mutations on cellular behavior. The potential consequences of these findings are enormous and provide an explanation for the source of tumor heterogeneity as well as a target for therapeutic intervention. Our understanding of the *VHL* gene and HIF signaling continues to evolve as well. Pathways are never as simple as they initially appear, and the intense focus on HIF-1α signaling associated with *VHL* mutation has gradually shifted to a focus on HIF-2α as the offending culprit in this disease, with definitive evidence now available. Sean Bailey and William Kim describe these findings in more detail.

RCC is increasingly being recognized as a metabolic disease, and key lesions in nutrient sensing and processing

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have been detected. These metabolic abnormalities provide protection for the tumor but also may be a source of vulnerability and therapeutic opportunity. James Brugarolas and Amato Giaccia describe this important network. The same is true for the recently described abnormalities in extracellular matrix engendered by loss of VHL function, which are elucidated by Ghada Kurban and Arnim Pause. VHL is increasingly being recognized as an important regulator of the primary cilium and, by extension, the cilia centrosome cycle. A better understanding of the role played by VHL in this pathway could potentially lead to insights into RCC carcinogenesis. Cheryl Walker provides a summary of this complex and intriguing function.

It is well established that *VHL* mutations lead to malfolded and poorly functioning VHL protein. A better understanding of VHL proteostasis may allow us to develop strategies to refold or otherwise refunctionalize point-mutated, full-length VHL. Eric Jonasch and Judith Frydman report on recent developments in this emerging field. Numerous biomarkers have emerged to clarify the presence of heterogeneity among tumors that can be exploited for prognostic value or intervention. Kimryn Rathmell reviews the emergence of a molecular classification for RCC, and Amado Zurita describes prognostic and predictive biomarkers under development. The goal of all of these scientific efforts is to offer patients better chances for survival and healthier lives. The therapeutic options for ccRCC have evolved rapidly in the last 6 years and continue to improve. Both targeted therapies directed at features uncovered in molecular and genetic studies and improved opportunities to redirect the immune system have great potential to improve the outlook for patients with ccRCC. Brian Rini describes current and emerging molecularly targeted agents, and Pam Sharma and Michael Atkins review the exciting new developments in immunotherapy for RCC.

Genetics

RCC is a collective term applied to a set of cancers that arise in the epithelium of renal tubules. It is comprised of 3 main histopathological entities. ccRCC is the dominant histology, accounting for ~65% of reported cases, followed by papillary RCC (pRCC) and chromophobe RCC, accounting for ~15% to 20% and 5% of cases, respectively. Other, rarer subtypes constitute the remainder of RCC cases, including collecting duct, mucinous tubular, spindle cell, renal medullary, and MiTF-TFE translocation carcinomas.

Hereditary RCC, which accounts for ~4% of cases, has been a relatively dominant area of RCC genetics. Causative genes have been identified in several familial cancer syndromes that predispose to RCC, including *VHL* mutations in von Hippel-Lindau disease that predispose to ccRCC (1), *MET* mutations in familial papillary renal cancer (2), fumarate hydratase (*FH*) mutations in hereditary leiomyomatosis and renal cell cancer (HLRCC) that predispose to pRCC (3), and folliculin (*FLCN*) mutations in Birt-Hogg-Dubé syndrome that predispose to primarily chromophobe RCC (4). In addition, germline mutations in the tuberous sclerosis complex (*TSC)1* and *TSC2* genes predispose to tuberous sclerosis complex. In the latter case, ~3% of patients develop ccRCC (5), and succinate dehydrogenase type B (*SDHB*) germline mutations in patients with paraganglioma syndrome give rise to an increased risk of developing multiple types of RCC (6). Moving away from rare monogenic disease to population-based RCC susceptibility, we note that results from a recent genome-wide association study of almost 6,000 RCC cases implicated loci on 2p21 and 11q13.3 in RCC susceptibility (7). 2p21 contains the *EPAS1* gene, which encodes a transcription factor that is operative in hypoxia-regulated responses, whereas the other region has no known coding genes.

However, comparatively less progress has been made in elaborating the somatic genetics of sporadic RCC. By far, the most studied somatically mutated gene is *VHL*, which follows the classic tumor-suppressor gene paradigm of a germline cancer susceptibility gene that also manifests as a somatically mutated gene in the sporadic form of cancer (8). *VHL* is somatically mutated in up to 80% of patients with ccRCC (9). The majority of these mutations are protein-terminating mutations with loss of the wild-type (WT) allele via large-scale loss of heterozygosity of chromosome 3p. A small proportion of patients (5%–10%) have no apparent somatic mutations that methylate the locus, and thus are functionally *VHL* null (9, 10). Following a similar theme of congruence of germline and somatic genetics, albeit with a diminished magnitude of effect, a dominantly activating kinase domain *MET* mutation has been reported in 4% to 10% of cases of sporadic pRCC (2). Conversely, somatic mutations in *FLCN* in chromophobe RCC are rare (11), and somatic *FH* mutations in sporadic papillary renal cancers have not been found (11–13). Similarly, somatic mutations of *TSC1/2* and *SDHB* have not been identified in sporadic RCC (12, 13). Recently, however, somatic mutations in *TSC1* were found in sporadic ccRCC (14). *TSC1* mutations occur in 5% of ccRCCs and may predict for extraordinary sensitivity to mTORC1 inhibitors clinically (14).

Further investigation of RCC somatic genetics has included evaluation of cancer genes that are important in other adult epithelial cancers. Taking all histologies combined, the COSMIC database (http://www.sanger.ac.uk/genetics/CGP/cosmic/) reports somatic point mutations in *TP53* in 10% of cases, *KRAS/HRAS/NRAS* combined $\leq$ 1%, *CDKN2A* 10%, *PTEN* 3%, *RB1* 3%, *STK11/LKB1* $\leq$ 1%, *PIK3Ca* $\leq$ 1%, *EGFR* 1%, and *BRAF* $\leq$ 1%. *MYC* has been reported to be amplified in pRCC (15), and rare cases of RCC have been reported with *EGFR* amplification (16). Focusing on the most prevalent histology, ccRCC, the contribution of cancer genes that are commonly mutated in other tumor types provides limited insight into which additional somatic genetic events contribute to pathogenesis.

With this as a background, investigators have undertaken systematic approaches to elaborate the somatic genetics of ccRCC. A screen of 3,544 protein-coding genes via PCR-based exon resequencing in 101 cases of ccRCC identified several new cancer genes in RCC (17, 18). Remarkably, 4 out of 5 genes with robust statistical support for being new

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cancer genes encode proteins involved in histone methylation/demethylation. Truncating mutations were identified in *KDM6A/UTX*, *SETD2*, and *KDM5C/JARID1C* which encode a histone 3 lysine 27 (H3K27) demethylase, H3K36 methyltransferase, and H3K4 demethylase, respectively. *MLL2*, an H3K4 methyltransferase, was also found to be mutated at a significant rate. These data implicate deregulation of histone H3, which is known to be a major regulator of euchromatin/transcription, as a new area of RCC biology for exploration. Of note, and further confirming the utility of large-scale systematic approaches, *NF2* truncating mutations were unexpectedly identified in a significant proportion of the small subset of ccRCCs that are *VHL* WT. Altogether, however, these genes are mutated in <15% of ccRCCs, suggesting the existence of additional cancer genes.

In a study involving solution capture and sequencing of the coding exons of 20,000 protein-coding genes, Varela and colleagues (19) used next-generation sequencing technologies to investigate ccRCC somatic genetics more comprehensively. They identified a second major somatically mutated cancer gene in ccRCC, and thus substantially reshaped the field of RCC genetics. Truncating mutations in the *PBRM1* gene were identified in a remarkable 41% (92/227) of ccRCCs (19). *PBRM1* encodes the Baf180 protein, a chromatin-targeting subunit of the SWI/SNF chromatin remodeling complex that has been implicated in multiple chromatin/transcriptionally mediated processes through interaction with histone H3 (20, 21), reinforcing the striking theme of deregulated chromatin in ccRCC biology. Of note, *VHL*, *SETD2*, and *PBRM1* are all located on chromosome 3p, thus providing a likely explanation for the near-pathognomonic loss of 3p seen in ccRCC. Indeed, half of all cases with a demonstrable *VHL* point mutation in this series had a *PBRM1* truncating mutation, and 9/9 cases with a *SETD2* mutation also had concurrent *VHL* and *PBRM1* mutations. This work framed important new areas for ccRCC basic and clinical research.

Recent work involving deep sequencing on samples from a variety of locations in individual large tumors and metastatic lesions showed that considerable heterogeneity exists within these tumors, suggesting a branched pattern of evolution (22). Mutational events, such as the VHL mutation, were ubiquitous to all samples; however, certain mutations were present only in the primary tumor or the metastatic lesions, and many mutations were private. Of particular interest, different phylogenetic branches showed distinct SETD2 mutations, indicating a convergent pattern of selection for certain genotypic events. More work to understand this process and the implications for biomarker development is needed.

Given the findings of these recent studies, it is certain that other RCC cancer genes and driver mutations remain to be identified. To that end, international efforts are under way [by the International Cancer Genome Consortium (http://www.icgc.org) and The Cancer Genome Atlas (http://cancergenome.nih.gov)] to sequence large numbers of RCCs at the whole-genome level, coupled with transcriptomic and epigenomic analyses. This work is proceeding at a rapid pace, and thus the comprehensive structure of the somatic architecture of RCC should be revealed in the next few years.

Epigenetics

Together with long-standing insights into HIF deregulation through VHL loss, recent findings suggest that RCC development may represent a nexus of epigenetic and transcriptional deregulation, and exploration of epigenetic modification could reveal critical biological properties and offer clues to novel therapeutic approaches.

Genetic alterations in epigenetic regulators

As described above, high-throughput genetic studies of RCC have identified recurrent mutations in genes encoding several epigenetic regulators. Mutated genes have been implicated in chromatin regulation through nucleosome repositioning and histone tail modification. *PBRM1*, which was found to be mutated in nearly 40% of human RCCs (19, 23), is a component of the Polybromo BRG1-associated factor complex (PBAF, SWI/SNF-B). PBAF, like SWI/SNF, functions as a nucleosome remodeler and was shown to be involved in transcriptional regulation (24–26). Less common mutations were also identified in 2 methyltransferases, *SETD2* and *MLL2*, and 2 demethylases, *UTX* (KDM6A) and *JARID1C* [KDM5C (Fig. 1; ref. 17)]. Deletion of 3p, a common finding in ccRCC associated with the loss of VHL at 3p25, can also affect *SETD2* and *PBRM1*, both at 3p21 (27). SETD2 mediates the trimethylation of H3K36 (28), a histone mark that is placed during transcription and may be important for maintaining faithful transcription (29, 30), whereas MLL2 mediates H3K4me3, a mark associated with active transcription. UTX demethylates H3K27me3 (32–34), a histone mark associated with repressed chromatin. Of interest, UTX associates with MLL2 (31, 34), suggesting that demethylation of repressive marks is linked to placement of marks associated with transcriptional activation. JARID1C demethylates H3K4 (35). The finding of mutations in *MLL2* and *JARID1C*, which act oppositely on the same residue, suggests that the genomic effects of mutations in these genes are likely to be complex (Fig. 1). Although some mutations may result in widespread epigenetic variation, others may induce effects in specific regions of the genome (36).

HIF- and hypoxia-mediated epigenetic regulation

The hypoxia response pathway has been shown to have a direct effect on histone modification. HIF was shown to activate several chromatin demethylases, including JMJD1A (KDM3A), JMJD2B (KDM4B), JMJD2C (KDM4C), and JARID1B (KDM5B), all of which are directly targeted by HIF (37–40). Reexpression of pVHL in *VHL*-deficient cell lines increased H3K4me3 levels associated with decreasing levels of JARID1C, a target of HIF2α (23). Silencing of JARID1C in *VHL*-deficient tumor cells augmented tumor growth in a xenografted mouse model, suggesting that JARID1C acts as a tumor suppressor. In contrast, hypoxia

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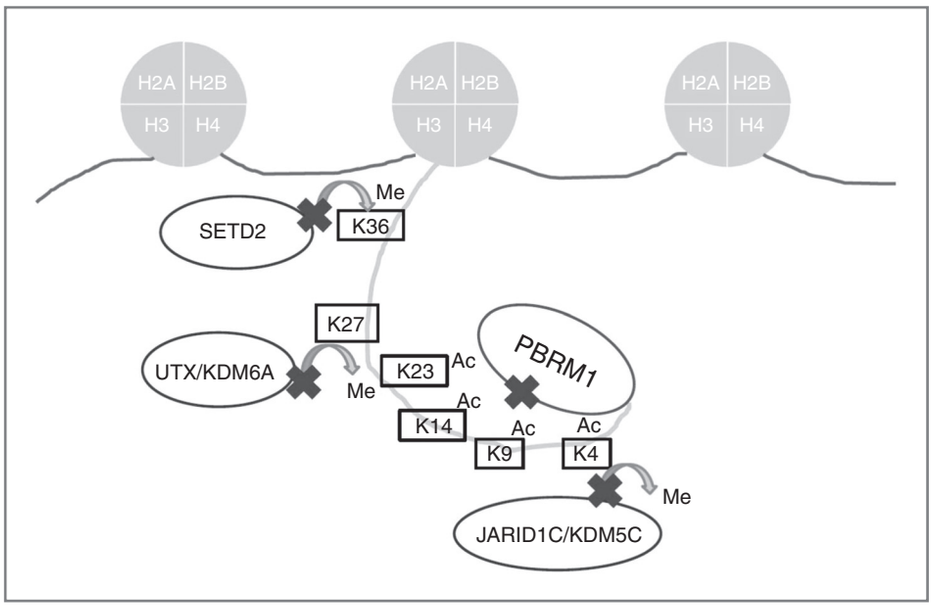

Figure 1. A number of histone-modifying genes are mutated in RCC. These include the H3K36 trimethylase SETD2, the H3K27 demethylase UTX/KDM6A, the H3K4 demethylase JARID1C/KDM5C, and the SWI/SNF complex component PBRM1, shown in this cartoon to represent their relative activities on histone H3.

may increase methylation through HIF-independent mechanisms. Like HIF prolyl hydroxylase (PHD, EGLN3), histone demethylases are members of the dioxygenase superfamily, which requires oxygen as well as iron and 2-oxoglutarate for activity (41, 42). In a manner analogous to stabilization of HIF via decreased hydroxylation, hypoxia was shown to suppress JARID1A (KDM5A) activity, resulting in increased H3K4me3 levels (43). This suggests the hypothesis that loss of demethylases (and, by analogy, increased histone methylation) is part of a hypoxia phenotype that is selected for in RCC. This hypoxia phenotype, which is mimicked by *VHL* loss, would also be mimicked by loss of histone demethylase activity, which, as noted above, is a high-frequency event in RCC.

Chromatin organization also influences HIF function. Studies of HIF induced under conditions of hypoxia showed preferential targeting of HIF to previously nucleosome-depleted chromatin regions (26). Moreover, the coexpression of SWI/SNF components BRG1, BAF170, and BAF57 augmented HIF activity from an HIF responsive reporter (25). This study showed that BRG1, but not BRM silencing, decreased HIF responsiveness, suggesting that PBAF may be more critical for HIF function than SWI/SNF.

The extent to which mutations of epigenetic regulators influence chromatin or HIF targeting remains unknown. Because of the direct influence of hypoxia on demethylase activity, it is likely that the relationship between epigenetic variation and HIF targeting differs under conditions of hypoxia in primary cells and in the context of specific epigenetic alterations in tumor cells. Altering the activity of an individual epigenetic regulator that functions as part of a complex may result in pleiotropic effects resulting from alterations in the stoichiometry of active complexes.

In addition to epigenetic regulation through histone tail modification, DNA methylation in RCC is well recognized. Studies of tumors, urine, and RCC-derived cell lines have shown hypermethylation of several tumor suppressor genes. RASSF1 may be hypermethylated in more than half of RCCs, with less common hypermethylation of VHL and CDKN2A (10, 44–47). Additional studies have identified methylation and silencing of other genes, including tissue inhibitor of metalloproteinase 3 (TIMP3) and secreted frizzled-related protein 2 (48–50). Genome-wide assays of methylation and studies of differential methylation will likely identify many more loci that are methylated in ccRCC (51, 52); however, the relationship between DNA hypermethylation and histone modification in the context of RCC remains unclear.

Therapeutic implications

Epigenetic differences may predict variation in patient outcome. Global decreases in H3K4 methylation and H3K18 acetylation have been associated with decreased patient survival (36, 53). Because epigenetic alterations and transcriptional deregulation are central to RCC, employing agents with predicted epigenetic influences may have an effect on disease outcomes. In preclinical studies, treatment with the histone deacetylase inhibitor vorinostat augmented the activity of the mTOR inhibitor temsirolimus to induce apoptosis in xenografted RCC cell lines (54). However, a phase II trial of a different HDAC inhibitor, panobinostat, in patients with refractory metastatic RCC failed to show an objective response (55). A more precise understanding of the role of epigenetic alterations could indicate other targetable strategies.

HIFs and HIF Target Genes

The HIFs are a family of transcription factors that contain a basic helix-loop-helix domain and function in a heterodimeric complex (56). HIFα has 3 subunits (HIF-1α, HIF-2α, and HIF-3α) that heterodimerize with their binding

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partner, ARNT (HIF-1β), to transcriptionally regulate target genes containing hypoxia response elements (HRE). HIF-1α and HIF-2α are the best characterized and are known to regulate transcriptional programs associated with cellular and physiological adaptation to hypoxia, such as erythropoietin (EPO), VEGF, and carbonic anhydrase 9 (CA9) (57). Although there is significant overlap in genes that are transcriptionally activated by HIF-1α and HIF-2α, it is thought that each HIF family member also transactivates unique target genes (58). For example, HIF-1α has been linked to regulating genes in pathways associated with glycolytic metabolism [e.g., SLC2A1 (GLUT1), LDHA, and autophagy BNIP3], whereas HIF-2α is uniquely responsible for transcriptionally activating genes associated with proliferation (TGFα) and dedifferentiation (cyclin D1 and Oct4).

VHL regulation of HIF

An important realization regarding the molecular pathogenesis of *VHL*-deficient RCC was that under conditions of normoxia, the pVHL complex binds to and polyubiquitinates HIFα subunits, resulting in their targeting and destruction by the proteasome (56). The interaction between HIF and pVHL is mediated by an enzymatic, post-translational hydroxylation of HIF on conserved prolyl residues by a family of HIF PHDs (or EGLNs). In keeping with the notion that regulation of HIF is an important function of pVHL, the majority of disease-associated *VHL* mutations are predicted to abolish the interaction between pVHL and HIF (59). Moreover, studies in mice suggest that HIF activation (in particular HIF-2α) mediates the majority of the phenotypes seen in the setting of *VHL* loss (60–62).

Role of HIF in RCC

Early *in vitro* and cell-line xenograft studies suggested that although HIF-2α is both necessary and sufficient for the growth of transformed RCC cell lines (63–65), HIF-1α is not (66), indicating that HIF-1α is expendable for RCC growth. However, it seems that HIF-1α is not merely dispensable in the context of RCC but actually functions as a tumor-suppressor gene. Several lines of evidence support this hypothesis. First, targeted exon sequencing of RCC has shown (albeit rarely) inactivating mutations in HIF-1α, although copy-number analyses of RCC cell lines and primary tumors suggest that the HIF-1α locus is frequently lost along with the long arm of chromosome 14 [14q (17, 67)]. Second, although all *VHL*-defective ccRCCs seem to overexpress HIF-2α, and approximately one third of these tumors seem to lack HIF-1α expression as well (68). Finally, functional studies *in vitro* and *in vivo* suggest that overexpression of HIF-1α in *VHL* WT cells restrains tumor growth, whereas suppression of HIF-1α in *VHL*-deficient cells enhances tumor growth (67, 69). Together, these studies provide support for HIF-1α as tumor-suppressor gene in renal cancer development and HIF-2α as a key driver of renal cancer progression.

Although there are a number of possible explanations for the contrasting properties of HIF-1α and HIF-2α in RCC pathogenesis, one intriguing observation is that HIF-1α and HIF-2α have opposing roles in the regulation of c-Myc activity. Specifically, HIF-1α acts to suppress c-Myc activity, whereas HIF-2α promotes the transactivation or transrepression of c-Myc–specific target genes (58, 68, 70). In keeping with this notion, RCC tumors that exclusively express HIF-2α have increased proliferation rates. Furthermore, intriguingly, a subset of ccRCC tumors seem to have copy-number amplification of 8q24, where c-Myc resides (71).

VHL Proteostasis

Two pVHL isoforms (a 213 amino acid, 30 kDa form, and a 160 amino acid, 19 kDa, form) exist in the cell (72). In order to function, pVHL must fold to its native conformation. The proper folding and functionality of pVHL require its tight association with elongins B and C to give rise to a VHL-elongin BC complex (herein termed the VBC). Failure of pVHL to fold and to interact with elongin BC results in misfolding and proteolytic degradation of pVHL (73). In this section we discuss pVHL protein homeostasis (also called proteostasis), and how disease-causing mutations affect pVHL stability and functionality.

Molecular chaperones are essential mediators of protein folding and quality control of most proteins in the cell. Following synthesis on ribosomes, folding of functional pVHL protein is the result of a complex interplay between nascent pVHL and cellular chaperones. Nascent pVHL is shuttled from the ribosomal machinery with the assistance of heat shock protein 70 [HSP70 (74)]. pVHL is then folded into its tertiary structure via association with the chaperonin TCP-1 ring complex [TRiC; also called chaperonin-containing TCP-1 (CCT)] (74–77). This hetero-oligomeric complex consists of 2 stacked rings with a central chamber in which unfolded polypeptides bind and fold. TRiC is responsible for folding a number of key proteins that, like pVHL, are also subunits of oligomeric complexes (75, 76, 78, 79). Hsp70 likely functions to stabilize nonnative forms of pVHL, whereas TRiC/CCT facilitates pVHL folding, which is coupled to its incorporation into assembly of VBC (75, 80, 81). Upon binding of VHL to elongin BC to form a mature VBC, pVHL is released from TRiC (Fig. 2; ref. 74).

Binding of VHL to TRiC occurs at amino acids 114–119 and 148–155 [called Box 1 and Box 2, respectively (82)]. Both motifs, located in adjacent strands of the β-domain, harbor tumor-causing mutations that disrupt association with TRiC and lead to misfolding of newly translated pVHL. Mutations that block pVHL incorporation into a well-folded VBC seem to result in destabilization and lower intracellular levels of pVHL, although residual functionality is maintained in some cases (83). Further analysis of how specific mutations affect the interaction of pVHL with chaperones and chaperonins provides insight into targetable mechanisms of pVHL protein destabilization. Disease-causing mutations in TRiC Box 1 and Box 2 binding sites (82, 84, 85) prevent association of pVHL to TRiC, resulting in a malfolded protein and the absence of a mature VBC in

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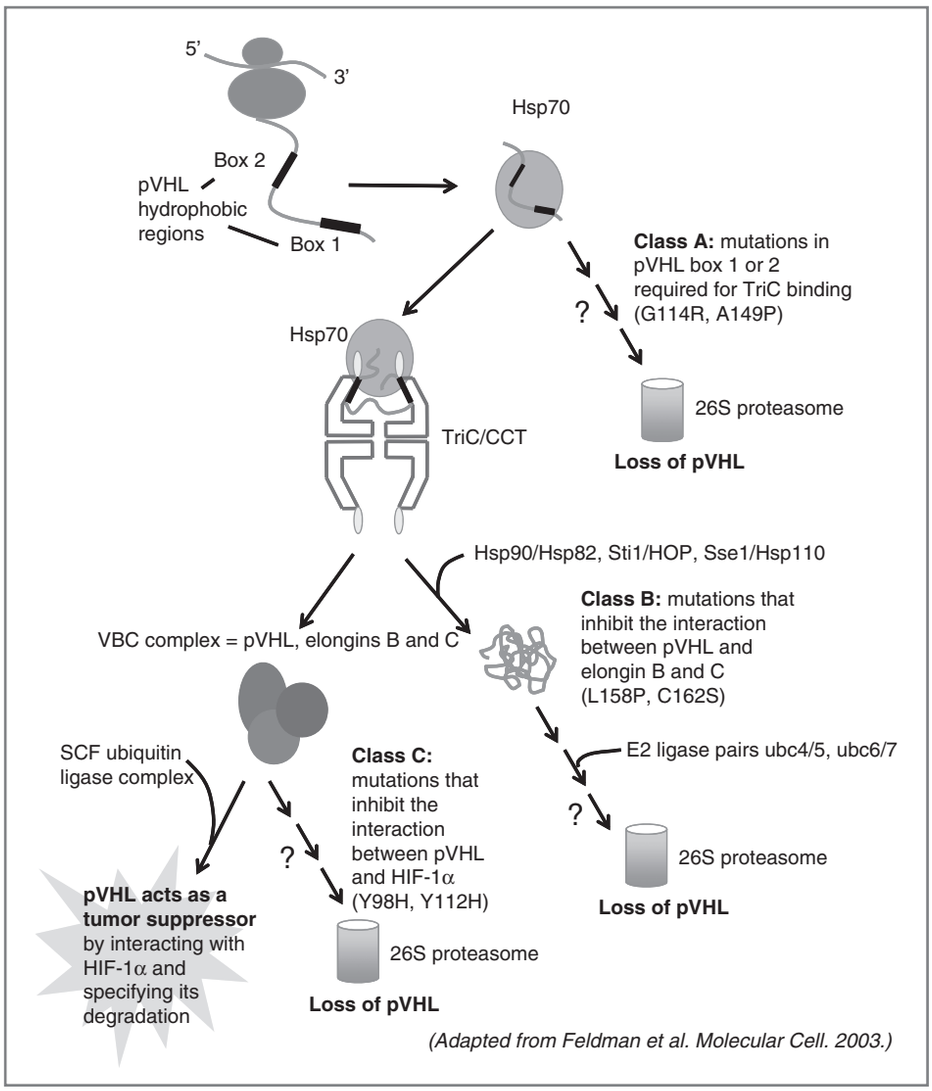

Figure 2. VHL proteostasis involves the chaperone-mediated translocation of nascent VHL peptide from the ribosome to the TRiC/CCT chaperonin, where folding occurs in an ATP-dependent process. The VBC complex is formed while VHL is bound to TRiC, and the mature complex is then released. Mutations are divided into three different classes: Class A mutations prevent binding of VHL to TRiC, and abrogate folding into a mature complex. Class B mutations prevent association of elongins C and B to VHL. Class C mutations inhibit interaction between VHL and HIF-1α.

the cell. Disease-causing mutations also occur in the amino acid 155–181 elongin C binding region (84, 85). This class of mutants can bind to TRiC but cannot stably bind to elongin BC. Loss of elongin C binding capacity seems to prevent pVHL release from TRiC (82), resulting in a lack of mature VBC.

Failure to generate a properly folded pVHL or a mature VBC will result in pVHL degradation through the ubiquitin-proteasome system. Chaperones are also involved in this quality control process (82, 86). pVHL degradation specifically requires another chaperone, Hsp90, which does not participate in pVHL folding (86). The identification of 2 distinct pathways of chaperone interactions for pVHL, one leading to folding and one to degradation, suggests that the fate of pVHL may be controlled by a hierarchy of chaperone interactions. Understanding the mechanism of how destabilized pVHL mutants are targeted for proteasomal degradation may lead to strategies for refolding and stabilization of a pVHL that is functional and competent to complex with elongins B and C. Bortezomib and MG132 are capable of increasing levels of VHL, and a cell-based Prestwick compound screen identified several compounds that upregulate point-mutated VHL (87). Efforts to analyze the functional consequences of pVHL upregulation using these compounds, as well as an expanded screening effort, are under way.

In summary, our evolving understanding of proteostasis will allow new therapeutic approaches to be developed for VHL disease. Recalibrating the interaction between point-mutated pVHL and the chaperones and chaperonins may alter the disease phenotype and provide a benefit for patients with lesions possessing either germline or sporadic *VHL* mutations.

RCC: One of the Ciliopathies

Together with polycystic kidney disease (PKD), TSC and VHL syndrome are considered ciliopathies (88). In PKD, TSC2, and VHL deficiency, renal cysts develop following

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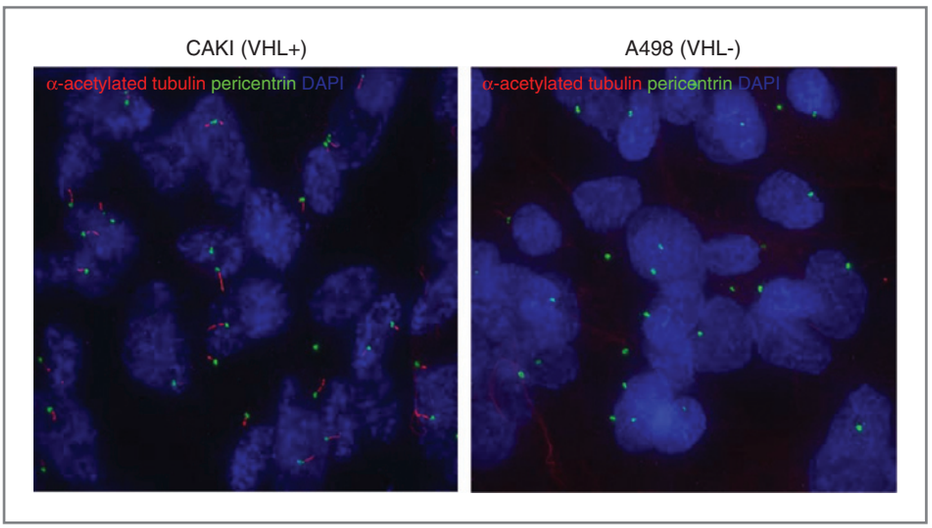

Figure 3. Immunofluorescent images of primary cilia in VHL+ and VHL− cells using the ciliary marker α-acetylated-tubulin (red) and the centrosomal marker anti-pericentrin (green), counterstained for DNA with DAPI (blue). The left panel shows a 3-color merge of VHL+ cells, and the right panel shows the absence of cilia in VHL− cells. DAPI, 4′, 6 diamidino 2 phenylindole.

loss of gene function, often as preneoplastic lesions. One of the hallmarks of cysts is dysfunctional primary cilia. All cells possess a single primary cilium, a nonmotile organelle that consists of a central microtubule axoneme anchored by the basal body, surrounded by the ciliary membrane (Fig. 3). In the kidney, the primary cilium projects from the apical surface of renal epithelial cells into the kidney lumen, where it responds to fluid flow and acts as a chemo-, osmotic, mechano-sensor of the environment. Loss of primary cilia results in dysregulated cell signaling, and cystogenesis in the kidney and several other organs, and is one of the hallmarks of many types of cancer, including RCC.

Several cell-signaling pathways that have been linked to tumorigenesis [e.g., Wnt, Hedgehog, and platelet-derived growth factor (PDGF) signaling] localize specifically to the primary cilium and/or are spatially regulated by this organelle (88, 89). In addition to aberrant signaling, the microtubule organizing center (MTOC) that forms the foundation of the primary cilium, the basal body, also functions in the cell during mitosis as the centrosome (90). The fact that this MTOC must shuttle between functioning as a basal body for the primary cilia and a centrosome for the mitotic spindle means that the cilia-centrosome cycle must be tightly coupled to cell division to maintain genomic stability. The cilia-centrosome cycle is important for maintaining genomic stability. Centrioles of the basal body that serve as the MTOC for the ciliary axoneme also serve as the MTOC for the mitotic spindle. There, they function as the centrosome, which is comprised of a pair of centrioles that are responsible for spindle formation during mitosis. Thus, the centrioles serve 2 distinct and mutually exclusive functions in the cells, serving as the MTOC for either the mitotic spindle (during M phase) or the primary cilium (during G_0–G_1). The fact that this MTOC shuttles between these 2 functions means that the cilia-centrosome cycle must be tightly regulated to guarantee the fidelity of centrosome replication, spindle formation, and genomic stability. Defects in cilia-centrosome cycle checkpoints have the potential to cause inappropriate centrosome replication, supernumerary centrosomes, and ultimately aneuploidy. Of interest, it was recently shown that VHL localizes to the mitotic spindle in mammalian cells, and causes spindle misorientation and chromosomal instability when it is defective or absent (91).

pVHL-, TSC-, and PKD-associated proteins also share a common function: regulation of the structure and function of the primary cilium. These renal cystoproteins are localized at the primary cilium, where they exert a variety of cellular responses (92, 93). For instance, PKD-1 plays a critical role at the primary cilium, where it is involved in ciliary mechanotransduction. Several studies have indicated that VHL is also involved in the biogenesis and function of the primary cilia (92, 94, 95), and biallelic inactivation of this gene is associated with loss of cilia (96). Consistent with this observation, RCC of the clear cell type, associated with loss of pVHL, showed markedly reduced cilia formation when compared with papillary carcinoma (97). In addition, pVHL binds to microtubules (91, 98) and colocalizes with the acetylated tubulin in the cilia, where its mobility is dependent on its association with Kif3A (99). In recent studies linking TSC2 deficiency to ciliary defects, loss of TSC2 was linked specifically to the development of aberrant primary cilia (100). This abnormal ciliary phenotype was also associated with loss of TSC1, which localizes to the basal body.

Regulation of the Extracellular Matrix

The extracellular matrix (ECM) is a complex structural component that surrounds the cells and provides support. It is composed of proteoglycans, hyaluronic acid, and glycoproteins such as fibronectin and many types of collagens (101, 102). Disruption of its regular architecture has been associated with tumor growth, angiogenesis, and metastasis. pVHL plays an important role in the regulation of the ECM. It was shown to interact directly with fibronectin and collagen IV, resulting in their assembly into the ECM and suppression of tumorigenesis, angiogenesis, and cell invasion (103–107). Most pVHL mutants fail to bind and degrade

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HIF-α; however, all pVHL mutants tested to date fail to bind fibronectin and collagen IV, and lose the ability to assemble an ECM (103–109). The interaction of pVHL with fibronectin is mediated by pVHL neddylation, which acts as a molecular switch in conferring selectivity to fibronectin binding over CUL2 (107, 110), whereas its interaction with collagen IV is dependent on endoplasmic reticulum (ER) hydroxylation (105). The VHL-collagen IV interaction was shown to occur at the ER membrane, with pVHL binding to a 70 kDa fragment of the collagen IV amino terminus that protrudes out of the ER into the cytosol (105). The mechanistic significance of these interactions is still not clear, but it was shown that pVHL did not affect fibronectin and collagen IV production or secretion, and did not result in collagen IV proteosomal degradation (104, 105).

The role of pVHL in ECM regulation is independent of its role in HIF-α regulation. Indeed, it was shown that inactivation of the VHL-ECM assembly pathway results in tumors that are highly vascularized, have a remodeled fibronectin and collagen IV matrix, and show increased invasive ability. Loss of the VHL-HIF-α regulation pathway resulted in tumors with high VEGF levels but with decreased angiogenesis, a tightly assembled fibronectin and collagen IV matrix, and low invasive capacity. Therefore, although both pathways cooperate in supporting tumorigenicity, ECM remodeling may promote angiogenesis by providing a path for blood vessels to infiltrate tumors (104).

Tumor cell invasion is dependent on adhesion and proteolytic remodeling of the ECM, both of which are influenced by pVHL activity. It was shown that pVHL regulates adhesion molecules, and its inactivation leads to downregulation of the adherens junction protein E-cadherin and stimulation of invasion in RCC (111–113). Loss of pVHL function also leads to downregulation of the tight junction proteins occludin and claudin in an E-cadherin–independent manner (114). In these studies, disruption of both adherens and tight junctions were mediated by loss of the pVHL-HIF-α regulation pathway. In another study, pVHL was found to downregulate integrins in an HIF-α–independent manner, and this correlated with restoration of tight and adherens junctions (115). Cells lacking pVHL also fail to form β1 fibrillar adhesions, possibly contributing to the increased cell motility and invasiveness seen in the absence of a functional pVHL (116).

VHL pathways also regulate matrix metalloproteinases (MMPs), a family of matrix-degrading enzymes that are involved in ECM turnover. RCC cell lines lacking pVHL showed increased invasiveness in growth factor–reduced Matrigel, overproduced MMP-2 and -9, and displayed an extensive branching morphogenesis phenotype in response to hepatocyte growth factor/scatter factor as compared with those with WT pVHL (117). Activation of MMPs upon loss of pVHL activity can be attributed to a disruption of both VHL-ECM and VHL-HIF-α pathways. Loss of VHL-ECM pathway regulation in RCC cells resulted in increased cell invasiveness and activation of MMP-2 (104), and HIF-α was also shown to influence RCC cell invasiveness by regulating membrane type-1 MMP expression (118, 119). Proteolytic remodeling of the ECM by MMPs was shown to expose cryptic sites in collagen IV, normally hidden within the triple helical structure, leading to loss of integrin α1β1 binding and a gain of binding to the αvβ3 integrin, resulting in stimulation of angiogenesis (120). Antibodies directed toward collagen IV cryptic sites led to inhibition of angiogenesis, tumor growth, and metastasis *in vivo*, suggesting the importance of collagen IV matrix remodeling in these processes (120–123).

The role of pVHL in maintaining ECM integrity and suppression of tumorigenesis, angiogenesis, and invasiveness is multifaceted and complex. It may result from the interplay of several mechanisms that remain unresolved. It is possible that pVHL mediates fibronectin and collagen IV modification, allowing their proper assembly into the ECM. Loss of these interactions would lead to an aberrant ECM, activation of MMPs, ECM remodeling, release of ECM-sequestered growth factors, and stimulation of tumorigenesis, angiogenesis, and invasion. Disruption of integrins and cell-adhesion molecule regulation would further enhance the invasive RCC phenotype. Understanding the mechanisms of ECM regulation by pVHL could lead to additional or alternate therapies [distinct from tyrosine kinase inhibitors [TKI]) for patients with RCC.

RCC and Metabolism

An intimate link between metabolism and renal cancer was established by the discovery that genes encoding enzymes of the Krebs cycle suppress tumor formation in kidney cells (124, 125). The Krebs cycle refers to 9 sequential enzymatic reactions implicated in oxidizing acetyl-CoA generated from glucose, fatty acids, and amino acids to CO_2 (Fig. 4). This cycle is essential to the process of mitochondrial ATP generation. SDH, a complex of 4 different polypeptides (SDHA-D) that is also involved in electron transfer, catalyzes the conversion of succinate to fumarate. Heterozygous germline mutations in *SDH* subunits predispose to pheochromocytoma/paraganglioma, and mutations in *SDHB* and *SDHD* have also been associated with RCC (6, 126).

FH catalyzes the next reaction of the Krebs cycle, the conversion of fumarate to malate. Heterozygous germline *FH* mutations cause HLRCC, a syndrome characterized by cutaneous and uterine leiomyomas as well as RCC (3, 127). RCCs occur in 20% to 50% of HLRCC families, are typically pRCC type 2 [pRCC-2 (128)], and tend to be very aggressive (129).

The *FH* and *SDH* genes function as 2-hit tumor-suppressor genes (54, 125). Loss-of-function mutations in the germline are usually accompanied by loss of heterozygosity in the tumor, causing truncation of the cycle and the accumulation of intermediates (130, 131). The accumulation of succinate or fumarate causes the inhibition of a family of 2-oxoglutarate-dependent dioxygenases normally implicated in HIF-α hydroxylation (132–134). In the absence of this modification, HIF-α evades recognition by pVHL and accumulates, leading to increased HIF activity and tumor

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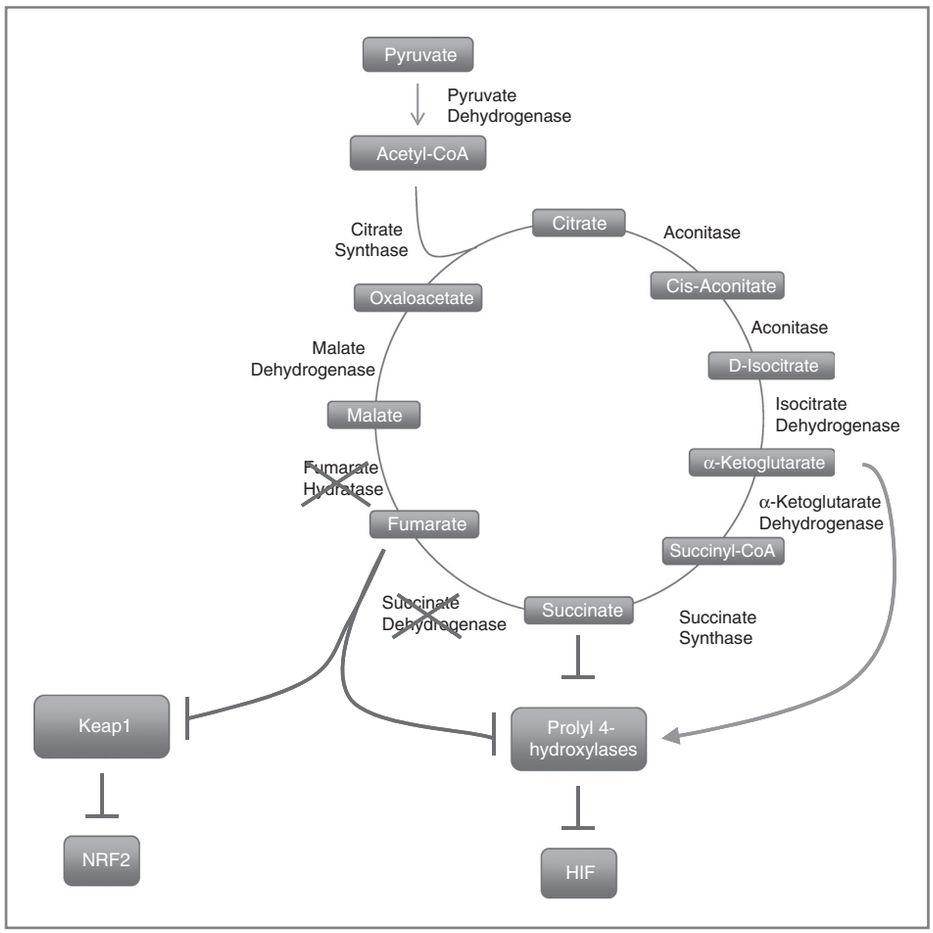

Figure 4. Regulation of PHDs by TCA cycle intermediates. PHDs use TCA cycle intermediates to help catalyze the oxygen-, iron-, and ascorbate-dependent addition of a hydroxyl side chain to a Pro^{402} and Pro^{564} of HIFα subunits, leading to VHL binding and degradation. Defects in either FH or SDH will drive up levels of fumarate and succinate, which competitively bind PHDs, and prevent HIF prolyl hydroxylation. This results in higher intracellular HIF levels.

development (56). In addition, the accumulation of succinate and fumarate results in the succination of proteins, such as Keap1 (135, 136). Keap1 is a component of an E3 ubiquitin ligase that targets NRF2 for degradation, and its succination blocks NRF2 degradation, resulting in its accumulation and the increased expression of stress-response and antioxidant genes (135–137).

Truncation of the Krebs cycle results in a compensatory increase in glucose uptake and glycolysis (130, 138–140). Accordingly, HLRCC-associated pRCC-2 are intensely fludeoxyglucose positron emission tomography (FDG-PET)–positive (140, 141). Unlike other tumor cells, FH-deficient pRCC-2 cells are unable to grow in low-glucose concentrations (140). This dependency on glucose offers an opportunity for therapeutic intervention, and we recently reported an attempt to treat an HLRCC patient with advanced pRCC-2 refractory to mTORC1 inhibition with an inhibitor of glycolysis (141).

Metabolic derangements are also associated with mutations in *VHL*. Germline *VHL* mutations predispose to ccRCC (1). [The term "clear cell" (cc) stems from the fact that the accumulation of lipid and glycogen gives a clear appearance to the tumor cells in the tissue after processing.] In contrast to *VHL*, *SDH* and *FH* genes are seldom mutated in the sporadic setting (11, 12). Of interest, although *Vhl* mutations do not cause RCC in the mouse, disruption in the liver phenocopies the accumulation of lipid and glycogen observed in ccRCC (60, 142–146). Thus, hepatocytes may serve as a model for studying the role of VHL in metabolism. Acute *Vhl* disruption in hepatocytes results in an HIF-dependent inhibition of mitochondrial respiration (146). Deprived of Vhl, glucose and ketone production by hepatocytes drops and the mice die within days (146). Although the relative contribution of HIF-1 and HIF-2 remains to be fully determined, HIF-2 may play an important role (145–147). If a similar inhibition of mitochondrial respiration occurs in ccRCCs, these tumors could be exquisitely sensitive to glycolysis inhibitors.

Vulnerabilities arising from *VHL* loss in ccRCC are also being exploited by means of synthetic lethal screens (148, 149). This was illustrated genetically in a study that screened *VHL*-deficient ccRCC cell lines with shRNAs against kinase targets (149). This screen identified several kinases that are synthetically lethal in the setting of *VHL* loss, including cyclin-dependent kinase 6 (CDK6), hepatocyte growth factor receptor (MET), and dual specificity mitogen-activated protein kinase kinase 1 (MEK1). Small-molecule inhibitors of CDK6 were also shown to reduce the viability

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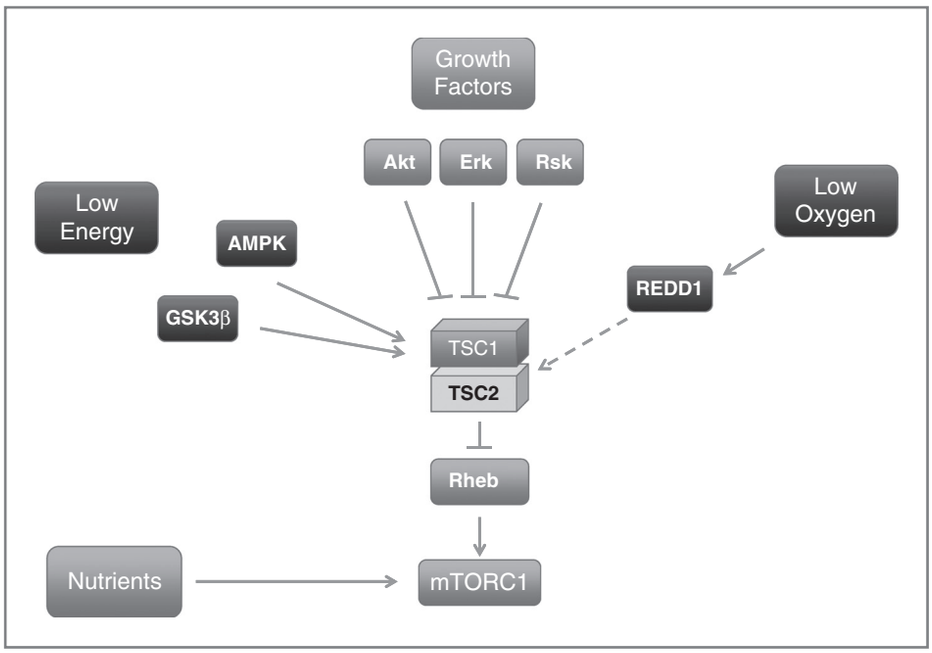

Figure 5. HIF regulation and mTOR pathway connections. Hypoxia blocks HIF expression in a TSC1/2- and REDD-dependent pathway (153). HIF1α seems to be both TORC1 and TORC2 dependent, whereas HIF2α is only TORC2 dependent (268). Signaling via TORC2 seems to upregulate HIF2α in an AKT-dependent manner (68).

of *VHL*-deficient ccRCC tumor cells (149). In addition to the shRNA approach, small-molecule screening has been fruitful in identifying new targets that exhibit enhanced cytotoxicity against *VHL*-deficient ccRCC. The compound STF-62247 significantly reduced the survival of *VHL*-deficient ccRCC in cell culture as well as in transplanted tumors in immunodeficient mice. STF-62247 induces autophagy and disrupts Golgi trafficking, which in *VHL*-deficient cells leads to cell death (148). From the same screen, a second compound, STF-31, was identified that also exhibits enhanced cytotoxicity against *VHL*-deficient ccRCC. STF-31 inhibits glucose uptake by the Glut-1 transporter and induces necrotic cell death in *VHL*-deficient ccRCC (150). The results obtained with this small molecule provide evidence that targeting glucose metabolism directly in *VHL*-deficient ccRCC could provide a therapeutic gain clinically.

Another pathway that has been implicated in RCC pathogenesis and plays an important role in metabolism is the mTORC1 pathway (Fig. 5). mTORC1 is the target of two U.S. Food and Drug Administration (FDA)-approved drugs, temsirolimus and everolimus, and is a master regulator of cell growth. mTORC1 integrates environmental and cellular cues with the cell growth machinery (151). Signals from energy stores (152), oxygen (153), and growth factors (154) are largely transduced to mTORC1 through a protein complex formed by the proteins TSC1 and TSC2. By contrast, nutrients regulate the subcellular localization of mTORC1 (155). Only in the presence of nutrients is mTORC1 receptive to signals funneled through TSC1/TSC2 (158, 159). The best characterized function of mTORC1 is to promote protein translation, a process that is mediated, at least in part, by the phosphorylation of S6K and the eukaryotic initiation factor 4E-binding protein 1 (155, 156, 160). However, mTORC1 also plays an important role in suppressing autophagy (155, 157) and regulating mitochondria (160). In addition, several transcription factors are regulated by mTORC1. mTORC1 regulates HIF-1 (161–166), thereby coupling trophic functions to angiogenesis. mTORC1 also regulates sterol regulatory element binding protein1 (SREBP1), a master regulator of lipogenesis (167, 168). Finally, we recently reported that mTORC1 regulates the transcription factor EB [TFEB (169)], a controller of lysosome biogenesis (170). Of interest, the TFEB gene is translocated in a subset of RCCs (171, 172), and the regulation of TFEB by mTORC1 may provide opportunities for therapeutic intervention.

Defining New Molecular Subtypes of RCC

It is becoming increasingly clear that ccRCC is an incredibly heterogeneous disease. Only recently have we learned how thoroughly distinct are the differences between ccRCC and non–clear-cell histologies, which in fact should probably be considered as distinct diseases in terms of biology, prognosis, and response to treatment (173–178). Indeed, even within the less common pRCC type, we see two distinct subtypes: pRCC-1 and pRCC-2 (179–184). These two papillary subtypes are associated with distinct familial syndromes: hereditary pRCC (associated with pRCC-1), caused by germline mutations in the Met proto-oncogene (2), and HLRCC [associated with pRCC-2 (125)], caused by FH, as discussed above. The underlying genetic events in sporadic versions of these two histologically defined subtypes are undergoing investigation.

Within the category of ccRCC, heterogeneity has also been widely appreciated, despite studies that revealed an increasingly tight connection with mutation of *VHL*. As discussed above, *VHL* mutation provides a permissive setting

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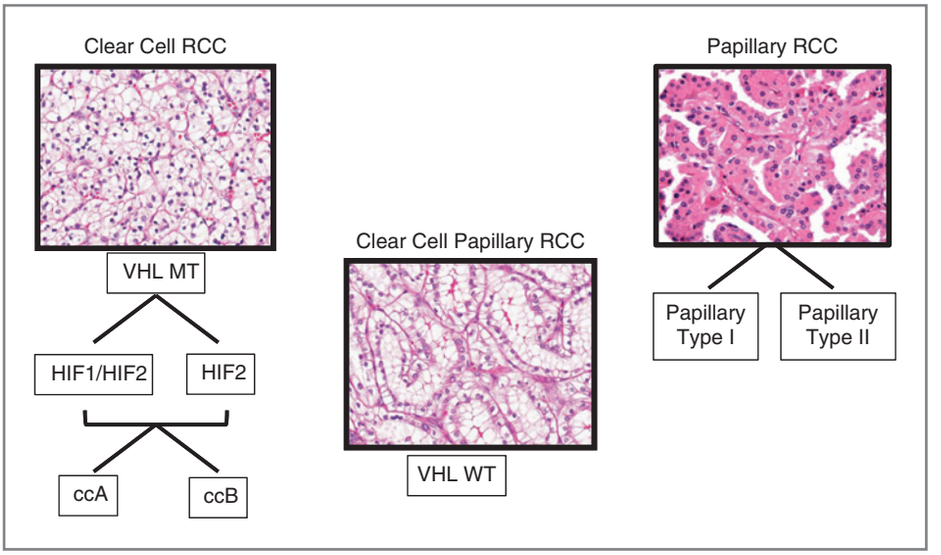

Figure 6. Different subtypes of ccRCC can be defined by HIF patterns as well as by transcriptomic expression as defined by ccA and ccB subtypes. pRCC also shows distinct histological subtypes. A recently described variant denoted as clear-cell pRCC is VHL WT; other clear-cell tumors are characterized by VHL mutation, loss, or inactivation (VHL MT).

for the deregulation of HIF family members, notably HIF-1α and HIF-2α (185). Therefore, tumors can be classified as tumors that express both factors (H1H2), express only HIF-2α (H2), or produce a functional pVHL (68). These definitions reflect distinct patterns of gene expression and signal transduction, and suggest that the HIF profile may be important for selecting therapy. This method of subclassifying ccRCC tumors has been hindered by the inconsistency of assays for the highly labile HIF proteins. It may be best to consider using a transcriptionally based instrument to assign H1H2 versus H2 status in future studies.

Indeed, ccRCC provides an outstanding tumor model for expression-based analyses, and numerous groups have laid the groundwork for defining the heterogeneity of this tumor classification based on transcriptional measurements. Studies based on supervised gene expression profiling of primary tumors versus metastases, early versus late recurrences, or short versus long survival have consistently shown differentially expressed genes (175, 186, 187). Recently, Rini and colleagues (188) reported on a transcriptional profile indicative of poor risk for recurrence developed from paraffin-embedded specimens, which indicates that expression-based biomarkers are ready for translation to the clinic for prospective evaluation.

In parallel, several groups have performed unsupervised analyses to determine whether inherent subtypes exist within ccRCC that can be defined by purely molecular means (189, 190). Two primary subgroups are found in relatively equal abundance in unselected tumors, suggesting that ccRCC may be represented by two major subclassifications, termed ccA and ccB in recent analyses (191). The ccA and ccB subclassifications share many similarities to the gene sets identified in good-risk or poor-risk tumors described above, respectively, in particular gene sets involved in local invasion, and epithelial-to-mesenchymal transition. Moreover, when the clinical outcomes are examined, groups of patients with ccA show a long median survival of 8.6 years, whereas their ccB counterparts have a median survival of only 2 years ($P = 0.002$). The advantages of these emerging strategies of subclassification include the potential to assign the profile of an individual tumor, capture of molecular information that is tied to genetic events that may be critical for selection of targeted therapy, and prognostic models that also consider clinically intermediate disease categories. A recent validation by meta-analysis confirmed the presence of these ccA and ccB subtypes, but also identified a subset defined by gene expression indicative of a WT *VHL* and variant histology consistent with the newly described clear-cell papillary subtype (192, 193).

In spite of the hurdles ahead, it seems likely that molecular strategies to classify individual tumors are on the horizon (Fig. 6). In fact, the emerging data from clinically supervised strategies to find risk-associated biomarkers, and molecularly driven strategies to identify patterns within unselected tumors suggest that these two different approaches (top-down and bottom-up) are leading to the same conclusion, i.e., that ccRCC is composed of two dominant subgroups that are closely aligned with clinical outcome. How this information will enable physicians and patients to make wise decisions in the management of ccRCC and eventually select the optimal pharmaceutical therapy remains to be seen, but in the light of many emerging targeted therapies, such information is likely to be highly valuable.

Biomarkers

The modern emergence of therapeutic options based on an increased understanding of the genetics and molecular biology of the RCC group of diseases has intensified the need for biomarkers to accurately assess prognosis, identify patients who are likely to benefit from therapy and specific drugs or classes of drugs, and elucidate the mechanisms of resistance. Here, we present a succinct overview of the most recent advances in the development of biomarkers for RCC,

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in particular for the clear-cell subtype. Although some are promising, it is important to note that none of these biomarkers are available for clinical testing at this time.

In clinically localized ccRCC, the emphasis has been placed on biomarkers of prognosis expressed in tumor tissue. Some of these biomarkers have been found to be independently prognostic, such as the HIF-1α–regulated hypoxia marker carbonic anhydrase IX (194), the antiapoptotic protein survivin (195–198), the cell proliferation protein KI-67 (199–202), and the immune inhibitory family of ligands B7-H (203–205), but their clinical value is still in question due to lack of independent and prospective validation. IMP3 (one of the insulin-like growth factor II mRNA binding proteins), whose immunohistochemical expression in tumor cells was found to be associated with short metastasis-free survival and overall survival (OS), is a rare exception because this finding was subsequently validated in an independent patient cohort (206, 207).

Cytogenetic and gene expression profiling studies have also shown some potential to deliver prognostic information in nonmetastatic ccRCC. In mostly small cohorts of patients, specific chromosomal abnormalities have been linked to good (5q gain) or poor (9p, 14q loss) prognosis (208–210). However, the relation of 9p loss with poor outcome (including prognostic value for small renal masses) has been repeatedly observed (211, 212), making this a logical candidate to incorporate into available prognostic algorithms. A number of potential biomarkers related to tumor development and progression have also emerged from gene expression analyses, several of which identified gene signatures associated with significant survival differences in patients (189, 213, 214). However, as with the immunohistochemical and cytogenetic markers, these signatures have not yet been validated. Work to validate these gene signatures to predict risk of recurrence is ongoing.

In patients with advanced ccRCC, the availability of effective treatments targeting the VEGF and mTOR pathways has shifted the focus toward a search for biomarkers, predominantly in tumor tissue but also in blood, that are capable of predicting therapy response and resistance. Although the analysis of *VHL* gene status has not resulted in consistent data to support either a prognostic or a predictive value (215–219), the activation state of the HIF subunits (68) and multiple HIF-responsive genes are being examined. One HIF target (VEGF) and other angiogenesis-related and tumorigenic factors in serum or plasma have been evaluated across multiple clinical trials of targeted agents in RCC. It has been established that higher baseline VEGF levels are associated with worse tumor stage and grade, performance status, and overall prognosis (220–225). Moreover, in a phase III trial of sorafenib versus placebo, patients with VEGF in the highest concentration quartile obtained greater relative benefit from sorafenib than those with lower concentrations (225). However, studies addressing whether VEGF is a predictive marker for identifying RCC patients who are likely to benefit from VEGF-targeted therapies have yielded inconsistent results (220, 225, 226). Preliminary evidence supports the premise that proteomic plasma profiling of cytokines and angiogenic factors (CAF) in plasma or serum can be used to develop prognostic and predictive biomarkers, and may also contribute to molecularly improve RCC classification (227). Using this approach, investigators identified 2 broad groups of patients with metastatic ccRCC patients: one predominantly expressing angiogenesis/hypoxia-related markers, and one showing an alternative expression of inflammatory markers. Regarding clinical benefit from VEGF inhibitors, a recent study in plasma samples collected in subsequent phase II and III studies of pazopanib identified low concentrations of interleukin (IL)-8, hepatocyte growth factor (HGF), outer membrane protein (OPN), and TIMP-1 with improved progression-free survival (PFS) on pazopanib (228). IL-8 was previously implicated in resistance to sunitinib (229). Unfortunately, no biomarkers that are predictive of differential benefit between available and active drugs in RCC have been validated. In a randomized phase II study of sorafenib versus sorafenib in combination with interferon that yielded no differences in PFS, a candidate 6-CAF signature consisting of markers in the angiogenic/hypoxia group (OPN, VEGF, collagen-IV, soluble CAIX, TRAIL, and soluble VEGF receptor-2) predicted for distinct PFS in the 2 arms (227). The results of similar analyses in larger patient sets are eagerly awaited.

Immunotherapy

The ability of some renal tumors to evoke an immune response, and the possibility that this may lead to spontaneous regression of metastatic RCC in some patients spurred the idea of developing immunotherapy as an effective treatment for patients with RCC (230–232). Various immunotherapeutic strategies have been tested, and many have shown some evidence of activity (233, 234). Established therapies consist of cytokines such as IFNα and IL-2. IFNα was reported to provide a survival benefit in a meta-analysis (235). High-dose (HD) IL-2 was shown to produce tumor responses in ∼10% to 20% of patients, with some patients achieving long-term response off treatment (236–240). The FDA approved HD IL-2 as a treatment for metastatic RCC in 1992 based on phase II data (236). However, both IFNα and HD IL-2 are associated with substantial toxicities that have limited their use (241, 242). In addition, due to the emergence of novel VEGF- and mTOR-targeted therapies that are comparatively easier to administer and better tolerated, and were shown to provide clinical benefit in phase III clinical trials (243, 244), the use of IFNα and HD IL-2 as a treatment for metastatic RCC has diminished. Clearly, however, there is a subset of patients who derive a substantial clinical benefit from immunotherapy. Efforts are ongoing to elucidate the mechanisms of action and identify predictors of response to cytokine therapies such as IFNα and HD IL-2 in an attempt to better select patients for treatment. In addition, novel immunotherapeutic strategies are being developed as a result of research in the field of basic immunology, which has provided strong scientific and preclinical data to enable successful immunotherapy trials.

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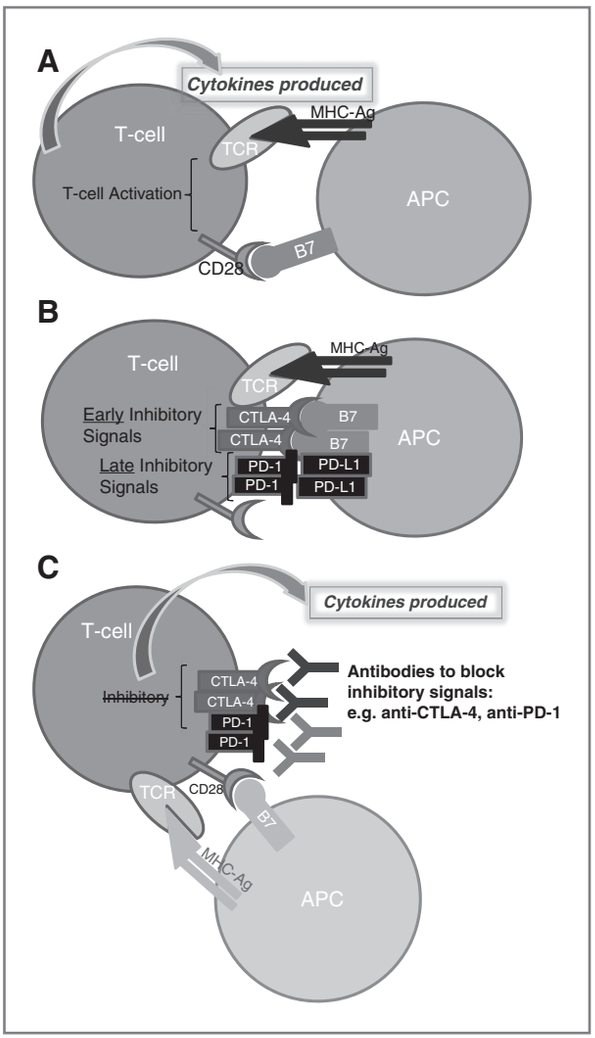

Figure 7. Immune regulation of renal tumor cells. A, when an APC engages a T cell via a cognate TCR and CD28, T-cell activation occurs. B, early and late T-cell inhibitory signals are mediated by CTLA-4 and PD-1 receptors upon engagement of the APC via B7 and PD-L1, respectively. C, inhibitory antibodies against CTLA-4 and PD-1 can overcome T-cell downregulation and once again allow cytokine production. APC, antigen-presenting cell; TCR, T-cell receptor.

An improved understanding of the various mechanisms by which T-cell activation can be positively or negatively regulated (Fig. 7) led to the development of agents that can enhance antitumor T-cell responses. The first such agent, and prototype, is anti-CTLA-4 antibody, which laid the foundation for the development of other immune checkpoint agents, such as anti-PD-1 antibody.

Upon engagement of the T-cell receptor with antigen bound by MHC (signal 1) and costimulation provided by CD28 interacting with B7-1 and B7-2 (signal 2), T cells become activated to produce cytokines and proliferate (245, 246). However, T-cell activity must be regulated to prevent damage to normal cells and tissues. Therefore, when T cells are turned "on," a series of signals within the cells also generate an "off" mechanism. This off switch is known as CTLA-4. CTLA-4 acts to limit T-cell responses (247, 248). The understanding of how CTLA-4 functions led to the idea that an antibody that blocks CTLA-4, thereby temporarily disengaging the off switch, would allow for enhanced T-cell responses against tumors. This idea was validated in preclinical models (249, 250) and then tested in clinical trials (251–253). Two phase III randomized clinical trials were completed, and showed a survival benefit for patients with metastatic melanoma who were treated with the anti-CTLA-4 antibody known as ipilimumab [Bristol-Myers Squibb (254, 255)]. On the basis of these data, ipilimumab was approved by the FDA in March 2011 as a treatment for patients with metastatic melanoma. Because anti-CTLA-4 targets a molecule expressed on T cells, as opposed to a molecule on tumor cells, this therapy is potentially applicable to multiple tumor types.

Anti-CTLA-4 has been evaluated in patients with metastatic RCC. In a phase II trial, 2 cohorts of patients with advanced RCC received 2 different dosing schedules of ipilimumab: a 3 mg/kg loading dose followed by either 1 mg/kg or 3 mg/kg maintenance doses every 3 weeks (256). Of the 21 patients who received the 1 mg/kg maintenance dose, 1 patient (4.7%) experienced a partial response. Of the 40 patients who were treated with the 3 mg/kg maintenance dose, 5 (12.5%) experienced partial responses. Of importance, responses were observed in patients who had failed prior HD IL-2 treatment, suggesting that there is no clear cross resistance. Given its recent FDA approval for use in melanoma, ipilimumab will likely be investigated further in patients with RCC.

PD-1 is another receptor that is expressed on activated T cells (257). Interactions with PD-1 and its ligands (PD-L1 and PD-L2) can serve to inhibit T-cell responses. PD-L1 was shown to be overexpressed in many RCCs, and greater expression was associated with worse prognosis (203). MDX-1106, a monoclonal antibody directed against PD-1, was recently assessed in phase I trials that included many patients with advanced RCC (258, 259). Antitumor activity was seen in a patient with RCC in the initial trial involving a single dose of the PD1 antibody (258). In a subsequent study (259), MDX-1106 was administered in doses of 1, 3, and 10 mg/kg given every 2 weeks. Of 16 patients with RCC treated at various doses, 5 patients (31%) achieved objective responses, including 1 complete response. This promising activity, coupled with a mild toxicity profile, prompted the initiation of a phase II trial of MDX-1106 in patients with advanced RCC. Over the next several years, agents such as ipilimumab and MDX-1106 will likely be assessed, possibly with cytokines and other therapies in various sequences and combinations, with the goal of achieving higher rates of durable responses than is possible with currently available therapies.

Molecularly Targeted Therapy

The biology of RCC as elucidated above has led to the development of multiple agents that target elements of the

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Table 1. Phase III trials of targeted therapy in metastatic RCC

Trial (ref.)	Number of patients	Clinical setting	RR (%)	PFS (mo)	OS (mo)
VEGF-targeted therapy					
*AVOREN bevacizumab + IFNα vs. IFNα (264)	649	First-line	31 vs. 12	10.2 vs. 5.5 ($P < 0.001$)	23.3 vs. 21.3 ($P = 0.129$)
*CALBG 90206 bevacizumab + IFNα vs. IFNα (265)	732	First-line	25.5 vs. 13	8.4 vs. 4.9 ($P < 0.001$)	18.3 vs. 17.4 ($P = 0.069$)
Sunitinib vs. IFNα (243)	750	First-line	47 vs. 12	11 vs. 5 ($P = 0.0001$)	26.4 vs. 21.8 ($P = 0.051$)
*TARGET sorafenib vs. placebo (266)	903	Second-line (post cytokine)	10 vs. 2	5.5 vs. 2.8 ($P < 0.01$)	17.8 vs.15.2 ($P = 0.88$)
Pazopanib vs. placebo (267)	435	First-line/second-line (post cytokine)	30 vs. 3	9.2 vs. 4.2 ($P < 0.0001$)	22.9 vs. 20.5 ($P = 0.224$)
*AXIS axitinib vs. sorafenib (263)	723	Second-line (post sunitinib, cytokine, bevacizumab, or temsirolimus)	19 vs. 9 ($P = 0.0001$)	6.7 vs. 4.7 ($P < 0.0001$)	Not reported
mTOR-targeted therapy					
*ARCC temsirolimus vs. Tem + IFNα vs. IFNα (244)	624	First-line, ≥3 poor-risk features[a]	9 vs. 5	3.8 vs. 1.9 for IFNα monotherapy ($P = 0.0001$)	10.9 vs. 7.3 for IFNα ($P = 0.008$)
*RECORD-1 everolimus vs. placebo (261)	410	Second-line (post sunitinib and/or sorafenib)	2 vs. 0	4.9 vs. 1.9 ($P < 0.0001$)	14.8 vs. 14.5

Abbreviations: ARCC, Advanced Renal-Cell Carcinoma; AVOREN, Avastin for Renal Cell Cancer; AXIS, Axitinib in Second Line; CALBG, Cancer and Leukemia Group B; OS, overall survival; PFS, progression-free survival; RECORD-1, Renal Cell Cancer Treatment with Oral RAD001 Given Daily; RR, response rate; TARGET, Treatment Approaches in Renal Cancer Global Evaluation Trial.

[a]Including a serum lactate dehydrogenase level >1.5 times the upper limit of the normal range, a hemoglobin level below the lower limit of the normal range, a corrected serum calcium level of >10 mg/dL (2.5 mmol/L), a time from initial diagnosis of RCC to randomization of <1 year, a Karnofsky performance score of 60 or 70, or metastases in multiple organs.

relevant VEGF and mTOR pathways (260). Table 1 outlines the major phase III trials of targeted therapy in RCC that led to regulatory approval of several agents. There are several points to be made about the key discriminating features of these agents. VEGF-targeted therapy produces more robust Response Evaluation Criteria in Solid Tumors (RECIST)-defined objective response rates than cytokine therapy, on the order of 30% to nearly 50% for the most active agents. Within the class of VEGF-receptor (VEGF-R) inhibitors, the response rate can vary from 10% to nearly 50%, with the higher rates observed for drugs that more potently inhibit VEGF-R. It is also recognized that antitumor activity, especially that of VEGF-targeting agents, is not entirely captured by size changes alone, as tumor necrosis (reduced perfusion on a contrast-enhanced CT scan) is thought to be indicative of drug effect and may or may not be accompanied by tumor size reduction. mTOR-targeted therapy in general produces more modest response rates of 2% to 10%, although to date mTOR-targeted and VEGF-targeted therapies have been studied in different populations (261, 262). With regard to the percentage of patients who experience at least some tumor burden reduction on therapy (including patients with a 1%–29% reduction, which does not meet the arbitrary 30% reduction required for a RECIST-defined response), VEGF-targeted therapy was shown to shrink tumors in ∼75% of patients, and mTOR-targeted therapy was shown to shrink tumors in ∼50% to 60% of patients.

PFS is generally doubled with targeted therapy compared with placebo/cytokines. Again, here we see important differences among the VEGF-R inhibitors, with the biochemically more potent agents producing a PFS of ∼11 months in untreated patients, compared with 5 months for the biochemically weaker agent sorafenib. Axitinib is the most biochemically potent of these inhibitors, but to date, only results from previously treated patients are available (267). Of note, in the subset of patients who were not exposed to prior VEGF-targeted therapy in the AXIS trial (the cytokine-refractory subgroup), the median PFS was >12 months. With regard to PFS, the mTOR-targeting agents have been studied in unique patient circumstances, i.e., poor-risk for temsirolimus and VEGF-R TKI-refractory RCC for everolimus (261, 262). The PFS for each was modest (∼5 months), but the effect of these agents in first-line use in good/intermediate-risk patients awaits further study. In addition, the clinical activity of these drugs was more robust

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in untreated patients than in cytokine-refractory patients, and less robust in patients who had already failed targeted therapy. The OS rate in these trials is notable for several reasons. The first-line trials of VEGF-targeted agents produced an OS of ~2 years, roughly double that of historical cytokine-treated controls. Nonetheless, no single trial (with the exception of the temsirolimus trial in poor-risk RCC) has shown a statistically significant OS benefit (despite a numerical advantage in the median OS). This is largely believed to be due to the high percentage of patients who cross over from initial therapy on trial (placebo or cytokine) and receive one or more active targeted therapies at progression. The efficacy of such a sequential salvage strategy has confounded interpretation of OS from these trials, although there is general consensus that targeted therapy has meaningfully extended the lives of patients with metastatic RCC.

There is no consensus regarding the best drug for initial therapy or the optimal sequence of agents. Ongoing trials are beginning to address these issues, but because of the multitude of agents available and the relative rarity of RCC, definitive trials are not possible at this time. Future research involving targeted therapy for RCC will focus on issues such as the relative toxicity/efficacy of various agents, the importance of the switching mechanism at progression, and biomarkers of response and resistance that may allow for improvement upon the current standard of an empiric sequence of monotherapies.

Conclusions

There has been a clear and important evolution in our understanding of RCC biology. We are now challenged with converting this newly acquired information into actionable items that will alter our approach to the prevention, diagnosis, and management of RCC. Several new rare types of cancers are now recognized to occur in the kidney, which will both challenge the urologic oncology community to maintain up-to-date guidelines for the management of these tumors and provide new opportunities to develop effective personalized therapies.

By comparing genomic, transcriptomic, and epigenetic data from precursor lesions and early ccRCC, we will be able to establish a roadmap of tumor ontogeny for this more common subtype. To achieve this goal, it will be essential to use material from patients with hereditary VHL disease. These data, in conjunction with epidemiological and laboratory-based studies, will allow investigators to identify driver mutations and epigenetic changes, and thus facilitate the development of markers that will permit early identification of ccRCC. In the same manner, studies of the cilia centrosome cycle and HIF regulation will provide a mechanistic, molecular biological understanding of early cancer development, with resultant opportunities for therapeutic intervention.

In more advanced disease, studies of genomics, transcriptomics, and molecular biology will enable investigators to gain insight into the mechanisms of tumor progression, especially if they are conducted in parallel with *in vitro* and *in vivo* models employing potential driver pathways that have been identified in ccRCC or other rare variant cancers. The hope is that by understanding both the cause and the consequence of the complex interactions between genomic and epigenetic changes, and assigning significance to the output of these alterations, we will be able to replicate RCC tumor diversity, identify subgroups, and develop more specific therapeutic interventions. Achievement of this goal will require a coordinated interaction among high-throughput platform experts, molecular biologists, and computational scientists who are capable of controlling and codifying the complex systems that arise from these collaborations. The recognition that the output of these changes is profoundly influenced by host genomic and phenotypic characteristics, and that the tumor microenvironment varies as a function of these characteristics, necessitates the development of precise tools to measure the tumor microenvironment.

Finally, the renaissance of tumor immunology has been fueled by the recognition that tumors can take advantage of the innate regulatory pathways that are built into T cells and other immune effectors. As we begin to understand the impact of tumor biology on T-cell regulation, as well as on the recruitment of bone marrow–derived immunological precursors, significantly better treatments will become available for patients with ccRCC in the next few years. Understanding the interface between evolving tumor biology and the host genomic determinants of the stromal endothelial phenotype will further advance this field.

We are poised to make very significant advances in RCC research in the next few years. With the right team and the right tools, the achievement of a truly personalized approach to treatment is within reach.

Disclosure of Potential Conflicts of Interest

E. Jonasch received commercial research grants from Pfizer, GSK, Novartis, and Onyx, and is a member of the consultant/advisory boards of AVEO, BMS, GSK, and Pfizer. W.Y. Kim received honoraria from the Speakers Bureau of Novartis and is a member of the consultant/advisory board of Novartis. B.I. Rini received commercial research grants from Pfizer, GSK, and Immatics, and is a member of the consultant/advisory boards of Pfizer, GSK, AVEO, and Roche. P. Sharma is a member of the consultant/advisory boards of Bristol-Myers Squibb and Dendreon. M.B. Atkins is a member of the consultant/advisory boards of Genentech, BMS, Novartis, AVEO, Amgen, Prometheus, and Astra Zeneca. W.K. Rathmell received a commercial research grant from GSK and is a member of the consultant advisory board of AVEO. No other potential conflicts of interest were disclosed by the other authors.

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