Minireview

Pathobiology, Prognosis, and Targeted Therapy for Renal Cell Carcinoma: Exploiting the Hypoxia-Induced Pathway

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Abstract

Historically, clinical factors have been used as prognostic markers for patients with renal cell carcinoma (RCC). Recent advances in the understanding of the pathogenesis, behavior, and molecular biology of RCC have paved the way for developments that may enhance early diagnosis, better predict tumor prognosis, and improve survival for RCC patients. This report reviews the molecular mechanisms of the hypoxia-induced pathway that play an essential role in angiogenesis, glycolysis, and apoptosis of common cancers and may be responsible for the ability of the cancers to adapt to a hypoxic environment and also for their resistance to radiation and chemotherapy. The hypoxia-induced pathway has been linked genetically to RCC through the von Hippel-Lindau tumor suppressor gene, which is inactivated in a majority of clear cell RCCs. Therefore, RCC is a particularly attractive clinical model to exploit the hypoxia-induced pathway for new therapeutic interventions. von Hippel-Lindau, hypoxia inducible factor 1α, carbonic anhydrase IX, vascular endothelial growth factor, and other important members of the hypoxia-induced gene family, provide new molecular targets for diagnosis, prognosis, and immunotherapy of RCC.

Introduction

Cancer of the kidney or renal pelvis afflicted an estimated 31,800 United States patients in 2002 with 11,600 estimated deaths (1), and these statistics seem to be rising annually (2). In the United States, there has been a 126% increase in the incidence of RCC since 1950, accompanied by a 36.5% increase in annual mortality. This increase in the incidence of RCC occurred in all age groups, with the greatest increase occurring in patients with localized tumors, suggesting a migration toward earlier stages at diagnosis as a result of earlier detection (3). This phenomenon may be explained in part by the increased number of asymptomatic, incidental tumors being detected as a result of the widespread use of noninvasive abdominal imaging modalities such as ultrasound, computerized tomography, and magnetic resonance imaging (4). However, the number of advanced cases, including those with regional extension and distant metastases, also increased in all race and sex categories. Despite the increase in the annual number of advanced cases, the rise in incidence for all stages over this time period has increased three times more than the rise in the rate of mortality. Consequently, the 5-year survival for all cases has nearly doubled from 34% in 1954 to 62% in 1996 (2), and the estimated annual percentage change in the United States renal cancer death rate actually fell by 0.2% during the period 1992–1999 (5), reflecting on an improved outlook for patients with RCC. However, although subsets of patients with advanced disease have experienced improvements in survival, the long-term survival of all patients with distant RCC remains low, with only 8.9% 5-year survival for patients of all sexes and races during this same time period (5).

This overall improved outlook reflects a number of trends, including improvements in radiological imaging, leading to earlier diagnosis and improved staging, refinements in surgical techniques and perioperative care, and enhanced understanding of the immunobiology of solid tumors. Significant achievements in the basic sciences have led to a greater knowledge of the underlying molecular genetics of RCC, which hold the promise of increased sophistication in attempts to individualize patient prognostication and for future treatment strategies. Improved outlook for patients with advanced and metastatic RCC is related to a better understanding of the role and timing of cytoreductive nephrectomy (6, 7) and the introduction of immunotherapeutic treatment approaches. A thorough comparison of the numerous agents and combination of agents available in diverse immunotherapy regimens is beyond the scope of this review, and several excellent recent reviews have already accomplished this task (8–10). However, regardless of the specific immunotherapy regimen used, patients included in immunotherapy clinical trials have experienced improved prognosis when compared with patients treated historically by other clinical protocols in which no immunotherapy was given (11). At UCLA, the median
survival of patients with metastatic RCC has improved in the 1990s by close to 12 months when compared with similar patient cohorts in the 1970s and 1980s. The optimum dose of IFN and/or IL-2 has not been definitively proven. High-dose bolus i.v. IL-2 is the only Food and Drug Administration-approved treatment for good performance status metastatic RCC. Response rates are 15–20%, median duration of response is 54 months, and median duration of response for complete responders is approaching 10 years (12). Moderate dose IL-2 given alone, in combination with IFN, or with cell-based therapy, has reported comparable response rates (8–10). Preliminary reports of two randomized trials have demonstrated higher response rates for high-dose bolus IL-2 when compared with outpatient IL-2/IFN α (13) or lower-dose inpatient bolus IL-2 or outpatient IL-2 monotherapy (14). Follow-up for these studies is too short to assess differences in durability of response across the different regimens. However, high-dose bolus IL-2 is often accompanied by very high toxicity and other adverse reactions. Therefore, newer, innovative strategies for therapy are continuously being sought.

**Prognostic Factors for RCC.** Advances in our understanding of the pathogenesis, behavior, and importance of prognostic factors for RCC have paved the way for increased sophistication in its classification and staging. The clinical behavior of RCC reflects its underlying genetic abnormalities. Throughout the 1990s, advances in the understanding of the genetic alterations underlying the pathogenesis of RCC reinforced the concept that there were distinct subtypes of RCC, each with its own associated genetic abnormalities (15, 16). Genetic alterations affect cellular biology differently, leading to different tumor morphology and behavior. In 1996, the Heidelberg Classification of Renal Cell Tumors was proposed, which sought to integrate an understanding of these genetic alterations with readily recognizable histological criteria (15). In many ways, the Heidelberg genetics analysis gave support for the cytomorphic-based Mainz classification. There is some evidence that such an approach can provide useful prognostic and clinical information (17).

There are four main histological subtypes of RCC: clear cell, papillary, chromophobe, and collecting duct carcinomas. Each subtype of renal tumor has distinguishing characteristics and patterns of disease that may be associated with prognosis. Clear cell carcinoma (also known as conventional or nonpapillary) is the most common type of renal tumor with malignant potential, accounting for 70–80% of cases (15, 18). Clear cell RCC is thought to arise in the proximal renal tubule and comes in both a hereditary and sporadic form. Hereditary clear cell RCC occurs in patients with VHL disease as a result of germ-line mutations in the VHL gene (a classic tumor suppressor gene), which resides on the short arm of chromosome 3 (3p25) (19). Somatic mutations in the VHL gene are thought to play a role in the development of sporadic conventional RCC, because a high frequency of patients display loss of one allele and either mutations or inactivation by hypermethylation of the other allele (20, 21). Mutations of the VHL gene, which occur exclusively in clear cell RCC (15), apparently develop as an early lesion. Papillary RCC is the second most common RCC histology, further divided into two types (22, 23). Type 1 papillary RCC has been found to behave less aggressively than type 2. Like clear cell tumors, papillary RCCs are thought to arise in the proximal renal tubular epithelium and to occur in both a hereditary and sporadic form (24). Hereditary papillary renal cancer is a familial cancer syndrome characterized predominantly by type 1 papillary RCC. The syndrome is associated with activating mutations of c-MET proto-oncogene on 7q34 (25). The characteristic cytogenetic findings for papillary RCC is trisomy of, most commonly, chromosomes 7, 16, and 17 as well as loss of the Y chromosome. Recently, Launonen et al. (26) described a new familial renal cancer syndrome named “hereditary leiomyomatosis and renal cell cancer,” an autosomal dominant disease related to germ-line mutations mapping to1q42–44 and involving the fumarate hydratase gene, encoding fumarate hydratase, an enzyme that catalyzes the conversion of fumarate to malate in the Krebs cycle. This syndrome is characterized by cutaneous and uterine leiomyomas and aggressive type II papillary renal cancers (26). Genetically, chromophobe RCC is characterized by monosomy of multiple chromosomes (including 1, 2, 6, and 10) as well as hypodiploidy (15). Chromophobe RCCs have been proposed to originate in the intercalated cells of the renal-collecting ducts (27). Collecting duct carcinoma, or Bellini’s duct carcinoma of the kidney, is an aggressive RCC variant, accounting for <1% of surgical cases. As a result of their rarity, there is little information available concerning the genetics of collecting duct carcinoma. Microscopically, they appear to arise in the medullary-collecting ducts (28). Recently, another new familial RCC syndrome has been described in association with the autosomal dominant Birt-Hogg-Dube genodermatosis (29). A number of unique histologies are associated with this syndrome, including chromophobe, clear cell, hybrid neoplasms, and oncocytomas. Microscopic oncocytosis has been found in the renal parenchyma of most patients, suggesting that this lesion may be an early precursor lesion for RCC.

**Integrated Staging Algorithms.** Genotypic changes lead to phenotypic and molecular changes that ultimately result in microscopic and macroscopic pathology. A comprehensive understanding and appreciation for the factors that impact on the biological behavior of RCC is essential for understanding the natural history of the disease in patients. The pathobiology of RCC is complex and is influenced by factors other than pathological stage. Patient- and tumor-related factors have been proposed as additional prognostic factors. It is clear that the clinical behavior of RCC results from complex interactions between these multiple prognostic factors. This realization has led to an increasing interest in integrated staging systems that predict outcome by combining other pathological and clinical variables (30). The future of RCC prognostication will extend this direction further back from the macroscopic to beyond the microscopic, and will involve the integration of molecular and genetic markers.

For RCC, tumor grade, tumor stage, and patient performance status remain the most useful, clinically available predictors of patient outcome (2). However, there are other important patient- and tumor-related clinical, radiographic, and pathological features that contribute to our understanding of the often unpredictable behavior of RCCs. A recent report suggests that VHL status may have prognostic significance for patients with sporadic clear cell RCC (31). A VHL alteration (mutation or hypermethylation) was detected in 108 of 187 RCC tumor
samples, and VHL alterations were strongly associated with better cancer-free survival and cancer-specific survival for 134 patients with stage I–III clear-cell RCC treated by radical nephrectomy. Importantly, VHL alterations remained an independent prognostic factor for patients with stage I–III tumors after adjustment for sex, age, stage, grading, and symptomatic presentation.

A number of clinical characteristics have been identified as having an impact on the clinical behavior and subsequent survival in patients with advanced RCC. These include, in addition to initial performance status, time from diagnosis to metastasis, location and number of metastatic sites, weight loss, and whether the patient has undergone nephrectomy (32–36). In 1988, Elson et al. (35) developed a scoring system to determine prognosis for patients with advanced RCC, stratifying patients into five groups based on ECOG performance status, time from diagnosis to metastasis, weight loss, prior chemotherapy, and number of metastatic sites. Recently, Motzer et al. (11) have developed a model based on the study of 670 patients with advanced RCC treated at Memorial Sloan-Kettering Cancer Center, defining the relationship between pretreatment clinical features and survival. Five pretreatment features were identified to be associated with a shorter survival as determined by multivariate analysis. These included low Karnofsky performance status, high serum LDH (>1.5 times normal), low hemoglobin (less than the lower limit or normal), hypercalcemia (>10 mg/dl), and absence of prior nephrectomy. These five prognostic variables defined three subgroups each having unique survival characteristics. Poor risk patients with three or more risk factors had a median survival time of only 4 months, whereas median survival improved to 20 months in patients with zero risk factors. Many of these older prognostic algorithms did not take into account molecular features or pathological subtypes of RCC, whereas more recent systems such as the Mayo Clinics SSSIGN (stage, size, grade, necrosis) score (37) and the latest Memorial Sloan-Kettering Cancer Center nomogram (23), both of which relate only to patients with localized RCC, have begun to include pathological subtype as a prognostic feature.

The UCLA Kidney Cancer Program has developed and refined an integrated staging system to better stratify patients into prognostic categories using statistical tools that can accurately define an individual patient’s probability of survival (30). Initially evaluated were a multitude of factors in 661 patients, including age, sex, tumor grade, TNM stage, tumor size alone, ECOG performance status, laterality, bilaterality, smoking, number of presenting symptoms, weight loss alone, tumor histological type, administration of immunotherapy, inferior vena cava involvement, number of metastatic sites, sites of metastases, and time interval between nephrectomy and tumor recurrence to determine which factors were having the greatest impact on RCC patients’ survival. The initial UISS contained five groups based on the most significant explanatory variables, namely TNM stage, tumor grade, and ECOG performance status. The UISS used the four-tier grading scheme based on nuclear and nucleolar size, shape, and content, as proposed by Fuhrman et al. (38), that remains the most commonly used system for grading RCC in North America. The projected 2- and 5-year survival, respectively, for patients in UISS groups are: I, 96% and 94%; II, 89% and 67%; III, 66% and 39%; IV, 42% and 23%; V, 9% and 0%. This novel system for staging and predicting survival for patients with RCC is simple to use, superior to stage alone in differentiating patients’ survival, and may prove to be an important prognostic tool for counseling patients with various stages of kidney cancer. The system is versatile and flexible enough to tie together metastatic as well as nonmetastatic patients in the same integration. The UISS was internally validated using a bootstrap technique. Recently, the UISS was validated using an expanded database of patients treated at the multidisciplinary Kidney Cancer Program at UCLA between 1989 and 2000 (39) and with external data from 576 RCC patients treated at the M. D. Anderson Cancer Center (Houston, TX) and in Nijmegen, the Netherlands (40, 41) using several methods of comparison, including direct comparison of survival rates for each UISS stage, analyzing hazard ratios describing the separation of UISS groups, and the formulation of concordance indices. Mathematical equations have subsequently been developed for estimating survival after radical nephrectomy for RCC predicted by use of Nadas equations that faithfully describe the actual survival based on the UISS Kaplan-Meier curves (42). The resulting formulas are capable of better tailoring survival estimates for a specific patient.

The UISS was further modified into a simplified system, based on separate stratification of metastatic and nonmetastatic patients into LR, IR, and HR groups (43). Decision boxes integrating TNM staging, tumor grade, and performance status were compiled to determine a patient’s risk group. LR, nonmetastatic patients experienced a 91% disease-specific survival at 5 years, lower recurrence rates, and a better disease-free survival compared with IR and HR, nonmetastatic patients. Fifty percent of HR, nonmetastatic patients progressed. Disease-specific survival of HR, nonmetastatic patients who received IMT for recurrent disease was similar to that of LR, metastatic patients treated with cytoreductive nephrectomy and IMT. Time from recurrence to death for HR, nonmetastatic patients was inferior to that of LR, metastatic patients. After IMT, approximately 25% of LR, metastatic and 12% of IR, metastatic patients had long-term progression-free survival. HR, metastatic patients did poorly despite IMT. For each of the risk groups, a relevant set of clinical outcome data were generated, including overall survival, disease-specific survival, freedom from recurrence in nonmetastatic patients, outcome after recurrence in nonmetastatic patients (Fig. 1), and freedom from progression in metastatic patients undergoing cytoreductive nephrectomy and IMT. Stratifying RCC patients into HR, IR, and LR subgroups provides a clinically useful system for predicting outcomes and provides a unique tool for risk assignment and outcome analysis. Subclassifying RCC into well-defined risk groups should allow better patient counseling and the identification of both HR, nonmetastatic subgroups that may benefit from adjuvant treatment and nonresponders that need alternative, experimental therapies. These classification systems are already being used by multicenter clinical trials of adjuvant therapy for HR, nonmetastatic RCC patients to establish patient eligibility criteria. Likewise, risk factor stratification is being used to define more homogeneous populations in the design of recent targeted therapy trials of metastatic RCC patients, an approach championed by Memorial Sloan-Kettering Cancer Center and others.
Tumor Tissue Banking: Providing New Opportunities for Discovery. In the past decade, a tremendous wealth of human tissue samples and clinical data have been accumulated from patients with kidney cancer. The Kidney Cancer Program at UCLA serves as an illustration of these trends and opportunities. Since 1989, over 1400 frozen and paraffin-embedded tumor specimens, including patients treated on experimental IMT protocols, have accumulated and are now associated with mature clinical data, offering the opportunity to analyze genetic and molecular features associated with multiple clinical variables. Over 1000 of these tumor specimens have banked matched normal control tissues. These specimens include primary tumors, lymph nodes, metastases to multiple distant sites, salvage-resected specimens obtained after IMT or chemotherapy, and biopsies before and after therapy. Several hundred specimens were obtained from patients with metastatic disease who were treated with IMT and can be evaluated for treatment response and survival, including over 100 patients who underwent cell-based therapy. Many of these tumors have been used to construct tissue microarrays, allowing the analysis by immunohistochemistry or in situ hybridization of hundreds of tumors simultaneously. Thus far, five blocks containing tissue for 417 kidney cancer patients have been arrayed. All arrayed patients have a full set of clinical data within the clinical databases for correlation with disease progression, treatment response, and survival. The UCLA Kidney Cancer Clinical Database contains data on over 1300 patients treated for RCC between 1989 and 2002. Two hundred sixty-three variables are recorded for each patient, including demographics, risk factors, laboratories, tumor histology and staging, treatment response, and outcomes.

![UCLA Integrated Staging System Risk Group Assignment for Patients with Renal Cell Carcinoma](image)

**LOCALIZED DISEASE (N0M0)**

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* From failure to death with immunotherapy treatment.

Fig. 1 Decision box and reference stable for the UISS for patients with localized RCC [Reprinted with permission from the American Society of Clinical Oncology from Zisman et al. (42)].
analysis. Clinical information on each patient sample has been encoded and is stored with anonymized numerical links to stored tumor specimens. Data from the results of gene expression, cytogenetics, and tissue microarray molecular marker analysis are linked to the clinical databases through a Web-based platform that can be exported to statistical software packages for analysis. All patient data and tissue samples banked are provided either retrospectively through an IRB exemption or prospectively with an IRB-approved submission and informed consent. This database structure allows the integration and analysis of multiple data sources based on any combination of clinical parameters. The UCLA Advanced Research Computing Cooperative is working to develop a comprehensive set of applications supporting the activities of the Kidney Cancer Program. This system will eventually provide results of molecular tissue analysis while ensuring effective methodology for patient privacy and security, and this approach will allow for the development of inter-institutional collaboration methodologies including data entry templates, rules for querying inter-institutional data and tissue banks, and scientific inquiry through protocol development of retrospective and prospective analysis. Supplying this data through distributed and synchronized data centers will afford rapid discoveries providing effective demographic, clinical, pathological, imaging, and molecular characterization, improving the evaluation of therapies and eventually allowing for an optimization of individual therapy.

Molecular Profiling: The Future of Patient Prognostication, Staging, and Treatment. Like other solid tumors, molecular tumor markers are expected to have an enormous impact on the future diagnosis, prognostication, and selection of therapeutic targets for RCC. Tumor markers provide not only prognostic information to aid in the identification of patients at risk for recurrence or metastasis but could also hold the key to targeted therapeutic interventions as well. The completion of the human genome project and development of the microarray technology promises rapid identification and validation of novel diagnostic and molecular markers. The development of a new genomic technology using DNA and tissue arrays has created a powerful tool that will enable researchers to evaluate hundreds to thousands of tumors simultaneously with histological, immunohistochemical, and chromosomal analyses. Currently, many markers relating to tumor proliferation, growth, angiogenesis, and loss of cell adhesion, among others, are being evaluated for their potential as prognostic factors. Until now, there have been no molecular markers for RCC that meet the College of American Pathologists criteria for a marker to be used generally in patient management or even to have been studied sufficiently biologically to provide for any degree of acceptance (44). Some are promising, but clinical trials are needed to validate their usefulness in clinical practice. Several markers, however, have been studied sufficiently clinically to suggest some level of evidence for their use. These latter markers include proliferating cell nuclear antigen (45), Ki-67 (46), and silver-staining nucleolar organizing regions (47), all of which have been shown to correlate with survival in small studies of patients with RCC. Ki-67 and proliferating cell nuclear antigen are both markers of cellular proliferation, and increased staining of these antigens has been shown to be associated with a poor prognosis in RCC. Argyrophilic nucleolar proteins, which are another measure of cellular activity and are thought to reflect DNA transcriptional activity, were shown by Delahunt et al. (48) to be an independent prognostic indicator. Other markers examined have included proteins involved in cellular signal transduction, control of transcription, apoptosis, cell adhesion, cytoskeletal regulation, tumor suppression, immune regulation, and angiogenesis. Enough information exists for many of these markers to legitimate further research into their functional significance and prognostic value.

Current efforts are to integrate molecular information from tissue microarrays into this system to generate a Molecular Integrated Staging System. Currently, the following targets have been examined in RCC: CA IX (49), CA XI, gelsolin (50), PTEN, EpCAM (51), CD10, p53, sodium potassium ATPase subunits, Vimentin, and Ki-67 (50). With the recognition that single-gene analysis has thus far provided only limited information and that predicting prognosis based on the immunohistochemical results of a single molecular marker has proved inadequate, clustering models are being generated to combine multiple markers into unique patient groupings. With further refinement of the use of these markers as well as development of more objective grading criteria, these histological parameters may eventually replace TNM staging as the definitive prognostic indicators for RCC. At the very least, they will serve as extremely useful, specific tools that, when used in conjunction with the TNM system, will provide crucial information about both the treatment and prognosis of RCC. Although the integration of molecular markers with traditional staging has not yet been accomplished, statistical methods of integrating pathological information on the anatomical extent of the tumor with other important clinical and pathological variables capable of improving the prediction of outcome probability in an individual patient and stratifying patients into prognostic categories with greater powers of discrimination is already underway.

We have proposed one such molecular marker, CA IX. CA IX protein, a member of the carbonic anhydrase family, is thought to play a role in the regulation of cell proliferation in response to hypoxic conditions and may be involved in oncogenesis and tumor progression (52, 53). The original designation, G250, referred to a mAb that was raised more than 10 years ago by immunization of mice with human RCC homogenates (54). The RCC-associated transmembrane protein designated G250 has since proven to be identical to MN/CA IX, a tumor-associated antigen originally identified in HeLa cells and expressed in cervical cancer (55). Previous studies using a mAb against CA IX have shown that CA IX is induced constitutively in certain tumor types but is absent in most normal tissues, with the exception of epithelial cells of the gastric mucosa (54, 56). Furthermore, previous immunohistochemical studies of malignant and benign renal tissues revealed that CA IX was also highly expressed in RCC, suggesting that CA IX expression may be a useful diagnostic biomarker (57). Clinical trials with radiolabeled mAb G250 in patients with RCC have demonstrated selective and specific delivery of mAb to renal cancer sites with both primary and metastatic RCC deposits being capable of being targeted and imaged (58, 59). However, the relationship between CA IX expression and RCC survivorship is unknown. Using the clinical and data resources of the UCLA Kidney Cancer Program, we investigated whether CA IX is associated
with progression and survival (49). Immunohistochemical analysis using a CA IX mAb was performed on tissue microarrays from patients treated by nephrectomy for clear cell RCC. CA IX staining was correlated with response to treatment, clinical factors, pathological features, and survival. CA IX staining was present in 94% of clear cell RCCs. Survival tree analysis determined that a cutoff of 85% CA IX staining provided the most accurate prediction of survival. Low CA IX staining was an independent poor prognostic factor for survival for patients with metastatic RCC, with a hazard ratio of 3.10 (P < 0.001). CA IX significantly stratified patients with metastatic disease when analyzed by T stage, Fuhrman grade, nodal involvement, and performance status (P < 0.001, P = 0.001, P = 0.009, and P = 0.005, respectively). For patients with nonmetastatic RCC and at high risk for progression, low CA IX predicted a worse outcome similar to patients with metastatic disease (P = 0.058). Overall expression of CA IX decreased with development of metastasis, as demonstrated by the lower CA IX staining levels in metastatic lesions relative to matched primary tumor specimens (P = 0.036). All complete responders to IL-2 IMT (8%) included patients with high CA IX (>85%) staining. On the basis of these data, CA IX seems to be the most significant molecular marker described in kidney cancer, to date. Decreased CA IX levels are independently associated with poor survival in advanced RCC. Significantly, studies by Ivanov et al. (60) showed a direct correlation between CA IX (G250) expression and loss of functional VHL gene product, postulated to be the cause of near universal CA IX expression in clear cell RCC. However, CA IX is expressed by tumors in many organ sites as a function of tumor hypoxia (see below), including breast (61), head and neck (62) non-small cell lung (63), and cervix (64). Thus, it is not clear whether the expression of CA IX in RCC is a function of VHL gene mutation, tumor hypoxia, or a combination of the two. However, CA IX clearly reflects significant changes in tumor biology, which may be useful to predict clinical outcome and identify HR patients in need for adjuvant IMT and CA IX-targeted therapies.

Targeting the Hypoxia-induced Pathway. Historically, tumor hypoxia, or the insufficient supply of oxygen to tissues to maintain basic biological function (65), was considered in the seminal work of Thomlinson (66), Gray et al. (67), and others to be a therapeutic problem, when it was realized that hypoxia renders solid tumors more resistant to the effects of radiotherapy. Since the 1990s, evidence has continued to accumulate demonstrating that hypoxia is also a common consequence of the rapid growth of many solid tumors, including tumors such as kidney cancer (65, 68). Studies of renal cell biology also provide evidence that hypoxia is an important regulator of a network of gene expression, with the ability to both stimulate and inhibit individual genes and to effect their expression on both posttranscriptional and posttranslational levels (65). In 1991, Lorentz et al. (69) reported that loss of blood was correlated with a response of increased erythropoietin concentration in blood. The promoter of the erythropoietin gene was studied, where the so-called HRE was discovered. HRE was responsive to the binding of a DNA-binding protein, HIF-1. HIF-1 is a heterodimer of HIF-1α and HIF-1β. HIF-1β is constitutively expressed, whereas the intracellular concentration of HIF-1α is controlled at the biosynthesis level as well as the posttranslational level. Regulation of HIF-1α plays an important role of the entire hypoxia-induced pathway (reviewed in Refs. 70 and 71).

Biosynthesis of HIF-1α is induced by growth factors through the PI3K-AKT-mTOR signal transduction pathway (Fig. 2). For example, in breast cancer, HER2 signaling induced by overexpression in mouse 3T3 cells or heregulin stimulation of human MCF-7 breast cancer cells results in increased HIF-1α protein and VEGF mRNA expression that is dependent on activity of PI3K, AKT (also known as protein kinase B), and the downstream kinase mTOR (mammalian target of rapamycin, or FRAP, FKBP-rapamycin-associated protein; Ref. 72). It is known that increased PI3K and AKT activity stimulated by other growth factors through their associated receptors, such as sarcoma inducible gene of Rous sarcoma virus, insulin-like growth factor, and epidermal growth factor, or resulting from a defective PTEN tumor suppressor gene, which is frequently observed in a number of human tumors, also increases HIF-1α expression through this signal transduction pathway (73).

In addition to the regulation of HIF-1α at the biosynthesis stage, the intracellular level of HIF-1α is significantly controlled at the posttranslational stage by hypoxia through the VHL suppressor protein (reviewed in Refs. 70 and 71). Under normoxic conditions, HIF-1α is hydroxylated at two proline residues, 402 and 564, as well as one asparagine residue, 803. The prolyl hydroxylated form of HIF-1α binds to VHL, the recognition component of an E3 ubiquitin ligase, which leads to ubiquitination and rapid degradation of HIF-1α. Therefore, HIF-1α has a very short half-life and is usually undetectable under normoxic conditions. In addition, the asparagine-hydroxylated form of HIF-1α is unable to initiate HIF transcription function, which normally leads to a cascade of subsequent transcriptional events. In contrast, under hypoxic conditions, the unhydroxylated form of HIF-1α does not bind to VHL and, thus, is not subject to degradation by the ubiquitin-proteasome pathway. Therefore, HIF-1α overexpression is commonly observed in cancerous cells and their metastases but rarely in normal tissues (74). In addition to hypoxia, recent evidence has indicated that the mutation, deletion, or hypermethylation of the VHL suppressor gene gives rise to defective ubiquitination of HIF subunits and, hence, causes the intracellular accumulation of HIF-1α, even in the absence of hypoxia (75, 76). VHL mutation or gene loss occurs in >50–80% of sporadic clear cell RCCs, suggesting a potential VHL-HIF tumorigenetic pathway for clear cell RCC (21, 77). Recent evidence has demonstrated that competitive inhibition of the VHL substrate recognition site with a peptide derived from the HIF-1α protein recapitulates the tumorigenic phenotype of VHL-deficient cancer cells, providing additional evidence of a possible link between VHL inactivation and elevated HIF-1α (77). Kondo et al. (78) recently published evidence that HIF is a critical downstream target of VHL and is important for its tumor-suppressive activity, by showing that VHL tumor suppression can be overridden by a HIF variant that escapes VHL control.

HIF-1 is a central transcriptional factor that sits at a key junction in the hypoxia pathway, through which hypoxia regulates the expression of a battery of genes, and the products of which are critical components of tumor angiogenesis (e.g.,
VEGF), glucose transport (e.g., glut1, glut 3), glycolysis (e.g., 6-phosphofructose 2-kinase), pH control (e.g., the carbonic anhydrase family), epithelial proliferation (e.g., transforming growth factor-α/EGF) and epidermal growth factor (EGF) receptors stimulate the PI3K-AKT-mTOR pathway. mTOR also senses intracellular nutrients such as amino acid and ATP. These pathways lead to the phosphorylation of S6K1 and 4E-BP1, activating the former and inactivating the latter. S6K1 and 4E-BP1 are critical components of the general translation machinery, which leads to the translation of HIF-1α protein. This pathway is negatively regulated by the tumor suppressor PTEN targeting AKT and by rapamycin analogue CCI-977 targeting mTOR. The hypoxia-induced pathway is linked to the VHL loss in RCC. In addition to hypoxia, the status of the VHL gene contributes directly to the posttranslational regulation of HIF-1α. The intracellular level of HIF protein plays an important role in regulating the expression of an array of genes that encode proteins essential to cancer cell functions under hypoxia conditions, such as glucose transport, angiogenesis, glycolysis, and pH control. The effects of these proteins on hypoxic tumor cells are indicated. Physiological conditions affecting the pathway are italic. Molecular targets currently exploited for therapeutic intervention are bold.

Fig. 2 Regulation of HIF-1α and points of drug intervention currently being exploited. Activation of receptor tyrosine kinases (RTK), such as SRC and the her2, and insulin-like growth factor (IGF) and epidermal growth factor (EGF) receptors stimulate the PI3K-AKT-mTOR pathway. mTOR also senses intracellular nutrients such as amino acid and ATP. These pathways lead to the phosphorylation of S6K1 and 4E-BP1, activating the former and inactivating the latter. S6K1 and 4E-BP1 are critical components of the general translation machinery, which leads to the translation of HIF-1α protein. This pathway is negatively regulated by the tumor suppressor PTEN targeting AKT and by rapamycin analogue CCI-977 targeting mTOR. The hypoxia-induced pathway is linked to the VHL loss in RCC. In addition to hypoxia, the status of the VHL gene contributes directly to the posttranslational regulation of HIF-1α. The intracellular level of HIF protein plays an important role in regulating the expression of an array of genes that encode proteins essential to cancer cell functions under hypoxia conditions, such as glucose transport, angiogenesis, glycolysis, and pH control. The effects of these proteins on hypoxic tumor cells are indicated. Physiological conditions affecting the pathway are italic. Molecular targets currently exploited for therapeutic intervention are bold.

Among the genes that are under the control of HIF-1, CA IX is particularly important for RCC, having a potential role in RCC diagnosis, determination of prognosis, and as a therapeutic target (79). CA IX is a transmembrane enzyme that contains carbonic anhydrase activity and is capable of catalyzing the reversible hydration of CO2 to form HCO3- and H+. It has been proposed that CA IX may allow tumors to accommodate to an acidic and hypoxic environment, thereby allowing cancer cells to further proliferate and metastasize. Since CA IX and other genes of the hypoxia-induced pathway play essential roles in tumor metabolism under the hypoxic tumor environment, gene products of the hypoxia-induced pathway may contribute to the overall resistance to chemotherapy and radiation therapy, the increased aggressiveness of hypoxic tumor, as well as tumor-related mortality. As described above, CA IX seems to be one of the most significant molecular markers described in kidney cancer, to date. Decreased CA IX levels are independently associated with poor survival in advanced RCC. In addition to RCC, CA IX has been found in cervical carcinoma, lung cancer, and breast cancer as an independent prognostic marker, in which increased expression is correlated with increased mortality (61, 64, 81). Other genes of the pathway have also been studied with regard to cancer prognosis. As described previously, VHL alteration status has been shown to be strongly associated with better cancer-free survival and cancer-specific survival for patients with stage I-III clear cell RCC treated with radical nephrectomy (31). In a study of epithelial ovarian cancers, HIF-1α overexpression was correlated with microvessel density. HIF-1α overexpression was also associated with an increased apoptotic rate in the majority of the ovarian cancer cases involved in the study, especially in low malignant potential tumors. Moreover, the combination of p53 mutation and HIF-1α accumulation was associated with a highly significant increase in the risk of patient mortality (82). These studies suggest that gene products of the hypoxia-induced pathway may be critical prognostic factors that...
may aid in outcome prediction and novel therapies to improve survival of cancer patients.

**Exploiting the Hypoxia-induced Pathway for Molecular Therapeutic Intervention of RCC.** On one hand, tumor cells that have adapted to a hypoxic environment are more aggressive in growth and are resistant to radiation and chemotherapy, complicating the treatment of solid tumors; in contrast, hypoxia represents a clear difference between tumors and normal tissues and, therefore, is potentially exploitable as a target in cancer treatment. The hypoxia pathway has many steps that may serve as unique targets for cancer therapy (Fig. 2). A number of agents directed against molecular targets in the hypoxia pathway are in various stages of development and clinical testing. One general strategy is to slow down tumor growth and adaptation to hypoxia by inhibiting the signal transduction pathway of PI3K-AKT-mTOR. For example, rapamycin and rapamycin analogues such as CCI-779 (Wyeth, Madison, NJ) can target the protein kinase mTOR and inhibit its activation of substrates such as S6 kinase and 4E-BP1 by phosphorylation. The inhibition of S6 kinase and 4E-BP1, both critical components of the mRNA translation machinery, leads to arrest in tumor growth and inhibition of HIF-1α synthesis (83); thus, CCI-779 is currently being investigated in clinical Phase I and II trials as a drug in a number of cancers, and antitumor responses and/or stable disease have been noted in patients with several drug-refractory cancers, including RCC. Mutations in the PTEN tumor suppressor gene have also correlated with increased S6 kinase activity and phosphorylation of ribosomal S6 protein, providing evidence for activation of the FRAP-mTOR pathway in these cells. In accordance, PTEN mutation makes tumors dramatically more responsive to treatment with CCI-779 than tumors with normal PTEN (84). PTEN is mutated in more than half of human glioblastoma and endometrial cancers (85) and in 20% of metastatic prostate cancers (86). On the basis of the preclinical results, patients with PTEN mutations are expected to respond better to the drug than those without. At UCLA, a tissue array study is under way to determine the frequency of PTEN mutations in RCC and to analyze other related hypoxia pathway molecules to better evaluate the potential of CCI-779 as a potential targeted therapy for RCC.

RCC has been linked genetically to the hypoxia-induced pathway through VHL inactivation, and, therefore, RCC may represent one of the best clinical models for directed therapies based on an understanding of the hypoxia-induced pathway. Both VHL-deficient mice and VEGF-knockout mice die in utero because of defective vasculogenesis (87, 88), a shared phenotype that might be explained by the fact that the VHL protein is linked tightly to angiogenesis via up-regulation of VEGF regulation through the action of HIF. Recently, a Phase II clinical trial to evaluate the activity of bevacizumab, a neutralizing antibody to VEGF, was performed in patients with metastatic RCC (89). With 116 patients randomized (40 to placebo, 37 to low-dose antibody, and 39 to high-dose antibody), there was a significant prolongation of time-to-progression in patients receiving high-dose antibody versus placebo (hazard ratio, 2.55; \( P = 0.0002 \)) and a small difference of borderline significance between low-dose antibody and placebo (hazard ratio, 1.26; \( P = 0.053 \)). The probabilities of being progression free for patients given high-dose antibody, low-dose antibody, or placebo were 64, 39, and 20% at 4 months and 30, 14, and 5% at 8 months, respectively. There were four partial responses, all to high-dose antibody (response rate, 10%; 95% confidence interval, 2.9–24.2%). At last analysis, there were no significant differences in overall survival between arms (all \( P > 0.20 \)). Another agent targeting HIF downstream targets is SU 11248 (Sugen, San Francisco, CA), a p.o. bioavailable indolinelone that works as a signal transduction inhibitor of several enzymes, including VEGF receptor, platelet-derived growth factor receptor, and c-kit tyrosine kinase. Phase I studies have shown activity in a range of tumors, including gastrointestinal and renal tumors that had not responded to IMT. A Phase II study of SU 11248 in RCC is set to begin. YC-1, a benzylindazole developed for circulatory disorders and that can inhibit platelet aggregation and vascular contraction, is another potential antiangiogenic anticancer agent that is unique in that it can directly inhibit HIF-1 activity in vitro through blockade of HIF-1α expression at the posttranscriptional level (90). YC-1 has been shown to decrease the growth of Caki-1 renal carcinoma xenografts in immunodeficient mice (90). One final agent with the potential to target HIF is PS-341 (Millenium, Cambridge, MA), a dipeptide boronic acid derivative that inhibits the proteasome by stabilization of its active site and by reversible inhibition of its chymotripsin-like activity. The ubiquitin-proteasome pathway plays an essential role in the degradation of many intracellular proteins, including HIF (91), and early phase clinical trials have suggested RCC to be one potential cancer type that may respond to proteasome inhibition. It is unclear at this time whether the relevant target of proteasome inhibition for RCC involves HIF, or one of the other myriads of proteins degraded by the proteasome, such as p53 or NFKB (91). Gene therapy approaches, expressing genes under the control of the HRE promoter (92) or the SP1/SP3 and HRE in the CA IX promoter (93), are still in their infancy.

Historically, IL-2-mediated therapy has been shown to play an important role in treating RCC. Currently, several tumor cell- and dendritic cell-based vaccine trials are in Phase I and Phase II clinical stages (94–96). In addition, nonmyeloablative stem cell transplantation has also showed some encouraging efficacy in HLA-matched donors, although the toxicity caused by graft versus host disease was severe (97). To date, targeted IMT has been tried rarely in the clinic. It is, therefore, conceivable that cellular-immune therapy targeting the hypoxia-induced pathway may also be exploitable in addition to blocking the hypoxia pathway by chemical and biological agents. Because of its critical role in RCC biology, CA IX has been exploited as a potential target for IMT. As noted above, CA IX staining has been found predominantly on the plasma membrane. Studies have reported that CA IX was present in >80% of primary and metastatic tumors and absent or minimally expressed in normal tissues. In addition, CA IX-derived CTL and CD4+ T-cell epitopes have been described that are immunogenic and can induce CA IX-specific T cells in vitro (98, 99). Therefore, CA IX represents an attractive candidate for vaccine development. Because none of the immunogenic CTL epitopes from CA IX have been confirmed to be present on RCC cell surface, vaccines encoding the full-length CA IX gene instead of individual epitope are being developed. To enhance the immunogenicity of CA IX protein, GM-CSF was fused with CA IX (100).
A purified recombinant FP as well as an adenovirus encoding the FP was tested for their in vitro immunogenicity. GM-CSF-CA IX FP was found to be effective in promoting the maturation of dendritic cell and HLA expression. The cytotoxic activity of PBMCs stimulated weekly with various combinations of cytokines with and without FP was compared using primary and metastatic tumors as well as normal kidney as targets. The addition of the FP containing the CA IX protein into PBMC significantly enhanced the antitumor activity against primary and metastatic tumor. A series of blocking experiments using antibodies directed against HLA molecules suggested that FP-induced cytolysis was HLA class I restricted. Similar data of inducing CTL responses in vitro was also obtained by stimulation of PBMC using adenovirus encoding the FP.3

The immune modulator effect of the FP was examined in severe combined immunodeficient mice (101). Both mock- and fusion gene-transfected R11 (CA IX-negative RCC line) showed similar in vitro growth activity, however, the growth of the tumor resulting from s.c. injection of fusion gene-transfected R11 was significantly retarded compared with mock-transfected R11 in severe combined immunodeficient mice. After depleting of the natural killer population with ASGM1, the inhibitory effect of GM-CSF-CA IX was reduced, suggesting that GM-CSF-CA IX effects were mediated in part by natural killer/macrophage activities. In addition, an influx of macrophages/monocytes was detected in the slow growing tumors formed by fusion gene-transfected R11 but not mock-transfected R11. These observations suggested that the fusion gene-transfected RCC line could induce immune modulation even in mice lacking T cells and was capable of producing a significant antitumor immunity. Because of the critical role of CA IX in tumor metabolism and predicting RCC progress, vaccines targeting CA IX may represent a widely applicable IMT approach that will benefit the treatment of RCC.

Conclusions

The last 10 years have witnessed the gradual transition from the use of solitary clinical factors as prognostic markers for patients with RCC, to the introduction of systems that integrate and synthesize multiple factors simultaneously, and to the beginning of the use of molecular and genetic markers. These markers will eventually enhance our ability to predict the behavior of an individual tumor and to stratify patients into more sophisticated risk categories and will ultimately permit the goal of moving from nonspecific treatments to designing and targeting therapies specifically for targeted populations of patients. These advances will culminate in a better understanding of the causes of renal cancer, its prevention, and, finally, its cure. Here, we considered the molecular mechanisms of the hypoxia-induced pathway, which plays an essential role in angiogenesis, glycolysis, and apoptosis. The hypoxia-induced pathway is intrinsically linked to RCC through the VHL tumor suppressor gene, which is inactivated in both familial and sporadic clear cell RCC, and may serve as the source of new rational treatment strategies based on the design of small molecule inhibitors, as well as vaccine, gene, and antibody therapies directed against targets such as VHL, HIF-1α, CA IX, and VEGF.

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References


3 H. Mukoyama, unpublished observations.


