Protein Expression Profiles in Renal Cell Carcinoma: Staging, Prognosis, and Patient Selection for Clinical Trials

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Abstract

Attempts to predict survival in patients with renal cell carcinoma (RCC) have traditionally relied on standard clinical variables, such as tumor-node-metastasis stage, histologic grade, and performance status. An accurate method for predicting patient survival is useful for patient counseling, planning follow-up, and selecting patients most likely to benefit from novel and established therapies. Furthermore, an improved prognostic system will allow for more accurate comparisons of clinical trials based on varying inclusion criteria. A large number of potential prognostic markers have recently been identified from methods based on gene arrays, which screen for differential expression of thousands of genes. The accepted method of clinical validation of novel markers is on formalin-fixed and paraffin-embedded specimens using immunohistochemistry. The development of tissue microarrays as a high-throughput technique has allowed for thousands of different cores of pathologic tissue to be assessed simultaneously in a timely and cost-efficient manner. This technology has enabled the analysis of protein expression profiles on specimens to determine their potential clinical significance and role in RCC biology. This article reviews the protein expression profiles in RCC and their association with pathobiology, prognosis, and response to treatment as well as their role in serving as potential molecular targets for therapy of RCC.

Recent advances in the understanding of the pathogenesis, behavior, and molecular biology of renal cell carcinoma (RCC) have paved the way for developments that may enhance early diagnosis, improve prognostication, and prolong survival. Reliable predictive factors are essential for the stratification of patients into clinically useful risk groups to provide patients with counseling regarding prognosis, select treatment modalities, and determine eligibility for clinical trials. The tumor-node-metastasis staging system has been the most extensively used staging system for RCC. New comprehensive staging modalities have since emerged in an attempt to improve prognostication by combining other pathologic and clinical variables (1). The recent identification and potential incorporation of molecular tumor markers into current staging systems are expected to revolutionize the staging of RCC (2). In addition to the use of molecular markers in the diagnosis and prognostication of RCC, they will be expected to play an important role in the selection of patients for emerging targeted therapies and a more accurate comparison of clinical trials based on varying inclusion criteria.

Molecular Profiling in RCC

Methods based on gene arrays, which screen for differential expression of thousands of genes, have identified large numbers of new, potentially important prognostic markers (3–7). The evaluation of protein expression in a high-throughput tissue microarray is a natural extension to the efforts for molecular staging. The potential of microarray technology in clinical research is enormous. This technology can be used for cancer diagnosis; identification of diagnostic markers through screening and comparing gene and/or protein expression profiles from normal, premalignant, and malignant tissues from the same organ; and the identification of gene and/or protein sets associated with metastasis or response to treatment. Moreover, it may now be possible to predict clinical outcome based on gene and/or protein expression patterns (3, 4, 6, 8–10). Furthermore, classification of patients into high-risk and low-risk subgroups based on a prognosis profile may be a useful means of guiding adjuvant therapy in patients (11). This approach should improve the selection of patients who would benefit from adjuvant systemic treatment, reducing the rate of both overtreatment and undertreatment. It may be also possible to predict which patients will benefit from extirpative surgical procedures. Finally, gene and/or protein expression signatures may be used to predict the clinical response to both conventional and targeted therapies.

The tissue microarray is an ordered collection of small tissue cores arranged in a single paraffin block, from which multiple sections can be cut and evaluated allowing for high-throughput data acquisition. Tissue microarray sections can be used for all
types of in situ analyses, including immunohistochemistry, RNA in situ hybridization, or fluorescence in situ hybridization. The most important prerequisite is the availability of a large collection of well-characterized tissues ideally with attached clinical data. Tissue microarray construction involves extracting cores of tissue, which vary in diameter (0.6-3.0 mm), from different donor paraffin blocks and inserting them in an organized manner into a blank recipient block. The range of tissue microarray applications is broad. Prevalence tissue microarrays contain tumor samples from one or several tumor entities without further clinicopathologic information and can be used to study the epidemiology of molecular features in tumor entities of interest. Progression tissue microarrays contain tissue samples at different stages of one particular disease process. Prognosis tissue microarrays contain tissue samples taken from patients with known clinical outcomes. Other tissue microarrays include those used in an experimental or a preclinical drug development context that contain cell lines or xenograft tissue. A potential limitation of tissue microarrays is that the small cores sampled may not be representative of whole tumors, particularly in heterogeneous cancers. This issue has been critically evaluated in numerous validation studies comparing tissue microarrays with whole mount sections in a variety of malignancies, with the vast majority of these studies revealing a high level of concordance between tissue microarrays and standard histopathologic techniques (12–27). In addition, these studies have shown that two or three samples provided more representative information compared with a single sample (20, 21, 25–27), and that the addition of more than four or five samples would not lead to a large improvement of the concordance level (20, 25).

Prognostic models based on protein expression profiles using high-throughput tissue microarray technology representing all stages of localized and metastatic clear cell RCC have been shown to perform better than standard clinical predictors (28, 29). On a custom tissue microarray, immunohistochemical analysis of Ki-67, p53, gelsolin, carbonic anhydrase IX (CA-IX), CA-XII, phosphatase and tensin homologue deleted from chromosome 10 (PTEN), epithelial cell adhesion molecule, and vimentin was done (28). For each molecular marker, the optimal cutoff for the staining scores to stratify disease-specific survival was determined using the default settings of the recursive partitioning function (RPART) in the freely available R statistical software (30, 31). Two prognostic nomogram models to predict survival after nephrectomy were created Cox proportional hazards regression analysis and calibrated. One was primarily based on using molecular markers (marker model), and the other one was based on using a combination of clinical variables and marker data (clinical/marker model). These models were shown to be accurate to within 10% of the actual 2- and 4-year disease-specific survival rates using bootstrap bias–corrected estimates. The predictive ability of the various models was quantified by calculating the concordance index (30), which showed that prognostic systems based on protein expression profiles for clear cell RCC did better than standard clinical predictors, such as tumor-node-metastasis stage, histologic grade, and performance status. The marker model included metastasis status, p53, CA-IX, gelsolin, and vimentin as predictors and had high discriminatory power (concordance index = 0.75; ref. 28). Furthermore, the clinical/marker model that included metastasis status, T stage, performance status, p53, CA-IX, and vimentin as predictors had a concordance index of 0.79, which was significantly higher (P < 0.05) than that of prognostic models based on grade alone (concordance index = 0.65), tumor-node-metastasis stage alone (concordance index = 0.73), or the University of California-Los Angeles integrated staging system (concordance index = 0.76; ref. 28). Although these prognostic nomograms are useful for visualizing predictive models, validation with independent patient populations is needed before being applied to patient care.

Defining Histologic and Molecular Correlates for Patient Selection in Clinical Trials for RCC

**Hypoxia-inducible pathway.** The molecular mechanisms of the hypoxia-inducible pathway play an essential role in angiogenesis, glucose transport, glycolysis, pH control, epithelial proliferation, and apoptosis of common cancers and may be responsible for the ability of cancers to adapt to hypoxic environment and their resistance to radiation and chemotherapy (32, 33). Hypoxia-inducible factor-1 (HIF-1) is a heterodimer of HIF-1α and HIF-1β. HIF-1α is constitutively expressed, whereas the intracellular concentration of HIF-1α is controlled at the biosynthesis level and the posttranslational level. Biosynthesis of HIF-1α is induced by growth factors through the phosphatidylinositol 3-kinase/protein kinase B/mammalian target of rapamycin (PI3K/AKT/mTOR) signal transduction pathway (33). In addition to the regulation of HIF-1α at the biosynthesis level, HIF-1α and the related protein HIF-2α are controlled at the posttranslational level by hypoxia through the von Hippel-Lindau (VHL) suppressor protein (34). Under normoxic conditions, HIF-1α and HIF-2α are hydroxylated and bound to VHL, which leads to ubiquitination and rapid degradation of HIF-α. In contrast, under hypoxic conditions, the unhydroxylated forms of HIF-1α and HIF-2α do not bind to VHL and thus are not subject to degradation by the ubiquitin-proteasome pathway. Mutation, deletion, or hypermethylation of the VHL suppressor gene also gives rise to defective ubiquitination of HIF subunits and causes the intracellular accumulation of HIF-1α and HIF-2α even in the absence of hypoxia (34, 35). VHL mutation or gene loss occurs in >50% of sporadic clear cell RCC, suggesting a potential VHL-HIF tumorigenic pathway for clear cell RCC (36). Up-regulation of HIF-α is a common feature in VHL gene alterations in clear cell RCC.

Among the genes that are under the control of HIF, CA-IX is particularly important for RCC (37). Immunohistochemical analysis done on tissue microarrays from patients treated by nephrectomy at the University of California-Los Angeles for RCC showed that CA-IX staining was present in 94% of clear cell RCC (8). Furthermore, survival tree analysis determined that a cutoff of 85% CA-IX staining provided the most accurate prediction of survival, and low CA-IX staining was an independent poor prognostic factor for survival in patients with metastatic RCC (hazard ratio, 3.10; P < 0.001; ref. 8). Another study has evaluated the role of CA-IX and Ki-67 independently and as a combined variable for predicting survival in patients with RCC (9). High Ki-67 staining and
low CA-IX staining correlated significantly with poor median survival (21 months, \( P < 0.001 \) and 22 months, \( P = 0.011 \), respectively). Furthermore, when Ki-67 and CA-IX were combined into a single variable, RCC tumors could be stratified into low-risk, intermediate-risk, and high-risk groups with a median survival of >101, 31, and 9 months, respectively \( (P < 0.001) \). In addition, the combined variable consisting of Ki-67 and CA-IX was an independent predictor of survival \( (P < 0.001) \) and was able to displace histologic grade. In addition, two studies have reported that patients with high CA-IX (>85%) expression of the primary RCC tumor were more likely to respond to high-dose interleukin-2 immunotherapy \( (8, 36) \). Although these findings are intriguing and have important prognostic implications for improving the selection of patients with metastatic RCC best suited for immunotherapy, this test still remains investigational and is currently used only in the context of clinical trials.

A revolutionary advance contributing to an improved understanding of pathways underlying RCC has been recognition of the vascular endothelial growth factor (VEGF) and VEGF receptor (VEGFR) tyrosine kinase family that comprise distinct pathways signaling angiogenesis and/or lymphangiogenesis. Clinical activity has been reported in RCC using any one of several agents that target VEGF (bevacizumab) or its receptor VEGFR (sunitinib, sorafenib; ref. 39). Understanding the expression of these markers will individualize the selection of target-specific therapies based on the tumor biology and optimize the benefit of agents that target these pathways in RCC.

Immunohistochemical analysis has been done with antibodies directed against VEGF-A, VEGF-C, VEGF-D, VEGFR-1, VEGFR-2, and VEGFR-3 on tissue microarrays constructed from paraffin-embedded clear cell and papillary RCC nephrectomy specimens in a study at the University of California-Los Angeles to evaluate the role of the VEGF-VEGFR family in different histologic subtypes of RCC. Protein expression was analyzed within the tumor epithelium and in tumor-associated endothelium \( (40) \). This data may shed light on whether other signaling pathways aside from VHL may be promoting VEGF production, and if so, consideration should be made for the inclusion of patients with histologic non–clear cell subtypes for therapies that target the VEGF ligands and/or their receptor tyrosine kinases. In addition, survival and metastatic pattern according to expression of VEGF-VEGFR family in clear cell RCC has also been evaluated. Analysis of these markers may allow for insight in predicting sites of metastatic spread and providing additional markers for determining disease-specific survival \( (41) \).

**mTOR Pathway**

The mTOR pathway is up-regulated in many human cancers in response to a diverse set of stimuli, including various growth factors and certain nutrients and amino acids. At the cell membrane, ligand-mediated activation of receptor tyrosine kinases recruits PI3K, a lipid kinase, to the cell membrane. PI3K converts phosphatidylinositol bisphosphate to phosphatidylinositol 3,4,5-triphosphate, which further recruits phosphoinositide-dependent kinase 1 and AKT to the membrane where phosphoinositide-dependent kinase 1 activates AKT \( (42) \). Once activated, AKT phosphorylates a myriad of proteins with the net result of increased cellular survival, proliferation, growth, and metabolism. One downstream target of AKT phosphorylation is mTOR, a process that occurs through inactivation of the tuberous sclerosis complex (TSC) \( (43–45) \). mTOR is therefore strategically placed at the intersection of multiple growth factor (e.g., insulin, insulin-like growth factor, and platelet-derived growth factor) and nutrient (e.g., amino acids) signaling networks, allowing it to function as a gatekeeper, regulating cell growth, metabolism, and proliferation. Preclinical data have suggested that agents that target the mTOR pathway may have greater activity in tumors that lack the PTEN tumor suppressor \( (23–28) \). Tissue microarrays containing nephrectomy specimens from patients with RCC at the University of California-Los Angeles were used to evaluate the potential and limitations of targeting the mTOR pathway in kidney cancer \( (46) \). Immunohistochemistry was done with antibodies directed against PTEN, phosphorylated AKT, phosphorylated S6 kinase, p27, and HIF-1α. Loss of PTEN was increased in all RCC compared with normal tissue, with the greatest loss occurring in clear cell RCC \( (25%) \) and tumors with sarcomatoid features \( (34%) \). Because PTEN loss can occur as a consequence of DNA mutation, transcriptional silencing of mRNA, or protein instability, these estimates may be low. Phosphorylated AKT staining frequency was greatest in collecting duct \( (89%) \) followed by clear cell \( (58%) \), suggesting activation of pathways other than PTEN loss. Phosphorylated S6 kinase was highly expressed by tumors with sarcomatoid features \( (61%) \) and clear cell tumors \( (41%) \) and was expressed more in high-grade \( (73% \) versus 31%) and high-stage \( (50% \) versus 30%) tumors. PTEN expression was found to correlate with phosphorylated AKT \( (P = 0.028) \) and HIF-1α expression \( (P < 0.0001) \). These results suggest that not all RCC tumor types are equally amenable to treatment strategies that target the mTOR pathway, but most patients have at least one component of the mTOR pathway affected \( (PTEN, phosphorylated\ AKT, and HIF-1α) \).

Preclinical data have suggested two parallel tumor suppressor pathways that depend on the translational rheostat, mTOR. Although inhibitors of mTOR (rapamycin, CCI-779) have antiangiogenic properties \( (47) \), findings from isogenic human kidney cancer cell lines and xenografts predict that mTOR inhibitors should be active in VHL-deficient RCC due to subsequent up-regulation of the HIF pathway through stabilization of HIF-1α \( (48) \). These findings have important implications as VHL status could serve as a biomarker for subject selection and provides a rationale for exploring combination therapy using inhibitors of mTOR and the VEGF-VEGFR pathway in RCC to block the effects of the activation of the HIF pathway. In addition, studies in prostate and brain tumors have shown that tumors that lack PTEN would make the tumors more mTOR dependent and be particularly sensitive to the growth inhibitory effects of agents that target mTOR \( (49–51) \). Thus, clear cell RCC may present the best opportunity to maximize enrollment of potentially susceptible patients from both of these tumor suppressor pathways.

Phosphorylated S6 kinase is also of particular interest because it may serve as a surrogate for mTOR activity. After receiving proliferative upstream signal mediated by the PI3K/AKT pathway, mTOR phosphorylates and activates S6 kinase. In turn, S6 kinase phosphorylates and activates the 40S ribosomal S6 protein, facilitating the recruitment of the 40S ribosomal subunit into actively translating polysomes, in particular enhancing mRNA translation with the 5′TOP sequence \( (52) \). Regulation of the S6 kinase is more complicated than simple
activation through mTOR because activation also seems to occur through a number of mTOR-independent pathways, such as phosphoinositide-dependent kinase 1, which phosphorylates AKT at the cell membrane, and also directly phosphorylates S6 kinase in an AKT-independent manner. In addition, mTOR may indirectly influence S6 kinase activity by repressing a serine-threonine phosphatase that dephosphorylates rapamycin-sensitive sites on S6 kinase. Regardless of the precise mechanism by which S6 kinase activation is perturbed in tumors, the fact remains that mutations in PTEN and TSC1/2 as well as amplification of the AKT gene all have a common downstream target, S6 (Fig. 1). Furthermore, mTOR regulates the translation of HIF through S6 (48).

p21 is a cell cycle and apoptosis regulating protein with multiple disparate functions in both normal and tumor cells. p21, originally described as an inhibitor of cell cycle progression through its attenuation of cyclin/cyclin-dependent kinase interaction, has since been shown to have additional functions associated with tumor progression and resistance to chemotherapy (55). The seemingly contradictory actions of the p21 protein may be in part dictated by its subcellular localization (e.g., cytoplasm or nucleus). For example, it has been shown that cytosolic-localized p21 is pro-proliferative in vascular smooth muscle cells (56), whereas its cyclin/cyclin-dependent kinase interaction occurs when p21 is in its “usual” intranuclear location (57). p21 is phosphorylated and stabilized by the downstream products of PI3K activation (58), and, as noted above, recent work has shown that PTEN-deficient tumors are sensitized to mTOR inhibition (49). Consequently, PTEN attenuation also results in increased levels of p21 (59). Immunohistochemical analysis of tissue microarrays containing nephrectomy specimens of clear cell, papillary, chromophobe, and collecting duct carcinomas has been done (60). Nuclear p21 staining was found to be highest in collecting duct carcinoma compared with all other RCC types and lowest in oncocytoma. Cytosolic p21 staining, conversely, was highest in oncocytoma and lowest in clear cell RCC. In addition, high cytosolic p21 expression in the primary tumor was an independent predictor of poor prognosis in patients with metastatic disease at the time of nephrectomy (hazard ratio, 1.02; 95% confidence interval, 1.01-1.04; P = 0.004), whereas high nuclear p21 expression in the primary tumor was associated with better prognosis in patients with localized disease at time of nephrectomy (P = 0.04). These data suggest that p21 may be a useful prognostic marker and may be useful for identifying selected patients for specific therapeutic approaches. In regard to the latter, a recent study on the mechanism of RAD001 (everolimus) induced sensitization of DNA-damaging chemotherapeutic agents showed that this effect is mediated by attenuation of p21 translation, which suggests that combination therapy with DNA-damaging agents and mTOR inhibitors may be a potential therapeutic regimen for selected patients with metastatic RCC (61).

![Fig. 1. Role of mTOR in the PI3K/AKT/PTEN pathway and targeting HIF. Growth factor receptor tyrosine kinases recruit PI3K, with subsequent activation of AKT. AKT phosphorylates and inactivates the TSC1/2 complex, resulting in constitutive activation of mTOR, which leads to the downstream activation of p70 S6 kinase (p70S6K) and the inactivation of the eukaryotic initiation factor 4E binding protein 1 (4E-BP1), ultimately driving translation of proteins that play a role in cellular growth and survival. Ras homologue-enriched in brain (Rheb) functions in this pathway downstream of TSC2 and upstream of mTOR. Inhibition of mTOR by rapamycin and its analogues CCI-779 or RAD001 results in down-regulation of these signals, including HIF-1. Loss of VHL, results in constitutive expression of HIF-1, with resultant up-regulation of genes encoding for cell cycle progression, invasion, metastasis, angiogenesis, pH regulation, and glucose metabolism. PI3K, phosphatidylinositol 3,4,5-trisphosphate; IGF-2, insulin-like growth factor 2; PDK1, phosphoinositide-dependent kinase 1; TNF-α, tumor necrosis factor-α.](image-url)

**Summary**

The last decade has witnessed the gradual transition from the use of clinical factors as prognostic markers for patients with RCC to the introduction of integrated staging systems that combine multiple factors to the beginning of molecular and genetic markers. Molecular markers will eventually enhance our ability to predict individual tumor behavior and to stratify patients into more sophisticated risk groups, ultimately permitting the goal of moving from nonspecific treatments to designing and targeting therapies for enriched patient populations. Novel agents that recognize defined molecular targets in the HIF and mTOR pathways are in clinical trials, and multitargeted receptor tyrosine kinase inhibitors have significant effects on response and progression. Pivotal clinical trials are under way with targeted agents to define the extent of clinical benefit. Future approaches will include combinations of targeted agents with cytokines, chemotherapies, and combinations of multiple targeted agents. These advances will culminate in a better understanding of the causes, prevention, and the successful treatment of RCC.

**Open Discussion**

Dr. Atkins: We’re getting much better at selecting who should get interleukin-2 (IL-2) therapy. Our response rates are 30% to 40%. Is that your experience or was this a select group of patients? Regarding CA9 selection, the benefit was not so much response rate but that all the long-term responders were in the high CA9 group.

Dr. Figlin: We are much better at selecting who gets therapy. Those of us who treat kidney cancer patients are removing from trials patients who are not going to benefit. The difference will be that complete responders having more
duration will require a different design and power of a clinical trial if that is the primary end point than if you are only trying to show a difference in response rate between expressers and nonexpressers. As we accumulate more data that suggest that complete responses are occurring in the population that overexpress CA9, then we have to ask how to design a trial in terms of numbers of patients.

Dr. Wood: The only issue I have with your study design is that it suggests that alteration of those molecular pathways somehow correlates with response and outcome. You seem to be suggesting that in an 8-week period you are going to be able to fully evaluate this drug. I would argue that even if you do not see a response by traditional criteria, it does not mean the drug is bad, and even if you do see it does not mean the drug is good.

Dr. Figlin: What I’m trying to find is a window of opportunity to predict how the biology as measured by molecular markers will be correlated with standard clinical criteria.

Dr. Wood: At least at this early stage, the trial needs follow-up to make those correlations. Retrospectively, CA9 is a great marker for response in IL-2, but prospectively, it is not so good. The markers that you have shown are validated internally in your own data set, but without external validation, I would have difficulty offering or denying someone therapy.

Dr. Kwon: The fact that their hypothesis is not yet supported with CA9 being a predictor of response to IL-2 does not negate its ability to be a prognostic marker for a high-risk population.

Dr. Rosman: Can you give us an idea of how the study is set up statistically to correlate the biologic effect and clinical benefit?

Dr. Figlin: The current iteration is the resubmission iteration. The first submission was a phase 1 trial evaluating the optimal biologic dose of TOR inhibition to produce clinical benefit. Between the first and second submissions, data arose out of the UCLA prostate group from a neoadjuvant trial in prostate cancer, showing that when you go from low levels of mTOR inhibition to 5 mg you optimally inhibit. As a result, we turned the trial from a phase 1 directly to a phase 2.

Dr. Atkins: We’re wrestling with our neoadjuvant trial, which is using sorafenib. We are struggling with whether to do this test in the adjuvant setting, where there are more patients and no approved therapy. Would you ever consider that with your trial?

Dr. Figlin: I would love to do it in the adjuvant setting, but the problem in the adjuvant setting is I would not have a clinical readout.

Dr. George: To me the biggest concern is how well the metastatic biopsy correlates with the primary biopsy and ultimately the clinical response, which is judged in the metastatic sites. Have you correlated primary tumors to metastatic tumors and how well do they correlate?

Dr. Figlin: In the few patients we have, it correlates very well. When you see the B7-H1 data and localized versus metastatic, you get a sense that what is happening in the mets is also happening in the primary tumor.

Dr. George: Selecting patients with synchronous metastasis is probably enriching for patients who have biology similar between metastatic and primary; therefore, this seems to be the right trial design.

References

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