Tissue Array-Based Predictions of Pathobiology, Prognosis, and Response to Treatment for Renal Cell Carcinoma Therapy

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ABSTRACT
Renal cell carcinoma is the most lethal of the common urologic malignancies, with ~40% of patients eventually dying of cancer progression. Approximately one third of patients present with metastatic disease, and up to 40% treated for localized disease have a recurrence. Historically, clinical factors have been used as prognostic markers for patients with renal cell carcinoma. Recent advances in the understanding of the pathogenesis, behavior, and molecular biology of renal cell carcinoma have paved the way for developments that may enhance early diagnosis, better predict tumor prognosis, and improve survival for renal cell carcinoma patients. Furthermore, reliable predictive factors are essential for the stratification of patients into clinically meaningful categories, which can be used to provide patients with counseling regarding prognosis, select treatment modalities, and determine eligibility for clinical trials. This has led to the creation of integrated staging systems that predict outcome by combining pathological and clinical variables. Although staging has been improved with the development of integrated systems, molecular tumor markers are expected to revolutionize the staging of renal cell carcinoma in the future. The development of methods based on gene and tissue arrays has created a powerful tool for evaluating hundreds to thousands of tumors simultaneously with histologic, immunohistochemical, and chromosomal analyses. Gene array analysis permits rapid molecular profiling, and tissue arrays enable the analysis of protein expression profiles on specimens to determine their potential clinical significance and role in renal cell carcinoma biology. This article reviews the tissue array-based predictors of pathobiology, prognosis, response to treatment, and potential molecular targets for therapy of renal cell carcinoma.

INTRODUCTION
Cancer of the kidney or renal pelvis is estimated to account for 35,710 new cases and 12,480 deaths in the United States in 2004 (1) and is steadily increasing at a rate of ~2.5% per year across population groups (2, 3). Renal cell carcinoma accounts for 3% of all adult malignancies and is the most lethal of the common urologic cancers (2). More than 40% of patients with renal cell carcinoma will die of their cancer compared with the ~20% mortality rates associated with prostate and bladder cancers (1). Approximately 20% to 30% of patients present with metastatic disease, and 20% to 40% of patients undergoing nephrectomy for clinically localized renal cell carcinoma will develop metastases (4). Significant advances in the diagnosis, staging, and treatment of patients with renal cell carcinoma during the last 2 decades have resulted in improved survival of a select group of patients and an overall increase in the natural history of the disease (2). Despite advances in biological and immune-based therapies, response rates for patients with metastatic renal cell carcinoma remain at ~15% to 30% (5–10). Significant achievements in the basic sciences have led to a greater knowledge of the underlying molecular genetics of renal cell carcinoma, which hold the promise of increased sophistication in attempts to individualize patient prognostication and for future treatment strategies. An enhanced ability to predict patient survival will allow for better selection of patients most likely to benefit from systemic therapies and for more accurate comparison of clinical trials based on varying inclusion criteria.

INTEGRATED STAGING ALGORITHMS
Renal cell carcinoma has traditionally been staged according to anatomic staging systems. The Tumor-Node-Metastasis (TNM) staging system is currently the most extensively used staging system; however, new comprehensive staging modalities have emerged from the University of California, Los Angeles and other institutions in an attempt to improve prognostication by combining other pathological and clinical variables (11–15). Tumor stage, tumor grade, and patient performance status remain the most useful, clinically available predictors of patient outcome for renal cell carcinoma (16). In addition, a number of other clinical and pathological characteristics have been identified as having an impact on the clinical behavior and subsequent survival in patients with localized and advanced renal cell carcinoma (16–20). The University of California, Los Angeles Integrated Staging System was developed to better stratify patients into prognostic categories using statistical tools that accurately define the probability of survival of an individual patient (11). Initially evaluated were a multitude of factors in 661 patients, including age, sex, Fuhrman grade, TNM stage, tumor size alone, Eastern Cooperative Oncology Group performance status, laterality, bilaterality, smoking, number of presenting symptoms, weight loss alone, tumor histologic type, administration of immunotherapy, inferior vena cava involve-
ment, number of metastatic sites, sites of metastases, and time interval between nephrectomy and tumor recurrence to determine which factors had the greatest impact on the survival of patients with renal cell carcinoma. The initial University of California, Los Angeles Integrated Staging System contained five groups based on TNM stage, Fuhrman grade, and Eastern Cooperative Oncology Group-performance status. Projected 2- and 5-year survival for patients in the University of California, Los Angeles Integrated Staging System groups I to V are 96% and 94%, 89% and 67%, 66% and 39%, 42% and 23%, and 9% and 0%, respectively. The University of California, Los Angeles Integrated Staging System was internally validated using a bootstrapping technique and then using an expanded database of patients treated at the University of California, Los Angeles between 1989 and 2000 (21), with external data from 576 renal cell carcinoma patients treated at M. D. Anderson Cancer Center (Dallas, TX) and in Nijmegen, the Netherlands (22, 23) and most recently with 4,202 renal cell carcinoma patients from eight international centers (24). The University of California, Los Angeles Integrated Staging System has been subsequently modified into a simplified system, based on separate stratification of patients with metastatic and nonmetastatic disease into low-risk, intermediate-risk, and high-risk groups (25). This provides a clinically useful system for predicting postoperative outcome and a unique tool for risk assignment and outcome analysis to help determine follow-up regimens and eligibility for clinical trials.

MOLECULAR PROFILING IN RENAL CELL CARCINOMA: THE FUTURE OF PATIENT PROGNOSTICATION, STAGING, AND TREATMENT

Prior attempts to predict patient survival have relied on traditional clinical parameters as described previously (11–15). More recently, methods based on gene arrays, which screen for differential expression of thousands of genes, have identified large numbers of new, potentially important prognostic markers (26). The evaluation of protein expression in a high-throughput tissue array is a natural extension of the efforts for molecular staging. Sections of the microarray provide targets for parallel in situ detection of DNA, RNA, and protein targets on the array and allow the rapid analysis of hundreds of molecular markers in the same set of specimens, which can be correlated to clinical data with respect to disease progression, treatment response, and survival.

Molecular tumor markers are expected to have an enormous impact on the future diagnosis, prognostication, and selection of therapeutic targets for renal cell carcinoma. Currently, markers relating to tumor proliferation, growth, angiogenesis, and loss of cell adhesion among others are being evaluated for their potential as prognostic factors. Increased staining of proliferating cell nuclear antigen (27), Ki-67 (28), and silver-staining nucleolar organizing region (29) have all been shown to correlate with poor survival in small studies of patients with renal cell carcinoma. Argyrophilic nucleolar proteins have also been shown to be an independent prognostic indicator (30). Other markers have included proteins involved in cellular signal transduction, control of transcription, apoptosis, cell adhesion, cytoskeletal regulation, tumor suppression, immune regulation, and angiogenesis.

A great deal of attention has been paid to carbonic anhydrase IX (CA IX). CA IX, a member of the carbonic anhydrase family, is thought to play a role in the regulation of intracellular and extracellular pH during periods of hypoxia in tumor cells (31). CA IX is one of several genes under the control of hypoxia inducible factor 1 in the hypoxia-inducible pathway that is particularly important for renal cell carcinoma (32, 33). Hypoxia inducible factor 1 is regulated at the biosynthesis stage through the phosphatidylinositol 3’-kinase-AKT-mTOR signal transduction pathway (34) and controlled at the post-translational stage by hypoxia through the von Hippel-Lindau (VHL) suppressor protein (35). Previous studies using a monoclonal antibody 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 (36, 37). Furthermore, previous immunohistochemical studies of malignant and benign renal tissues revealed that CA IX was highly expressed in renal cell carcinoma, suggesting that CA IX expression may be a useful diagnostic biomarker (38, 39). Clinical trials with radiolabeled monoclonal antibody against CA IX in patients with renal cell carcinoma have demonstrated selective and specific delivery of monoclonal antibody to renal cancer sites, with both primary and metastatic renal cell carcinoma deposits being capable of being targeted and imaged (40–42).

Immunohistochemical analysis performed on tissue microarrays from patients treated by nephrectomy at the University of California, Los Angeles for renal cell carcinoma demonstrated that CA IX staining was present in 94% of clear cell renal cell carcinomas (43). 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 renal cell carcinoma, with a hazard ratio of 3.10 (P < 0.001; ref. 43). 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 renal cell carcinoma 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 lower CA IX staining levels in metastatic lesions relative to matched primary tumor specimens (P = 0.036). Furthermore, all of the complete responders (8%) to interleukin (IL) 2 immunotherapy included patients with high CA IX (>85%) staining of the primary tumor.

A recent study from Harvard has corroborated these findings demonstrating that response to IL-2 immunotherapy was twice

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<th>Table 1</th>
<th>Clinical and molecular factors demonstrated to be prognostic indicators in the clinical/marker model</th>
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<td>Clinical</td>
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<td>T stage</td>
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Abbreviations: ECOG-PS, Eastern Cooperative Oncology Group performance status; CA IX, carbonic anhydrase IX.
as likely for patients with high CA IX (>85%) expression of the primary tumor (44). These findings have important prognostic implications for the immunotherapy of renal cell carcinoma patients, because non-clear cell renal cell carcinomas either do not express or express low levels of CA IX, whereas the majority of clear cell renal cell carcinomas express CA IX, and a subset express high levels of CA IX. These observations may partially explain why patients with papillary and chromophobe subtypes respond poorly to IL-2 immunotherapy and why some patients with clear-cell renal cell carcinoma respond better than others (45). Other genes of the hypoxia-inducible pathway have been studied with regard to cancer prognosis. In agreement with the findings of the University of California, Los Angeles study, a study of 187 clear-cell renal cell carcinoma patients indicated that VHL alteration (mutation or hypermethylation) was strongly associated with better cancer-free survival and cancer-specific survival for the 134 patients with stage I to III clear-cell renal cell carcinoma treated with radical nephrectomy (46). The association was more statistically significant among patients with relatively advanced disease (stage III or stage II).

Current efforts at the University of California, Los Angeles are aimed at integrating molecular information from tissue microarrays into the University of California, Los Angeles Integrated Staging System to generate a Molecular Integrated Staging System. Some of the expression profiles that have been examined in renal cell carcinoma include CA IX (43, 47), CA XII (48), gelsolin (49), PTEN (48), epithelial cell molecule adhesion (EpCAM; ref. 50), CD10 (50), p53 (48), sodium-potassium ATPase subunits (52), vimentin (48), Ki-67 (47), CXCR4 (53), vascular endothelial growth factor (VEGF; ref. 53), androgen receptors (55), bcl-2 (56), α-catenin (57), cadherin-6 (58), CA-125 (59), epithelial membrane antigen (59), CD44 (60), insulin-like growth factor 1 (61), caveolin-1 (62), and cyclin A (63). Immunohistochemical analysis of Ki-67, p53, gelsolin, CA IX, CA XII, PTEN, EpCAM, and vimentin was performed on a custom tissue microarray using clear-cell renal cell carcinoma from 318 patients, representing all stages of localized and metastatic renal cell carcinoma (48). Increased staining for Ki-67, p53, vimentin, and gelsolin correlated with worse survival, whereas the inverse was true for CA IX, PTEN, CA XII, and EpCAM. In a multivariate analysis, only metastatic status (M = 1 or n > 0), gelsolin, p53, and metastatic status/CA IX remained significant predictors of survival and were used to create a prognostic model (marker model) (Table 1). Using a similar approach, a prognostic model was constructed using a combination of clinical variables and marker data (clinical/ marker model) (Table 1). In a multivariate analysis, CA IX, vimentin, and p53 were statistically significant predictors of survival independent of the clinical variables, T stage, metastatic status, Eastern Cooperative Oncology Group-performance status, and grade. Both nomograms were calibrated, using bootstrap bias corrected estimates, to be accurate to within 10% of the actual 2- and 4-year survival rates. The predictive ability of the various models was quantified by calculating the C index, which demonstrated that prognostic systems based on protein expression profiles for clear-cell renal cell carcinoma perform better than standard clinical predictors such as TNM stage, histologic grade, and performance status. The predictive accuracy of the marker model for renal cell carcinoma was comparable with the University of California, Los Angeles Integrated Staging System, and the clinical/marker model was significantly more accurate than the University of California, Los Angeles Integrated Staging System. Although these nomograms are useful for visualizing our predictive models, they need to be tested on independent patient populations before being applied to patient care.

**DISCUSSION**

The last decade has witnessed the gradual transition from the use of solitary clinical factors as prognostic markers for patients with renal cell carcinoma to the introduction of systems that integrate multiple factors to the beginning of the use of molecular and genetic markers. A number of classification and staging systems have been proposed that use different combinations of prognostic factors in an effort to refine the ability to predict the clinical behavior of renal cell carcinoma and to evaluate potential outcomes. These classification and staging systems provide patients with counseling regarding prognosis, guide the selection of proper treatment modalities, and determine the eligibility for enrollment in clinical trials. In addition, once treatment has been initiated, these factors can assist with the adoption of patient-specific surveillance criteria to efficiently identify tumor recurrence and progression while avoiding unnecessary tests.

The potential of microarray technology in clinical research is enormous, because it allows for the capacity to evaluate hundreds of genes and/or proteins in a single experiment. 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. Sophisticated molecular classifiers for a broad range of human cancers have been developed recently by several groups (64–67). Gene and/or protein expression profiles can be derived through microarray technology to potentially allow for diagnosis of a particular cancer and cancer subsets, without examining the histology. This may not only eliminate the diagnostic category of the unknown primary cancer but may also improve the diagnostic accuracy of current approaches by using immunohistochemical analyses combined with classic histopathological techniques. Moreover, it is now possible to predict clinical outcome on the basis of gene and/or protein expression patterns (48, 68, 69). Microarray technology will be applied clinically to predict accurate diagnosis, prognosis, and possibly even therapeutic options. Classification of patients into high-risk and low-risk subgroups on the basis of a prognosis profile may be a useful means of guiding adjuvant therapy in patients. 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 even 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.

Molecular markers will eventually enhance our ability to predict the behavior of an individual tumor and to stratify...
patients into more sophisticated risk categories, ultimately permitting the goal of moving from nonspecific treatments to designing and targeting therapies for targeted patient populations. Two prognostic models, one based primarily on molecular markers and the other on a combination of clinical and molecular predictors, have been generated for predicting survival in patients with clear-cell renal cell carcinoma. Prognostic models based on protein expression profiles of renal cell carcinoma were shown to perform better than standard clinical predictors, such as TNM stage, histologic grade, and performance status. This technology offers a glimpse into the potential future of renal cell carcinoma staging in which prognosis and clinical decision-making could be derived from a decision tree structured from clinical information, anatomic extent, histopathologic criteria, and molecular expression. With the additional refinement of the use of these molecular markers as well as development of more objective grading criteria, these histologic parameters may eventually replace TNM staging as the definitive prognostic indicators for renal cell carcinoma. At the least they may serve as extremely useful, specific tools, which, when used with the TNM system, may provide crucial information on the treatment and prognosis of renal cell carcinoma. These advances will culminate in a better understanding of the causes of renal cancer, its prevention, and finally its successful treatment.

OPEN DISCUSSION

Dr. Michael Atkins: Did most, if not all, of those patients with stage IV disease have received IL-2? Are you therefore looking at the impact of not just CA IX but also CA IX with IL-2 therapy?

Dr. Robert Figlin: That is correct. This distinction can only be determined with prospective trials. CA IX expression may change the biology of the tumor independent of response and may also be a predictor of response to IL-2.

Dr. Robert Motzer: In your studies, did all of the patients have clear cell cancers?

Dr. Figlin: In the first analysis, we looked at 500 patients independent of histology. In the second, we examined 318 clear cell carcinoma patients.

Dr. Motzer: Because CA IX (G250) is a marker for clear cell cancer, what percent of clear cell cancers looked at by your pathologists are negative for G250?

Dr. Figlin: CA IX is not just a marker for clear cell tumors. CA IX is a HIF-inducible gene. So if you turn on HIF through a mechanism other than VHL mutation, you will get CA IX expression. It is highest in clear cell tumors, but 50% of papillary tumors have CA IX. So is it possible that papillary tumors really induce HIF through a VHL-independent mechanism, and is that why you get CA IX expression?

Dr. Atkins: UCLA has also shown that there is less expression in metastases than in the primary (Cin. Cancer Res., 9: 802–11, 2003), so there may be an evolution that happens as the disease progresses. Since the UCLA data set has a lot of patients who never metastasized, the percentage of the stage IV population that expresses high levels of CA IX may be considerably less. In our hands, only about 60% of tumors had > 5% cells stain for CA IX.

Dr. Avigan: Has anyone looked at IL-2 therapy as patients progress? You intimated that there may be antigenic loss over time and that may be associated with a worsening prognosis.

Dr. Atkins: When you say antigen, I don’t think we know whether or not CA IX is an antigen.

Dr. Figlin: We don’t know that it is an antigen at least in terms of the immune response, but we do know that if we use an in vitro system we can generate CA IX-specific CTL. Now whether or not that is occurring in humans remains to be examined.

Dr. Lipton: What is the function of CA IX, and why do people who have high levels have a worse prognosis or response?

Dr. Figlin: CA IX controls intracellular pH. It takes water and ions and makes something that can be excreted from the cell. Why patients with high CA IX staining do better or worse is unknown.

Dr. WalterStdalder: Two words of caution. First, as you look at more markers and get more things on your microarray, you are going to find factors that correlate with prognosis response simply as a statistic anomaly. Second, I agree that we need to characterize these patients and tumors molecularly before we put them on clinical trials. But we also need to be careful about what we do and don’t know. We have these kinase inhibitors that we say are an inhibitor of x. Yet that drug may inhibit a lot more targets that we may not know about. We have to keep an open mind that these drugs may be active against something that is completely and totally unexpected.

Dr. Atkins: In many cancers, high expression of CA IX and other HIF targets is associated with bad prognosis. For reasons that are unclear, the opposite appears to be the case for CA IX expression in renal cancer, at least as it relates to immunologic therapy.

Dr. Figlin: Unless, of course, it actually is an antigen that elicits an immunologic response.

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