Molecular Signatures of Localized Clear Cell Renal Cell Carcinoma to Predict Disease-Free Survival after Nephrectomy

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Abstract

Purpose: To identify the molecular signature of localized (N0M0) clear cell renal cell carcinoma (RCC) and assess its ability to predict outcome.

Methods: Clinical characteristics and pathologic records of 170 patients with localized clear cell RCC who underwent nephrectomy were reviewed. Immunohistochemical analysis was done on a tissue microarray of all primary tumors using a kidney cancer–related panel of protein markers, which included CAIX, CAIXII, CXCR3, gelsolin, Ki-67, vimentin, EpCAM, p21, p27, p53, p56, PTEN, HIF-1α, pAkt, VEGF-A, VEGF-C, VEGF-D, VEGFR-1, VEGFR-2, and VEGFR-3. Associations with disease-free survival (DFS) were evaluated with Cox models, and a concordance index assessed prognostic accuracy.

Results: Median follow-up was 7.1 years. The final multivariate Cox model determined T classification, Eastern Cooperative Oncology Group performance status, and five molecular markers (Ki-67, p53, endothelial VEGFR-1, epithelial VEGFR-1, and epithelial VEGF-D) to be independent prognostic indicators of DFS. The molecular signature based on these markers predicted DFS with an accuracy of 0.838, an improvement over T classification of 0.746, and the University of California-Los Angeles Integrated Staging System of 0.780. A constructed nomogram combined the molecular, clinical, and pathologic factors and approached a concordance index of 0.904.

Conclusions: A molecular signature consisting of five molecular markers (Ki-67, p53, endothelial VEGFR-1, epithelial VEGFR-1, and epithelial VEGF-D) can predict DFS for localized clear cell RCC. The prognostic ability of the signature and nomogram may be superior to clinical and pathologic factors alone and may identify a subset of localized patients with aggressive clinical behavior. Independent, external validation of the nomogram is required.

Introduction

Between 20% and 30% of patients with clinically localized (N0M0) renal cell carcinoma (RCC) develop metastases after undergoing a potentially curative nephrectomy (1). Once metastatic, RCC yields poor prognosis with a median survival time of 1 to 2 years (2). The newly approved agents sorafenib, sunitinib, and temsirolimus show promise; however, responses are partial and the majority of patients succumb to their disease (3-5). Adjuvant therapy is not approved for localized RCC and careful observation remains the postoperative standard of care. Patients undergoing resection of an isolated recurrence show long-term survival and those with limited disease burden respond better to systemic therapy (6, 7).

Identifying this high-risk group of patients remains a clinical challenge. Conventional pathologic and clinical factors such as tumor-node-metastasis stage, Eastern Cooperative Oncology Group performance status (ECOG PS), and nuclear grade provide robust prognostic information; however, alone, they cannot accurately predict disease progression. Several prognostic models combine independent prognostic factors to improve risk group assessment, but the reported accuracy indices vary (1, 8-12).

The incorporation of molecular markers into conventional models is anticipated to enhance their predictive accuracy. The advent of tissue and gene arrays has allowed analyses of multiple tumors and individual markers. Evaluation of multiple prognostic markers leads to better understanding of tumor behavior and the construction of prognostic models. For melanoma and lung, breast, and prostate cancer, gene expression profiles can divide patients with localized disease into risk groups (13-16). Additionally, tissue and gene arrays in medulloblastoma are useful in predicting survival (17-19).

In most solid malignancies, molecular models fail to show an improvement over existing clinicopathologic nomograms. However, the promise of molecular models has recently been realized in breast cancer as gene signatures improve on the existing clinicopathologic systems. High- and low-risk groups are based on a

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70-gene molecular signature, which has been validated using large patient cohorts (20, 21). Whether the molecular information improves outcome is uncertain; however, an ongoing multicenter, international study is under way.

A wide variety of molecular markers influence prognosis in localized RCC, including mediators of cellular proliferation, the hypoxia-inducible pathway, cell cycle regulators, and adhesion molecules (22-29). The aberrant expression of several of these pathways, including the hypoxia-inducible pathway and mammalian target of rapamycin pathway, leads to altered expression of downstream products and serves as a target of the newly approved agents (3, 4, 30).

We hypothesized that protein expression profiling can improve postoperative risk stratification for localized clear cell RCC. To test this hypothesis, we evaluated 29 markers, many relevant to the hypoxia-inducible and mammalian target of rapamycin pathways, in a defined, mature cohort treated at a single RCC referral center.

Materials and Methods

Patient Selection and Clinicopathologic Variables.

Our study cohort consisted of 170 randomly selected patients who underwent radical or partial nephrectomy for sporadic, clinically localized (N0M0) clear cell RCC at the University of California-Los Angeles (UCLA) Medical Center between 1989 and 2000. After approval by the UCLA Institutional Review Board, a retrospective assessment gathered all demographic, clinical, and pathologic data for each patient. ECOG PS was assigned to each patient at the time of diagnosis. T classification was defined according to 2002 American Joint Committee on Cancer criteria and the nuclear grade according to Fuhrman’s grading scheme. Additional data collected included tumor size and UCLA Integrated Staging System (UISS) risk group classification (1).

Tissue Microarray Construction.

Formalin-fixed paraffin-embedded primary tumor specimens were obtained from the Department of Pathology and Laboratory Medicine. Three core tissue biopsies, 0.6 mm in diameter, were taken from selected morphologically representative regions of each paraffin-embedded specimen and precisely arrayed using methods described previously (31). Representative 4-μm-thick sections of the resulting tumor tissue microarray block were transferred into glass slides using the paraffin sectioning aid system (adhesive-coated slides PSA-CS4x, adhesive tape, UV lamp, Instrumented, Inc.) to support the cohesion of 0.6-mm array elements.

Immunohistochemistry.

Immunohistochemical staining was done with a Dako Envision or Vectastain Elite avidin-biotin complex method (Vector) staining system, as described previously (28, 32). The primary antibodies used targeted gelsolin (final concentration, 3.8 μg/mL; Sigma Chemical Co.), HIF-1α (0.1 μg/mL; R&D Systems), p56 (0.125 μg/mL; Cell Signaling), pAkt (1.5 μg/mL; Cell Signaling), PTEN (2 μg/mL; Zymed), VEGF-A (4 μg/mL; Santa Cruz Biotechnology), VEGF-C (3 μg/mL; Zymed), VEGF-D (3 μg/mL; R&D Systems), VEGFR-1 (1 μg/mL; Santa Cruz Biotechnology), VEGFR-2 (2 μg/mL; Santa Cruz Biotechnology), and VEGFR-3 (2 μg/mL; a gift from Dr. Kari Alitalo, University of Helsinki, Helsinki, Finland). All staining protocols have been published previously in detail (22-24, 28, 33-36). Each antibody was individually evaluated using varying incubation times, antigen retrieval protocols, and dilution series, with final adjustments and optimization being done on test arrays before final testing on our RCC array. Part of this optimization process is done to minimize background staining (e.g., by quenching endogenous peroxidase by incubating in 0.5% H2O2 for 10-30 min after fixation). Data on antibody stains that have not met quality assurance criteria have not been included in this article. Each immunohistochemical assay was done using standardized positive and negative antibody controls to ensure specificity of staining.

A single pathologist (D.B.S.), blinded to clinicopathologic variables and clinical outcome, did the quantitative assessment of protein expression. The extent of expression (“staining frequency”) was recorded as percentage of the entire tumor sample that stained positive, without consideration of staining intensity. The overall score used for subsequent statistical analysis was the pooled mean from the three spots of the same tumor. Expression of pAkt, p21, and p27 was evaluated in both the nucleus and cytoplasm. VEGF-A, VEGF-C, VEGF-D, VEGFR-1, VEGFR-2, and VEGFR-3 were assessed separately in tumor epithelium (epithelial) and in the endothelium of tumor-associated vessels (endothelial).

Outcome Measures.

The end point of this study was disease-free survival (DFS) time, which was calculated from the date of nephrectomy to the date of local or distant recurrence or last contact. Local or distant recurrence was determined either clinically based on surveillance imaging or histologic evaluation of a metastatic site.

Statistical Analysis.

Survival probabilities of DFS were estimated by Kaplan-Meier methodology. Univariate and multivariate Cox proportional hazards models were fit to determine clinical and pathologic features and protein expression associated with DFS. Only variables that showed a significant (P < 0.05) relationship with DFS in univariate Cox proportional hazards analysis were included in multivariate modeling. The predictive accuracy of a Cox proportional hazards model was assessed by the concordance index (C-index). The 95% confidence interval (95% CI) of the C-index was calculated by bootstrapping (i.e., by testing 200 bootstrap resamples, each involving the entire data set with replacement). Continuous and nondichotomized marker expressions were used in the Cox models to protect against overfitting. The proportional hazards assumption was tested by the Schoenfeld test. To visualize the relationship of DFS with clinical, pathologic, and
molecular predictors, we constructed a nomogram for a Cox model that only contained significant variables. Data were all analyzed using the statistical software package R v2.4.4. A $P$ value of $<0.05$ was considered statistically significant.

Results

Characteristics. Our study cohort consisted of 108 men (64%) and 62 women (36%) with a median age of 64 years (range, 27-89) at nephrectomy. An ECOG PS of 0 was assigned to 90 patients at presentation (53%). Radical and partial nephrectomy was done in 124 and 46 cases, respectively. Surgical margins were negative in all patients and no patient received adjuvant therapy. Patient characteristics are summarized in Table 1.

Predicting DFS. The median follow-up was 7.1 years (range, 0.6-16.9 years), during which time 33 patients (19%) developed recurrence. Univariate associations between clinicopathologic factors and molecular marker expression with DFS are summarized in Table 2. Of the 29 molecular markers, expression was associated with DFS in six, including Ki-67, p53, nuclear p21, endothelial VEGFR-1, epithelial VEGFR-1, and epithelial VEGF-D. The significant clinicopathologic factors and molecular markers were combined in a multivariate Cox proportional hazards model. Because of the high correlation of T classification with tumor size, as well as UISS with ECOG PS, T classification, Fuhrman grade, and UISS group were all associated with DFS. Of the 29 molecular markers, expression was retained as independent prognostic factors of DFS.

Table 1. Patient and tumor characteristics

| Characteristic       | No. | %    |
|----------------------|-----|------|
| Tumor location       |     |      |
| Right sided          | 92  | 46   |
| Left sided           | 78  | 54   |
| ECOG PS              |     |      |
| 0                    | 90  | 53   |
| $\geq 1$             | 80  | 47   |
| T classification     |     |      |
| T1                   | 94  | 55   |
| T2                   | 18  | 11   |
| T3                   | 54  | 32   |
| T4                   | 4   | 2    |
| Tumor size           |     |      |
| Median               | 4.7 |      |
| Range                | 0.8-18.0 |  |
| Fuhrman grade        |     |      |
| G1                   | 36  | 21   |
| G2                   | 94  | 55   |
| G3                   | 39  | 23   |
| G4                   | 1   | 1    |
| UISS group           |     |      |
| Low risk             | 52  | 31   |
| Intermediate risk    | 88  | 52   |
| High risk            | 39  | 18   |

Table 2. Univariate Cox regression analysis

| Clinicopathologic factors | HR (95% CI) | $P$  |
|---------------------------|-------------|------|
| ECOG PS                   | 4.610 (2.077-10.23) | $<0.001$ |
| T classification          | 2.889 (1.914-4.352) | $<0.001$ |
| Tumor size                | 1.234 (1.147-3.129) | $<0.001$ |
| Fuhrman grade             | 2.073 (1.215-3.337) | 0.008  |
| UISS group                | 5.032 (2.856-8.865) | $<0.001$ |

| Molecular markers         | HR (95% CI) | $P$  |
|---------------------------|-------------|------|
| p33                       | 1.042 (1.020-1.065) | $<0.001$ |
| VEGF-D (epithelial)       | 0.980 (0.968-0.992) | 0.002  |
| VEGFR-1 (endothelial)     | 1.038 (1.014-1.063) | 0.002  |
| VEGFR-1 (epithelial)      | 1.017 (1.004-1.029) | 0.008  |
| Ki-67                     | 1.041 (1.004-1.079) | 0.029  |
| p21 (nuclear)             | 0.980 (0.962-0.999) | 0.037  |
| p27 (nuclear)             | 0.984 (0.966-1.002) | 0.079  |
| p66                       | 1.009 (0.999-1.019) | 0.087  |
| CAIX                      | 0.990 (0.978-1.002) | 0.099  |
| VEGF-A (epithelial)       | 1.009 (0.997-1.020) | 0.158  |
| EpCAM                     | 0.987 (0.969-1.006) | 0.173  |
| p21 (cytoplasmic)         | 0.979 (0.942-1.016) | 0.263  |
| VEGF-D (endothelial)      | 0.833 (0.600-1.162) | 0.286  |
| Gelsolin                  | 1.005 (0.996-1.014) | 0.312  |
| pAkt (nuclear)            | 0.990 (0.969-1.011) | 0.340  |
| VEGF-A (endothelial)      | 1.007 (0.993-1.021) | 0.350  |
| VEGF-C (epithelial)       | 1.007 (0.993-1.021) | 0.358  |
| Vimentin                  | 1.006 (0.993-1.019) | 0.358  |
| CXCR3                     | 0.996 (0.984-1.008) | 0.497  |
| pAkt (cytoplasmic)        | 1.004 (0.992-1.016) | 0.535  |
| VEGFR-3 (endothelial)     | 1.003 (0.991-1.016) | 0.612  |
| VEGFR-2 (epithelial)      | 1.003 (0.992-1.014) | 0.613  |
| CAIX                      | 1.003 (0.991-1.015) | 0.651  |
| PTEN                      | 1.003 (0.990-1.016) | 0.653  |
| VEGF-C (endothelial)      | 1.006 (0.977-1.036) | 0.702  |
| p27 (cytoplasmic)         | 0.995 (0.966-1.024) | 0.723  |
| VEGFR-3 (epithelial)      | 1.005 (0.973-1.037) | 0.778  |
| VEGFR-2 (endothelial)     | 1.004 (0.971-1.038) | 0.816  |
| HIF-1a                    | 0.999 (0.985-1.013) | 0.909  |

NOTE: ECOG PS, T classification, tumor size, Fuhrman grade, UISS group, Ki-67, p33, nuclear p21, epithelial and endothelial VEGFR-1, and epithelial VEGF-D expression were all associated with DFS.

Table 3. Multivariate Cox proportional hazards regression model

| Factor               | HR (95% CI) | $P$  |
|----------------------|-------------|------|
| ECOG PS              | 5.749 (1.862-16.09) | 0.002  |
| T classification     | 1.851 (1.109-3.090) | 0.019  |
| Fuhrman grade        | 0.922 (0.480-1.771) | 0.808  |
| Ki-67                | 1.053 (1.010-1.099) | 0.016  |
| p33                  | 1.039 (1.012-1.067) | 0.005  |
| p21 (nuclear)        | 0.994 (0.971-1.018) | 0.646  |
| VEGFR-1 (endothelial) | 1.050 (1.019-1.081) | 0.002  |
| VEGFR-1 (epithelial) | 1.025 (1.007-1.042) | 0.007  |
| VEGF-D (epithelial)  | 0.970 (0.955-0.985) | $<0.001$ |

NOTE: T classification, ECOG PS, Ki-67, p33, epithelial and endothelial VEGFR-1, and epithelial VEGF-D expression were retained as independent prognostic factors of DFS.

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Kaplan-Meier survival estimates based on total points assigned for each patient by the nomogram. Calibration plots showed that the nomogram did well compared with an ideal model for prediction of 1-, 2-, and 5-year DFS (Fig. 2). The low-risk group was classified by ≤120 points and showed a DFS of 100% at 5 years. The intermediate risk group (points, 121-175) showed a 1-, 2-, and 5-year DFS of 100%, 96%, and 87%, respectively. The

Figure 1. A. Nomogram for predicting DFS using T classification, ECOG PS, and the five molecular markers Ki-67, p53, epithelial and endothelial VEGFR-1, and epithelial VEGF-D expression. To read the nomogram, one should draw a vertical line from the status of each factor to the axis-labeled points. The sum of the points gives the total points and corresponds to a 1-, 2-, and 5-y DFS probability. The predictive accuracy of the nomogram was 0.904. B. Kaplan-Meier survival estimates according to the total points assigned to each individual patient by the nomogram. Patient groups were defined according to total points assigned by the nomogram. P values comparing the curves were calculated with log-rank tests. The numbers of patients at risk are specified.
high-risk group was defined by >175 points and 1-, 2-, and 5-year DFS was 69%, 62%, and 47%.

Analysis of the five molecular markers alone predicted DFS with an accuracy of 0.838 (95% CI, 0.813-0.863), which was more accurate than T classification (0.746; 95% CI, 0.734-0.759) and UISS (0.780; 95% CI, 0.776-0.784). The constructed nomogram containing T classification, ECOG PS, and the five molecular markers approached a predictive accuracy of 0.904 (95% CI, 0.875-0.932).

Discussion

Several recent models improve risk stratification after nephrectomy for localized RCC by combining clinical and pathologic variables. The UISS and the SSIGN (stage, size, grade, and necrosis) are integrated staging systems to predict survival for localized and metastatic RCC (1, 8). Both have been externally validated with large multicenter cohorts and showed good predictive accuracy with C-indices of 0.809 (UISS, localized RCC; ref. 12) and 0.90 (SSIGN; ref. 37). Kattan and colleagues (9) introduced a nomogram to predict DFS for localized RCC. The calculated C-index in the original report was 0.74 (9); however, in a recent study, the nomogram did poorly (C-index, 0.607; ref. 11).

Despite considerable achievements understanding conventional clinical and pathologic factors, prediction of postoperative biology remains difficult and many patients develop unexpected recurrence. We hypothesized that integration of molecular markers, in the form of tissue arrays, into models would improve the prediction of DFS. Although many molecular markers have been evaluated thus far, few are linked with outcome, and fewer have achieved independent predictor status in multivariate analysis (23-29).

We analyzed molecular markers important in kidney cancer development, many of which are involved in the hypoxia-inducible pathway plays a critical role in clear cell RCC and leads to alterations in the downstream VEGF pathway. The importance of VEGF to angiogenesis in RCC is well defined and serves as a target of new systemic therapies. Higher VEGF-A expression portends poor prognosis in cohorts of localized and metastatic RCC (29, 45). In the current study, VEGF-A did not prove to be an independent predictor of outcome for localized tumors; however, higher expression of its receptor, VEGFR-1, both in the tumor epithelium and tumor-associated vessels yielded a worse prognosis. VEGF-D is involved in lymphangiogenesis pathways and binds VEGFR-2 and VEGFR-3 (46-48). One would expect that higher VEGF-D expression correlates with increased lymphangiogenesis and a higher metastatic potential; however, we found the opposite. Our findings are further corroborated by a study of Lam et al. (29), which showed that low VEGF-D expression increases the risk of death from RCC. Perhaps, decreased expression of VEGF-D down-regulates lymphangiogenesis and subsequently up-regulates angiogenesis.

Our study confirms the importance of VEGF pathways in aggressive phenotypes of clear cell RCC. Endothelial and epithelial VEGFR-1 as well as VEGF-D were
independent predictors of outcome. As both sunitinib and sorafenib inhibit VEGF receptors (49, 50) and overexpression of these proteins was associated with poorer prognosis in the current study, our data generate the hypothesis the patients with higher expression of VEGFR-1 may be candidates for adjuvant treatment with these agents. This hypothesis should be tested in future studies.

Molecular markers alone provided better risk stratification than the clinicopathologic variables. Using the five markers independently associated with DFS (Ki-67, p53, epithelial and endothelial VEGFR-1, and epithelial VEGF-D), our prognostic model was a better predictor of DFS than tumor-node-metastasis stage or the UISS. Taken in context with the gene profiling in breast cancer, this reinforces the idea that the molecular signature is a more accurate representation of tumor behavior than clinicopathologic features alone. The incorporation of the classic predictors, T classification and ECOG PS, enhanced the predictive accuracy to a C-index of 0.904. Our nomogram may serve as an example to incorporate molecular information into conventional prognostic systems. Other molecular markers that will add prognostic power will be tested, including DNA and RNA expression data.

Several limitations must be acknowledged, including the small cohort and limited subgroups. Ideally, two patient cohorts are necessary: one to develop and a second to validate the nomogram. Although feasible with clinicopathologic factors using large databases, it is difficult and costly with multimarker, tissue-based studies. We attempted to reduce bias by bootstrapping and the use of continuous rather than dichotomized marker expression. External, independent validation of the nomogram and a standard protocol for scoring protein expression are necessary before application to clinical practice. Further, more than one pathologist may be necessary to evaluate staining to reduce subjectivity of scoring. Potentially, this subjectivity could be reduced by computer-based systems; at present, however, they are not broadly used and far from being an alternative to evaluation by eye.

Conclusions

The molecular signature derived from the expression of five molecular markers (Ki-67, p53, endothelial VEGFR-1, epithelial VEGFR-1, and epithelial VEGF-D) found in the primary tumor may improve risk stratification following nephrectomy for localized clear cell RCC. Inclusion of clinicopathologic variables enhances the predictive accuracy of the molecular signature alone and the proposed nomogram may identify a subgroup of patients at a high risk of recurrence. Independent, external validation of the nomogram is required.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Molecular Signatures of Localized Clear Cell Renal Cell Carcinoma to Predict Disease-Free Survival after Nephrectomy

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