Prognostic Relevance of the mTOR Pathway in Renal Cell Carcinoma

Implications for Molecular Patient Selection for Targeted Therapy

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BACKGROUND. The mammalian target of rapamycin (mTOR) pathway is up-regulated in many human cancers, and agents targeting the mTOR pathway are in various stages of clinical development. The goal of the study was to evaluate the potential and limitations of targeting the mTOR pathway in renal cell carcinoma (RCC).

METHODS. Immunohistochemical analysis using antibodies against pAkt, PTEN, p27, and pS6 was performed on a tissue microarray constructed from paraffin-embedded specimens from 375 patients treated by nephrectomy for RCC. The expression was associated with pathological parameters and survival.

RESULTS. The mTOR pathway was more significantly altered in clear-cell RCC, high-grade tumors, and tumors with poor prognostic features. PS6 and PTEN showed the strongest associations with pathological parameters. Survival tree analysis regarding expression of cytoplasmic pAkt, nuclear pAkt, PTEN, cytoplasmic p27, and pS6 identified staining percentages of 40%, 10%, 75%, 7%, and 70%, respectively, as ideal cutoff values for stratification, with corresponding *P*-values of .03, .001, .02, .005, and <.0001, respectively. Interestingly, high nuclear pAkt expression was associated with a favorable prognosis, whereas high cytoplasmic pAkt expression was associated with a poor prognosis. In multivariate Cox regression analysis, ECOG PS, T classification, N classification, M classification, cytoplasmic Akt, nuclear pAkt, PTEN, and pS6 were independent prognostic factors of DSS.

CONCLUSIONS. Components of the mTOR pathway are significantly associated with pathological features and survival. Not all RCC tumor types seem to be equally amenable to mTOR targeted therapy. PTEN, pAkt, p27, and pS6 may serve as surrogate parameters for patient selection and predicting prognosis. Patients with a highly activated mTOR pathway should benefit most from this therapy. External validation of our results is recommended. ***Cancer* 2007;109:2257–67.** © *2007 American Cancer Society.*

KEYWORDS: mTOR, S6, Akt, PTEN, p27, survival.

With over 38,000 new cases, representing an annual increase of 2% to 3%, and over 12,000 cancer-related deaths in 2006 in the US,[1,2] renal cell carcinoma (RCC) represents a major therapeutic challenge. Only a small number of patients with metastatic RCC can be cured by existing therapies. Approximately 20% to 30% of patients present with metastatic disease and an additional 20% to 40% develop recurrence after undergoing curative surgery for localized RCC.[3,4] Advances in biological and immune-based therapies have produced response rates for patients with metastatic RCC of approximately 15% to 30%, with some long-term durable remissions. Recent

advances in understanding the changes associated with *von Hippel-Lindau (VHL)* gene inactivation have led to several angiogenesis inhibitors (sunitinib, sorafenib, bevacizumab) demonstrating enhanced response, improvement in progression-free survival, and trends toward improvement in overall survival for some of these agents administered in both the first- and second-line setting.[5–11] A comparison of sunitinb to interferon-alpha (IFN) in a recent phase 3 trial demonstrating improvement in progression-free survival has changed the paradigm for treatment of this disease.[11] Despite these advances, most responses are partial in nature, with the majority of patients ultimately succumbing to their disease.

Significant achievements in the basic sciences have led to a greater knowledge of the underlying signaling pathways in RCC,[12] including the mammalian target of rapamycin (mTOR) pathway (phosphoinositide 3-kinase/Akt pathway) (Fig. 1). The mTOR pathway has a central role in the regulation of cell growth and increasing evidence suggests its dysregulation in cancer.[13] Receiving input from multiple signals, including growth factors, hormones, nutrients, and other stimulants or mitogens, the pathway stimulates protein synthesis by phosphorylating key translation regulators such as ribosomal S6 kinase. The mTOR pathway also contributes to many other critical cellular functions, including protein degradation and angiogenesis. Hence, use of inhibitors of the pathway represents a new strategy for the targeted treatment of RCC.

Temsirolimus (CCI-779) is an inhibitor of mTOR and, in a phase 2 trial, showed antitumor activity in heavily pretreated patients with advanced RCC.[14] In a recent randomized phase 3 trial of patients with poor prognosis, previously untreated, metastatic RCC, temsirolimus demonstrated a statistically significant 49% improvement in overall survival when compared with IFN.[15]

With mTOR inhibitors, it is unclear what clinical parameters and/or molecular pathways will predict which patients will derive the greatest benefit. These agents might have clinical activity only in selected patient cohorts in whose diseases this pathway drives their biology. An enhanced ability to predict patient survival would allow patients most likely to benefit from mTOR targeting therapies to be selected. For patient selection, a wide spectrum of molecular biomarkers is currently available including upstream and downstream targets of mTOR. Hence, the goals of our study were 1) to evaluate the prognostic relevance of the mTOR pathway in RCC in a large patient cohort, and 2) to identify patients whose tumor biology would most likely benefit from mTOR targeting therapy. For these goals, we carried out an immunohistochemical study of the mTOR upstream and downstream targets phosphorylated Akt (pAkt), phosphorylated S6 ribosomal protein (pS6), and p27, as well as the tumor suppressor PTEN (phosphatase and tensin homolog deleted on chromosome 10), and correlated our findings with pathological parameters and survival.

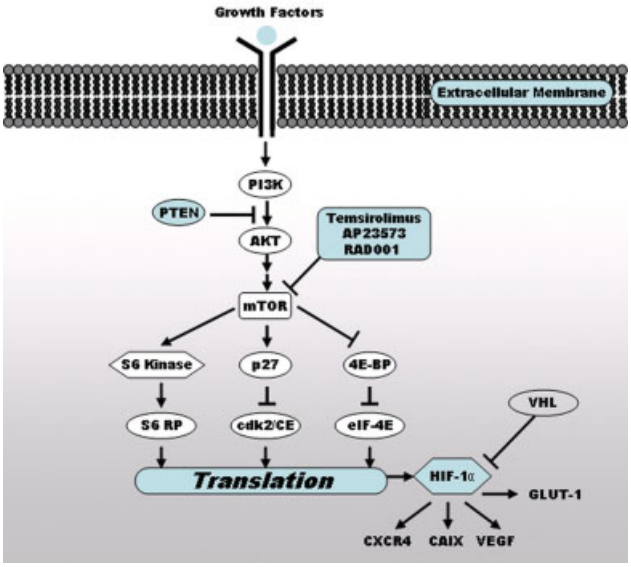

FIGURE 1. PI3K-Akt-mTOR pathway. Activation of the pathway leads to phosphorylation of S6 kinase and 4E-BP, activating the former and inactivating the latter. S6 kinase and 4E-BP are critical components of the general translation machinery. Cell cycle regulator p27 is also regulated through mTOR. The pathway is negatively regulated by PTEN and rapamycin analogs like temsirolimus.

MATERIALS AND METHODS

Patients

Our study cohort consisted of 375 patients who underwent radical or partial nephrectomy for sporadic RCC at the University of California, Los Angeles (UCLA) between 1989 and 2000. After approval by the UCLA Institutional Review Board, a retrospective study was performed with outcome assessment based on chart review of clinical and pathological data. Clinical data included age, gender, and Eastern Cooperative Oncology Group performance status (ECOG PS). Pathological data included TNM staging and histologic subtyping, which was performed according to the 1997 Union Internationale Contre le Cancer (UICC) and American Joint Committee on Cancer (AJCC) classification, as well as Fuhrman grade. Localized RCC was defined as N0M0 RCC, whereas metastatic RCC was defined if regional

lymph node metastasis and/or distant metastasis were present.

Tissue Microarray Construction

Formalin-fixed, paraffin-embedded tumor specimens from our patient cohort were obtained from the Department of Pathology. Three core tissue biopsies, 0.6 mm in diameter, were taken from selected morphologically representative regions of each paraffin-embedded RCC and precisely arrayed using a custom-built instrument as described previously.[16] Additional core tissue biopsies were taken from morphologically benign-appearing surrounding renal parenchyma tissue for each tumor. Sections of the resulting tumor tissue microarray block, 4 μm thick, were transferred to glass slides using the paraffin sectioning aid system (adhesive coated slides PSA-CS4x, adhesive tape, UV lamp, Instrumedics, Hackensack, NJ) to support the cohesion of 0.6 mm array elements.

Immunohistochemical Staining and Evaluation

Immunohistochemical staining was performed with a Dako Envision (Dako, Carpinteria, Calif) or Vectastain Elite ABC (Vector, Burlingame, Calif) staining system, as described previously.[17,18] Rabbit monoclonal antibody phospho-Akt Ser473 (Cell Signaling, Danvers, Mass) at a concentration of 1.5 μg/mL was used to stain for pAkt. Immunostaining for PTEN was performed using rabbit polyclonal antibody PN37 (Zymed, San Francisco, Calif) at 2 μg/mL. Mouse monoclonal antibody SX53G8 (Dako) was used at a concentration of 8 μg/mL to stain for p27. Staining for pS6 was performed with polyclonal rabbit antibody phospho-S6 ribosomal protein Ser 235/236 (Cell Signaling) at a concentration of 0.125 μg/mL.

The expression was evaluated by an anatomical pathologist (D.B.S.) in a blinded fashion to validate the diagnostic morphology of each array spot. The evaluation of expression involved site (subcellular localization) and degree of reactivity (staining intensity: 0 = negative, 1 = weak, 2 = moderate, 3 = strong, and staining frequency: percentage of positive cells). The overall score used for subsequent statistical analysis was the pooled mean from the 3 spots of the same tumor.

Statistical Analysis

The associations between protein expression and T classification, Fuhrman grade, metastatic status, and histologic subtype were evaluated using the nonparametric Mann-Whitney *U*-test (when 2 independent groups were compared) or the Kruskal-Wallis test (when more than 2 independent groups were compared). Correlations were determined using the Pearson coefficient. The primary endpoint was disease-specific survival time (DSS). The Kaplan-Meier method was used to generate the survival functions. To find appropriate cutoffs for classifying patients according to the amount of expression, we used the recursive partitioning function in the R software (www.r-project.org).[19] Subsequently, the dichotomized variable was used in univariate and multivariate survival analysis. Univariate survival analysis was performed by using the log-rank test and univariate Cox regression analysis. Independent prognostic variables of survival were identified with a multivariate Cox regression analysis. A significance level of .05 was used for all statistical tests. The statistical package SPSS (Chicago, Ill) was used for the analysis.

TABLE 1
Patient and Tumor Characteristics

	No.	%
ECOG PS		
0	149	39.7
1	207	55.2
2	16	4.3
X	3	0.8
Tumor size		
Median	6.5	—
Range	1–18	—
T classification		
T1	140	37.3
T2	49	13.1
T3	165	44.0
T4	21	5.6
N classification		
N0	323	86.1
N1	23	6.1
N2	29	7.7
M classification		
M0	216	57.6
M1	159	42.4
Fuhrman grade		
G1	49	13.1
G2	187	49.9
G3	126	33.6
G4	13	3.5
Histological subtype		
Clear cell	323	86.1
Papillary	40	10.7
Chromophobe	8	2.1
Collecting duct	4	1.1

ECOG PS indicates Eastern Cooperative Oncology Group performance status.

RESULTS

Our study cohort consisted of 375 patients, 252 men and 123 women with a median age of 61 years (range, 27–88 years). Characteristics of the patients are summarized in Table 1.

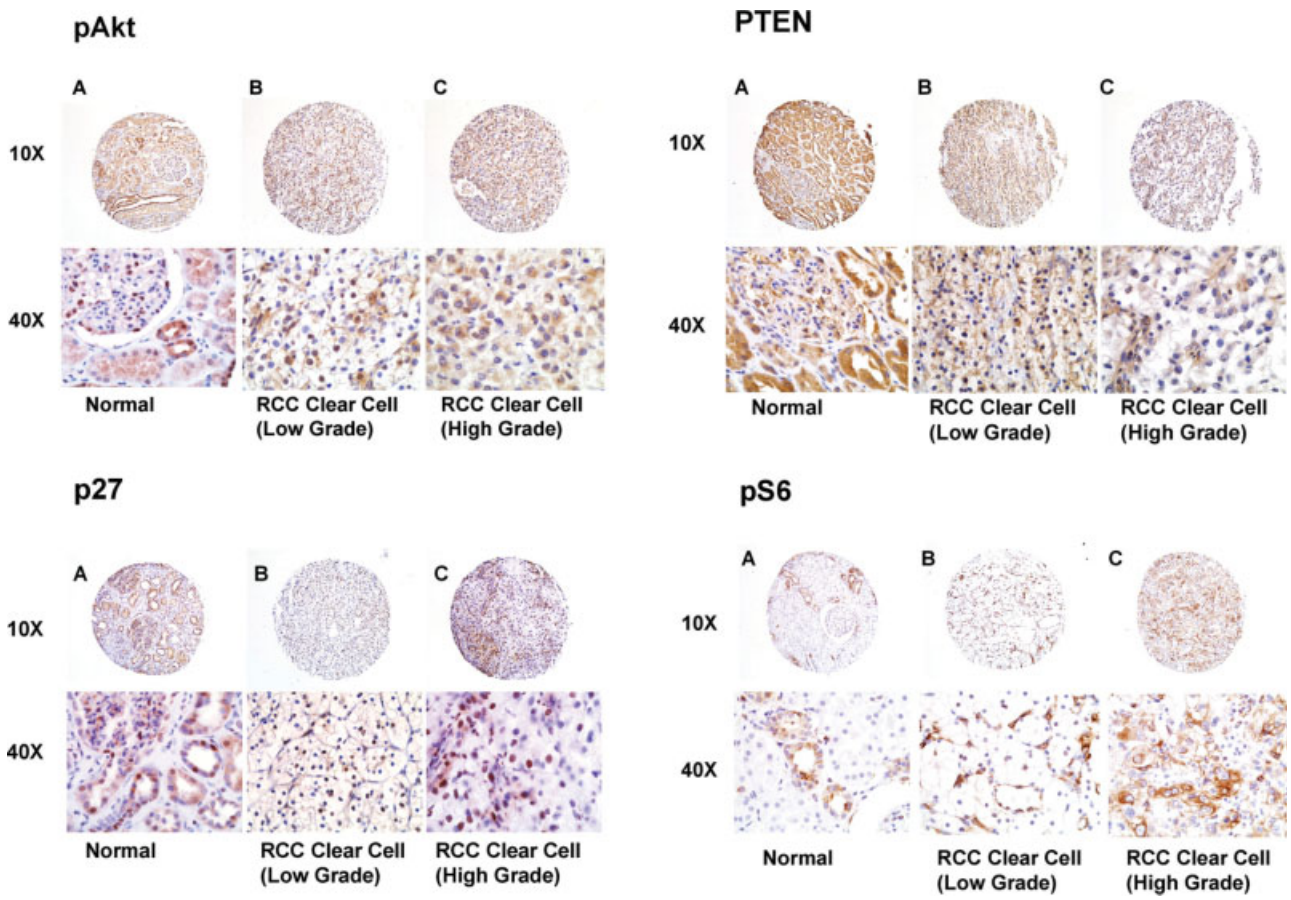

FIGURE 2. Immunohistochemical pattern of pAkt, PTEN, p27, and pS6 in (A) normal kidney tissue, (B) low-grade clear-cell renal cell carcinoma (RCC), and (C) high-grade clear-cell RCC.

Expression and Association With Pathological Variables (Figs. 2, 3)

Because staining intensity and frequency showed high intercorrelation (cytoplasmic pAkt: $R = 0.88$, nuclear pAkt: $R = 0.90$, cytoplasmic p27: $R = 0.84$, nuclear p27: $R = 0.91$, PTEN: $R = 0.61$, pS6: $R = 0.94$, each $P < .0001$), we restricted subsequent analyses to staining frequency.

pAkt

Anti-pAkt staining was seen in both cytoplasmic and nuclear cellular staining compartments. Cytoplasmic staining was detected in 93% of the RCCs. Higher cytoplasmic expression was noted in clear-cell than in nonclear-cell RCC, whereas no significant associations were found with other variables. Nuclear staining was positive in 61% of the tumors. Higher nuclear expression was observed in patients with localized disease. A significantly inverse correlation was found with tumor size ($R = -0.13$, $P = .01$).

PTEN

The anti-PTEN antibody stained the tissues of the array in the cytoplasmic cellular compartment in 96% of the tumors. Tumors showed a lower expression than normal renal tissues. PTEN expression was higher in tumors with lower T classification (T1/2), nonclear-cell subtypes, and in localized RCC.

p27

Cellular staining with anti-p27 antibody occurred in both nuclear and cytoplasmic compartments in 78% and 46% of the tumors, respectively. Nuclear expression was higher in clear-cell RCC and inversely correlated with tumor size ($R = -0.12$, $P = .02$). Cytoplasmic expression was higher in metastatic RCC.

pS6

Anti-pS6 staining was only seen in the cytoplasmic cellular compartment. Staining in tumors was noted in 85% of the RCCs, where it was generally increased

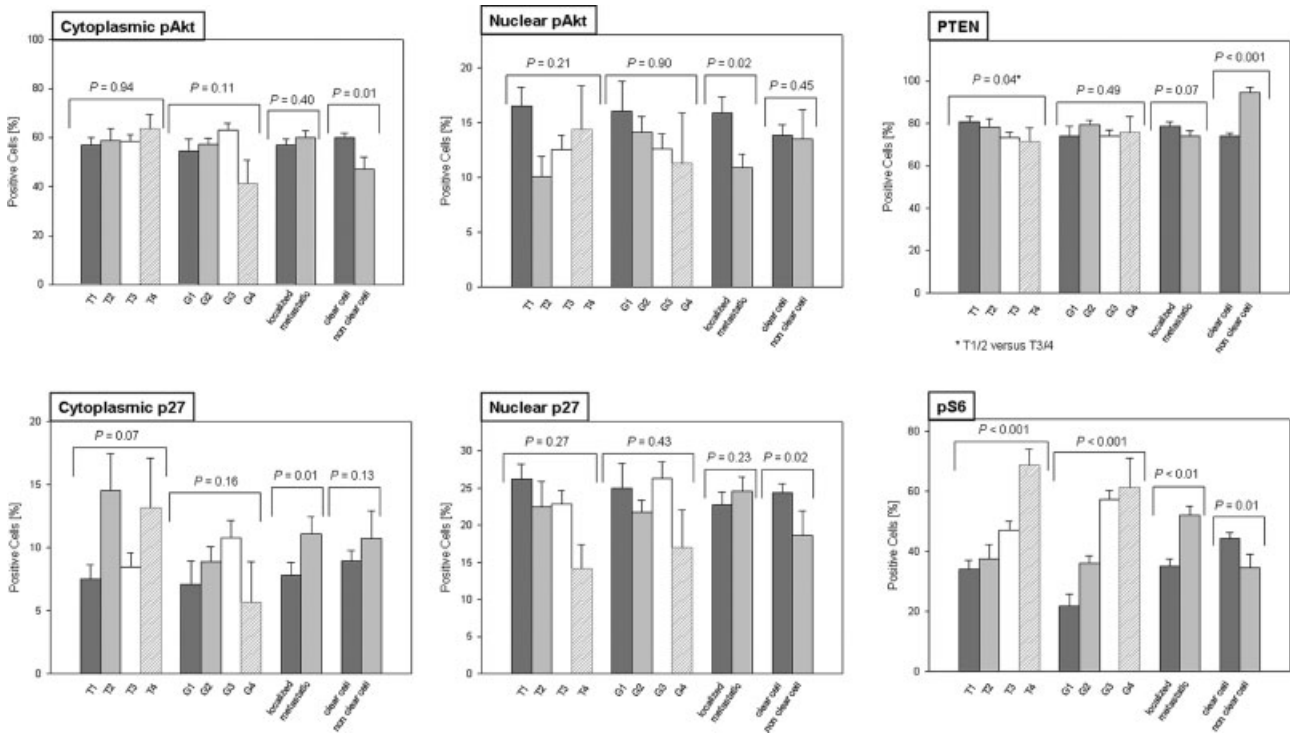

FIGURE 3. Relation of T classification, Fuhrman grade, localized/metastatic disease, and histologic type and mean expression frequency. The Kruskal-Wallis test was used to compare expression among T classification and Fuhrman grade. Mann-Whitney *U*-test was applied to compare expression between localized and metastatic renal cell carcinoma (RCC), clear-cell vs nonclear-cell RCC, and T1/2 vs T3/4 (PTEN).

compared with matched normal tissue. Significantly higher expressions were observed in tumors with higher T classification, higher Fuhrman grades, in metastatic disease, and clear-cell subtype.

Correlation Between Biomarkers

The mean expression of nuclear and cytoplasmic pAkt ($R = 0.25$, $P < .001$) and nuclear and cytoplasmic p27 ($R = 0.14$, $P = .01$) were significantly correlated with each other. In addition, cytoplasmic pAkt expression was significantly correlated with nuclear p27 ($R = 0.32$, $P < .001$), and pS6 ($R = 0.18$, $P = .001$). Nuclear pAkt expression was significantly correlated with nuclear p27 ($R = 0.38$, $P < .001$), PTEN ($R = 0.13$, $P = .02$), and pS6 ($P = -.18$, $P = .001$). PTEN expression was further correlated with nuclear p27 expression ($R = 0.12$, $P = .04$).

Survival Analysis

The median follow-up time was 56.9 months (range, 0.2–141.8) for the censored patients and 14.1 months (range, 0.4–115.0) for patients who died from RCC.

Performing survival tree analysis with regard to expression of cytoplasmic pAkt, nuclear pAkt, PTEN, cytoplasmic p27, and pS6 for all patients, we identified staining percentages of 40%, 10%, 75%, 7%, and 70%, respectively, as ideal cutoff values for further patient stratification. The corresponding *P*-values for the dichotomized patient cohort, calculated with the log-rank test, were .034, .001, .021, .005, and <.0001, respectively (Fig. 4). Notably, nuclear p27 expression was not associated with DSS. High nuclear pAkt expression was associated with favorable prognosis, whereas high cytoplasmic pAkt expression was associated with poor prognosis. In univariate Cox regression analysis, ECOG PS, T classification, N classification, M classification, Fuhrman grade, expression of cytoplasmic and nuclear pAkt, PTEN, cytoplasmic p27, and pS6 were all predictors of DSS. In multivariate Cox regression analysis, ECOG PS, T classification, N classification, M classification, Fuhrman grade, cytoplasmic and nuclear pAkt, PTEN, and pS6 were independent prognostic factors (Table 2).

We carried out further subanalysis dividing patients into localized and metastatic RCC at initial presentation. For patients with localized RCC (N0M0), pS6, nuclear p27, and nuclear pAkt expression provided additional prognostic information to ECOG PS, T classification, and Fuhrman grade (Fig. 5A). Using a cutoff value of 65%, lower staining of pS6 predicted a better prognosis than a higher staining ($P = .0001$). Higher nuclear p27 expression

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