

Cytoplasmic Sequestration of p27 via AKT Phosphorylation in Renal Cell Carcinoma

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Abstract **Purpose:** p27 localization and expression has prognostic and predictive value in cancer. Little is known regarding expression patterns of p27 in renal cell carcinoma (RCC) or how p27 participates in disease progression or response to therapy. **Experimental Design:** RCC-derived cell lines, primary tumors, and normal renal epithelial cells were analyzed for p27 expression, phosphorylation (T157 of the NLS), and subcellular localization. RCC-derived cell lines were treated with phosphatidylinositol 3-kinase (PI3K) and mammalian target of rapamycin (mTOR) inhibitors and effects on p27 localization were assessed. The potential contribution of cytoplasmic p27 to resistance to apoptosis was also evaluated. **Results:** p27 was elevated in tumors compared with matched controls, and cytoplasmic mislocalization of p27 was associated with increasing tumor grade. Cytoplasmic localization of p27 correlated with phosphorylation at T157, an AKT phosphorylation site in the p27 NLS. In RCC cell lines, activated PI3K/AKT signaling was accompanied by mislocalization of p27. AKT activation and phosphorylation of p27 was associated with resistance to apoptosis, and small interfering RNA knockdown of p27 or relocalization to the nucleus increased apoptosis in RCC cells. Treatment with the PI3K inhibitors LY294002 or wortmannin resulted in nuclear relocalization of p27, whereas mTOR inhibition by rapamycin did not. **Conclusions:** In RCC, p27 is phosphorylated at T157 of the NLS, with increasing tumor grade associated with cytoplasmic p27. PI3K inhibition (which reduces AKT activity) reduces T157 phosphorylation and induces nuclear relocalization of p27, whereas mTOR inhibition does not. Clinical testing of these findings may provide a rational approach for use of mTOR and PI3K/AKT pathway inhibitors in patients with RCC.

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p27 is a member of the Cip/Kip family of cyclin-dependent kinase inhibitors (CKI), which function to negatively regulate cell cycle progression. p27 interacts with cyclins D, E, and A-dependent kinases, facilitating nuclear import of cyclin D-cyclin-dependent kinase 4/6 complexes and inactivating cyclins A and E-cyclin-dependent kinase 2 complexes in the nucleus (1). Although aberrant cell cycle progression is a hallmark of tumorigenesis and defects in cell cycle regulation are common in cancer cells, mutations in the p27 gene in cancer are rare. Rather, p27 is regulated predominantly by posttranslational modifications, primarily phosphorylation, which determine protein stability and subcellular localization (2–4). Intriguingly, p27 protein degradation is mediated by different processes in the cytosol and nucleus, potentially contributing to cytoplasmic or nuclear accumulation (5).

In tumors, p27 activity is regulated via several different mechanisms. One of the primary regulatory mechanisms is post-translational modification, most commonly phosphorylation, which targets p27 for degradation or sequesters it in the cytoplasm, where it is unable to fulfill its nuclear function as a CKI (3, 4). p27 can be phosphorylated on T157 by AKT, causing it to localize to the cytoplasm (6, 7). The correlation

Translational Relevance

Currently, there are no molecular markers that meet the College of American Pathologists criteria for use in patient management or with sufficient biological or clinical validation for acceptance in RCC. Data presented in this report suggest that p27 expression and localization may be a useful biomarker for RCC, warranting further clinical validation. In addition, mTOR inhibitors have received great attention recently for preclinical efficacy and promise in clinical trials for RCC, and temsirolimus (Torisel) has now been approved for treatment of advanced RCC. However, the fact that PI3K/AKT but not mTOR inhibitors relocalize p27 to the nucleus and induce apoptosis suggests that therapeutics that inhibit PI3K/AKT may have improved therapeutic efficacy over inhibitors that target mTOR alone.

of high levels of AKT and cytoplasmic p27 in breast cancer may account for unrestricted cyclin-dependent kinase 2 activity in the nucleus of these tumors to enable continuous progression through the cell cycle (8). Recently, it has become appreciated that p27 sequestered in the cytoplasm exhibits gain-of-function in tumors, which, in contrast to the tumor suppressor function of nuclear p27, serves to inhibit apoptosis and regulate the decision to undergo an autophagic survival program (9, 10).

Renal cell carcinoma (RCC) cell lines and primary tumors exhibit activation of the phosphatidylinositol 3-kinase (PI3K)/AKT signaling pathway and elevated mammalian target of rapamycin (mTOR) activity (11–13). The PTEN tumor suppressor, which negatively regulates PI3K signaling, may also participate in aberrant PI3K/AKT signaling in RCC. Although PTEN mutations are rare in RCC, PTEN expression is frequently reduced (14, 15) and correlates with increased phosphorylation/activation of AKT in these tumors (16). However, although p27 is known to be a target of PI3K/AKT signaling, little is known about the effect of aberrant PI3K/AKT signaling on p27 localization and function in RCC.

We report here that activation of AKT signaling is a consistent feature of RCC-derived cell lines and primary tumors. AKT activation in RCC correlated with phosphorylation at T157 and cytoplasmic sequestration of p27. The presence of cytoplasmic p27 also correlated with tumor grade in RCC patients. In RCC cell lines, inhibition of PI3K/AKT signaling induced nuclear relocalization of p27, whereas mTOR inhibition had no effect on localization of this CKI. AKT activation and phosphorylation of p27 also correlated with resistance to apoptosis in RCC cell lines, with relocalization of p27 by PI3K/AKT inhibitors or small interfering RNA (siRNA) knockdown of p27 increasing sensitivity to apoptosis in these cells.

Materials and Methods

Antibodies and reagents. Phospho-S6 (S235/236), S6, phospho-AKT (S473), and AKT antibodies were purchased from Cell Signaling Technology. p27 (K5020) antibody was from BD Transduction Laboratories. β -Actin and lamin A/C antibodies were from Santa Cruz Biotechnology. Phospho-p27 T157, T198, and total p27 antibodies

were from R&D Systems, and LDH antibody was from Chemicon. Secondary antibodies conjugated to horseradish peroxidase were purchased from Santa Cruz Biotechnology and secondary antibodies conjugated to FITC and Texas red were purchased from Jackson ImmunoResearch Laboratories. LY294002 (20 μ mol/L), wortmannin (200 nmol/L), and rapamycin (0.2–2 μ mol/L) were purchased from Sigma and resuspended in DMSO as the vehicle.

Cell lines. VHL^{+/+} Caki-1 cells were cultured in McCoy's 5A and ACHN cells were grown in MEM supplemented with 10% fetal bovine serum (Hyclone). VHL^{-/-} RCC4 cells were cultured in DMEM and 786-O cells were maintained in RPMI with 10% fetal bovine serum. All cells were from the American Type Culture Collection and media were purchased from Invitrogen. Normal and tumor-matched UMRC2 and UMRC5 to UMRC7 renal cell lines were a kind gift from H. Barton Grossman (The University of Texas M. D. Anderson Cancer Center) and maintained in α MEM supplemented with 10% fetal bovine serum, nonessential amino acids, sodium pyruvate, and penicillin-streptomycin-amphotericin B at 37°C in a humidified atmosphere with 5% CO₂.

Western analysis and cell fractionation. Tissue lysates were made in radioimmunoprecipitation assay buffer (1 \times PBS, 0.1% SDS, 1% NP-40, and 12 mmol/L sodium deoxycholate) and cell lines were lysed in 1 \times lysis buffer [20 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% Triton X-100, and 2.5 mmol/L sodium pyrophosphate]. All buffers contained 1 \times complete protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktails 1 and 2 (Sigma).

For fractionation, cells were washed with ice-cold PBS and scraped into chilled hypotonic buffer [10 mmol/L HEPES (pH 7.2), 10 mmol/L KCl, 1.5 mmol/L MgCl₂, and 0.1 mmol/L EGTA] and broken by a Dounce homogenizer. After centrifugation at 3,000 rpm, the supernatant was collected (cytoplasmic fraction) and the crude nuclei pellet was further homogenized in hypotonic buffer and washed in nuclear washing buffer [10 mmol/L Tris-HCl (pH 7.4), 0.1% NP-40, 0.05% sodium deoxycholate, 10 mmol/L NaCl, and 3 mmol/L MgCl₂] and centrifuged at 3,000 rpm. Purified nuclei were lysed in high-salt lysis buffer [20 mmol/L HEPES (pH 7.4), 0.5 mol/L NaCl, 0.5% NP-40, and 1.5 mmol/L MgCl₂]. All buffers contained 1 \times complete protease inhibitor cocktail and phosphatase inhibitor cocktails 1 and 2.

Immunohistochemistry and tissue microarrays. Tissue microarrays were generated using a Beecher instrument with 0.6 mm cores taken from the donor block and placed into the recipient block in triplicate for each case. For each block, there were four observations: "involvement," "intensity," grade, and location. Tissue microarray images were acquired using the BLISS system (Bacus Laboratories) and were scored using the TAD system (Biostatistics Department, The University of Texas M. D. Anderson Cancer Center) and the Biogenex iVISION image analysis system. Cores were scored according to presence or absence of tumor, degree of tumor involvement (continuous variable), staining intensity (none, low, mid, and high), and location of staining (cytoplasmic, nuclear, or a combination thereof).

For immunohistochemistry, unstained slides were placed in a 60°C oven for 20 min, air dried for 5 min, deparaffinized and rehydrated by three changes of xylene and 100% ethanol and two changes of 80% ethanol, rinsed three times with deionized water, and placed in TBS. Slides were then placed in a 3% hydrogen peroxide blocking solution for 5 min, treated with heated 10 mmol/L citrate buffer (pH 6.0) for 45 min, cooled for 20 min, and rinsed with deionized water and TBS. The primary antibody was incubated with the tissue for 1 h, rinsed with deionized water three times, and placed in TBS. Secondary antibody was incubated for 30 min, rinsed three times with deionized water, and placed in TBS. Excess buffer was removed and DAB applied to tissue, incubated for 8 min, and rinsed with deionized water. DAB enhancer was followed with a rinse with deionized water and counterstained with H&E.

Immunocytochemistry. Localization of endogenous p27 was determined by immunofluorescence analysis with biotin-avidin signal amplification in RCC cell lines. Cells were plated onto glass chamber slides 18 to 24 h before treatment and treated as indicated. Cells

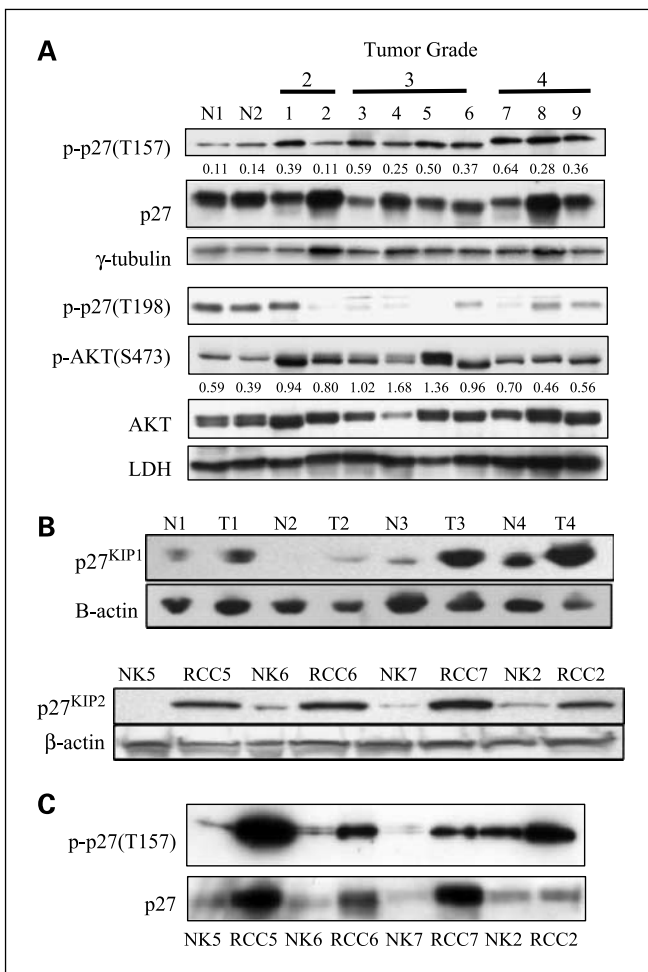


Fig. 1. p27 expression in RCC. **A**, Western analysis of p27, phospho-p27 (T157), AKT, and phospho-AKT (S473) in normal kidney and grades 2 to 4 RCC. Relative ratio of phosphorylation at T157 (p27) and S473 (AKT) was calculated using densitometric analysis. **B**, Western analysis of 4 representative pairs of normal and patient-matched stage II RCC (*top*) and cultured cells (*bottom*) showing increased p27 expression, which was observed in tumor cells of 6 of 8 patients. RCC5 and RCC7 are derived from stage III tumors, RCC6 is from stage I, and RCC2 is from stage IV. **C**, Western analysis for p27 phosphorylation at T157 in normal and patient-matched tumor cells from **B** showing increased p27 phosphorylation at T157 in tumor cells.

were then fixed in 4% paraformaldehyde in PBS for 30 min at 4°C and permeabilized in 0.5% Triton X-100/PBS for 10 min at room temperature. Nonspecific antigens were blocked for 1 h in PBS containing 7.5% bovine serum albumin at 37°C, and endogenous biotin-avidin was blocked using Avidin/Biotin blocking kit (Zymed Laboratories, Invitrogen). Subsequent staining was as described previously (17) with anti-p27 (K5020) primary antibody, biotin-conjugated donkey anti-mouse antibody (Jackson ImmunoResearch Laboratories), and FITC-conjugated streptavidin (Invitrogen).

Apoptosis assays. Cellular protein lysate (40 μ g) was added to a 200 μ L reaction mixture containing the caspase substrate, 50 μ mol/L Ac-DEVD-AFC (Biomol) in 1 \times caspase reaction buffer (25 mmol/L HEPES, 50 mmol/L NaCl, 0.05% CHAPS, 5 mmol/L DTT, 0.5 mmol/L EDTA, and 5% glycerol) for 90 min at 37°C. Production of cleaved AFC by activated caspase in lysates was measured using a FL6000 Microplate Fluorescent Reader (Bio-Tek) with an excitation wavelength of 400 nm and an emission wavelength of 505 nm. Fluorimetric units were converted to μ mol/ μ g protein. Results are presented as fold activation above the control.

siRNA experiments. Chemically synthesized siRNA SMARTpool of human p27 was purchased from Dharmacon (Thermo Fisher Scientific) and the negative control siRNA used was siCONTROL (RISC-free #1, D-001220-01). 786-O cells were plated in 6-well plates 24 h before transfection, and transfection was done as described previously (17). Knockdown efficiency was determined by Western analysis 48 h post-transfection.

Statistical methods. Descriptive statistical analyses were carried out, including percentage, proportion, mean, and SD. χ^2 and t tests were used in univariate analyses of categorical and continuous variables, respectively. For the data analysis on multiple observations from a subject, a mixed-effects model was used to assess the tumor grade with the correlated data, and a logistic regression model was used to assess intensity (high versus low) by the generalized estimating equation method (18), which is implemented in GENMOD procedure in SAS. This model allows the user to account for intrasubject correlations among repeated measurements on the same subject (the exchangeable working correlation matrix was specified in this study).

Results

Expression and cytoplasmic sequestration of p27 in RCC. Two series of primary tumors (The University of Texas M. D. Anderson Cancer Center and the Mayo Clinic) from RCC patients were evaluated for p27 expression. Initially, Western analysis was done to assess p27 expression in tumors relative to normal kidney. As shown in Fig. 1A, expression of p27 in primary tumors was high but heterogeneous, appearing comparable with that of normal kidney, which was confirmed by quantitative assessment of Western blot intensities relative to loading controls (data not shown). To investigate the possible contribution of interindividual differences to the observed heterogeneity in p27 expression levels, we also evaluated p27 expression in several patient-matched primary tumors and uninvolved kidney. As shown by representative patient-matched tissues in Fig. 1B, although p27 levels varied between tumors of multiple stages, p27 expression was higher in all tumors relative to patient-matched normal tissue. This pattern of elevated p27 expression was observed in 6 of 8 stage II RCC examined relative to matched uninvolved kidney. Concordant with these data from primary tumors, *in vitro* cultures of isolated tumor cells expressed abundant p27, whereas this CKI was undetectable or expressed at very low levels in patient-matched normal cells (Fig. 1B).

Interestingly, in the majority of tumors, phosphorylation of p27 at T157 was increased relative to normal kidney ($P \leq 0.03$, t test; Fig. 1A), a site in the NLS of p27 known to regulate cytoplasmic localization (5). T157 is an AKT phosphorylation site (3–6), and tumors were confirmed by Western analysis to express phosphorylated (active) AKT (Fig. 1A). Although tumors again exhibited heterogeneity in levels of phospho-AKT, overall phospho-AKT was higher in tumors relative to normal kidney ($P \leq 0.04$, t test), consistent with the observed elevated T157 p27 phosphorylation in these tumors (Fig. 1A). Elevated T157 phosphorylation of p27 was also observed in cultured tumor cells relative to patient-matched normal controls (Fig. 1C). In contrast, another phosphorylation site of p27, T198, known to be phosphorylated by AMPK (10) rather than AKT, exhibited no increase in phosphorylation in tumors compared with normal tissues (Fig. 1A).

It has been shown in other tumors that AKT phosphorylation at T157 in the p27 NLS causes it to become sequestered in the

cytoplasm, where it is unable to inhibit its cyclin-dependent kinase targets in the nucleus (5, 8). Abundant p27 expression in RCC accompanied by phosphorylation at T157 suggested that functional inactivation of this CKI could be occurring in these tumors due to cytoplasmic sequestration; therefore, we investigated p27 localization in these tumors. We observed that, in many RCC, determination of p27 localization patterns by immunohistochemistry was confounded by the fact that the glycogen accumulation characteristic of the clear cell RCC phenotype often appeared to be occluding p27 immunoreactivity from the majority of the cytoplasm, compressing it to the cell periphery. Nevertheless, in most cases, it was possible to discern cytoplasmic from nuclear staining in a significant proportion of cells within the tumors and score tumors based on their p27 localization pattern. As determined by immunohistochemistry of paraffin-embedded tumors (Fig. 2A) and tissue microarrays (Fig. 3; Table 1), the majority of tumors

exhibited cytoplasmic immunoreactivity for p27. Importantly, cytoplasmic versus nuclear localization of p27 correlated significantly ($P \leq 0.001$) with tumor grade, with loss of nuclear p27 increasing with tumor grade (Table 1). Immunohistochemistry also confirmed that, similar to what was observed by Western analysis, tumors expressed higher levels of phospho-AKT than uninvolved kidney (Fig. 2B). p27 localization was also compared with levels of AKT immunoreactivity using tissue microarrays. Specificity for AKT immunoreactivity was shown in normal kidney cores and between low-grade and high-grade RCC (Supplementary Fig. S1). Figure 3 illustrates staining patterns of tumors with predominately nuclear p27 and low AKT (total and phospho) activity (*top*) and tumors with predominately cytoplasmic p27 and high AKT (*bottom*). Although, in general, tumors with predominately cytoplasmic p27 had the highest scores for total AKT (26% of cores with cytoplasmic p27 had strong AKT immunoreactivity versus

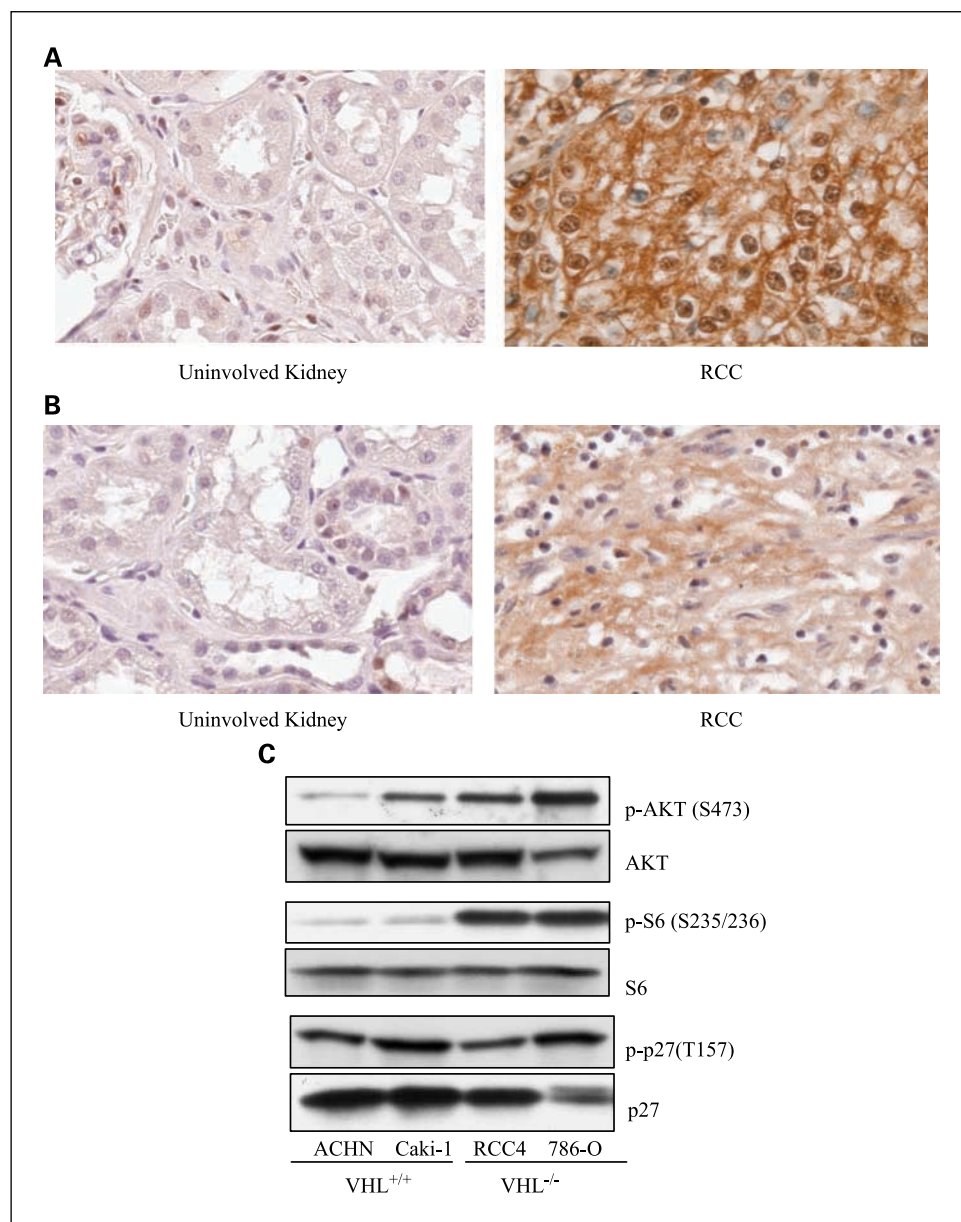
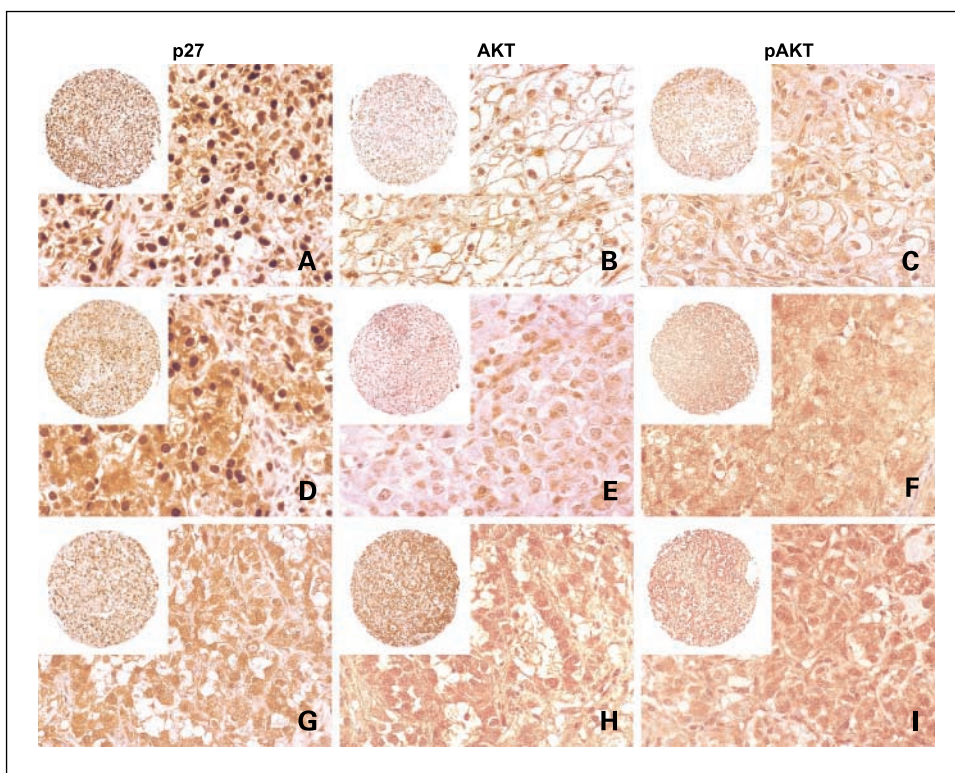


Fig. 2. p27 and AKT expression in RCC. **A**, immunohistochemistry of representative normal and patient-matched RCC illustrating expression and cytoplasmic p27 localization in tumor cells. **B**, immunohistochemistry of representative normal and patient-matched RCC illustrating elevated phospho-AKT expression. **C**, p27 and AKT expression and phosphorylation in RCC cell lines.

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Fig. 3. p27 and AKT immunoreactivity in RCC. *A to C*, low-grade RCC from a single block. p27 is predominantly nuclear. *D to F*, intermediate-grade tumors from a single block. Stronger cytoplasmic and weaker nuclear p27 staining is observed. AKT and phospho-AKT staining is more intense. *G to I*, high-grade tumors from a single block. p27 nuclear staining is absent, and AKT and phospho-AKT staining is high intensity.



13-0% of cores with nuclear p27) and phospho-AKT (33% of cores with cytoplasmic p27 had strong phospho-AKT immunoreactivity versus 23-10% of cores with nuclear p27) staining (Supplementary Table S1), intratumor and intertumor heterogeneity within the 20 cases examined prevented these data from reaching statistical significance. Therefore, we turned to RCC-derived cell lines to further investigate a possible linkage between AKT activity and p27 phosphorylation and cytoplasmic localization in RCC.

p27 phosphorylation at T157 correlates with localization to the cytoplasm in RCC cell lines. To determine if cytoplasmic sequestration of p27 in RCC was a result of AKT phosphorylation at T157, we first determined if, like primary tumors, AKT was active and p27 localized to the cytoplasm in RCC-derived cell lines. p27 was phosphorylated at T157 in all four RCC cell lines examined (Fig. 2C), indicating that, similar to what was observed in primary RCC and tumor cell explants, phosphorylation of p27 at T157 was a consistent feature of RCC cell

lines. As shown in Fig. 2C, AKT was phosphorylated (active) in both VHL-negative cell lines 786-O and RCC4 and VHL-positive Caki-1 and ACHN RCC cell lines. The highest levels of AKT phosphorylation were observed in RCC4 and 786-O cells, which also had the highest levels of phospho-S6, indicating that these cell lines also had the highest levels of mTOR signaling, a downstream effector of AKT.

We next examined p27 localization in RCC cell lines. Subcellular fractionation (with lamin A/C and LDH as controls for nuclear or cytoplasmic localization, respectively) revealed that p27 was sequestered in the cytoplasm in these cells (Fig. 4A and B). ACHN, Caki-1, and RCC4 expressed abundant p27 that was exclusively cytoplasmic, as did 786-O cells, although the amount of total p27 expressed by 786-O cells was lower than the other cell lines (Figs. 2C and 4A). Because small proteins such as p27 can leak from the nucleus during subcellular fractionation, cytoplasmic localization of p27 was also confirmed by immunocytochemistry. Endogenous p27 was

Table 1. Nuclear localization of p27 decreases with increasing tumor grade

p27 localization	No. samples	No. (%) samples by tumor grade			Fitted mixed model	
		2	3	4	Mean (SD)	P
C	21	2 (4)	7 (12)	12 (23)	3.40 (0.17)	<0.001
CN	45	1 (2)	19 (33)	25 (47)	3.23 (0.16)	
N	30	20 (43)	10 (18)	0 (0)	2.80 (0.16)	
NC	60	23 (50)	21 (37)	16 (30)	2.83 (0.15)	

NOTE: Cores from 20 patients with 3 blocks each and 3 cores per block were used to record effect of grade on p27 localization. For grade 2 tumors, 43% had nuclear-only staining, whereas none of the grade 4 tumors had nuclear-only staining.

Abbreviations: C, cytoplasmic only; CN, predominantly cytoplasmic with some nuclear; N, nuclear only; NC, predominantly nuclear with some cytoplasmic.

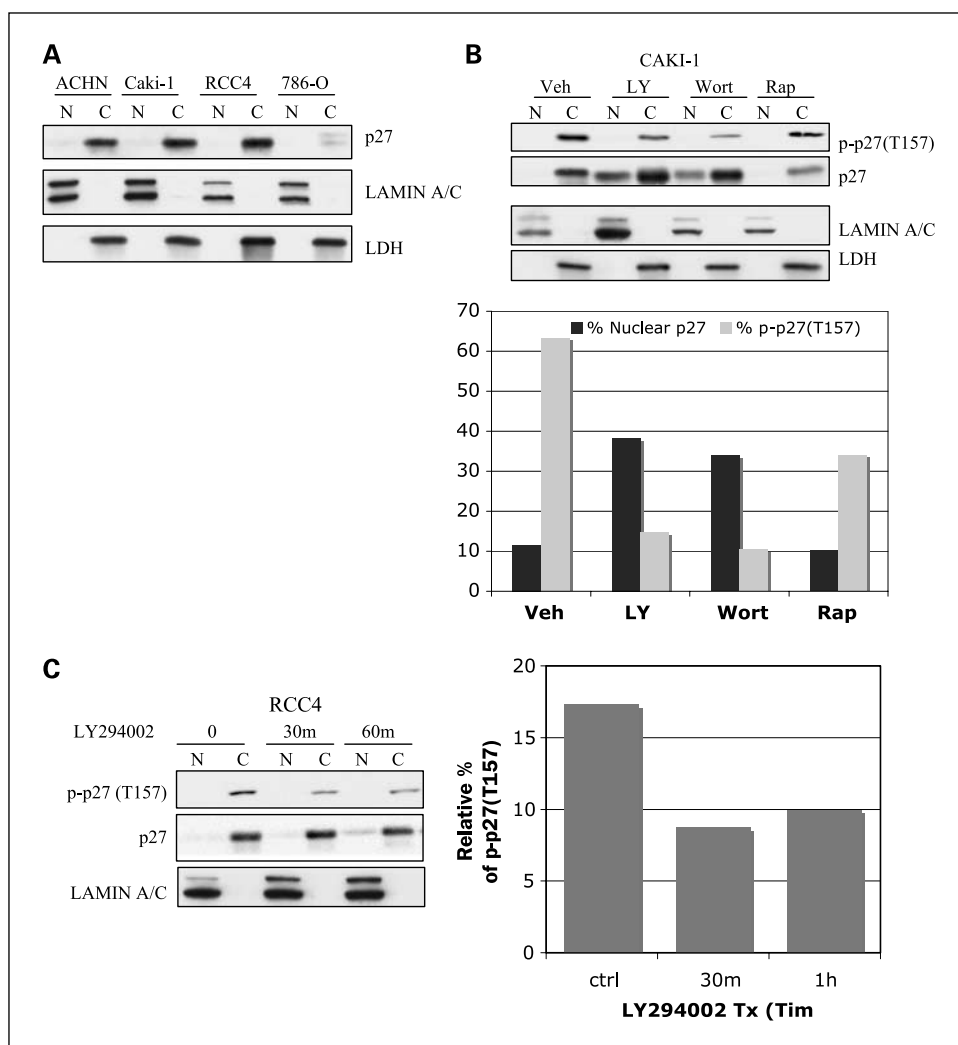


Fig. 4. PI3K inhibitors reduce phosphorylation at T157 and restore nuclear localization of p27. **A**, localization of p27 in RCC cell lines. All cell lines tested exhibit cytoplasmic localization of p27. Lamin A/C and LDH are used as nuclear and cytoplasmic markers, respectively. **B**, LY294002 and wortmannin treatment reduce phosphorylation of p27 at T157 and relocalize p27 to the nucleus, whereas rapamycin, a mTOR inhibitor, does not. The densitometric analysis done to measure the ratio of nuclear p27 or phospho-p27 versus total p27 also confirms that PI3K/AKT inhibition but not mTOR inhibition relocates p27 into nuclei, which correlates with decreased p27 phosphorylation at T157. **C**, phosphorylation of cytoplasmic p27 at T157 is rapidly (≤ 30 min) decreased after LY294002 treatment in RCC4 cells, concomitant with nuclear relocalization of p27. Similar data were obtained with other RCC cell lines (data not shown).

diffusely localized in RCC cells, with only an occasional cell ($<5\%$) exhibiting strong nuclear immunoreactivity (Fig. 4E; data not shown), confirming results obtained by subcellular fractionation demonstrating nuclear exclusion of p27.

Inhibition of PI3K/AKT but not mTOR signaling restores nuclear localization of p27. Because phosphorylation of p27 by AKT at T157 has been shown to sequester p27 in the cytoplasm (3–5), we next determined if PI3K/AKT signaling in RCC might be responsible for exclusion of p27 from the nucleus. Although none of the inhibitors altered the cell cycle profile of treated cells at 24 h (Supplementary Fig. S2), as shown in Fig. 4B, inhibition of PI3K by the pharmacologic inhibitors LY294002 or wortmannin (Supplementary Fig. S3) reduced cytoplasmic levels of p27 phosphorylated at T157, whereas the mTOR inhibitor, rapamycin, had no effect on p27 phosphorylation. Importantly, treatment with PI3K inhibitors that reduced T157 phosphorylation caused a rapid increase in nuclear p27 (Fig. 4B–D), which was detectable as early as 30 min after treatment with LY294002 or wortmannin (Fig. 4C and D; data not shown). In addition, whereas LY294002 and wortmannin increased the total amount of p27 expressed between 1 and 24 h (Fig. 4B and D), densitometric analysis showed that the fold increase of p27 in the nucleus (~ 30 -fold)

greatly exceeded that of cytoplasmic p27 (<5 -fold) in RCC cells (Fig. 4E). Moreover, inhibition of AKT (Supplementary Fig. S3) and nuclear relocalization of p27 by PI3K/AKT inhibitors was inversely correlated with levels of p27 phosphorylation at T157 (Fig. 4B).

Immunocytochemistry confirmed cellular fractionation data and showed reappearance of p27 in the nucleus of cells treated with either LY294002 or wortmannin. For 786-O, RCC4, Caki-1, and ACHN cells, relocalization of p27 to the nucleus on PI3K/AKT inhibition was quantitated in response to treatment with these inhibitors. As shown in Fig. 4E, treatment with both LY294002 and wortmannin significantly increased p27 localization to the nucleus. In 786-O and RCC4 cells, the percentage of cells with nuclear p27 increased from 12% and 13%, respectively, to 42% and 52%, following treatment with LY294002. Wortmannin induced a similar increase in nuclear p27 in treated cells. Caki-1 and ACHN also exhibited relocalization of p27 in the presence of these inhibitors, with LY294002 increasing the percentage of Caki-1 cells with nuclear p27 from 5% to 61% and wortmannin also increasing the percentage of cells with nuclear p27 to 30%. ACHN cells appeared to be most sensitive to the effect of inhibitor, with LY294002 and wortmannin increasing the percentage of cells

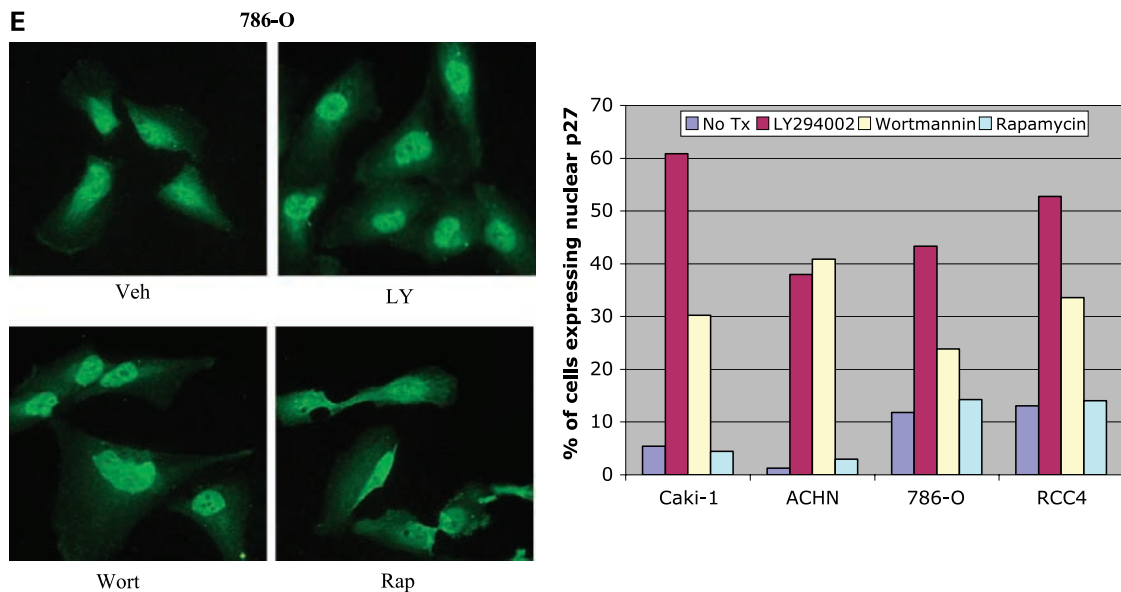
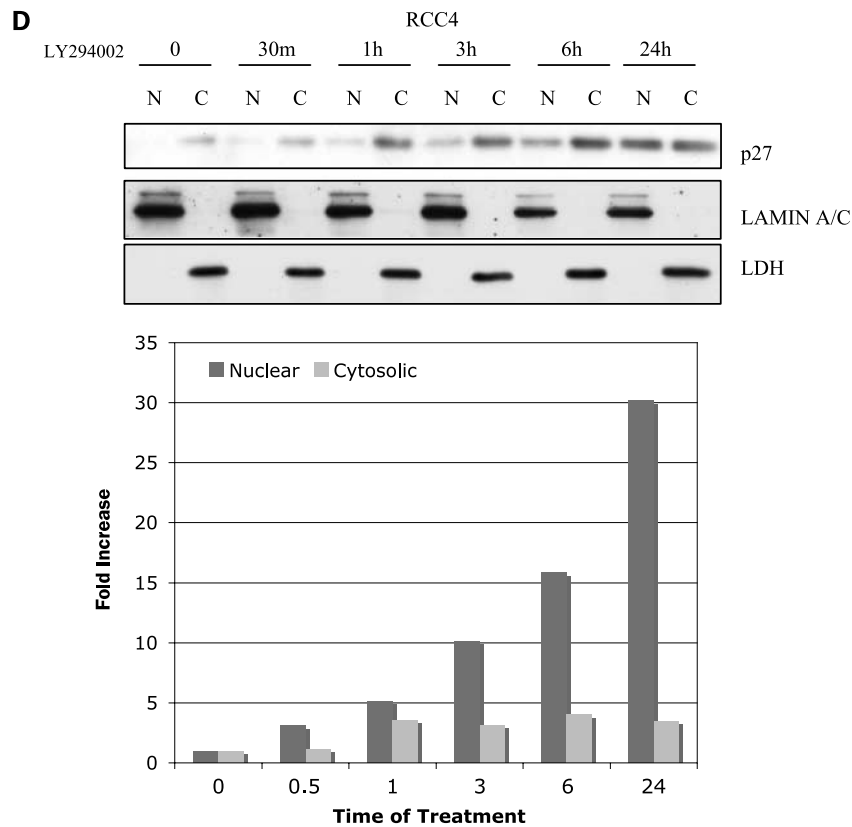


Fig. 4 *Continued.* *D*, nuclear relocalization of p27 after LY294002 treatment increases as a function of time in RCC4 cells. Similar data were obtained with other RCC cell lines (data not shown). The increase in nuclear p27 is much greater than that of cytoplasmic p27. *E*, immunohistochemistry of p27 showing increased percentage of nuclear staining with PI3K/AKT but not mTOR inhibition. The percentage of cells shows nuclear staining in each treatment group. Representative experiment ($n = 2$; >500 cells counted per cell line).

with nuclear p27 from 2% to 38% and 41%, respectively. Importantly, in contrast to PI3K/AKT inhibitors, the mTOR inhibitor rapamycin had no effect on p27 localization in RCC cells as determined by cell fractionation (Fig. 4B) or by

immunocytochemistry (Fig. 4E). There was no difference in cell cycle distribution of 786-O cells treated with LY294002, wortmannin, or rapamycin 24 h after treatment (Supplementary Fig. S2), showing that differences in p27 localization did

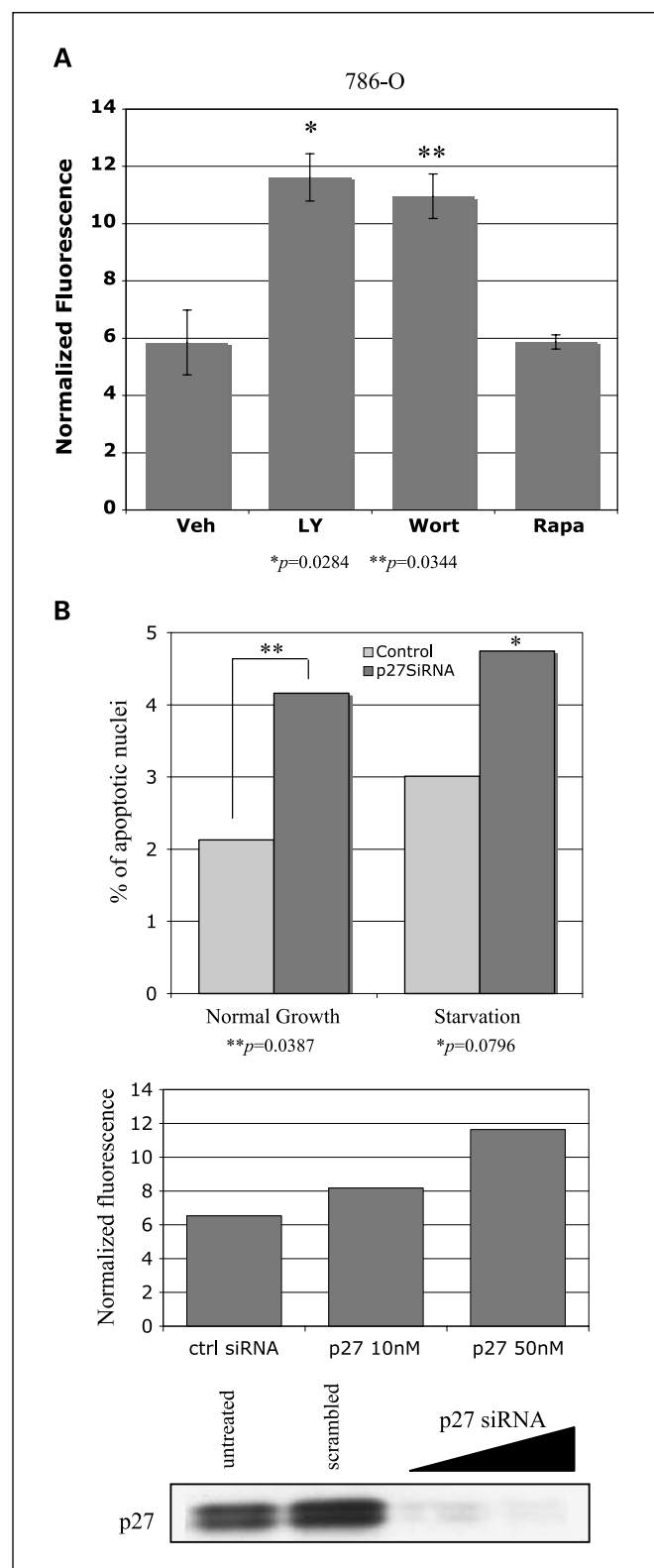


Fig. 5. Cytoplasmic p27 correlates with resistance to apoptosis in RCC cell lines. *A*, caspase activity (as determined by fluorescence of cleaved caspase substrate) is increased in 786-O cells after 6 h treatment with either LY294002 (20 $\mu\text{mol/L}$) or wortmannin (200 nmol/L) but not with rapamycin (2 $\mu\text{mol/L}$). *B*, knockdown of p27 induces caspase activity in 786-O cells. Western blot of p27 verifying siRNA knockdown of p27. Increased apoptosis in p27 siRNA-treated 786-O cells is shown by increased apoptotic nuclei (*top*) and increased fluorescence of caspase-cleaved substrate (*middle*).

not occur as a result of differential induction of cell cycle arrest by these compounds.

p27 confers resistance to apoptosis in RCC cells. p27 has been reported to exhibit gain-of-function and serve an antiapoptotic role in tumors when phosphorylated and sequestered away from the nucleus (9, 10). To investigate a potential antiapoptotic function for p27 in RCC, we investigated the relationship between cytoplasmic sequestration of p27 out of the nucleus and resistance to apoptosis in RCC cell lines. Treatment of 786-O cells with either LY294002 or wortmannin (which inhibits AKT and relocalizes p27 to the nucleus; see above) caused a significant increase in apoptosis as assessed by caspase cleavage that was not observed with rapamycin (which does not relocalize p27 to the nucleus; Fig. 5A). Similar results were obtained with Caki-1 cells (data not shown). To show that p27 was involved in resistance to apoptosis in these cells, siRNA experiments were done to knockdown p27 in 786-O cells. As shown in Fig. 5B, caspase cleavage increased with p27 depletion under both basal and serum starvation conditions, as did the number of apoptotic nuclei, indicating that p27 directly contributes to resistance to apoptosis in RCC cells.

Discussion

We found that elevated p27 expression and activation of AKT were consistent features of RCC, with cytoplasmic mislocalization of this CKI significantly correlating with higher tumor grade. RCC-derived cell lines, which also exhibited activated AKT and cytoplasmic sequestration of p27, were used to show that phosphorylation of p27 at T157 of the p27 NLS correlated with cytoplasmic localization and that PI3K inhibitors that inhibited AKT activity reduced T157 phosphorylation and relocalized p27 to the nucleus. Importantly, the mTOR inhibitor rapamycin, which did not decrease AKT activity, was ineffective at reducing p27 phosphorylation at T157 and reversing cytoplasmic mislocalization of p27.

Understanding the role of PI3K/AKT signaling and activation of mTOR in RCC is important, as mTOR inhibitors have shown preclinical efficacy (13) and promise in clinical trials for RCC (19–23), with temsirolimus (Torisel) being recently approved for treatment of advanced RCC. RCC primary tumors and tumor-derived cell lines exhibit activation of PI3K/AKT signaling (11–13), and whereas PTEN mutations are rare in RCC, PTEN expression is frequently reduced (14, 15) and decreased PTEN expression is associated with increased AKT activation in RCC (16). Although activation of PI3K/AKT and mTOR signaling has been considered in the context of activation of hypoxia-inducible factor in RCC (24), the effect of activation of this signaling pathway on p27 function, and the downstream prognostic and therapeutic implications in RCC, have not been investigated.

AKT is antiapoptotic, directly activating several cell survival pathways; recently, we and others have shown that, under certain conditions, p27 is also antiapoptotic (9, 10). The present study and similar data in rodent models indicate that, as a result of activation of kinases that phosphorylate p27, this CKI is sequestered from the nucleus and acquires a gain-of-function antiapoptotic role (25). Thus, it is interesting to speculate that, in conjunction with AKT activation, in RCC, p27 phosphorylated by this kinase may contribute to cell survival and resistance to drugs that induce apoptosis. Although p27

was not evaluated, Sourbier et al. (12) also found that PI3K inhibitors, which we have shown to decrease p27 phosphorylation and relocalize it to the nucleus, reduced AKT phosphorylation and induced apoptosis in 786-O cells *in vitro*.

These data suggest the interesting possibility that therapeutics that inhibit AKT and relocalize p27 to the nucleus may have improved therapeutic efficacy over mTOR inhibitors alone (which do not inhibit AKT or relocalize p27 to the nucleus) in patients where p27 contributes to tumor survival.

Prognostic factors for RCC include histologic subtype, tumor grade and stage, and clinical characteristics such as performance status, time from diagnosis to systemic therapy, anemia, lactate dehydrogenase levels, hypercalcemia, and location and number of metastases (26–29). As defined by Steeg and Abrams (30), clinical markers must be (a) reproducible, (b) provide prognostic information independent from and better than other conventional pathologic criteria, and (c) provide information that can influence treatment decisions. Currently, there are no molecular markers for RCC that meet the College of American Pathologists criteria for use in patient management or that has sufficient biological or clinical validation for acceptance (31). However, several prognostic and predictive biomarker candidates are in various stages of validation for RCC including proliferating cell nuclear antigen, Ki-67, silver staining of nucleolar organizing regions (32–35), molecular markers such as VHL status (36), carbonic anhydrase (37, 38), PTEN (39), and protein expression profiles (40).

Data that loss of nuclear p27 correlates with tumor grade suggest that p27 expression and/or localization may also be useful biomarker(s) for RCC, warranting further clinical validation. The utility of p27 as a prognostic marker has been validated for many cancers (for a review, see ref. 41), including breast, gastrointestinal, prostate, lung, and ovarian cancers. Recently, p27 has received interest as a potential prognostic marker for RCC, although previous studies have generally scored p27 as present or absent rather than its localization within the cell (42–47). Although these data are not altogether concordant, in general, the absence of p27 confers a worse prognosis. However, our data suggest that localization may also be important and that some of the discrepancies in the literature could be attributable to differences between p27-positive tumors where p27 is cytoplasmic (and presumably nonfunctional) and p27-positive tumors where p27 is nuclear. Interestingly, a recent study examining expression of p27 and Skp2 (the E3 ligase that targets p27 for degradation in the nucleus) found that, in general, Skp2 activity increased with stage and grade in clear cell carcinoma, whereas p27 immunoreactivity decreased with stage and grade (46). Although this study noted that there was no correlation between p27 and Skp2 expression in

individual tumors, as Skp2 would be responsible for degradation of nuclear but not cytoplasmic p27, additional analysis as to whether p27 expressed in Skp2 positive tumors was in the nucleus versus cytoplasm would likely lend clarity to the interpretation of these data. Importantly, in a recent report from Pantuck et al. (48), in a series of tissue microarrays, 48% of RCC had cytoplasmic p27 and cytoplasmic localization of p27 was higher in metastatic RCC. In the cohort of patients examined in that study, higher nuclear p27 also predicted a more favorable outcome. Similar to our analysis, these authors also concluded that cytoplasmic mislocalization of p27 is a poor prognostic finding and possibly an important variable for patient selection for targeted therapy.

Additionally, up-regulation of AKT in primary RCC tumors is associated with poorer outcome (49). This observation underscores the potential prognostic significance of AKT activation in RCC and is concordant with our hypothesis that part of the phenotype associated with activated AKT is phosphorylation and cytoplasmic mislocalization of p27. Our data suggest that, in RCC, AKT pathway inhibition can relocalize p27 to the nucleus, and this effect is VHL independent. These findings have potential clinical implications in the choice of agents for patients with RCC. Recent data suggest that temsirolimus is relatively more active in patients with papillary versus clear cell RCC (50). It is possible the lack of response in these patients is not due to VHL status *per se* but the functional status of p27. Thus, additional studies are warranted to evaluate the effect of p27 status on response and resistance to rapamycin or other targeted therapy in RCC.

In summary, we have shown that, in RCC, nuclear exclusion of p27 and AKT phosphorylation confers resistance to apoptosis and preferential sensitivity to AKT inhibition compared with mTOR inhibition. These data provide important insights regarding the mechanism of p27 mislocalization in RCC and its potential utility as a predictive biomarker. Furthermore, p27 status may be relevant for choosing tyrosine kinase inhibitors alone or in combination with mTOR inhibitors in the clinical management of RCC. Additional studies are now warranted to validate these preclinical observations and test these hypotheses in RCC patients treated with targeted agents.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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