Low Expression of $p27^{Kip1}$ Is Associated with Tumor Size and Poor Prognosis in Patients with Renal Cell Carcinoma

Toshiro Migita, M.D.[1]
Yoshinao Oda, M.D., Ph.D.[1]
Seiji Naito, M.D., Ph.D.[2]
Masazumi Tsuneyoshi, M.D., Ph.D.[1]

[1] Department of Anatomic Pathology, Kyushu University, Fukuoka, Japan.

[2] Department of Urology, Kyushu University, Fukuoka, Japan.

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Address for reprints: Masazumi Tsuneyoshi, M.D., Ph.D., Department of Anatomic Pathology (Second Department of Pathology), Pathological Sciences, Graduate School of Medical Sciences, Kyushu University, Maidashi 3-1-1, Higashi-ku, Fukuoka 812-8582, Japan; Fax: +81-92-642-5968; E-mail: masazumi@surgpath.med.kyushu-u.ac.jp.

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BACKGROUND. Proliferative activity in tumors depends on regulation of the cell cycle. $p27^{Kip1}$ (p27) plays a pivotal role as a negative regulator of the cell cycle. A decrease in p27 expression has been reported in many kinds of tumors, but little is known regarding p27 in patients with renal cell carcinoma (RCC).

METHODS. Expression of p27 and the related cyclins (cyclin A, cyclin E, and cyclin D1) was examined immunohistochemically in 67 patients with of clear cell RCC. The Ki-67 labeling index (MIB-1 LI) and clinicopathologic parameters related to a poor prognosis also were analyzed. To determine their prognostic significance, univariate and multivariate survival analyses were performed.

RESULTS. In tumors, there was considerable immunoreactivity for cyclin A, cyclin D1, and MIB-1, and the mean values for each were 1.08%, 16.1%, and 1.5%, respectively. Cyclin E expression was rare. The expression of p27 was correlated strongly with the expression of cyclin A (correlation coefficient, 0.432; $P < 0.0004$) and cyclin D1 (correlation coefficient, 0.476; $P < 0.0004$). Also, an inverse correlation was present between p27 expression and tumor size ($P = 0.0377$). In univariate analysis, the unfavorable prognostic factors were high TNM stage ($P < 0.0001$), large tumor size ($P = 0.0016$), high histologic grade ($P = 0.0104$), and low p27 expression ($P < 0.0001$). In multivariate analysis, high TNM stage ($P = 0.0035$) and low p27 expression ($P = 0.0235$) were independent prognostic factors for disease specific survival in patients with RCC.

CONCLUSIONS. The results of this study suggest that low p27 expression may be a significant and independent, unfavorable prognostic factor in patients with renal cell carcinoma. ***Cancer* 2002;94:973–9.** © *2002 American Cancer Society.*
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KEYWORDS: renal cell carcinoma, $p27^{Kip1}$, cyclin dependent kinase inhibitor, immunohistochemistry.

Numerous studies concerning the prognostic markers for renal cell carcinoma (RCC) have been reported; however, there is still no consensus with regard to the prognostic significance of these markers, with the exception of nuclear grade and tumor stage.[1] Cell proliferation is strictly regulated by a cell cycle control mechanism that depends on the activities of the G1 cyclins and the cyclin dependent kinase (CDK) complexes. These complexes are regulated both positively and negatively. Cyclin A, in association with CDK2, is expressed from the S-phase to the M-phase. Cyclin D1, in association with CDK2 and CDK4, is expressed from the late phase of G1 to M. Cyclin E, in association with CDK2, is expressed at the G1-to-S transition. Ki-67, as a useful proliferative marker, is expressed in cells at all stages of the cell cycle, but not in G0 cells. Correlation between the expression of

cyclins and tumor progression has been reported in many kinds of malignant tumors, including RCC.[2–5] Conversely, $p27^{Kip1}$ (p27), a member of the Cip/Kip family proteins, regulates the CDKs and is a major negative regulator of the G1-to-S transition in the cell cycle. The role of p27 as a diagnostic or prognostic marker has been reviewed in human malignancies[6] but has not been reported in RCC. The objective of the current study was to evaluate the expression of p27 and the related cyclins in RCC and to analyze these proteins and clinicopathologic factors to determine the prognostic significance of cell cycle parameters. This is the first report to suggest that p27 may be an independent prognostic marker in RCC.

MATERIALS AND METHODS

Tumor Specimens and Survival Data

Two hundred fifty-six patients with renal cell neoplasms underwent radical nephrectomy between 1989 and 2000, and specimens from these tumors were registered in the Department of Anatomic Pathology, Graduate School of Medical Science, Kyushu University. Each sample had been fixed in formalin, routinely processed, and embedded in paraffin. Sixty-seven clear cell carcinoma specimens that were available for immunohistochemistry and survival analysis were collected for this study. The patients consisted of 40 men and 27 women (average age at diagnosis, 60.7 years). Sixteen patients died of their disease, but 51 patients are still alive. The mean follow-up for the 51 surviving patients was 35 months (range, 2–94 months). Nineteen patients had infiltration of the renal vein, and 12 patients had distant metastases (to the lung in 8 patients, the lymph nodes in 4 patients, bone in 1 patient, and adrenal gland in 1 patient) at the time of surgery. The histopathology and immunohistochemistry of all patients were reviewed by two pathologists (T.M. and M.T.) who were blind to the clinical features and outcome of the patients. All tumor specimens were diagnosed histopathologically as clear cell carcinoma (also known as conventional, common, or nonpapillary carcinoma). All tumors were staged according to the TNM-International Union Against Cancer classification system[7] and were classified and graded according to the World Health Organization criteria.[8] Two tumors that were diagnosed as clear cell carcinoma with sarcomatoid features were included.

Immunohistochemistry

Paraffin sections from representative tumors containing nonneoplastic kidney tissue were deparaffinized and microwave treated for antigen retrieval according to standard procedures. For the immunohistologic detection of p27, cyclin A, cyclin D, and Ki-67, a combination of the standard streptavidin-biotin-peroxidase complex method (Histofine SAB-PO kit; Nichirei, Tokyo, Japan) was performed. Positive reaction was observed with hydrogen peroxide containing 3,3′-diaminobenzidine. Hematoxylin was used as a counterstaining medium. The mouse monoclonal antibodies used were cyclin A (NCL-cyclin A; Novocastra Laboratories, United Kingdom) at a 1:50 dilution, cyclin D1 (NCL-cyclin D1; Novocastra Laboratories) at a 1:25 dilution, cyclin E (Ab-1; Calbiochem, Cambridge, MA) at a 1:100 dilution, p27/Kip 1 (NCL-p27; Novocastra Laboratories) at a 1:20 dilution, and Ki-67 (MIB-1; Dako, Glostrup, Denmark) at a 1:100 dilution. All nuclei that exhibited a distinct strong reactivity were considered positive. In each complete section, 1000 tumor cells were counted manually, and the percentage of positive cells was calculated. We referred to previously reported[3] and well-prespecified criteria regarding the immunohistochemical expression of p27 in various types of tumors and, accordingly, divided the tumors into a group that had p27 expression with a labeling index (LI) $\geq$ 50% (high p27 expression) and a group that had p27 expression with an LI $<$ 50% (low p27 expression). In addition, the tumors were divided into two groups with regard to the other cell cycle-related proteins: high expression ($\geq$ 1%) and low expression ($\leq$ 1%) for cyclin A,[3] high expression ($\geq$ 10%) and low expression ($<$ 10%) for cyclin D1,[4] and high expression ($\geq$ 1%) and low expression ($<$ 1%) for MIB-1.[3]

Statistical Analysis

Statistical analysis was performed using the chi-square test with a Yates continuity correction and the Mann–Whitney *U* test. Correlations between variables were tested according to the Spearman correlation test. The survival calculations were illustrated with Kaplan–Meier curves, and univariate and multivariate survival analyses were performed by using the log-rank test or the Cox proportional hazards regression model. Stepwise selection of variables was used to determine the best predictors. In addition, the other investigated prognostic factors were classified as follows; age at diagnosis ($\geq$ 65 years vs. $<$ 65 years), gender (female vs. male), tumor size ($\geq$ 7 cm vs. $<$ 7 cm), histologic grade (Grade 1, Grade 2, or Grade 3), and TNM stage (Stage I and II vs. Stage III and IV). Values of $P < 0.05$ were considered statistically significant. All calculations were performed using the StatView J software package (version 5.0; Statsoft, Tulsa, OK).

RESULTS

Expression of p27, Cyclin A, Cyclin D1, Cyclin E, and MIB-1

In nonneoplastic renal tissue, the distal tubules, glomerular epithelial cells, and lymphoid cells showed

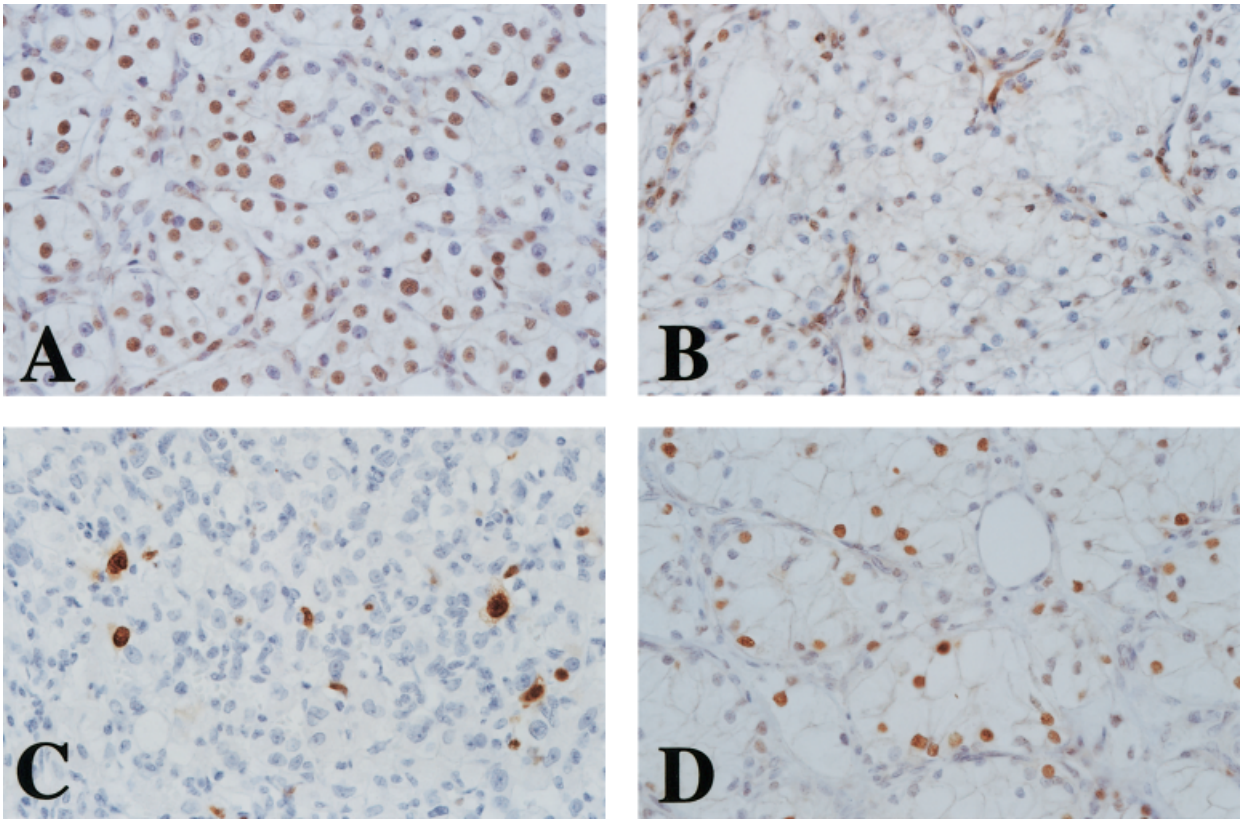

FIGURE 1. Immunohistochemical staining of p27^{Kip1} (p27), cyclin D1, and cyclin A in clear cell renal carcinoma (RCC) using the monoclonal antibody for each protein. (A) There is strong nuclear staining of p27 in the majority of tumor cells. This tumor was categorized in the group with high p27 expression. (B) In another tumor, immunoreactivity for p27 was low. This tumor was categorized in the group with low p27 expression. (C) The cyclin A staining pattern is seen in clear cell RCC. There is strong nuclear staining in various nuclear-graded tumor cells. (D) The cyclin D1 staining pattern is seen in clear cell RCC. There is strong-to-moderate nuclear staining with heterogeneous intensity (original magnification, ×400 in A–D).

intense nuclear staining for p27 protein. A cytoplasmic staining pattern was not observed in any renal tissue. In tumor tissue, there was considerable heterogeneity with regard to the p27 LI (Fig. 1A,B). The mean ± standard deviation p27 LI was 57.7% ± 28.4% (range, 0–94%). Cells located at the periphery of the tumor tended to show a higher p27 LI and more intensity compared with cells located in the center of the tumor.

Conversely, the nonneoplastic kidney epithelium was exclusively negative for cyclin A, cyclin D1, cyclin E, and MIB-1. In tumor tissue, there was considerable immunoreactivity for cyclin A (Fig. 1C), cyclin D1 (Fig. 1D), and MIB-1, and the mean ± standard deviation cyclin A LI, cyclin D1 LI, and MIB-1 LI was 1.08% ± 1.61% (range, 0–7%), 16.1% ± 23.8% (range, 0.1–81.0%), and 1.5% ± 7.5% (range, 0–58%), respectively. Nuclear reactivity with cyclin E was present in some tumors but represented < 1% of cells. For this reason, we performed no additional analysis on cyclin E in accordance with a previous report.[5] Cells with positive staining for cyclin D1 and MIB-1 were noted mainly at the periphery of the tumor, whereas cells with positive staining for cyclin A were identified both in the center and at the periphery of the tumor.

The correlations between the expression of cell cycle-related proteins and clinicopathologic factors are summarized in Table 1. There was a significant correlation between low p27 expression and high TNM stage (Stage III and IV; $P = 0.006$) or large tumor size (≥ 7 cm; $P = 0.009$). In addition, there was a significant correlation between high cyclin A expression and high TNM stage ($P = 0.015$).

Specific Correlations with Staining Patterns

Based on the known correlations between cell cycle regulators and the proliferation rate, the immunohistochemical results (LI) were evaluated as follows (Table 2): The expression of p27 was related to that of cyclin D1 (correlation coefficient, 0.476; $P = 0.0001$) and cyclin A (correlation coefficient, 0.432; $P = 0.0004$). No specific correlation between p27 and

TABLE 1
Cell Cycle-Related Proteins and Clinicopathologic Parameters in 67 Patients with Renal Cell Carcinoma

	p27			Cyclin A			Cyclin D1			MIB-1		
Characteristic	High	Low	*P* value	High	Low	*P* value	High	Low	*P* value	High	Low	*P* value
Total	46	21		20	47		29	38		8	59	
Age												
< 65 yrs (38)	25	13	N.S.	13	25	N.S.	16	22	N.S.	5	33	N.S.
≥ 65 yrs (29)	21	8		7	22		13	16		3	26	
Gender												
Female (27)	17	10	N.S.	10	17	N.S.	13	14	N.S.	4	23	N.S.
Male (40)	29	11		10	30		16	24		4	36	
Tumor size (cm)												
< 7 (45)	36	9	0.009[a]	11	34	N.S.	22	23	N.S.	7	38	N.S.
> 7 (22)	10	12		9	13		7	15		1	21	
Histologic grade												
G1 (20)	16	4	N.S.	5	15	N.S.	12	8	N.S.	3	17	N.S.
G2 (36)	24	12		12	24		13	23		3	33	
G3 (11)	6	5		3	8		4	7		2	9	
TNM stage												
I–II (43)	35	8	0.006[a]	8	35	0.015[a]	19	24	N.S.	5	38	N.S.
III–IV (24)	11	13		12	12		10	14		3	21	

N.S.: not significant.

[a] Statistical analysis was performed by the chi-square test with Yates continuity correction. $P < 0.05$ was considered significant.

TABLE 2
Correlation Coefficients among Tumor Size and Expression Frequencies of the Related Proteins[a]

Factor	p27	Cyclin A	Cyclin D1	MIB-1	Tumor size
p27	—	0.432[b]	0.476[b]	0.192	−0.258[c]
Cyclin A	—	—	0.347[d]	0.389[d]	0.163
Cyclin D1	—	—	—	0.117	−0.223
MIB-1	—	—	—	—	0.039
Tumor size	—	—	—	—	—

[a] Positive ratios of tumor cells on immunohistochemical staining in each tumor were analyzed with the Spearman rank test. $P < 0.05$ was considered significant.

[b] $P < 0.001$.

[c] $0.01 < P < 0.05$.

[d] $0.001 < P < 0.01$.

MIB-1 expression was identified ($P = 0.118$). The expression of cyclin A was related to that of cyclin D1 (correlation coefficient, 0.347; $P = 0.0051$) and MIB-1 (correlation coefficient, 0.389; $P = 0.0016$). Tumor size was related inversely only with p27 expression (correlation coefficient, −0.258; $P = 0.0377$), and, when p27 expression was divided into two groups of high expression and low expression, there was a significant difference in tumor size between the two groups ($P = 0.0009$) (Fig. 2).

Survival Analysis

Of 67 patients with RCC who were included in the survival analysis, 51 patients were alive at the end of follow-up, and 16 patients died of disease. In the univariate analysis, the Kaplan–Meier survival curves showed that high cyclin A expression, low cyclin D1 expression, and high MIB-1 expression tended to shorten the survival (results not shown) but with no statistical significance (Table 3). Conversely, the Kaplan–Meier survival curves for high TNM stage versus low TNM stage showed a highly significant separation ($P < 0.0001$), as did the curves for high p27 expression versus low p27 expression ($P < 0.0001$) (Fig. 3), large tumor size versus small tumor size ($P = 0.0016$), and high tumor grade versus low tumor grade ($P = 0.0104$). When a multivariate Cox proportional hazards model was constructed, TNM stage ($P = 0.0035$) and p27 LI ($P = 0.0235$) both were indepen-

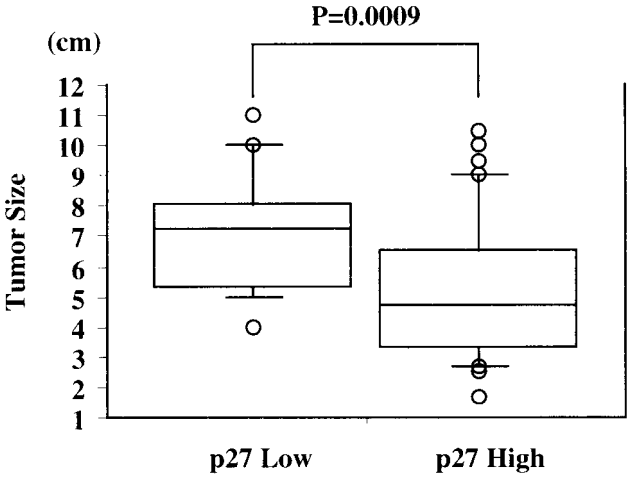

FIGURE 2. Scattergram of tumor size with regard to high $p27^{Kip1}$ (p27) expression (≥ 50% positive tumor cells) and low p27 expression (< 50% positive tumor cells). The median tumor size was significantly greater in tumors with low p27 expression (7.2 cm) than in tumors with high p27 expression (4.7 cm) (Mann–Whitney *U* test, $P = 0.0009$).

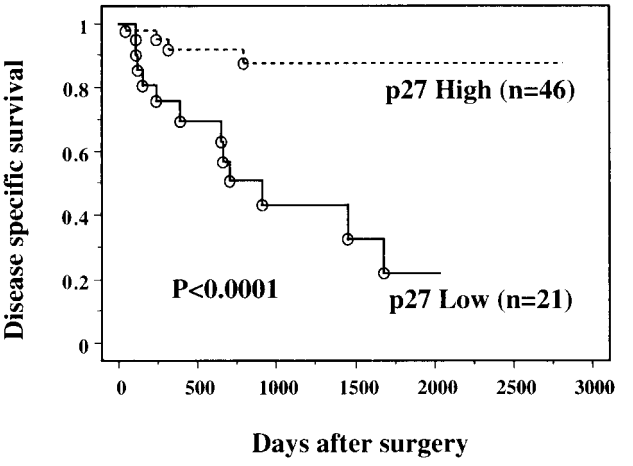

FIGURE 3. Kaplan–Meier survival curves of patients with clear cell renal carcinoma stratified by $p27^{Kip1}$ (p27) expression level. Patients who had tumors with low p27 expression (< 50% positive tumor cells) had a significantly poor outcome (log-rank test; $P < 0.0001$).

TABLE 3
Univariate and Multivariate Survival Analysis in 67 Patients with Renal Cell Carcinoma

	P value on survival analysis	
Variable	**Univariate[a]**	**Multivariate[b]**
Age	0.556	0.8201
Gender	0.0744	0.1858
Tumor size	0.0016[c]	0.3714
TNM stage	< 0.0001[c]	0.0035[c]
Histological grade	0.0104[c]	0.5829
p27 LI	< 0.0001[c]	0.0235[c]
Cyclin A LI	0.5204	0.9324
Cyclin D1 LI	0.213	0.4152
MIB-1 LI	0.1157	0.1656

LI: labeling index.
[a] Statistical analyses were performed by the log-rank test.
[b] Statistical analyses were performed by Cox proportional-hazards regression model.
[c] $P < 0.05$ was considered significant.

dent prognostic factors for disease specific survival in patients with RCC.

DISCUSSION

p27 is a member of the Cip/Kip family of CDK inhibitory proteins that negatively regulate cell proliferation. p27 regulates progression from G1-phase into the S-phase by binding to and inhibiting the cyclin E/CDK2 complex. Recent studies have demonstrated that p27 abundance is regulated mostly at a post-transcriptional level by ubiquitin-proteasome-mediated proteolysis.[9,10] Loss of p27 protein is correlated with adverse pathologic features or poor survival in patients with a variety of neoplasms, including carcinoma of the prostate,[11–13] breast,[2,14–16] gastrointestinal system,[16–18] lung,[19–21] and liver.[22,23] Some reports have shown that determining the expression of p27 using immunohistochemical techniques was reliable, suggesting that it may become a routine part of the evaluation and management of patients with malignant disease.[2,14,24] Here, we have reported the results of our immunohistochemical analysis of p27 and related cyclins in patients with clear cell RCC.

Our first objective was to demonstrate the expression of p27 and related cyclins in RCC and to examine the correlation between p27 and the cyclins as well as the proliferative potential, as represented by the MIB-1 LI. An inverse correlation between p27 expression and cell proliferation generally was seen; however, this inverse correlation has not always been observed in some tumor cell lines.[16,25,26] Recent studies have reported that high levels of p27 were associated with the levels of cyclin D1 and cyclin E in patients with esophageal carcinoma[25] and breast carcinoma.[26] This positive correlation between the expression of p27 and cyclin D1 suggests the existence of a homeostatic feedback mechanism that may prevent the potentially toxic effects of excessive cyclin kinase activity.[25–27] In the current study, the expression of p27 was associated significantly with the expression of cyclin A and cyclin D1, but not with MIB-1. Some studies in vivo that showed no correlation between tumor cell proliferation and p27 have been reported[15,17,20]; however, the abundance of p27 reflects its function as a

CDK2 kinase inhibitor by Western blot analysis,[17] and loss of p27 was an independent and unfavorable prognostic marker.[15,17] Accordingly, p27 seems to have some role to play in tumor cells that is not related simply to cell cycle progression.[6,26,28]

Our second objective was to compare the expression of cell cycle-related proteins with clinicopathologic parameters and to identify prognostic factors in patients with RCC. We found that, as reported previously,[1] TNM stage ($P < 0.0001$), tumor size ($P = 0.0016$), and nuclear grade ($P = 0.0104$) were strong predictive prognostic markers. In patients with RCC, there have been some reports suggesting that low cyclin D1 expression, high cyclin A expression, and high Ki-67 expression indicate a poor clinical outcome.[3,29,30] Renshaw et al.[5] reported that there was a positive correlation between cyclin A and Ki-67, but neither of these proliferative markers was correlated with tumor grade or stage in 33 patients with RCC. In contrast, other investigators have reported that increased cyclin A is a powerful and independent, unfavorable prognostic marker and that increased cyclin D1 is a favorable prognostic marker in patients with RCC.[3,29] However, the former study included RCC tumors of all histologic types,[3] and the latter study employed Western blot analysis,[29] in contrast with our methods. We also found that high cyclin A expression and low cyclin D1 expression had a tendency to shorten survival, but we could not demonstrate with any significance that they were independent prognostic factors in patients with clear cell RCC.

Conversely, we demonstrated that p27, a CDK inhibitor, is a powerful and independent prognostic factor in patients with RCC, as also noted in the other malignancies.[6] It has been shown that mutation of the p27 gene is a rare event in human malignancies.[31,32] Therefore, p27 expression in vivo may reflect the inhibitory activity of CDKs. Assessment of p27 using immunohistochemistry may be useful in predicting the prognosis of individual patients with RCC.

Furthermore, we found that the p27 LI was related inversely to tumor size and TNM stage. In vivo, p27 knockout mice develop generalized hyperplasia and pituitary tumors.[33–35] Many reports have demonstrated the role of p27 as a prognostic marker in human malignancies and have shown that decreased p27 tends to contribute to an increase in tumor size.[18,23,36,37] In patients with RCC, the tumor size, at least in intrarenal tumors, is correlated with survival,[38–40] and p27 may function as a proliferative marker in smaller sized RCC tumors and may diminish as the tumor grows.

We also examined p27 expression in six chromophobe cell carcinomas and four papillary RCC tumors in our preliminary study; however, those tumor types rarely showed p27 expression, irrespective of the clinical outcome (data not shown). Thus, p27 may play different biologic roles in the pathogenesis of each of the following major histologic categories: papillary RCC, chromophobe cell carcinoma, and clear cell carcinoma. Further examination of p27 expression in vitro is required for the determination of its precise biologic and morphologic roles in RCC.

In conclusion, we have studied the correlations between the expression of p27 or related cyclins and clinicopathologic factors in patients with RCC. Low p27 expression was associated with tumor size and stage. In multivariate analysis, p27 was a novel prognostic marker, the low expression of which was associated with a poor clinical outcome. The results from this study suggest that p27 immunohistochemical assessment predicts biologic behavior in individual patients with RCC, and, in the future, this may provide us with new therapeutic strategies, such as p27 gene therapy, for the treatment of patients with advanced RCC.

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