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Reduced Expression of the Cell Cycle Regulator p27^{Kip1} within the Invasion Front of Renal Cell Carcinomas Proved to be a Significant Marker for Disease-specific Survival

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Abstract. *Background: The expression of the negative cell cycle regulator p27^{Kip1} is frequently found to be deregulated in various human cancer types. Whether the expression of p27^{Kip1} can be used as a prognostic marker for renal cell cancer patients still remains to be clarified. Therefore, in the present investigation the expression of protein within different tissue areas obtained from renal cell carcinomas, their invasion front and corresponding histologically benign renal parenchyma was determined and statistically correlated with several tumor and patient characteristics including the disease-specific long-term survival following surgical treatment. Patients and Methods: For analysis of p27^{Kip1} expression in 420 tumor nephrectomy specimens obtained from 420 consecutively included patients, tissue microarrays were used comprising 1260 tissue samples each obtained from the tumor itself, the invasive front as well as the non-malignant surrounding parenchyma. A sufficient follow-up after surgical therapy was available in 251 cases In total, 88 out of 251 patients (35%) had died from tumor progression after a median follow-up of 138 (36-240) months. Results: In univariate survival analysis, decreased expression of p27^{Kip1} within tissue cores obtained from the invasion front was significantly correlated with the patients' disease-specific long-term survival ($p=0.02$, log rank test). In contrast, expression of p27^{Kip1} within the primary tumors was not identified to reveal any prognostically important information. In Cox regression analysis, histological stage and grade ($p<0.01$), the presence of regional lymph node ($p<0.01$) or distant metastases at the time of surgery ($p<0.01$) as well as decreased expression of p27^{Kip1} ($p=0.04$) within the invasion front tissue samples independently predicted the disease-specific long-term survival following surgery. Conclusion: Our analysis demonstrated that p27^{Kip1} is heterogeneously expressed in renal cell carcinomas. Moreover, the results of the present study supports the prognostic value of p27^{Kip1} protein expression for patients diagnosed with renal cell carcinoma.*

Renal cell cancer (RCC) comprises about 2-3% of all malignancies. A review of the patients gathered in the Connecticut Tumor Registry indicates a six-fold increase of RCC from 1935 to 1989 (1). Currently, regardless of the tumor stage at primary diagnosis, the average 5-year survival rate of RCC patients has improved by only 50% (2). This may be due to the fact that, up until now, no sufficient adjuvant therapeutic options for the treatment of locally advanced or metastasized RCC are available. The introduction of new imaging methods such as ultrasound and computed tomography (CT-scans) into the clinical routine, together with their further refinement during the last few decades, has resulted in the diagnosis of a higher number of early stage renal cell tumors. Although the latter development has contributed to an improvement of the patients' clinical prognosis in general, even organ-confined malignancies of comparable stage and grade can reveal an absolutely diverging biological behavior that either results in a high or a low tendency towards tumor progression and systemic metastatic spread. Additionally, the development of metastases can be observed in a substantial number of RCC patients with tumors initially classified as T1b or T2 disease, even more than five years after the initial treatment. Therefore, prognostically important parameters that would allow to predict the biological behavior of the individual tumor in addition to established patient and tumor

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characteristics, like tumor stage and histological grading are urgently needed to either select patients for effective adjuvant treatment regimens that might be available in the future or to include them in timely extended follow-up programs.

The cyclin - dependent kinase (CDK) inhibitor $p27^{Kip1}$ belongs to the WAF/Kip family of CDK inhibitors and is involved in cell cycle control at the G1-S transition. $p27^{Kip1}$ is supposed to be functionally related to different cellular processes such as tumor suppression, promotion of apoptosis, hypoxia (2), cellular differentiation, drug resistance of tumors and protection against inflammatory injury (3). While miscoding mutations and epigenetic alteration, *i.e.,* aberrant methylation of the $p27^{Kip1}$ promoter region, have been inconsistently detected in only few human cancer types (4-7), the deregulation of normal $p27^{Kip1}$ proteolysis seems to be more frequently associated with the development of a stable malignant transformation of somatic cells (8, 9).

The important role of $p27^{Kip1}$ in cell cycle control has led to numerous studies indicating that a decreased p27Kip1 level could serve as an independent prognostic marker for a variety of human malignancies (3, 10). However, so far, only few studies have investigated different aspects of the potential prognostic significance of $p27^{Kip1}$ in RCC (11-15).

To further clarify the prognostic value of altered $p27^{Kip1}$ expression for surgically treated renal cell cancer patients, the present study utilized the high-throughput tool of tissue microarray analysis for the detection of the $p27^{Kip1}$ protein by immunohistochemistry, thus facilitating the analysis of a significant number of patients. For patients with colorectal carcinomas, the expression of a mucin not in the primary tumor specimens but exclusively within their invasion front could be identified as a variable that independently predicted the clinical course of the disease during multivariate statistical analysis (22). Therefore, in the present study, the expression analysis of $p27^{Kip1}$ both in tumor and invasion front tissue specimens was correlated with several tumor and patient characteristics including the disease-specific long-term survival following surgical treatment of RCC patients.

Patients and Methods

Patients. Four hundred and twenty renal cancer patients treated between 1980 and 1998 by radical nephrectomy were included in the present investigation. A sufficient postoperative follow - up was available for a subgroup of 251 RCC specimens from 251 different patients (159 males and 92 females). The primary tumors were localized within the right or left kidney in 125 and 126 cases, respectively. The median age of the patients was 58 years (range 32-99 years). After a median follow-up of 138 (36-240) months, 88 out of 251 patients (35%) had died from tumor progression. The pathological stages of the tumors, defined according to the TNM system (1997), were as follows: stage T1, 9 tumors; stage T2, 137 tumors; stage T3, 96 tumors; stage T4, 9 tumors. The histological grading was G1 in 68 cases, G2 in 166 cases and G3 in 17 cases. At the time of first diagnosis, regional lymph node or distant metastases were observed in 67 patients. An infiltration of lymph or blood vessels within the primary tumor specimens was identified in 64 cases. Patients were followed by computed tomography (CT) scans of the abdomen and X-rays of the lungs, every 3 months during the first year after tumor nephrectomy and every 3-6 months thereafter.

Tissue microarrays. All archival tissues were formalin fixed and embedded in paraffin blocks according to standard methods. On respective H&E - sections to these blocks, a representative tumor or desired tissue area was selected by the pathologist. These slides were positioned on the cutting side of the corresponding archival paraffin block and the same area was marked here. In the next step, tissue cores of about 1.4 mm in diameter were punched out off the marked areas utilizing a biopsy needle. All tissue cores were melted together in a checkerboard pattern into a new tissue array block, covering up to a maximum of 96 cores. These tissue arrays could be routinely cut and serial sections were mounted on self-coated slides (poly-L-lysine, Sigma, Deisenhofen, Germany) and the procedure was continued as with standard paraffin blocks. For each patient, the tissue microarray slides contained a tissue core from the histopathological normal renal tissue within the tumor-bearing kidneys, from the primary tumor itself as well as from the invasion front; the latter defined as the area where the primary tumor borders the microscopically non-malignant parenchyma (16).

Immunohistochemical analysis. Formalin-fixed and paraffin-embedded tissue microarrays were pretreated by incubation with citric acid in order to achieve demasking of sections. Internal negative and positive controls and the ABC staining procedure were carried out as described previously (17). For the detection of the $p27^{Kip1}$ protein, the monoclonal anti-$p27^{Kip1}$-antibody (clone G173-524, IgG_1; Pharmingen, San Diego, CA, USA) was applied as primary antibody (dilution 1:50 in PBS, incubation 1 h in a moist chamber at room temperature). For the determination of the proliferative index of the tumors, the Ki67 epitope was analyzed by utilization of a mouse monoclonal anti-mib1 antibody (Dako, Glostrup, Denmark). Visualization of both antigens was carried out using the standard streptavidin-biotin method (Vectastain, Burlingame, CA, USA) which was applied according to the manufacturer's instructions.

The TMA slides were reviewed and classified by three independent investigators (M.K., A.M., M.M.). For the classification of the immunohistochemical staining reaction, 400 - 500 tumor cells were manually counted in two microscopic fields (magnification 240 - fold) per tissue core. The percentage of positively-stained tumor cells within each tissue core was estimated in correlation to the total number of tumor cells identified. For each patient, the highest category obtained was considered. Tumors were initially classified into six groups: (1) negative reaction, (2) <5% positivity regarding the relative amount of positively-stained tumor cells (3) 5% up to 25% positivity, (4) >25% up to 50%, (5) >50% up to 75% positivity and (6) >75% positivity.

Statistical analysis. The Friedman nonparametric correlation analysis of paired samples was applied for analysis of statistical relationships between staining results within different tissue cores obtained from a single nephrectomy specimen following $p27^{Kip1}$ immunostaining. A pair-wise comparison of the tissue specimens investigated was carried out by the Wilcoxon signed ranks test. For the determination of a statistically relevant relationship between $p27^{Kip1}$ and the histological grading, the tumor stage as well as the presence of

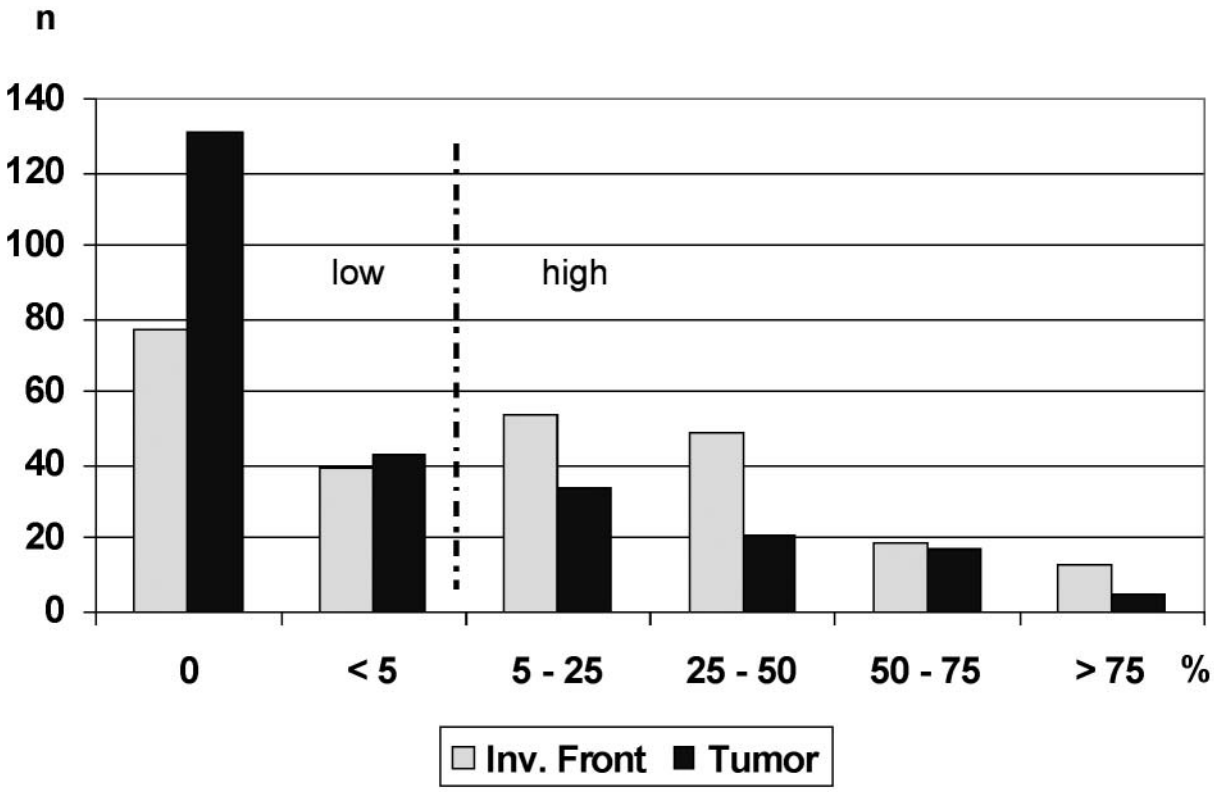

Figure 1. *Distribution of relative amounts of tumor cells exhibiting a positive immunohistochemical staining reaction for p27^{Kip1} protein. Tissue samples were either obtained from the tumor center or the invasion front of 251 renal cell carcinoma specimens.*

regional lymph node or distant metastases, Pearson's Chi-square test was applied.

Survival was calculated from the time between radical nephrectomy and death from tumor progression or the end of follow-up. For univariate and multivariate survival analyses of p27Kip positivity, a cut-off value of 5% for the relative amount of positively-stained cells was used. The log-rank test was employed to individually determine the significance of each of the biological parameters for survival. Finally, Cox regression analysis was applied to disclose whether any of the tumor or patient characteristics investigated, *e.g.* age, gender, tumor stage, lymph node status, presence of distant or regional lymph node metastases, histological grade and reaction for p27^{Kip1}, either within the normal renal tissue, the primary tumors or the invasion front, could be identified as a parameter of independent prognostic relevance.

Results

Immunohistochemical analysis of p27^{Kip1} protein levels. For each of the 251 renal cancer specimens investigated for expression of the p27 protein, the tissue microarray slides contained a tissue core obtained from normal renal tissue, the primary tumor itself as well as the so called invasion front, latter defined as the area where the primary cancer specimen borders the histologically normal renal parenchyma within the tumor-bearing kidney. Taking the tissue cores obtained from histologically normal renal parenchyma into consideration, intense nuclear staining for the p27 protein was observed within the distal tubules and glomerular epithelial cells.

For the primary cancer specimens, a negative (negative reaction or <5% positivity) or positive staining reaction was observed in 174 and 77 cases, respectively. The distribution of the immunohistochemical staining reaction was : negative reaction, 131 patients ; <5% positivity, 43 patients ; 5-25% positivity, 34 patients ; 25-50% positivity, 21 patients ; 50-75% positivity, 17 patients ; >75% positivity, 5 patients.

For the invasion front tissue, 116 tumors exhibited a negative and 135 renal cancer specimens a positive (≥5% positivity for the relative amount of positively-stained tumor cells) immunohistochemical staining reaction that was classified in detail as follows: negative reaction, 77 patients; <5% positivity, 39 patients; 5-25% positivity, 54 patients; 25-50% positivity, 49 patients ; 50-75% positivity, 19 patients; >75% positivity: 13 patients. In summary, most of the tissue specimens either obtained from the primary cancer specimens or the invasion front showed a p27^{Kip1} positivity of less than 5% (Figure 1).

Comparison of p27^{Kip1} positivity in primary cancer, invasion front and normal tissue specimens, respectively. In tissue

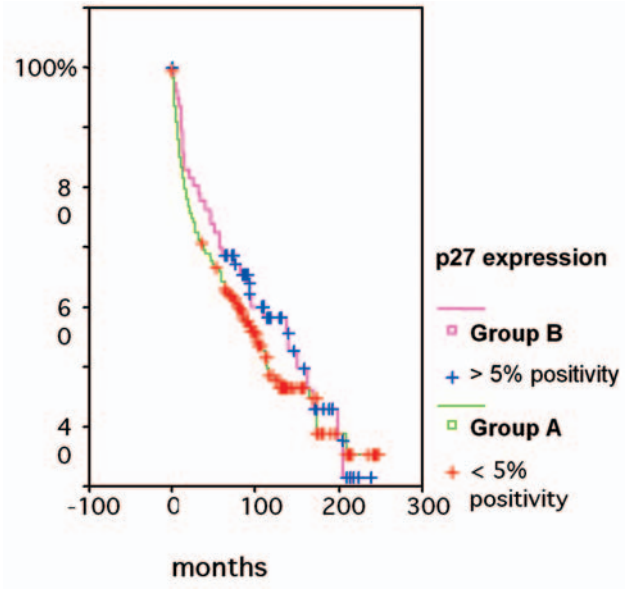

Figure 2. *Correlation of p27^{Kip1} protein expression within the primary renal cell cancer specimens with the long-term survival of 251 patients undergoing radical nephrectomy for the treatment of renal cell cancer. The difference between Group A and B was not statistically significant (p=0.42).*

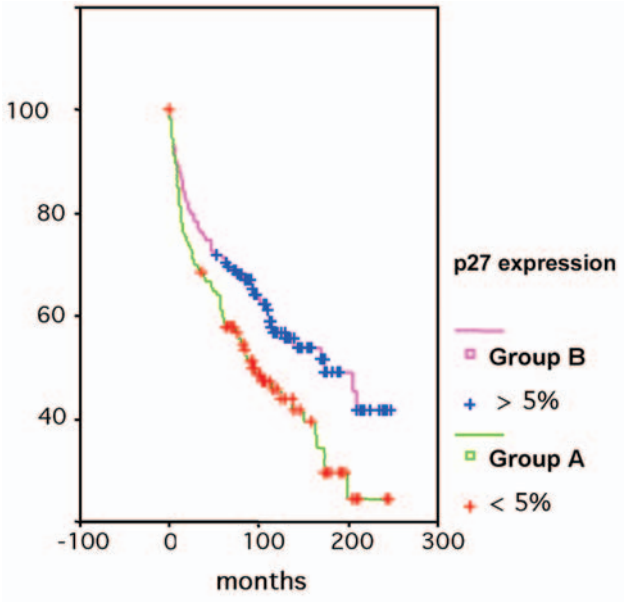

Figure 3. *Correlation of p27^{Kip1} protein expression within the invasion front with the long-term survival of 251 patients undergoing radical nephrectomy for the treatment of renal cell cancer. The difference between Group A and B was statistically significant (p=0.02).*

samples obtained from the invasion front of tumors, the highest staining score (mean staining class 1.8) regarding the relative number of tumor cells positively stained for the p27^{Kip1} protein was observed when compared with the primary tumors (mean staining class 1.4) or the histologically benign renal parenchyma (mean staining class 1.1). Statistical comparison of mean staining classes observed within the latter tissue areas demonstrated a highly significant difference (Friedman test, $p<0.001$). The pair-wise comparison of the staining reactions utilizing the Wilcoxon signed ranks test also demonstrated a significant difference for each of the constituted pairs, *e.g.* tumor *vs.* invasion front ($p=0.014$), tumor *vs.* normal ($p=0.032$) and invasion front *vs.* normal renal tissue ($p=0.000$).

Correlation of p27^{Kip1} with additional patient and tumor characteristics. The nonparametric correlation analysis according to Spearman demonstrated that the expression pattern following staining for the protein was independent of tumor grade as well as of the presence of regional lymph node or distant metastases (data not shown).

Correlation between p27^{Kip1} positivity and disease-specific long-term survival of RCC patients following surgical treatment. For statistical analysis of the relationship between patient postoperative disease-specific long-term survival and a positive immunohistochemical staining reaction for the p27^{Kip1} protein within the primary tumors, the invasion front or peritumoral tissue samples, patients were classified into two groups according to the relative amount of tumor cells positively stained for p27 (Group A: <5%; Group B: ≥5% positivity within the respective tissue area). In primary cancer specimens, low and high positivity was observed in 174 and 77 cases, respectively. According to the Kaplan-Meier analysis, a median survival of 62 (36-248) and 67 (64-238) was observed for patients from Group A and B, respectively (log-rank test, $p=0.42$) (Figure 2). In the invasive front tissue, low and high positivity was detected in 116 and 135 cases, respectively. The corresponding median survival times were 51 (36-244) for the low and 74 (53-248) months for the high expression group, a difference that was statistically significant (log-rank test, $p=0.02$) (Figure 3).

Regarding the relationship between classical clinico-pathological parameters and the disease specific long-term survival of patients following tumor nephrectomy, no significant correlation between p27^{Kip1} protein expression and any of the other variables, including age (log rank test, $p=0.11$), gender ($p=0.23$) and tumor stage ($p=0.41$) could be detected. However, the histological grading ($p<0.01$), vascular invasion by the tumor as well as the presence of regional lymph node ($p<0.01$) or distant metastases ($p<0.01$) were identified to be significant correlated with the patients' long-term survival (see Table I).

In a multivariate step-wise logistic regression analysis, histological grade ($p<0.01$), vascular invasion within the primary tumor specimens, the presence of regional lymph

node ($p<0.01$) or distant metastases ($p<0.01$) as well as a low p27^{Kip1} expression value within the invasion front ($p=0.04$, Hazard ratio 1.8) were identified as independent prognostic parameters for the long-term survival of the patients (Table I).

Table I. *Results of univariate and multivariate survival analysis.*

Parameters investigated	Univariate survival analysis *p*-value[a]	Multivariate survival analysis *p*-value[b]
Staining for p27Kip1		
Normal tissue	$p=0.08$	$p=0.08$
Tumor	$p=0.42$	$p=0.31$
Invasion front	$p=0.02$	$p=0.04$
Age	$p=0.11$	$p=0.13$
Gender	$p=0.23$	$p=0.22$
Tumor stage	$p<0.01$	$p<0.01$
Lymph node status	$p<0.01$	$p<0.01$
Distant metastases	$p<0.01$	$p<0.01$
Histological differentiation (G)	$p<0.01$	$p<0.01$

[a]Log-rank test.
[b]Cox proportional hazard regression.

Discussion

For a variety of human malignancies, low or absent p27^{Kip1} protein expression was suggested to predict the likelihood for disease progression and a worsened clinical prognosis (3, 10). Accordingly, the prognostic relevance of an altered p27^{Kip1} expression for RCC patients was recently indicated (11-15).

In the present study, the tissue microarray technique (TMA) served as a high -throughput tool for the immunohistochemical detection of the p27^{Kip1} and Ki67 protein in renal cell cancer specimens. Moreover, in addition to tissue specimens that were obtained from histomorphologically well-defined areas within the primary tumors and the surrounding non-malignant parenchyma, tissue samples representing the invasion front were subjected to immunohistochemical analysis.

For the comparison between the primary tumors and histologically-benign tissue samples, significant differences with regard to p27^{Kip1} protein expression were expected, However, taking previous reports into consideration (11-15), an increased level of p27^{Kip1} protein in the invasion front tissue, which was statistically independent from that observed within the corresponding primary tumor specimens, has not, to our knowledge, been demonstrated before.

Interestingly, a similar finding was recently described for colorectal cancer and basal cell carcinomas, such that up-regulation of a member of the CDKI family, the p16^{Ink4a} gene, was associated with an impaired proliferation within the invasion front tissue specimens when compared with findings in the corresponding primary tumors (18, 20). Accordingly, Jung *et al*. (21) investigated the expression of cyclin D1, p16INK4, Ki67 and beta-catenin. Beta-catenin revealed a positive regulatory influence on cyclin D1 in primary human colorectal adenocarcinomas as well as their invasion front. Regardless an increased expression of cyclin D1, p16INK4 and beta-catenin within the invasion front tissue samples, it was suggested that the function of cyclin D1 as a cell-cycle promoter be reconsidered according to low proliferative activity in the invasion front tissue as indicated by a low Ki67 expression (21). Taking the aforementioned observations into account, it was hypothesized that tumor cells may switch from a proliferative status in the tumor center to a more invasive status, characterized by decreased proliferation, in the invasive front tissue (18). Although the previous results seem to correspond with our observations, a more detailed and preferably functional analysis is required focusing on renal cell cancer.

The absence of correlation between p27^{Kip1} protein levels and tumor grade observed in the present investigation confirm the outcome of three previous studies including a total of 244 different RCC specimens (11-13). In contrast, p27 positivity also decreased when histological dedifferentiation increased (total number of patients investigated in 2 studies: 372) (14, 15). In concordance with the data presented herein, three investigations covering a total of 331 renal cancer patients suggested that p27^{Kip1} protein levels were not correlated with tumor stage (11, 12, 14). In contrast, one report described a significant difference in p27^{Kip1} protein expression when tumors of stage I/II were compared with RCC classified as stage III/IV (13). In conclusion, the p27^{Kip1} protein level in RCC seems less likely to be associated with tumor stage, whereas a possible correlation with the histological grading of tumors remains to be clarified.

In the present investigation, reactivity for p27^{Kip1} exclusively within the invasion front revealed independent prognostic information regarding the patients' postoperative disease-specific long-term survival. Therefore, the different clinical outcomes observed for patients demonstrating high or low p27^{Kip1} protein levels in the invasive front tissue samples support our hypothesis that RCC contain differentially regulated areas, which in turn may exhibit a diverging biological potential towards disease progression. Interestingly, a similar finding was recently described for colorectal carcinomas. The immunohistochemical detection of a mucin exclusively within the invasion front tissue was correlated with the patients' clinical prognosis during multivariate survival analysis (19).

In contrast to our observation that the signals obtained exclusively from the invasion front tissue samples revealed an independent prognostic information regarding the disease-

specific long-term survival of RCC patients, previous investigations identified altered $p27^{Kip1}$ protein expression within the primary tumors as a biological variable of independent prognostic value (11, 13, 15). In addition to technical aspects and differences concerning the higher number of patients included in the present investigation compared with former analyses, the statistical evaluation presented here could refer to a significantly extended follow-up period. As the Kaplan-Meier analysis of the p27 protein expression data obtained from the primary tumors demonstrates, the survival curves for patients revealing either a low or high staining score tended to correspond with increasing follow-up time (Figure 3). Thus, the diverging results between the present and previous studies might be due to the different lengths of follow-up periods.

In conclusion, the present analysis of the $p27^{Kip1}$ protein levels in the tumor, the invasive front and the peritumoral tissue samples obtained from RCC patients reveals evidence that the invasive front tissue exhibits a specific status of cell cycle regulation which in turn affects the patients' long-term survival after surgery. Moreover, our results support previous reports on the promising value of $p27^{Kip1}$ as a biological marker of prognostic relevance for this tumor entity.

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