

MIB-1 (KI-67) Proliferation Index and Cyclin-Dependent Kinase Inhibitor p27^{Kip1} Protein Expression in Nephroblastoma

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ABSTRACT

Purpose: A number of studies have indicated that the tumor proliferation marker MIB-1 and cell cycle inhibitor p27^{Kip1} expression are of prognostic importance in a variety of cancers. The present study was performed to evaluate the prognostic value of these molecules in Wilms' tumors.

Experimental Design: MIB-1 and p27^{Kip1} expressions were investigated by the means of immunohistochemical analysis of 62 Wilms' tumor. Patients were preoperatively treated by chemotherapeutic agents and had a mean follow-up of 5.7 years.

Results: MIB-1 and p27^{Kip1} were expressed in normal kidney tissues and in the three main components of Wilms' tumor, *i.e.*, the blastemal, epithelial, and stromal cells. In Wilms' tumors, the percentage of MIB-1-positive cells in the blastema ranged between 0 and 42% (mean, 9.4%) and in the epithelial component between 0 and 53% (mean, 19.9%), with a significant difference ($P < 0.01$). The percentage of blastemal p27^{Kip1}-positive cells ranged between 3 and 85% (mean, 55.1%) and for the epithelial component between 1 and 87% (mean, 59%). There was a significant inverse relationship between blastemal MIB-1 and p27^{Kip1} expression in Wilms' tumor. Univariate analysis showed that blastemal MIB-1 and p27^{Kip1} expression were indicative for clinical progression and tumor-specific survival. In a multivariate analysis, blastemal MIB-1 and p27^{Kip1} protein expression proved to be an independent prognostic for clinical progression besides stage.

Conclusions: It was concluded that both MIB-1-based proliferative activity and p27^{Kip1} protein expression in the blastema have prognostic impact in Wilms' tumor.

INTRODUCTION

Wilms' tumor is a pediatric malignancy of the kidney and one of the most common solid tumors in children (1). Currently, the prediction of outcome is based mainly on the histopathology and stage of disease at the time of resection (2). Identification of factors predictive of the aggressive growth of this malignant tumor would enable stratification of patients for optimal strategy. Predicting the clinical behavior of Wilms' tumor can be difficult; one approach is to identify molecular prognostic markers (3).

Advances in cell cycle research have led to the identification of protein markers responsible for the regulation of cell proliferation. The Ki-67 monoclonal antibody has been developed and used in evaluating cellular proliferation rates of malignant tumor (4–7). More recently, the monoclonal antibody, MIB-1, has been developed using recombinant portions of the Ki-67 nuclear antigen as immunogen. MIB-1 recognizes the Ki-67 nuclear antigen, which is associated with cell proliferation and is found throughout the cell cycle (G_1 , S, G_2 , and M phases) but not in resting (G_0) cells (8). The clinical value of proliferation markers for prognostication of nephroblastoma is still subject of debate (9–11).

The p27^{Kip1} protein is a cyclin-dependent kinase inhibitor, affecting the G_1 -S traverse in response to extracellular signals (12–14). In experimental models, the p27^{Kip1} protein is conspicuously present in quiescent cells and in cells undergoing terminal differentiation, whereas this marker is absent during cell division (15). There is increasing evidence that low expression of p27^{Kip1} is an important clinical marker for disease progression in many tumor types (16–23).

In the present study, we investigated the expression and the prognostic value of the Ki-67 (MIB-1) proliferative index (PI) and p27^{Kip1} expression in nephroblastoma using immunohistochemistry on paraffin-embedded material.

MATERIALS AND METHODS

Patients. During the period 1987–1999, 62 patients with nephroblastoma were treated by neoadjuvant chemotherapy and subsequent tumor nephrectomy. Twenty-six patients (42%) were female, and 36 patients (58%) were male. Patients were treated according to Société Internationale d'Oncologie Pédiatrique (SIOP) protocol 9 and some according to 93-01, receiving actinomycin D and vincristine. After treatment, patients were followed regularly, and all data concerning diagnosis, treatment and follow-up were stored in a database. The mean overall follow-up period was 5.7 years, and the mean age at operation was 4.7 years. Clinical progression was defined as histologically or cytologically proven local recurrence or the

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appearance of distant metastases. Tumor death was defined as death due to direct effect of metastases.

Sample Selection. All nephrectomy specimens were fixed in 10% buffered formalin and embedded in paraffin. The tumor stage was done according to the SIOP trial protocol established in the SIOP meeting in Stockholm in 1994 (24). Among the tissue blocks of tumors from individual patients having the classical type of tumor, *i.e.*, tumor samples containing the three different cell types (blastema, epithelial, and stromal), different samples were selected throughout the tumor. In addition, adjacent normal kidney tissue ($n = 26$) was taken from each patient. Samples containing any aspect of nephroblastosis were excluded from this series.

Antibodies. These primary antibodies were used as follows: mouse monoclonal antibodies against Ki-67 (MIB-1; Immunotech, Marseille, France) and against p27^{Kip1} (clone 1B4; Novocastra Laboratories Ltd., Newcastle, United Kingdom). The specificity and characteristics of these antibodies have been published elsewhere (8, 13, 14).

Immunohistochemistry. The peroxidase-antiperoxidase immunohistochemistry technique was used and applied to serial sections (5 μ m) from all samples, which were mounted on 3-aminopropyl-triethoxysilane (Sigma Chemical Co., St. Louis, MO) coated glass slides and subsequently incubated overnight at 60°C incubator. To enhance antigen exposure, slides were microwaved at 700 W in 0.1 M citrate buffer at pH 6.0 for 15 min. Sections were incubated with 10% normal rabbit serum (Dako) in PBS (PBS) 5% BSA (BSA) for 15 min and subsequently incubated with the primary antibody MIB-1 for 30 min at room temperature and overnight with p27^{Kip1} at 4°C. The antibodies were diluted in PBS/5%BSA at 1:20 for MIB-1 and p27^{Kip1}. After being incubated with rabbit-antimouse antibody, the peroxidase-antiperoxidase complex (Dako) was diluted in PBS/5%BSA and incubated for 30 min, after which antigen-antibody binding was visualized with diaminobenzidine tetrahydrochloride dihydrate (Fluka, Neu-Ulm, Germany). Replacing the primary antibody by PBS/5%BSA included negative controls.

Immunostaining Analysis (Quantification). The slides were evaluated by two independent observers, using a standard light microscope with a $\times 40$ objective and equipped with an ocular grid. Cells were considered positive regardless of the intensity or location of nuclear staining. Stromal cell staining was excluded from the counting process. Also, the tumor-infiltrating lymphocytes cells were avoided in the MIB-1 evaluation. The subjective assessment of the distribution of positively labeled cells was identical in all cases. MIB-1 and p27^{Kip1} expression (*i.e.*, the percentage of MIB-1 and p27^{Kip1}-positive tumor cells) was derived by counting at least 1000 tumor cells

in five randomly selected fields of view. Fields containing areas of extensive necrosis were excluded from evaluation. For MIB-1 PI, two categories were defined according to the percentage of stained nuclei PI < 5% and PI \geq 5%. The cutoff value of 5% was statistically known to be the lowest value at which discrimination with a significant probability value was achieved. On the basis of the previous studies, for p27^{Kip1} protein, a cutoff value of 50% of the tumor cell was used (19, 20).

Statistical Analysis. Statistical analysis was performed using the SPSS 9 software package. The association between MIB-1 and p27^{Kip1} expression and clinicopathological features was analyzed using χ^2 test. MIB-1 and p27^{Kip1} expression in normal kidney was studied using the Spearman rank correlation test because the data were not normally distributed. For analysis of survival data, Kaplan-Meier curves were constructed, and the log-rank test was performed. Multivariate analysis was performed using Cox's proportional hazards model with $P < 0.05$ considered statistically significant.

RESULTS

Clinicopathological Findings. The pT-stage distribution was T₁ in 22, T₂ in 19, and T₃ in 21 patients. Clinical progression occurred in 14 patients (23%) and 7 patients (11%) died from their tumor. At the end of the follow-up period, 55 patients were alive.

MIB-1 Expression in Normal Kidney and Wilms' Tumor Tissues. The PI of normal renal tissue, *i.e.*, the percentage of MIB-1-positive cells ranged between 0 and 3% (1.5 ± 1 ; mean \pm SD; Table 1 and Fig. 1A). In Wilms' tumor, immunostaining was localized in the blastemal and epithelial nuclei. The frequency of positive nuclei varied from specimen to specimen and among tumors having the same stage. In 19% of cases, the highest PI was observed in the peripheral zone of malignant tissues close to the supportive stroma. The mean percentage of blastemal MIB-1-positive cells was 9.4 ± 10 (range, 0–42%), whereas for epithelial cells, MIB-1 PI was $19.9 \pm 18.6\%$ (range, 0–53%; Table 1). In the lesions studied, the PI for the epithelium was significantly higher than those found for the blastema (Spearman rank correlation coefficient, $P < 0.01$). In 7 of 62 (11%) specimens, derived from tumors of various stages, no labeling with MIB-1 antibody was found. At a cutoff PI of 5%, MIB-1-positive blastemal and epithelial cells were found in 38 (61%) and 41 (66%), respectively, of the Wilms' tumors studied. A correlation between MIB-1 and pathological stage was not found for blastema or epithelium (Table 2).

p27^{Kip1} Expression in Wilms' Tumor Tissues. The percentage of p27^{Kip1}-positive cells in normal renal tissue

Table 1 Distribution of MIB-1 and P27^{Kip1} expression in Wilms' tumor tissues and normal renal tissues

	Wilms' tumors tissues				Normal renal tissues	
	MIB-1		P27 ^{Kip1}		MIB-1	P27 ^{Kip1}
	Blastema	Epithelial	Blastema	Epithelial		
Mean \pm SD	$9.4 \pm 10\%$	$19.9 \pm 18.6\%$	$55.1 \pm 24\%$	$59 \pm 27\%$	$1.5 \pm 1\%$	$21 \pm 14.2\%$
Range	0–42%	0–53%	3–85%	1–87%	0–3%	0–55%

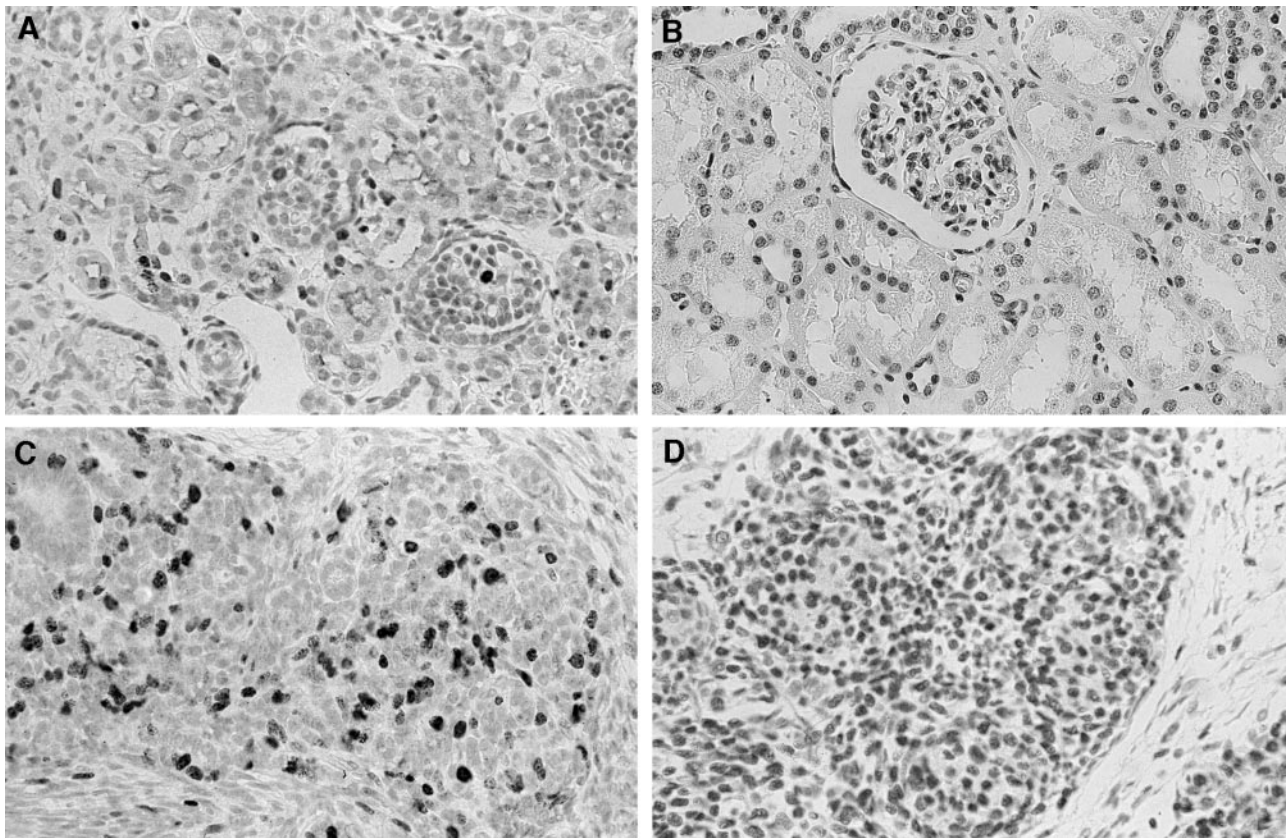


Fig. 1 Comparison of MIB-1 antigen and p27^{Kip1} in nonneoplastic and nephroblastoma tissues. Normal renal tissue sections and nephroblastoma tissues were immunostained for MIB-1 (A, C) and P27^{Kip1} (B, D), respectively. Slides were counterstained with hematoxylin (magnification, $\times 400$).

ranged between 0 and 55% (21 ± 14.2 ; Table 1 and Fig. 1B). In Wilms' tumors, p27^{Kip1} was expressed at variable levels in the blastemal and epithelial nuclei, usually with a low background and with a rather diffuse staining pattern (Fig. 1D). The number of cells expressing p27^{Kip1} as well as staining intensity varied from case to case. Infiltrating mature lymphocytes showing positive p27^{Kip1} staining served as an internal positive control. The mean percentage of blastemal p27^{Kip1}-positive cells was $55.1 \pm 24\%$ (range, 3–85%), and for the epithelium p27^{Kip1}, it was $59 \pm 27\%$ (range, 1–87%; Table 1). The p27^{Kip1} scores of blastema and epithelial tumor cells showed a significant inverse correlation compared with those found in normal tubular epithelial cells, *i.e.*, correlation coefficient of $r = -0.42$, $P < 0.05$,

$r = -0.58$, $P < 0.01$), respectively. Using the cutoff value of 50%, p27^{Kip1} positivity for blastemal and epithelial cells was found in 35 (57%) and 45 (73%), respectively, of the Wilms' tumors studied. A correlation between p27^{Kip1} and pathological stage was not found in either blastema or epithelium (Table 2).

Relationship between MIB-1 PI and p27^{Kip1} PI. In most specimens, the proportion of p27^{Kip1}-positive tumor cells was greater than the proportion of MIB-1-positive tumor cells. An inverse correlation between blastemal p27^{Kip1} expression and MIB-1-based proliferative activity was found, with a correlation coefficient of -0.311 ($P < 0.01$; Fig. 2). Notably, in the scatterplot shown in Fig. 2, a separate population of Wilms' tumor becomes apparent (indicated by arrows).

Table 2 Relationship between pT stage and blastemal and epithelial expression of MIB-1 and p27^{Kip1}^a

Stage	MIB-1		p27 ^{Kip1}	
	Blastemal	Epithelial	Blastemal	Epithelial
T1	16 (73)	15 (68)	10 (46)	14 (64)
T2	11 (58)	14 (74)	12 (63)	13 (68)
T3	11 (52)	12 (57)	13 (62)	18 (86)
Total	38 (61)	41 (66)	35 (57)	45 (73)
P	>0.05		>0.05	

^aData are presented as numbers of MIB-1-positive (cutoff value 5%) and p27^{Kip1}-positive (cutoff value 50%) Wilms' tumors, percentages between brackets.

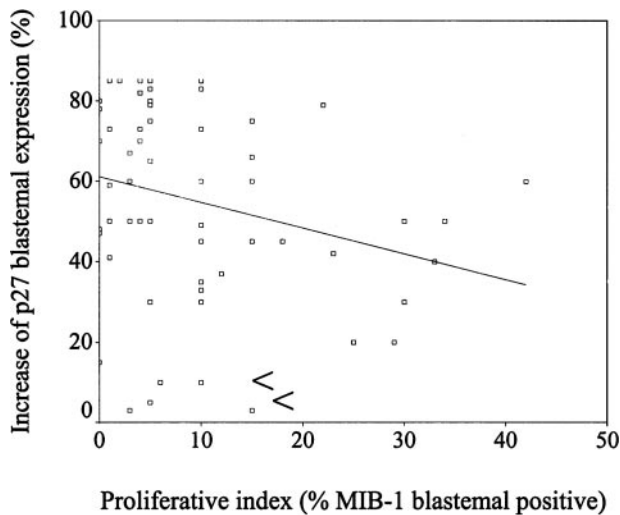


Fig. 2 Relationship between blastemal MIB-1 proliferative index (PI) and p27^{Kip1} expression in nephroblastoma. Scatterplot of MIB-1 PI versus p27^{Kip1} PI with regression line showing a correlation of the two cell cycle regulators using Spearman's correlation coefficient. Arrows indicate a separate population of Wilms' tumor.

Prognostic Value of MIB-1 and p27^{Kip1}. Univariate analysis, using the log-rank test, showed prognostic significance of blastemal MIB-1 PI and p27^{Kip1} expression for clinical progression and tumor-related death (Table 3, Figs. 3 and 4). In contrast, the epithelial MIB-1 PI and p27^{Kip1} expression did not show any prognostic value (Table 3). To assess whether MIB-1 and p27^{Kip1} expression had any prognostic impact, a multivariate Cox's regression analysis was done, including the parameters pT stage, MIB-1, and p27^{Kip1} expression. The parameters that were not dichotomic were dichotomized as follows: pT stage was divided as pT1–2 versus pT3; for MIB-1 was classified as PI < 5% versus PI > 5%; and for p27^{Kip1} as PI < 50% versus PI > 50%. Accordingly, both MIB-1 and p27^{Kip1} blastemal expression could be identified as independent prognostic variable for clinical progression besides stage (Table 4).

DISCUSSION

The assessment of the presence of cell cycle-related proteins may yield important information about the biological behavior of a tumor. A variety of methods have been used for estimation of the PI of human cancer. One of these methods is scoring of mitotic figures. However, mitosis represents only a

short phase in the active cell cycle, and accordingly, only 1–5% of all DNA-synthesizing cells is microscopically detectable (24). In recent years, MIB-1 (a true equivalent of Ki-67) has been proven to be the best proliferation marker for routine use in formalin-fixed and paraffin-embedded tissue sections (4, 7, 24). The present study was carried to investigate whether the proliferative activity represented by MIB-1 and the immunoreactivity of p27^{Kip1} has prognostic value in nephroblastoma. All patients in this study received chemotherapy before operation.

The prognostic value of the Ki-67 proliferative tumor marker as assessed by the MIB-1 antibody is well established in several types of tumors, *e.g.*, squamous cell carcinoma of the esophagus, non-Hodgkin's lymphoma, non-small cell lung cancer, cervical cancer, pancreatic head cancer, prostatic cancer (7, 25–29), as well as by Delahunt in Wilms' tumor (11). In the present study, a prognostic value of proliferative activity of the blastemal, *i.e.*, blastemal MIB-1 expression was found for clinical progression and tumor-specific survival. The epithelial component of individual tumors showed significantly higher MIB-1 scores than those found for the blastema, an observation that was also described by Khine *et al.* (10). The lack of correlation between PI as measured with MIB-1 antibody and tumor stage may be explained by the decrease in nuclear Ki-67 immunostaining as a result of hypoxia at increasing distances from the surrounding capillaries in tumors of larger size (30).

Lack of MIB-1 staining has been observed in 11% of all tumors studied. This very low proliferative activity in nephroblastoma tissue has also been reported by other investigators (10). Also, the observation that tumor stroma shows less proliferative activity than blastemal and epithelial components (10, 31) suggested that the stroma of Wilms' tumor is of dual character consisting of both desmoplastic and tumor elements. This proliferative activity is derived mainly from both neoplastic and nonplastic tissues. However, the precise histogenetic relation between the blastema and the stroma is as yet not conclusively established and needs further study. In general, this observation is in line with the notion that Wilms' tumor is composed of different tissues with a strong variation in proliferation and differentiation potentials and patterns of marker expression.

Alteration in the p27^{Kip1} expression has been described in a number of human tumors (32). In this study, we show that high levels of the cell cycle-dependent kinase inhibitor p27^{Kip1} are associated with poor survival in nephroblastoma. This is contradictory to most tumors, where a low p27^{Kip1} is associated with poor prognosis, *e.g.*, colorectal, epithelial, ovarian, breast, gastric, Barrett's, esophageal, non-small cell lung, and prostatic

Table 3 Univariate analysis of prognostic markers

Variable	Blastemal				Epithelial			
	Clinical progression		Tumor-specific survival		Clinical progression		Tumor-specific survival	
	χ^2	P^a	χ^2	P^a	χ^2	P^a	χ^2	P^a
MIB-1	5.08	0.02	4.02	0.05	2.9	0.09	0.15	0.71
p27 ^{Kip1}	8.24	0.004	4.12	0.04	0.05	0.82	0.15	0.70

^a Log-rank test.

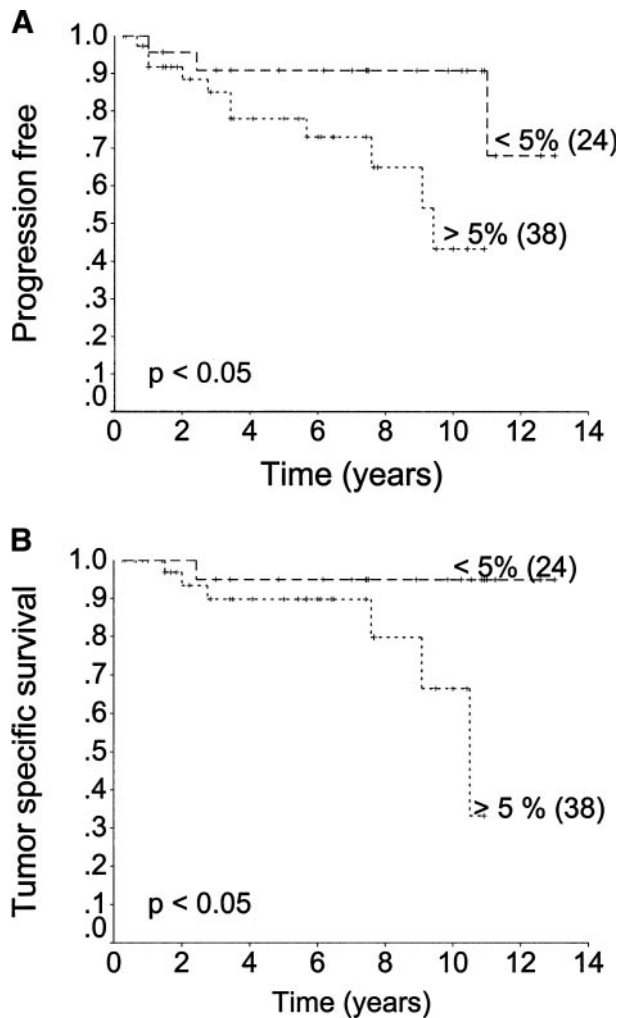


Fig. 3 Kaplan Meier curves showing a relationship between blastemal MIB-1 expression and clinical progression (A) and survival (B), respectively. Censored patients are indicated by a *tic marks* along their line. Number of patients/group is shown between brackets.

carcinomas (18–21, 32–37). The findings are, however, consistent with previous evidence that p27^{Kip1} is often expressed at relatively high levels in human cancer cell lines, which are additionally characterized by increased the expression of cyclin D1 or cyclin E (38, 39). Also, high levels of p27^{Kip1} are associated with poor survival in invasive cervical carcinoma (16)

High proliferative activity, as defined by expression of the Ki-67 analysis (MIB-1) antibody, has been shown to correlate with reduced p27^{Kip1} in lymphoid neoplasms, carcinoma of the oral cavity, and endocrine tumors, including pituitary, thyroid, and parathyroid gland hyperplasia (14, 40–43). In contrast, studies on colorectal and breast cancer showing no correlation between tumor cell proliferation and p27^{Kip1} have been reported (18, 21). The present study demonstrates that increased p27^{Kip1} expression correlates to some extent with decreased proliferative activity measured by MIB-1 (Fig. 2).

This study may indicate that MIB-1 and p27^{Kip1} protein contribute to or reflect cell proliferation by alteration of the

kinetic behavior in primary treated Wilms' tumor. Because in individual cases MIB-1 and p27^{Kip1} do not have an inverse correlation, other factors than p27^{Kip1} may also be involved in proliferative activity of Wilms' tumor. The small separate population of Wilms' tumors of mainly low p27^{Kip1} activity and low MIB-1 PI (Fig. 2) may thus indicate that in these tumors p27^{Kip1} does not play an important role in regulation of proliferation.

A limitation of this study could be that it has been performed on Wilms' tumors after pretreatment with chemotherapy. Chemotherapy is known to affect the cellular compartments of the Wilms' tumor, the blastemal component in particular. In the mean time, we have been able to perform a similar immunohistochemical study on material derived from patients that did not receive any therapy before surgery. Preliminary data of staining of a pilot group of these tumors demonstrated that overall scores of the blastemal as well as epithelial cells in these tissues significantly differ from the scores of the pretreatment

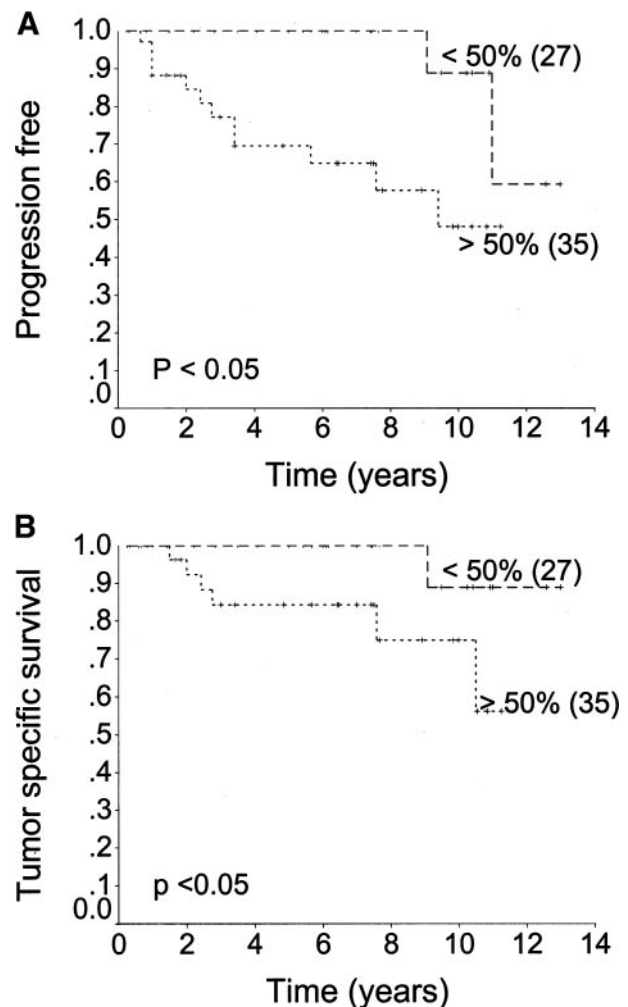


Fig. 4 Kaplan-Meier curves showing a relationship between blastemal p27^{Kip1} expression and clinical progression (A) and survival (B), respectively. Censored patients are indicated by a *tic marks* along their line. Number of patients/group is shown between brackets.

Table 4 Results of Cox's multiregression analysis

Outcome parameters	Variable	Hazard ratio	95% confidence limit	P
Clinical progression	MIB-1 (blastema)	4.9	1.2–25.3	0.03
	P27 ^{Kip1} (blastema)	7.5	1.9–41.4	0.006
	Stage	4.1	1.1–8.9	0.04
Tumor-specific, survival	MIB-1 (blastema)	3.7	1.0–69.4	0.06
	P27 ^{Kip1} (blastema)	3.6	1.0–64.8	0.06

group described in the present article. Because most of the patients responded well upon chemotherapeutic treatment, it was not to our surprise to observe that proliferation was affected, and MIB-1 scores were lower in the pretreatment group, whereas the contrary applied to the p27^{Kip1} marker.

Having access to material derived from a considerable number of patients with a good stage distribution and clinical data, the aim of the present study was to determine those factors that could predict the clinical outcome of patients after chemotherapy and surgery. In that way, the remaining proliferative activity in tumor tissue after chemotherapeutic treatment may be of prognostic value for predicting the course of disease after surgical removal of the tumor.

In conclusion, here, we report that there is an increase in the p27^{Kip1} protein levels in nephroblastoma tissues after chemotherapy compared with normal renal tissues. The blastemal expression of p27^{Kip1} showed a consistent and strong association with poor prognosis. The results support the conclusion that MIB-1 and p27^{Kip1} are relevant markers for assessing the proliferative activity and tumor cell dynamic of Wilms' tumor. Therefore, these biological predictors may potentially provide the clinical oncologist with a biological rationale in identifying patients at high risk of tumor recurrence and to guide the adjuvant chemotherapy and/or radiotherapy.

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