Expression and Prognostic Value of Wilms' Tumor 1 and Early Growth Response 1 Proteins in Nephroblastoma

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ABSTRACT

Wilms' tumor is one of the most common solid tumors of children. The protein product of the tumor-suppressor gene, Wilms' tumor 1 (WT-1), binds to the same DNA sequences as the protein product of the early growth response 1 (EGR-1) gene. There is experimental evidence that EGR-1 is involved in controlling cell growth. The expression of both genes in Wilms' tumor was studied by others, mainly at the mRNA level. The present study evaluates the prognostic value of WT-1 and EGR-1 in 61 Wilms' tumors of chemotherapeutically treated patients at the protein level, using an immunohistochemical approach. WT-1 was expressed in normal kidney tissues and in the blastemal and epithelial component of Wilms' tumor, whereas stromal tissue was negative. EGR-1 was expressed in normal kidney tissues and in the three main cell types of Wilms' tumor. In 59 and 56% of Wilms' tumor, the blastemal cells stained for WT-1 and EGR-1, respectively. The blastemal expression of WT-1 and EGR-1 and the epithelial expression of WT-1 were statistically significantly correlated with clinical stage. WT-1 immunoreactivity correlated with EGR-1 expression. Univariate analysis showed that blastemal WT-1 and EGR-1 expression were indicative for clinical progression and tumor-specific survival, whereas epithelial staining was of no prognostic value. Multivariate analysis showed that blastemal WT-1 expression is an independent prognostic marker for clinical progression other than stage. We conclude that a relationship exists between WT-1 and EGR-1 expression in clinical nephroblastomas. Blastemal WT-1 and EGR-1 expression is related to prognosis.

INTRODUCTION

Wilms' tumor is a pediatric malignancy of the kidney and one of the most common solid tumors in children (1). At present, the prediction of outcome is based mainly on histology and stage at the time of resection (2). Despite remarkable advances in elucidating molecular events involved in the pathogenesis of Wilms' tumor, an independent prognosticator has not been identified as yet (3).

The genetic basis of this tumor is highly complex, and several loci have been shown to be associated with tumor formation. The *WT-1*[2] gene (tumor suppressor gene) is localized at human chromosome 11p13. Estimates of the frequency of *WT-1* mutations are sometimes difficult to interpret because of varying methods of detection. The percentage of Wilms' tumors that have been shown to contain homozygous or heterozygous *WT-1* mutations is relatively low, however, and may be <25% and perhaps as little as 5% (4, 5). The WT-1 protein has been implicated in many processes, such as proliferation, differentiation, and apoptosis (6). Comparison of the amino acid sequence of the zinc fingers of WT-1 with the amino acid sequences of other zinc finger proteins revealed a high degree of similarity of WT-1 and early growth response genes such as *EGR-1* (7). EGR-1 expression is induced during the G_0-G_1 transition of the cell cycle in a variety of cell lines upon mitogenic stimulation, suggesting that EGR-1 is involved in controlling cellular proliferation. However, the effect of EGR-1 on cell growth may also be cell type specific (8).

The results of a number of transient transfection studies demonstrated that WT-1 represses transcription of promoters responsive to EGR-1 (9, 10). These results suggest that there may be a reciprocal expression between these two proteins: WT-1 may act as an antagonist of EGR-1 or may be a tissue-specific factor that is involved in maintaining a particular differentiated phenotype. The balance in the levels of EGR-1 and WT-1 proteins in the nucleus may therefore be critical, and inactivation of WT-1 could result in the onset of neoplasia. In the present study, the immunohistochemical expression patterns of WT-1 and EGR-1 were studied in Wilms' tumor tissues, and the prognostic value was determined in patients treated by preoperative chemotherapy and radical nephrectomy.
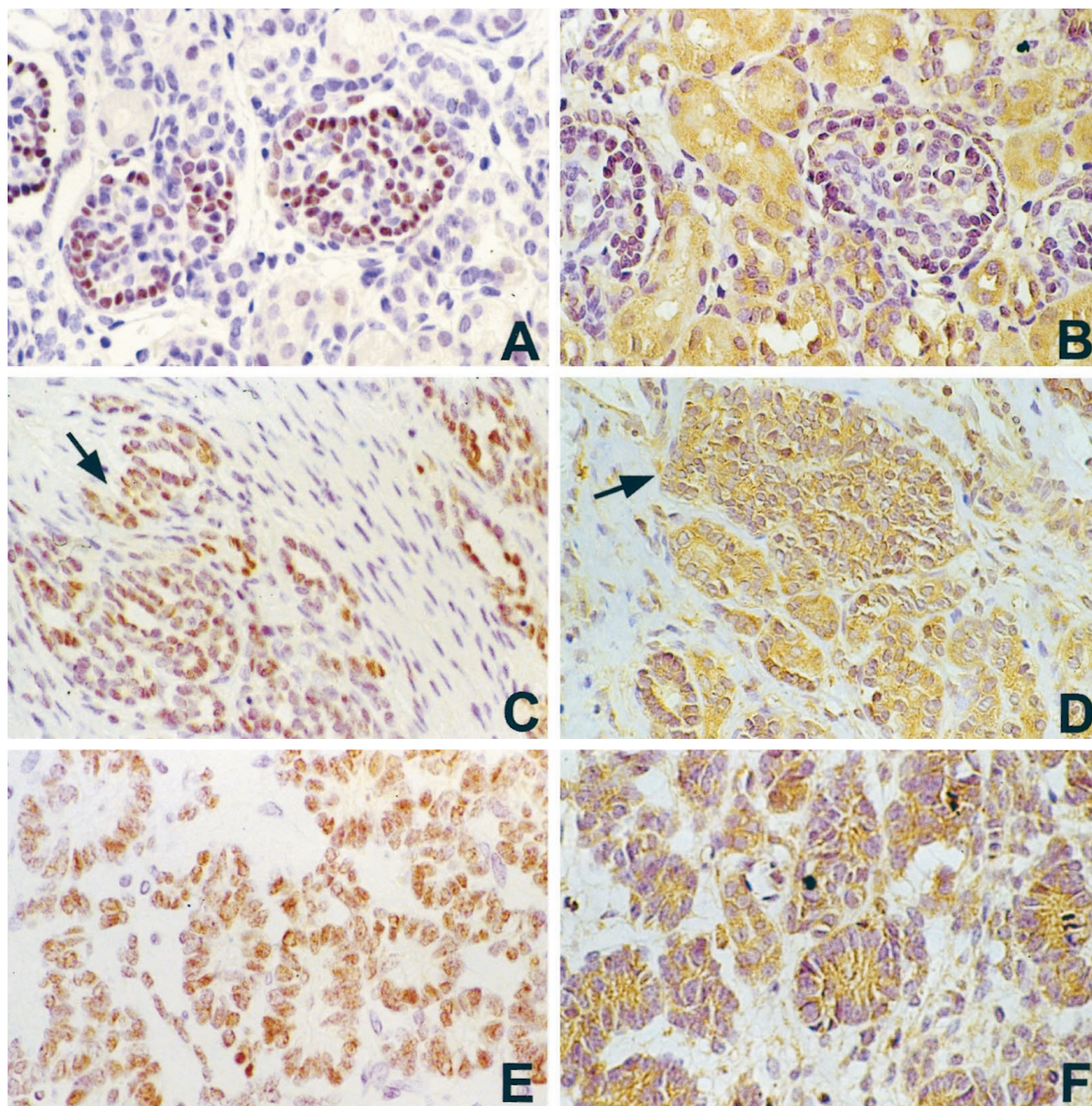
MATERIALS AND METHODS

Patients. During the period 1987–1999, 61 patients with nephroblastoma were treated by neo-adjuvant chemotherapy

[2] The abbreviations used are: WT-1, Wilms' tumor 1; EGR-1, early growth response 1.

Fig. 1 WT-1 and EGR-1 staining of normal kidney and clinical Wilms' tumor tissues. Glomerular visceral epithelium of normal kidney tissue showed positive nuclear staining of WT-1 (*A*) and tubular cytoplasmic staining of EGR-1 (*B*). Nuclear WT-1 expression was found in Wilms' tumor blastema (*C*) and epithelium (*E*), whereas cytoplasmic expression of EGR-1 was found in the blastemal (*D*) and epithelial (*F*) compartments. *Arrows* indicate stained blastemal cells in *C* and *D*, respectively. Slides were counterstained with hematoxylin (×400).

and, subsequently, with tumor nephrectomy. After treatment, the patients were followed regularly, and all data concerning diagnosis, treatment, and follow-up were stored in a database. Clinical progression was defined as histologically or cytologically proven local recurrence or the appearance of distant metastases. Tumor death was defined as death as a result of the direct effect of metastases.

Sample Selection. All nephrectomy specimens were fixed in 10% buffered formalin and embedded in paraffin. The H&E-stained slides were reviewed by an experienced pediatric pathologist (J. C. D. H.) to assess the stage according to the TNM classification (11). Among the tissue blocks available for individual patients, tumor samples containing the three different

cell types of Wilms' tumor were selected. In addition, adjacent normal kidney tissue was taken from each patient.

Immunohistochemistry. The following primary antibodies were used: F-6, a mouse monoclonal antibody against WT-1; and 588, rabbit polyclonal antibody against EGR-1, both from Santa Cruz Biotechnology (Santa Cruz, CA). The specificities and characteristics of these antibodies have been published elsewhere (7, 8). The peroxidase-antiperoxidase technique was used. Serial sections (5 μm) from all samples were mounted on 3-aminopropyle-trietoxysilane (Sigma Co., St. Louis, MO)-coated glass slides, which were incubated overnight, in a 60°C incubator. After dewaxing in fresh xylene for 10 min and rehydration in 100% methanol for 10 min, the sections were rinsed in methanol containing 3%

Table 1 Relationship between pT stage and blastemal expression

Data are presented as *n*, with percentages in parentheses.

Score	WT-1[a]			EGR-1[a]		
	T$_1$	T$_2$	T$_3$	T$_1$	T$_2$	T$_3$
<10%	4 (7%)	11 (18%)	10 (16%)	14 (23%)	10 (16%)	3 (5%)
10–25%	4 (7%)	2 (3%)			1 (2%)	4 (7%)
25–50%	13 (21%)	5 (8%)	10 (16%)	2 (3%)	5 (8%)	4 (7%)
>50%		2 (3%)		5 (8%)	4 (7%)	9 (15%)

[a] $P < 0.05$ (Pearson χ^2 test).

Table 2 Relationship between pT stage and epithelial expression

Data are presented as *n*, with percentages in parentheses.

Score	WT-1[a]			EGR-1		
	T$_1$	T$_2$	T$_3$	T$_1$	T$_2$	T$_3$
<10%	5 (8%)	8 (13%)	13 (21%)	3 (5%)	2 (3%)	3 (5%)
10–25%	8 (13%)	8 (13%)	5 (8%)	4 (7%)	6 (10%)	7 (12%)
25–50%	8 (13%)	2 (3%)	1 (2%)	10 (16%)	8 (13%)	9 (15%)
>50%		2 (3%)	1 (2%)	4 (7%)	4 (7%)	1 (2%)

[a] $P < 0.05$ (Pearson χ^2 test).

Table 3 Relationship between WT-1 and EGR-1 blastemal expression

Data are presented as *n*, with percentages in parentheses.

	Score	EGR-1[a]			
		<10%	10–25%	25–50%	>50%
WT-1[a]	<10%	14 (23%)	3 (5%)	3 (5%)	5 (8%)
	10–25%	2 (3%)		4 (7%)	
	25–50%	10 (16%)	2 (3%)	3 (5%)	13 (21%)
	>50%	1 (2%)		1 (2%)	

[a] $P < 0.05$ (Pearson χ^2 test).

Table 4 Univariate analysis of prognostic markers

	Outcome parameter							
	Blastema				Epithelium			
	Clinical progression		Tumor-specific survival		Clinical progression		Tumor-specific survival	
Variable	χ^2	P^a	χ^2	P^a	χ^2	P^a	χ^2	P^a
WT-1	9.07	0.003	5.3	0.02	0.64	0.42	0.04	0.85
EGR-1	7.24	0.007	4.23	0.04	0.41	0.52	0.03	0.86

[a] Log-rank test for trend.

hydrogen peroxide for 20 min to block endogenous peroxidase activity. The slides then were rinsed with distilled water. To enhance antigen exposure, the slides were microwaved at 700 W in 0.1 M citrate buffer (pH 6.0) for 15 min. After being cooled and rinsed with PBS, the slides were placed in a Sequenza immunostaining system (Shandon, Uncorn, United Kingdom). Sections were incubated with 10% normal rabbit serum if the monoclonal antibody was to be used or normal goat serum if the polyclonal antibody was to be used (DAKO A/S, Glostrup, Denmark). Slides were incubated in PBS-5% BSA for 15 min and subsequently overnight with the primary antibody at 4°C. The antibodies were diluted in PBS-5% BSA at 1:100 for WT-1 and 1:300 for EGR-1. Subsequently, the slides were rinsed with PBS-0.1% Tween, incubated for 30 min with rabbit antimouse antibody for the monoclonal antibody or goat antirabbit antibody for the polyclonal antibody (DAKO), and then rinsed with PBS containing 0.1% Tween. The peroxidase-antiperoxidase complex (PAP; DAKO) was diluted in PBS-5% BSA at 1:300, incubated for 30 min, and then rinsed with PBS. The antigen-antibody complex was visualized with diaminobenzidine tetrahydrochloride dihydrate (Fluka, Neu-Ulm, Germany) as chromogen. The sections were lightly counterstained with hematoxylin. Negative controls were included by replacing the primary antibody by PBS- 5% BSA. Normal kidney tissue, which was present in all clinical specimens, served as positive control.

Immunostaining Analysis (Quantification). The slides were examined at ×25 magnification without knowledge of the clinical outcome of the patients. Semiquantitative evaluation was performed by one of the authors (T. H. V. d. K.). The percentage of WT-1- and EGR-1-positive cells in a particular area was scored semiquantitatively as <10, 10–25, 25–50, and >50%. The specimens were regarded as positive when the percentage of positive cells was >10%. In addition, the amount of blastema was estimated by counting the number of low-power magnification fields of blastema.

Statistical Analysis. Statistical analysis was performed using the SPSS 9 software package. The association between WT-1 and EGR-1 expression and clinico-pathological features was analyzed using the Pearson χ^2 test. For analysis of survival data, Kaplan-Meier curves were constructed and the log-rank test for trend was performed. Multivariate analysis was performed using Cox's proportional hazards model, with $P < 0.05$ considered statistically significant.

Protein Extraction and Western Blot. To confirm the WT-1 and EGR-1 immunohistochemical data, Western analysis was performed with tissues from Wilms' tumor xenografts. Six different xenograft tissues were analyzed in total. Tissues 1–3 (see Fig. 6) originated from transplants of three individual patients, resulting in xenografts WT-7, WT-9, and WT-11, respectively, whereas tissues 4–6 (WT-15, WT-15LN, and WT-16) were from one individual patient, being specimens of a primary tumor in the right kidney (WT-15), lymph node metastasis (WT-15LN), and a primary tumor in the left kidney (WT-16), respectively. Morphologically, all six tissues contained the blastemal and stromal component, whereas in tissues 1 and 3, epithelial cells were also present.

Frozen tissues were crushed in a liquid nitrogen-chilled metal cylinder. The tissue homogenates were transferred to a lysis buffer consisting of 10 mM Tris (pH 7.4), 150 mM NaCl (Sigma), 1% Triton X-100 (Merck, Darmstadt, Germany), 1% deoxycholate (Sigma), 0.1% SDS (Life Technologies), 5 mM EDTA (Merck), and protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 mM aprotinin, 50 mg/liter leupeptin, 1 mM benzamidine, and 1 mg/liter pepstatin; all from Sigma). The samples were spun at 35,000 × g at 4°C for 10 min. The protein content of the supernatant was measured photometrically using the Bio-Rad, protein assay (Bio-Rad, Munich, Germany). The proteins were transferred to a SDS-
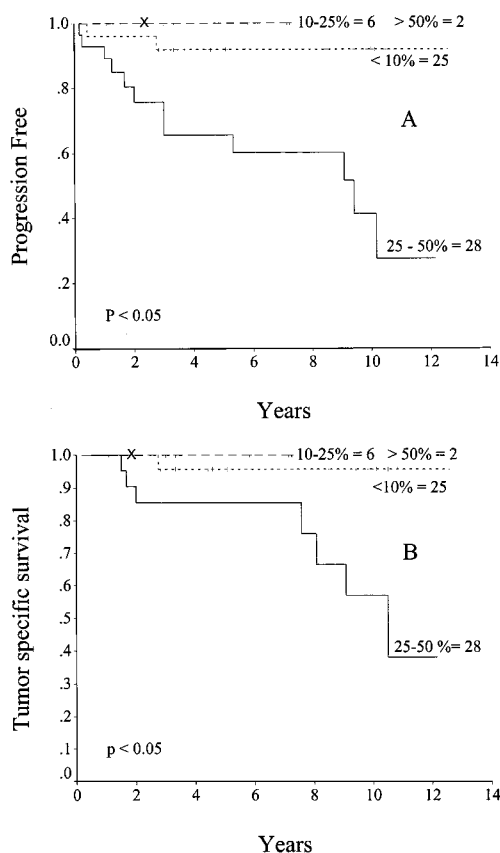
Fig. 2 Kaplan-Meier curves show a significant relationship between blastemal WT-1 expression and clinical progression (*A*) and survival (*B*). Censored patients are indicated by *tic marks* along their lines. The end of the follow-up of group >50% is indicated by an ✕. Numbers of patients per group are shown.

Fig. 3 Kaplan-Meier curves show a significant relationship between blastemal EGR-1 expression and clinical progression (*A*) and survival (*B*). Censored patients are indicated by *tic marks* along their lines. Numbers of patients per group are shown.

polyacrylamide gel, and electrophoresis was performed in 10× diluted tray buffer for 2 h. The gel was blotted to a 0.45 μm cellulose nitrate membrane (Schleicher & Schuell, Dassel, Germany). Prestained markers were used as size standards (Novex, San Diego, CA). The immunoblot was blocked for 1 h with 5% dry milk (Sigma) in 0.1% Tween 20 (Sigma). The antibodies were diluted 1:1000 in 5% dry milk and were applied overnight at 4°C. After rinsing with PBS-0.1% Tween, the blot was incubated with horseradish peroxidase-labeled goat antirabbit antibody (1:2000; DAKO) for 1 h. Subsequently, the blot was incubated for 1 min with a 1:1 mixture of luminol and oxidizing reagent (Chemiluminescence kit; DuPont NEN, Boston, MA). Excess reagent was removed by placing the blot on a piece of Whatman paper. Finally, the antibodies were visualized by exposure of the blot to an X-ray film for 30 s.

RESULTS

Clinicopathological Findings. The T-stage distribution was T_1 in 21, T_2 in 20, and T_3 in 20 patients. Clinical progression occurred in 14 patients (23%). Twenty-five patients (41%) had large amounts of blastema. The patient distribution was 29 (48%) females and 32 (53%) 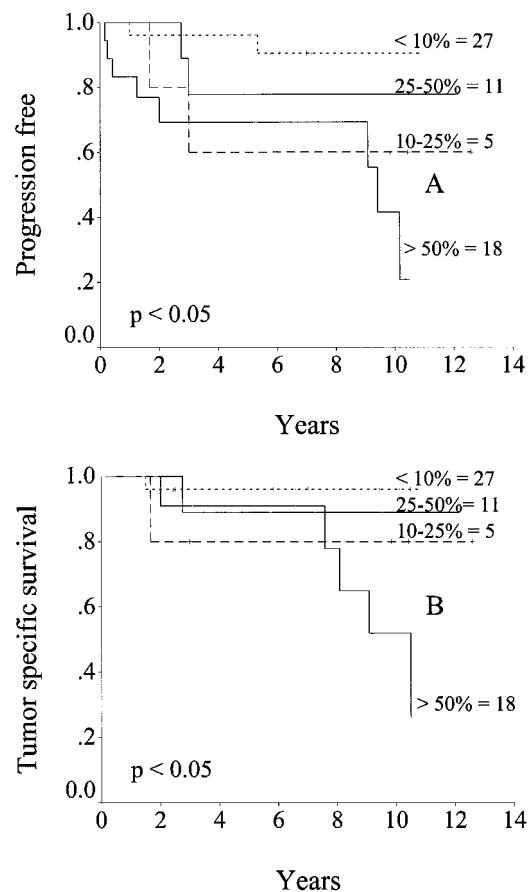males. The mean overall follow-up period was 5.7 years, and the mean age at operation was 4.2 years. Eight patients (13%) died from their tumors. At the end of the follow-up period, 53 patients were alive.

WT-1 Expression in Wilms' Tumor Tissues. WT-1 immunohistochemistry of normal kidney showed a very intense nuclear staining of glomerular visceral epithelial cells but faint staining of the tubules (Fig. 1*A*). WT-1 immunoreactive blastemal and epithelial cells were found in 59 and 57% of patients, respectively, whereas no expression was found in the stromal component. Immunostaining was localized in the nucleus (Fig. 1, *C* and *E*). A statistically significant correlation was found between WT-1 protein and pathological stage, both for blastema and epithelium (Tables 1 and 2). There was variability in the intensity of WT-1 staining in the same component and among the tumors having the same stage. Epithelial differentiation in tumors was accompanied by clear WT-1 expression (Fig. 1*E*).

EGR-1 Expression in Wilms' Tumor Tissues. EGR-1 immunohistochemistry of normal kidney showed a very intense cytoplasmic staining of proximal and distal convoluted tubules, but faint staining of the glomeruli (Fig. 1*B*). EGR-1 immunoreactivity was found in the blastemal, epithelial, and stromal components of nephroblastoma tumors. EGR-1-immunoreactive
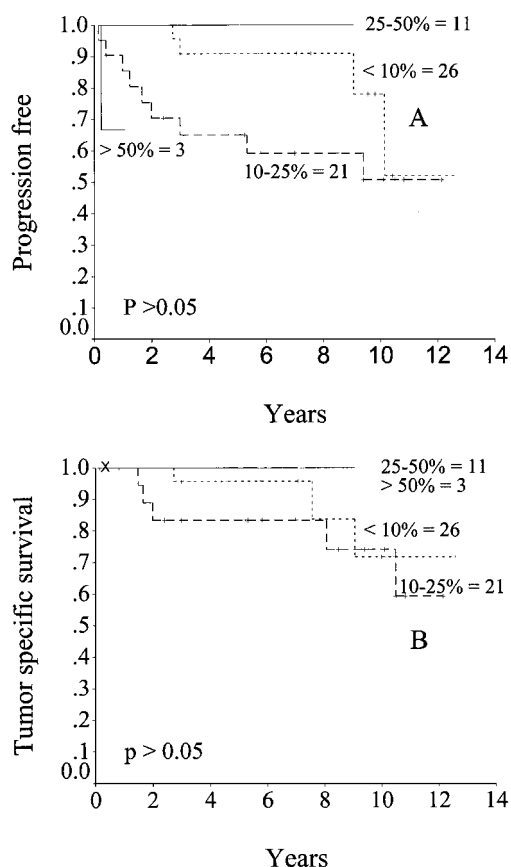
Fig. 4 Kaplan-Meier curves show a nonsignificant relationship between epithelial WT-1 expression and clinical progression (*A*) and survival (*B*). Censored patients are indicated by *tic marks* along their lines. The end of the follow-up of group >50% (*B*) is indicated by ×. Numbers of patients per group are shown.

Fig. 5 Kaplan-Meier curves show a nonsignificant relationship between epithelial EGR-1 expression and clinical progression (*A*) and survival (*B*). Censored patients are indicated by *tic marks* along their lines. Numbers of patients per group are shown.

blastema and epithelium were found in 56 and 87% of patients, respectively. Immunostaining was cytoplasmic (Fig. 1, *D* and *F*). The nephroblastoma sections showed intense expression of the epithelium with little expression in the stromal components (Fig. 1*F*). In contrast to WT-1, EGR-1 expression was more widely distributed and more intense. The blastemal expression of EGR-1 protein correlated with the pathological stage (Table 1). A statistically significant correlation was found between blastemal WT-1 and EGR-1 expression (Table 3).

Prognostic Value of WT-1 and EGR-1 Molecules. Univariate analysis using the log-rank test for trend showed a prognostic value of blastemal WT-1 and EGR-1 expression for clinical progression and tumor-related death (Table 4; Figs. 2 and 3). The epithelial expression of EGR-1 and WT-1 did not show any prognostic value (Table 4; Figs. 4 and 5). To test whether WT-1 and EGR-1 have any prognostic impact, a multivariate Cox's regression analysis was done that included the parameters pT stage and WT-1 and EGR-1 expression. The parameters that were not dichotomic were dichotomized as follows: pT1–2 *versus* pT3; and immunoreactive score <10% *versus* >10%. In that analysis, blastemal WT-1 could be identified as an independent prognostic marker for clinical progres-
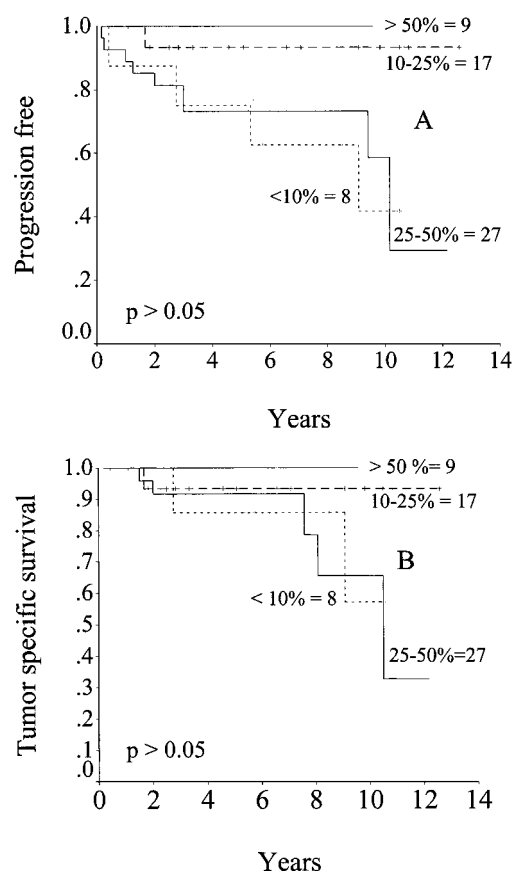
sion other than stage (Table 5). Regarding the amount of blastema, no prognostic value was found (data not shown).

Immunoblot Analysis. Immunoblot analysis of tissue lysates of a panel of human Wilms' tumor xenografts identified the specificity of the antibodies for detection of WT-1 and EGR-1 proteins (Fig. 6). Morphologically, all six tissues contained blastemal and stromal components, whereas in tissues 1 and 3, epithelial cells were also present. WT-1 was detected as a single band corresponding to a molecular size of 52 kDa, whereas EGR-1 was detected as a single 80-kDa band. Among this relatively small group of tissues, heterogeneous patterns of WT-1 and EGR-1 expression were found. As expected, the majority of tissues showed expression of WT-1, although it is remarkable that two tissues were devoid of any WT-1 protein. Interestingly enough, both of these tumors had relatively low EGR-1 expression. Clearly, there is no correlation between the expression of the proteins and the morphological characteristics of the tissues.

DISCUSSION

The interaction of the related proteins WT-1 and EGR-1 has been studied during recent years. The expression of both genes in Wilms' tumor was examined by others mainly at the mRNA level and was compared with clinical, histological, and

Outcome parameter	Variable	Hazard ratio	95% Confidence limit	P
Clinical progression	WT-1 (blastema)	9.2	2.0–42.2	0.004
	Stage	5.2	1.7–15.9	0.003
Tumor-specific survival	WT-1 (blastema)	7.4	0.91–60.3	0.06

Fig. 6 Immunoblot analysis of WT-1 (*A*) and EGR-1 (*B*) proteins in nephroblastoma tissues derived of six human xenograft models with approximate indication of size markers.

molecular features (12–14). The present study was carried out to investigate whether the expression of WT-1 and EGR-1 at the protein level has a prognostic value in specimens of clinical nephroblastoma, using paraffin-embedded tissue sections. All of the patients received chemotherapy before nephrectomy. In the present study, the clear expression of both WT-1 and EGR-1 in normal kidney suggests that the interaction of WT-1 with EGR-1 occurs well after the inductive events of the ureteral epithelial and metanephric blastema (15). Because normal kidney tissue was present in almost all sections, this could serve as an internal control for the immunostaining procedure.

WT-1 expression is restricted to certain cell types and to a specific stage of differentiation. Thus, the level of WT-1 expression in neoplasia would be expected to correlate with the relative amount of the specific cell type present. Tumors that exhibit primarily blastemal and epithelial differentiation show higher levels of WT-1 expression than those in which stroma is predominantly present (16, 17). In our present study, no correlation was found between the amount of blastema and prognosis. There was some heterogeneity in the intensity of signals both between cases and in tumors of similar stage. Variability in the intensity of immunostaining may be explained by the wide variation in the levels of WT-1 transcripts that has been noted in Wilms' tumors by Northern blot analysis (12, 16, 18). Epithelial differentiation in tumors was accompanied by diffuse WT-1 expression (Fig. 1*E*).

Stromal areas of the tumor did not express WT-1 (Fig. 1*C*). The negative stromal elements included differentiated mesenchymal tissue, in which adipose tissue and smooth muscle were seen. WT-1 mRNA has not been detected in the stromal component of Wilms' tumors, nor have recent morphological studies demonstrated the WT-1 protein in the stroma (19). These results suggest that there is pathogenetic heterogeneity in Wilms' tumors, with stromal-predominant tumors having complete loss of expression the gene.

WT-1 has been investigated in several human tumors other than Wilms' tumor. Testicular cancer has been demonstrated to produce decreased expression of WT-1 (20, 21). On the other hand, the *WT-1* gene was found to be overexpressed in leukemia (22), and ovarian cancer (23), in which WT-1 mRNA levels appeared to be much higher than in normal tissues. WT-1 expression was much lower in benign prostatic hyperplasia than in normal prostatic tissues, but no changes were seen in prostatic adenocarcinoma (24). In addition, altered expression of the *WT-1* gene was demonstrated in human breast cancer (25). These phenomena may be related to the ability of WT-1 to enhance transcription of growth factors and other genes when present in a mutated form (26) or in the presence of modulating factors (21). A correlation exists between the levels of WT-1 expression and poor prognosis in human hematopoietic malignancies (27, 28).
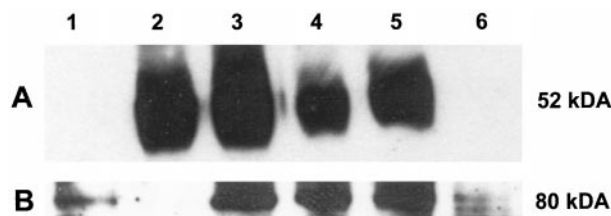
Very recent studies, using RNA slot blot analysis, showed expression of EGR-1 during murine nephrogenesis and overexpression in some clinical Wilms' tumor (14, 15). In the present study, at the protein level EGR-1 was expressed in the three components of Wilms' tumor (blastema, epithelium, and stroma). EGR-1 is expressed in a high percentage of the epithelial component of nephroblastoma specimens. Our study demonstrated a significant increase in the expression of EGR-1 in aggressive tumors. These observations support the hypothesis that EGR-1 plays a role in tumor cell proliferation and/or tumor formation.

EGR-1 expression has been shown to correlate with the transformed phenotype of B-lymphocytes immortalized with EBV, causing Burkitt lymphoma (29). Low expression of EGR-1 has been demonstrated in human breast cancer cells and tumor tissues, as well as in human lung cancer compared with normal lung tissues (30, 31), whereas EGR-1 is overexpressed in prostate cancer (32). The apparent contradictory role of EGR-1 in different human carcinomas could be attributable to the influence of the intracellular milieu and the presence of other proteins in a certain cell type (33, 34). EGR-1 expression in nephroblastoma was found to be of prognostic value in predicting clinical progression and tumor-specific death (Fig. 3). Increased expression of EGR-1 was associated with an increased risk.

Multivariate analysis showed that blastemal WT-1 expression independently predicted clinical progression. Furthermore, both WT-1 and EGR-1 bind to the same DNA response element, suggesting the possibility of stimulation of nephroblastoma growth via competition of EGR-1 with WT-1-binding elements on target DNA (12, 35). Although the mechanism by which EGR-1 counteracts the effect of WT-1 is unknown, WT-1 and EGR-1 may have the ability to stimulate carcinogenesis in the kidney through one or more pathways suggested above.

Our findings suggest that a relationship exists between WT-1 and EGR-1 proteins in clinical nephroblastoma. Expression in the blastema, which rather than the epithelium is the most malignant component of Wilms' tumor, is predictive of poor prognosis. Univariate analysis showed that WT-1 and EGR-1 blastemal expression is related to prognosis. WT-1 blastemal expression is an independent predictor for clinical progression other than stage.

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