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’ tumors, 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 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 EGR-1 (7). EGR-1 expression is induced during the G0-G1 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
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-aminopropyl-triethoxysilane (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%
stained with hematoxylin. Negative controls were included by using 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 χ² 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/ml leupeptin, 1 mM benzamidine, and 1 mg/ml 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-
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 T1 in 21, T2 in 20, and T3 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. 1A). 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. 1E).

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. 1B). EGR-1 immunoreactivity was found in the blastemal, epithelial, and stromal components of nephroblastoma tumors. EGR-1-immunoreactive
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. 1F). 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 progression 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
WT-1 and Nephroblastoma Prognosis

produce decreased expression of WT-1 (20, 21). On the other hand,
the gene.
stromal-predominant tumors having complete loss of expression
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Wilms' tumors, nor have recent morphological studies demon-
WT-1 mRNA has not been detected in the stromal component of
mal tissue, in which adipose tissue and smooth muscle were seen.
The negative stromal elements included differentiated mesenchy-
mal 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).

Table 5 Results of Cox's multi-regression analysis

<table>
<thead>
<tr>
<th>Outcome parameter</th>
<th>Variable</th>
<th>Hazard ratio</th>
<th>95% Confidence limit</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinical progression</td>
<td>WT-1 (blastema)</td>
<td>9.2</td>
<td>2.0–42.2</td>
<td>0.004</td>
</tr>
<tr>
<td></td>
<td>Stage</td>
<td>5.2</td>
<td>1.7–15.9</td>
<td>0.003</td>
</tr>
<tr>
<td>Tumor-specific survival</td>
<td>WT-1 (blastema)</td>
<td>7.4</td>
<td>0.91–60.3</td>
<td>0.06</td>
</tr>
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Very recent studies, using RNA slot blot analysis, showed
expression of EGR-1 during murine nephrogenesis and overexpres-
sion 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 trans-
formed 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-spe-
cific death (Fig. 3). Increased expression of EGR-1 was associated
with an increased risk.

Multivariate analysis showed that blastemal WT-1 expres-
sion 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 blast-
emal expression is an independent predictor for clinical progres-
sion other than stage.

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REFERENCES


