

Prognostic Markers
in
Nephroblastoma (Wilms' Tumor)

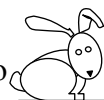
Mazen Ahmed Ghanem



Prognostic Markers in Nephroblastoma (Wilms' Tumor)

Prognostische Markers van het Nephroblastoom
(Wilms' Tumor)

Cover design: Gert-Jan van den Bemd/White Rabbit Photo



*Cover representing ancient Egyptian structures in a Dutch context.
The hole in one pyramid reflects the pathological process studied.*

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Prognostic Markers in Nephroblastoma (Wilms' Tumor)

Prognostische Markers van het Nephroblastoom
(Wilms' Tumor)

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To the soul of my father
To my mother
To my family
To Egypt
Whom I owe much more than I can give

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Abbreviations

Bcl-2	B-cell lymphoma
BLA-1	Blastemal-associated antigen
CA125	Carbohydrate antigen 125
CD44s	Cluster of Differentiated 44 standard
cDNA	Complementary Deoxyribo Nucleic Acid
CNWT	Complete necrotic Wilms' tumor
EGF	Epidermal Growth factor
EGR-1	Early Growth Response -1
FGF	Fibroblast Growth factor
GRB-7	Growth Factor Receptor bound
HGF	Hepatocyte Growth Factor
HSP70	Heat Shock Protein 70
hTR	Human Telomerase
IGF-I, II	Insulin Growth Factor I, II
IHC	Immunohistochemistry
kDa	Kilodalton
LOH	Loss of heterozygosity
MDR	Multidrug resistance
MK	Midkine
MSI	Microsatellite instability
NGF	Nerve Growth Factor
NWTS	National Wilms' Tumor Study
P53	Phospholipoprotein-53
Pgp	P-glycoprotein
PCNA	Proliferating Cell Nuclear Antigen
PCR	Polymerase chain reaction
PDGF	Platelet-derived Growth Factor
SIOP	Société Internationale d'Oncologie Pédiatrique
SSCP	Single strand conformation polymorphism
TGF	Transforming Growth Factor
TPA	Tissue Polypeptide Antigen
VEGF	Vascular Endothelial Growth Factor
WT-1	Wilms' Tumor gene 1

CHAPTER 1

General Introduction

1.1. Epidemiology

Rance, in 1814, apparently was the first to recognize renal tumors of infancy, called nephroblastoma, as a separate entity; Max Wilms, in 1899, further characterized this tumor composed of primitive cells and structures that has become associated with his name. Other more descriptive terms commonly used include mixed tumor of the kidney and embryoma of the kidney. Wilms' tumor (WT) is the most common malignant neoplasm of the urinary tract in children and is responsible for 8% of all solid tumors in children. This tumor comprises more than 80% of genitourinary cancers in children younger than 15 years (Young, 1978). WT occurs in approximately 1/10,000 children between 1 and 6 years of age. In about 75% of cases the diagnosis is made in children between 1 and 5 years of age with a peak incidence between 3 and 4 years. The male-to-female ratio is almost equal. Occasionally the tumor occurs in adults. Familial cases are rare and found in about 1% of patients (Olsen, 1970). WT may vary considerably in their histopathological pattern and composition, displaying various variants. Nevertheless, the pattern of variation in morphology is almost uniform worldwide, with little geographic variation (Innes, 1972). At diagnosis, the median age was 36.5 months for males and 42.5 months for females with unilateral disease. Bilateral, however disease was noted to become manifest earlier at median ages of 23.5 and 30.5 months, respectively (Breslow, 1982; 1988).

1.2. Tumor staging

A number of different staging systems have been advocated for WT. The clinical-pathologic staging system established by the NWTSS group (National Wilms' Tumor Study) and SIOP (Société Internationale d'Oncologie Pédiatrique) pathological TNM classification have been the most widely adopted (D'Angio, 1989) (table 1,2). As pre- and postoperative chemotherapy is the treatment of choice for children over 6 months of age treated according to SIOP protocols, pathological staging is performed after preoperative chemotherapy in this age group, whereas NWTSS staging identifies disease status at the start of treatment.

The assessment of regional nodes in the TNM is more extensive than in the SIOP staging. Presence of ascites and preoperative needle biopsy testing are included in TNM classification. Peritoneal metastases and tumor extension outside the normal contour of the kidney with a pseudocapsule between kidney and tumor are not included in this classification. On the other hand, SIOP staging assesses not only the extent of residual viable tumor but also the sites of extension of necrotic tumor. Other SIOP 93-01 staging criteria are similar to those of NWTSS-5, though there are some minor differences regarding the definition of stage I based on the extent of capsular penetration (Beckwith, 1989).

1.3. Tumor histopathology

WT is thought to be the result of an abnormal proliferation of metanephric blastema lacking a normal differentiation into tubules and glomeruli (Machin, 1980). The classical (triphasic) nephroblastoma consists of blastemal, epithelial and stromal components (Fig.1). All three components do not always present and biphasic and monophasic nephroblastomas are not rare at all. The morphologically distinct components of WT can also be demonstrated by employment of differentiation tissue-specific markers. The tubular or epithelial components express cytokeratin and epithelial membrane antigen (EMA), while the blastemal component is negative for both antigens (Garvin, 1987).

Table 1: Wilms' tumor staging

NWTS	Tumor staged following surgery and before chemotherapy
Stage I	- Tumor limited to the kidney and completely excised, with an intact capsule on histology. - The tumor has not ruptured before or during its extirpation nor has a biopsy been taken (fine needle biopsy excluded). - A tumor extending beyond the normal contours of the kidney but surrounded by an intact capsule or pseudocapsule. - Tumor infiltration of the capsule but not reaching the surface of the specimen. - Invasion limited to the pelvis should be classified as stage I, which is different from previous trials and studies (SIOP 1, 5, and 6).
Stage II	Tumor extending outside the kidney, but probably completely excised Local extension of the tumor occurs in the following areas: - a- Penetration beyond the tumor capsule into the peri-renal and/or peri-pelvic tissues. "Adhesion" histologically confirmed as due to tumor. - b- Invasion of the hilar nodes and/or peri-aortic nodes at the origin of the renal artery confirmed histologically. All the excised nodes must be carefully searched for tumor cells. Completely necrotic or xanthomatous lymph nodes have to be classified as positive. Metastatic nodes have to be investigated on radicality of the excision. - c- Invasion of the vessels outside the kidney or thrombosis caused by tumor in these vessels. - d- Invasion of the ureter.
Stage III	Incomplete excision, without haematogenous metastases. This stage occurs if one or several of the following conditions are present: - a- A tumor biopsy has been taken before or during surgery (fine needle biopsy excluded). - b- Tumor rupture before or during surgery. - c- Peritoneal metastases, which are not simple tumor adhesions of stage II. - d- Invasion of lymph nodes beyond the local regional nodes. - e- Incomplete excision (e.g. infiltration or a tumor thrombus in vena cava not lifted out). Incomplete excision or rupture of metastatic lymph nodes.
Stage IV	Distant metastases, involving lungs, liver, bones, brain, etc.
Stage V	Bilateral renal involvement at diagnosis
SIOP	Tumor staged following surgery preceded by chemotherapy (unless metastases present at presentation)
Same as NWTS-4 except	
Stage II	Extra-renal tumor extension by extracapsular invasion, regional lymph node invasion, extrarenal vascular extension or ureteral invasion completely excised.
Stage III	Incomplete surgical resection of extracapsular invasion, including preoperative biopsy, preoperative rupture, peritoneal implants or nodal metastasis beyond regional lymph nodes.

Table 2: pTNM Pathological Classification

T	T0	a. No evidence of tumor b. Intrarenal tumor completely necrotic or fibrous as result of pre-operative treatment.
	T1	a. Intrarenal and within the contours of the kidney c. Partly or mostly pseudo extrarenal with a pseudocapsule c. Invasion of the pelvis
	T2	Invasion beyond the capsule, tumor completely excised: a. Invasion of perirenal / perihilar fat tissue b. Invasion of extrarenal vessels c. Invasion of ureter
	T3	I. Pre/ peri- operative rupture: a. With spillage b. Without spillage II. Pre-operative biopsy (fine needle biopsy excluded) III. Tumor irradically excised: a. Microscopic residue b. Macroscopic residue c. Malignant ascites d. Tumor not resectable
N	N0	Regional nodes negative
	N1	Regional nodes positive a. Completely resected b. Incompletely resected c. Necrotic nodes completely resected d. Necrotic nodes incompletely resected
	NX	Nodes not received or not investigated by the pathologist
M	M0	No evidence of distant metastases
	M1	Distant Metastases: a. Organs b. Extra regional lymph nodes
	MX	Distant metastases can not be assessed

The tumor blastema is occasionally found positive or minimally reactive for vimentin. Especially the immature muscle fibers in the stromal component clearly express desmin (Garvin, 1985).

The SIOP group has changed the nomenclature and classification of Wilms' and non-Wilms' tumors at the Stockholm meeting in June 1994 (table 3). Instead of low (favorable), intermediate (standard) and high (unfavorable) grades of malignancy, the terms "low, intermediate and high" risk tumor have been introduced. Recently, the SIOP (Stockholm) working classification has been revised and now incorporates the results of the latest SIOP trials and studies. Some entities that existed in the previous classification have been excluded (nephroblastoma with fibroadenomatous structures), grouped with other types (highly differentiated epithelial nephroblastoma with epithelial type) or moved into another risk group (blastemal type nephroblastoma) (Vujanic, 1999a) (table 4).

However, there is no major difference between NWTS classification and the Stockholm Working Classification of Renal Tumors of Childhood. The prevalence of anaplasia as indicative for high-risk tumors in SIOP 9 (4.7%) is similar to that in NWTS studies (5%) (Faria, 1996; Vujanic, 1999b). Analysis of NWTS-1 identified extreme nuclear pleomorphism (anaplasia) of tumor cells as the most powerful predictor of poor prognosis in WT. The diagnosis of anaplasia is based on the following cytological criteria: markedly enlarged and usually multipolar mitotic figures, threefold nuclear enlargement compared with adjacent nuclei of the same cell type and hyperchromasia of the enlarged nuclei (Zuppan, 1998). The histogenesis of the mesoblastic nephroma, clear cell sarcoma and rhabdoid tumor of the kidney are uncertain, but it is believed that these are entities unrelated to nephroblastoma. However, they are all included in the new SIOP classification system since they represent typical renal tumors of childhood (table 3)

Table 3: The Stockholm working classification of renal tumors of childhood (1994)

I- Low risk tumor (“Favorable”) -Cystic partially differentiated nephroblastoma -Nephroblastoma with fibroadenomatous-like structures -Nephroblastoma of highly differentiated epithelial type -Nephroblastoma completely necrotic after pre-operative chemotherapy - Mesoblastic nephroma	
II- Intermediate risk tumor (“ Standard”) -Non-anaplastic Nephroblastoma with its variants -Nephroblastoma- necrotic but some features left (< 10%)	
III- High risk tumor (“Unfavorable”) -Nephroblastoma with anaplasia -Clear cell sarcoma -Rhabdoid tumor of the kidney	
IV- Other tumor or lesion -Cystic nephroma -Renal cell carcinoma (all variants) -Transitional cell carcinoma -Neuroepithelial tumor • Renal neuroblastoma • Renal carcinoid -Adenomas -Miscellaneous sarcomas -Renal lymphoma -Angiomyolipoma -Metastases from other sites -Other tumor and lesions	
V- Addendum Presence / absence of nephrogenic rests should be clearly stated	

Table 4: Revised SIOP working classification of renal tumor of childhood (2001)

A. For pretreated cases:	
I- Low risk tumor	- Mesoblastic nephroma - Cystic partially differentiated nephroblastoma - Completely necrotic nephroblastoma
II- Intermediate risk tumor	- Nephroblastoma- epithelial type - Nephroblastoma- stromal type - Nephroblastoma- mixed type - Nephroblastoma- regressive type - Nephroblastoma- focal type
III- High risk tumors	- Nephroblastoma- blastemal type - Nephroblastoma- diffuse anaplasia - Clear cell sarcoma of the kidney - Rhabdoid tumor of the kidney
B. For primary nephrectomy cases:	
I- Low risk tumors	- Mesoblastic nephroma - Cystic partially differentiated nephroblastoma
II- Intermediate risk tumors	- Non-anaplastic nephroblastoma - Nephroblastoma- focal anaplasia
III- High risk tumors	- Nephroblastoma- diffuse anaplasia - Clear cell sarcoma of the kidney - Rhabdoid tumor of the kidney

1.4. Histopathological changes after preoperative chemotherapy

Preoperative chemotherapy causes both macroscopical and histopathological changes in WT (Guarda, 1984; Zuppan, 1991; Boccon-Gibod, 2000) and at macroscopy extensive necrosis may be noted (Fig.2). The most dramatic chemotherapeutic change affects the stroma, being the first element to disappear. Next, the blastematos nodules become reduced in size and undergo necrosis or maturation, often into rhabdomyomatous structures. Studies dealing with pretreated (SIOP) and untreated tumor (NWTs), which included morphology, age, and frequency of the histological pattern, suggested that diffuse blastema is the more responsive histological component (Zuppan, 1991; Harms, 1995; Vujanic, 1999b). Differentiated elements appear to be more resistant to chemotherapy, as indicated by the lack of necrosis in tubules or glomeruloid structures (Bracken, 1982). Xanthomatous and fibrotic changes also are present. Some cytological changes (hyperchromatism, degenerative nuclear alterations) share some characteristics with anaplasia. Chemotherapy has the potential to extensively ablate the immature component of WT, with only scattered mature elements remaining occasionally (Bannayan, 1971; Becht, 1983; van Leeuwen, 1987). Alterations of tumor stage by preoperative therapy are a mixed blessing: while providing potentially important prognostic information they also complicate determination of true tumor stage, which may result in either undertreatment, or overtreatment of the individual patients.

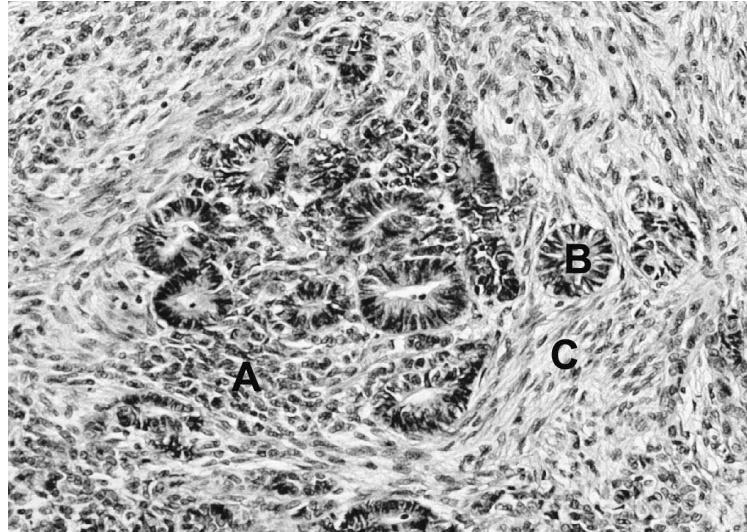


Fig.1
Nephroblastoma tissue with the triphasic component (blastemal (A), epithelial (B), stroma (C)).
The section was stained with hematoxyline and eosine (original magification x 400)



Fig.2
A longitudinal section of Wilms' tumor specimen after chemotherapeutic treatment with gross features of necrotic tissue mainly in the upper pole and middle zone (indicated by arrow)

1.5. Treatment

The management of WT varies geographically. Two approaches are well established and have their merits but the common goal of both is maximizing the cure rate and minimizing treatment morbidity. In Europe and many other countries the protocols of SIOP are followed (table 4) (de Kraker, 1995). Treatment is based on the results of the initial imaging evaluation and chemotherapy is then started before surgical extirpation of the tumor. In the current SIOP trial stage I tumors patients with favorable histology receive no postoperative therapy, whereas children with advanced stages are stratified into more intensive regimens.

Table 5: SIOP nephroblastoma trials and studies

SIOP 1 (Sept. 1971)	Preoperative radiotherapy significantly reduces incidence of tumor rupture and down staging the tumor Additional actinomycin D (6X) does not improve EFS* in either arm
SIOP 2 (Oct. 1974)	Confirmation of benefits of preoperative radiotherapy Postoperative chemotherapy for 6 months is as good as for 15 months
SIOP 5 (Jan. 1977)	Preoperative 2-drug chemotherapy is as effective as preoperative radiotherapy in avoiding rupture and improving the stage distribution.
SIOP 6 (July 1980)	Preoperative 2-drug therapy now leads to 56% stage I patients Postoperative treatment according to risk factors Stage I No difference in the two arms of different duration Stage II No significant difference in EFS for the R+ and R- arm Stage A significant difference in favor of the arm containing II+& III adriamycin
SIOP 9 (Nov. 1987)	8 weeks preoperative treatment does not produce a more favorable stage distribution than 4 weeks
SIOP-93 01 (July 1993)	Therapy has to be adapted to risk - The role of the pathologist has been enhanced A new SIOP (Stockholm) working classification close to that of NWTs has been developed

* EFS: Event-free survival

In the far majority of centers in the United States and Canada, under the protocols of NWTs, primary nephrectomy is done for all but the largest unilateral tumor and subsequent adjuvant therapy is based on surgical and pathological findings (D'Angio, 1989) (table 5). Presently, the only indications for pre-operative chemotherapy in the NWTs studies are bilateral tumor, massive non-resectable unilateral tumor and vena caval tumor thrombus above the hepatic veins.

Table 6: Summary of NWTS treatment protocols

Inclusion dates		Major conclusions
NWTS-1	1969-1973	In Stage I with favorable histology: postoperative flank radiation therapy is not necessary in children less than 2 years old In Stage II-III combination of vincristin + dactinomycin is more effective than either drug alone
NWTS-2	1974-1978	In Stage I with favorable histology 6 months of vincristin + dactinomycin is equivalent to 15 months. In stages II-IV addition of doxorubicin to vincristin + dactinomycin improved relapse-free survival
NWTS-3	1979-1986	In Stage II with favorable histology: 15 months of vincristin + dactinomycin without abdominal radiation was equivalent to protocols with radiation or with addition of doxorubicin In Stage III with favorable histology: (1) addition of doxorubicin to vincristin + dactinomycin was superior to 2-drug regimen, (2) 1,000 cGy. Abdominal radiation was equivalent to 2,000cGy. In Stage IV with favorable histology: addition of cyclophosphamide to doxorubicin + vincristine+ dactinomycin did not improve results. In Stage II-IV with unfavorable histology: addition of cyclophosphamide to doxorubicin + vincristin + dactinomycin is superior to 3-drug regimen (except in focal anaplasia)
NWTS-4	1986-1994	Pulse-intensive regimens as effective as standard regimens 6 months chemotherapy is as effective as 15 months for stage II-IV favorable histology and stages I-IV clear cell sarcoma of the kidney Examined economic impact of various protocols
NWTS-5	1994	

NWTS-5 (1995) started the assessment of the biological features of the tumor. This study has confirmed the preliminary findings that loss of heterozygosity (LOH) for chromosomes 16q and 1p are useful genetic markers in identifying patients who will have a relapse (Bardeesy, 1994) (table 6, 7).

1.6. Prognosis

During the past 50 years significant advances in the treatment of WT have been made and cure can be achieved in at least 90% of children. The survival of patients with complete necrotic Wilms' tumor (CNWT) after chemotherapy is very favorable. Five-year survival in CNWT patients is 98% versus 90% in non-necrotic WT patients (Boccon-Gibod, 1996). Despite the remarkable improvement in treatment, recurrence occurs in approximately 20% of patients with stage II and III disease and in over 50% of patients having stage IV disease or having mainly tumors with unfavorable histologic characteristics (Groot-Loonen, 1990). The majority of recurrences are seen in the first 2 years after nephrectomy but initial relapse has

been documented as late as 11 years post-operatively. The lungs are the most common sites of hematogenous metastases of WT, the second most common site of metastasis is the liver (D'Angio, 1989).

Table 7: Protocol for NWTs-5

		RT	Chemotherapy
Stage I	Favorable histology, less than 2 yrs.+ less than 550 gm	None	None
Stage I	Favorable histology, greater than 2 yrs.+ less than 550 gm Stage I anaplasia Stage II favorable histology	None	Pulse-intensive dactinomycin+ vincristin (18wks)
Stage III-IV	Favorable histology and focal anaplasia II-IV	1,080 Gy.	Pulse-intensive dactinomycin+ vincristine+ doxorubicin (24wks)
Stage II-IV	Diffuse anaplasia and clear cell sarcoma of kidney	Yes*	Dactinomycin+ vincristine+ doxorubicin + cyclophosphamide + etoposide
Stage I-IV	Rhabdoid tumor of kidney	Yes*	Carboplatin, etoposide + cyclophosphamide

RT: radiotherapy;

*Radiation therapy is given to all clear cell sarcomas.

Stage IV/favorable histology cases receive radiation based on the local tumor stage.

1.7. Prognostic markers

In the search for parameters other than histology and tumor stage for assessment of the long-term risks of WT, we have searched for tumor markers to establish an early diagnosis of WT recurrence in high risk group patients. Tumor markers may also allow the identification of subsets of patients requiring less aggressive therapy and consequently sparing unwanted effects of such therapy (Wen, 1997). A tumor marker can be defined as a qualitative or quantitative alteration or deviation from normal of a molecule, substance or process that can be detected by some kind of assay and that is correlated with prognosis (Hayes, 1996).

Tumor markers have played a significant role in the therapy of genitourinary neoplasms. A variety of relatively non-specific tumor-associated antigens has been identified in urine and serum of patients with WT and have been evaluated as potential markers for WT (Carpenter, 1990; Lin, 1995). Despite these efforts, reliable WT markers have remained elusive. None of them has proven to be a useful marker clinically, which made us to decide to further look for clinically relevant tumor markers.

The development of immuno-enzymatic techniques for identification of specific cell components or products has revolutionized tumor diagnosis. The most widely used method employs antibodies linked to various chromogens that are rendered visible by the action of enzymes, such as peroxidase (Hayes, 1996). By these techniques different tissue markers for WT have been investigated (Table 8).

Table 8: Potential prognostic markers for Wilms' tumor

Tumor suppressor genes	1- WT-1 2- WT-2 3- WT-3 4- p53
Apoptosis associated Markers	1- Bcl-2 2- Bax 3- Bcl-X
Proliferation markers	1- Proliferating cell nuclear antigen (PCNA) 2- Ki-67
Growth factors	1- Insulin-like growth factors (IGF-I, II) 2- Epidermal growth factor family (EGF, TGF- α , EGF-R) 3- Platelet- derived growth factor (PDGF) 4- Basic fibroblast growth factor (bFGF) 5- Midkine expression (MK) 6- Vascular endothelial growth factors (VEGF) 7- Nerve growth factors (NGFs)
Cell adhesion molecules (CDA) and Extracellular matrix proteins (ECM)	1- Integrins and Extracellular matrix proteins 2- Tenascin 3- Hyluronic acid
Nuclear acid content (DNA)	
Nuclear morphometry	
N-myc expression	
Total plasma renin	
Drug resistance markers	
Other potential tumor markers	1- PAL-E 2- Blastemal-associated antigen (BLA-1) 3- Human paired box gene (PAX8) 4- Tissue polypeptide antigen (TPA) 5- Carbohydrate antigen (CA) 6- Growth factor receptor bound (GRB-7) 7- Fibrillin-1 cDNA 8- RET proto-oncogen 9- TGF- β 10- Telomerase enzyme 12- Heat shock protein 70 (HSP70)

1.7.1. Tumor suppressor genes

1.7.1.1 WT-1

The WT-1 gene is localized on human chromosome 11p13. It spans about 50kb at the 11p13 locus, contains 10 exons and specifies a 3 kb mRNA. The WT-1 gene is expressed during the normal development in a strict temporal and spatial pattern (Rackley, 1993). WT-1 gene expression has been studied most extensively in WT at mRNA level. In a subset of these tumors the WT-1 gene was found to be homozygously deleted, suggesting that it might function as a tumor suppressor gene (Kikuchi, 1992). Tumors may also develop as a result of reduced WT-1 expression since suppression of tumorigenicity may be gene dose-dependent (Benedict, 1984). In contradiction with this hypothesis is the observation that heterozygous WT-1 mice appear normal and develop no tumors (Kreidberg, 1993). Thus, the role of WT-1 gene in the development of WT may be more complex than the two-hit model of Knudson and Strong. In this thesis, the prognostic value of WT-1 gene expression has been investigated in clinical WT at protein level.

1.7.1.2. WT2

A second locus at chromosome 11p15.5 has also been described in WT. 11p15.5 is an important tumor-suppressor gene region, showing LOH in WT. It is involved in Beckwith-Wiedemann syndrome that confers susceptibility to WT (Karnik, 1998) and therefore it seems relevant to study the prognostic value of 11p15.5 in WT at both mRNA and protein level.

1.7.1.3. WT3 (16q)

LOH on chromosome 16q in WT has been found in about 17% of cases (Grundy, 1996). The coincidence of microsatellite instability (MSI) in a tumor exhibiting 16q LOH suggests that the loss of genetic material may be caused by errors in DNA mismatch repair and may contribute to the pathogenesis of WT (Mason, 2000). A significant relationship between the loss of 16q and hypomethylation of 16q Sat2 DNA in WT was found. This molecular finding has been linked to a poor prognosis of WT (Grundy, 1994; 1998).

1.7.1.4. P53

In 1988, Baker showed that deletions of the short arm of chromosome 17 were associated with point mutations in the p53 allele on the homologous chromosome. Since then, much evidence has supported the view that p53 is a tumor suppressor gene involved in a wide range of human malignancies (Porter, 1992). The prevalence of p53 alterations in pediatric tumors is, however, less well established in comparison to other malignancies.

Conflicting reports regarding the possible involvement of p53 mutations in WT have emerged from various studies. Malkin et al (1994) detected p53 mutations in 2 of 21 and Lahoti et al (1996) found 4 mutations among 23 WT cases. Most studies have confirmed its correlation with anaplasia and advanced stage disease (Bardeesy, 1995; Cheah, 1996). By examination of a group of 38 WT cases with single-strand conformational polymorphism (SSCP) analysis, Waber et al (1993) could not demonstrate mutant p53 in any tumor. According to some authors p53 expression was not altered by preoperative chemotherapy (Lahoti, 1996; Govender, 1998), which is in contrast with the observation by Moll et al (1995) that chemotherapeutical treatment influenced p53 protein expression.

It should be emphasized that the immuno-histochemical detection of p53 is neither a perfect nor an accurate reflection of p53 mutations, and it has been recommended to verify the immunohistochemical p53 data with DNA analysis (Battifora, 1994).

1.7.2. Apoptosis- associated markers

Apoptosis or programmed cell death is an important cellular mechanism, which plays a role in a large number of physiologic processes such as embryonal development, selection of immuno-competent cells and tissue homeostasis. Cell death may occur by two processes, necrosis or apoptosis of which the cytological and biochemical characteristics are distinct. Nuclear and cytoplasmatic condensation, preservation of intracellular organelles, formation of membrane-bound apoptotic bodies, increased protein synthesis, normal ATP levels and oligonucleosomal DNA degradation to a size of 180-200 bp without inflammatory infiltration characterize apoptotic death and differentiate it from necrosis (Kerr, 1972). Apoptosis may be induced in tumor cells by treatment with tumor necrosis factor and in mature normal lymphocytes by the addition of glucocorticoids (Laster, 1988).

It has been shown that three varieties of apoptosis occur which could guide urinary tract embryogenesis; 1) phylogenetic apoptosis-cell death associated with vestigial structures (e.g. appendix testis in a male), 2) morphogenetic-cell death associated with sculpting of specific structures (e.g., glans urethra), and 3) histogenetic-cell death associated with cell differentiation (e.g., metanephrogenic mesenchyme) (Coles, 1993).

The control of apoptosis is complex and involves many genes including oncogenes and tumor suppressor genes. Thus, the product of the wild type tumor suppressor gene p53 may induce apoptosis after arresting the cell cycle. Recently, mutations of p53 have been observed in anaplastic areas of WT that was shown to result in attenuation of apoptosis in WT (Bardeesy, 1995). Bcl-2 is an oncogenic protein that inhibits apoptotic cell death and which is required for normal metanephrogenesis. In its absence, kidney size and nephron numbers are markedly reduced, which results in excessive apoptosis within developing metanephric blastema (Coles, 1993). The activity of Bcl-2 can be substituted or antagonized by respectively Bcl-X and Bax. The Bcl-X gene, through alternative splicing, generates two messages coding for two functionally distinct proteins (Tanaka, 1999): a short version, designated Bcl-X_s, which is a positive modulator of apoptosis, and a long version, Bcl-X_L, which is an inhibitor of apoptosis, even stronger than Bcl-2 (Boise, 1993). Pro-apoptotic action of Bax has been proposed to result from its ability to form inactive heterodimers with both Bcl-2 and Bcl-X_L (Chen, 1996). Therefore among several other proteins, the balance between Bcl-2, Bax, and Bcl-X ultimately controls apoptosis. The role and the prognostic significance of Bcl-2, Bcl-X and Bax in nephroblastoma are not known. However, Takamizawa et al (2000) found that mRNA pro-apoptotic genes (Bax, Bik, Bak, and Bcl-X_s) are expressed at higher level in “low risk” WT, whereas its expression is less in normal kidney and lowest in “high risk” WT. This suggests that tumors with unfavorable histology are rapidly proliferating, independent of pro-apoptotic mediators.

The effect of WT-1 expression on the apoptotic process may also contribute to its role in Wilms' tumorigenesis. Overexpression of WT-1 suppresses the growth of WT cells and mutational inactivation of WT-1 is present in 5-10% of sporadic WT specimens (Haber, 1993). In these cases loss of WT-1 mediated apoptosis during a critical developmental window may be responsible for initiating tumor growth. WT-1 protein may change p53 action, modulate its trans-activational properties and inhibit its ability to induce apoptosis. This effect may be due to the elevated levels of wild-type p53 protein that are observed in WT (Maheswaran, 1995).

It was found that WT-1 (-KTS) induced apoptosis could not be suppressed by co-expression of either the Bcl-2 or the BAG-1 or Mr proteins. Co-expression of either the

epidermal growth factor receptor or insulin receptor, however, partially rescued the cells from apoptosis (Menke, 1997).

Early Growth Response Gene-1 (EGR-1) suppressed transformation and counteracted apoptosis by the coordinated activation of TGF β 1, p21 and FAK, leading to enhanced cell attachment and reduced caspase activity. In the cell line HT1080, the survival effect of EGR-1 was dominant over the apoptotic effect of p53 (Belle, 1999). Measurement of the frequency of apoptosis or of molecules involved in apoptosis may after careful confirmatory research become a clinical useful parameter.

1.7.3. Proliferation markers

Tumor proliferation is a dynamic process that is difficult to assess. Several methods to measure the proliferative activity are available including counting of mitotic figures (Mourad, 1991) and flow cytometric analysis (Rainwater, 1987). The Ki-67 monoclonal antibody detects a nuclear non-histone protein of 395 and 345 kDa, encoded by a gene on chromosome 10 (Gerdes, 1983; Fonatsch, 1991). Using recombinant parts of this Ki-67 antigen as an immunogene, a new monoclonal antibody, MIB-1, was produced, which can be applied to formalin fixed, paraffin embedded tissue sections detecting the same nuclear antigen as defined by Ki-67 (Cattoretti, 1991). MIB-1 staining appears to be more sensitive in the detection of proliferating cells. More importantly, MIB-1 scores were about two times as high as Ki-67 scores in routinely processed tissues with a strong linear correlation between the two antibodies (Noordzij, 1995).

The application of antibodies directed to the proliferation related antigen (Ki-67, MIB-1) and proliferating cell nuclear antigen (PCNA) now appear practical markers of cellular kinetics applicable to archival tissues. There are few reports describing the activity of PCNA scores in children with WT. Nagoshi et al (1994) reported that PCNA scores and survival of the patients lacked a significant correlation. However, Delahunt et al (1994) reported that poor prognosis in post-chemotherapy WT has been weakly associated with high PCNA scores. In contrast, no prognostic impact of PCNA labeling was reported on tumors resected prior to the administration of chemotherapy.

It should be recognized that tumor growth is the net result of cell gain and loss. Studies of cell proliferation using MIB-1 in combination with expression patterns of p27^{kip1}, a marker for non-cycling cells (Polyak, 1994a, b) may therefore, provide more prognostic information.

1.7.4. Growth factors

Synthesis and actions of growth factors have been characterized in the developing metanephric kidney. Studies have identified a number of abnormalities of growth factor expression in WT (Durbeej, 1993; Tsutsui, 1993; Donovan, 1994; Alpers, 1995)

1.7.4.1. Insulin-like growth factors (IGFs)

Insulin-like growth factors comprise a class of peptides, two members of this family have been identified: IGF-I and IGF-II. In vitro cultured WT cells produce and release various forms of “large-size IGF-II” with molecular masses between 10 and 25 kDa (Schmitt, 1997). It is well documented that WT tissue expresses high levels of IGF-II (Haselbacher, 1987) with levels similar to those of fetal kidney (Scott, 1985). Furthermore, Yun et al (1993) found that expression of IGF-II genes in WT is highly elevated compared to adjacent normal kidney and

adult tissues. An in situ hybridization study by Paik et al (1989) suggested that IGF-II mRNA expression is inversely associated with normal epithelial differentiation, and that this differentiation may be deregulated in WT. On the other hand, Little et al (1987) demonstrated in two xenograft models of WT that enhanced expression of IGF-II is not a necessary event in WT progression.

IGF-I is produced in cortical and medullary collecting ducts of the kidney where IGF-I receptors are present (Hammerman, 1989). Receptors for IGF-I are also found in WT (Gansler, 1988). Antibodies to the IGF receptor inhibit the growth of WT in vitro and of tumors transplanted to athymic mice, consistent with a role for IGF in oncogenesis (Gansler, 1989). However, the exact role of IGFs in WT growth is unclear. Since WT cells can produce IGF-II and carry receptors for this growth factor, an autocrine loop may be hypothesized.

1.7.4.2. Epidermal growth factor (EGF) family

EGF is a mitogenic polypeptide hormone which, via its receptor EGF-R, stimulates the growth of epithelial and stromal cells (Cohen, 1962; 1976). In normal human kidney, both EGF immunoreactivity and EGF mRNA were localized in tubular profiles corresponding to Henle's loop and, to a lesser extent, to distal convoluted tubules (Gesualdo, 1996). EGF may play a role in regulating the growth of some cancer cells in vivo because of its known mitogenic effects in vitro (Hamburger, 1981; Stoscheck, 1986). The EGF-R is a transmembrane glycoprotein with tyrosine kinase activity which binds EGF as well as TGF- α . Both TGF- α peptide and receptors for TGF- α are present in fetal human renal tissue and its mRNA is present in the mouse mesonephros (Rogers, 1992). *c-erb B-2* is a receptor that shows considerable homology with EGF-R, but does not bind EGF to be activated. By dimerization with EGF-R, it enhances its activity (Semba, 1985).

An immuno-histochemical study of the expression of members of the EGF family e.g. EGF, TGF- α and their receptor (EGF-R) may yield information about potential prognostic value of these factors in WT patients. Until now, the expression of these growth factors and their receptors (EGF-R) in nephroblastoma has not been characterized as yet.

1.7.4.3. Platelet-derived growth factor (PDGF)

PDGF is the principal mitogen of serum acting on cultured mesenchymal cells. It is composed of two distinct but homologous polypeptide chains (A and B), linked by disulfide bonds (Antoniades, 1983; Waterfield, 1983). Both cultured WT cells and human fetal kidney cells produce PDGF, which is consistent with its role in normal fetal growth and in neoplastic renal cell transformations (Fraizer, 1987). PDGF A-chain expression has been identified in epithelial elements of WT (Alpers, 1995). Clarification of the potential role of PDGF in Wilms' tumorigenesis and its prognostic value in this malignancy still needs further studies.

1.7.4.4. Basic fibroblast growth factor (bFGF)

FGFs are potent angiogenic peptides implicated in the growth and metastasis of solid tumor. The FGF family consists of nine members (FGF-1 - FGF-9). Skoldenbeg et al (2001) showed FGF-2 (bFGF) nuclear staining in the epithelial and stromal compartments of WTs. In patients with WT, elevated preoperative urinary bFGF levels raise the suspicion of aggressive disease, while elevated post-operative levels may indicate recurrence of disease (Carpenter, 1990). According to the available data the influence of FGFs and their receptors on WT growth is still not fully understood.

1.7.4.5. Midkine expression (MK)

A new family of heparin-binding growth/differentiation factors has been described (Kadomatsu, 1988; Tomomura, 1990). Midkine, the first to be discovered, is a product of a retinoic acid responsive gene (Murata, 1988). A significant level of MK gene expression was observed in all samples of WT examined. The inhibition of WT cells growth by an anti-MK antibody implies that MK is involved in growth regulation of these cells. This suggests a role of MK in biological behavior of WT and its potential use as a diagnostic marker (Tsutsui, 1993).

1.7.4.6. Vascular endothelial growth factors (VEGF)

The close relationship between tumor vascularity and tumor growth is well established (Senger, 1993). Previous studies indicated that VEGF and its receptor Flt-1 could play an important role in the process of tumor metastases. Similarly, in several tumors the levels of VEGF and Flt-1 were shown to be associated with poor prognosis (Dvorak, 1995; Takanmi, 1997). Tumor angiogenesis, as reflected in microvessels density (MVD), has also been reported to have possible prognostic implications in a variety of malignancies (Weidner, 1991; Wiggins, 1995; Hollingsworth, 1995).

With regard to WT one single study, using a murine model, reported the overexpression of VEGF in a highly metastatic WT cell line (Kayton, 1999). However, the clinical relevance of this animal study has not yet been demonstrated.

1.7.4.7. Nerve growth factors (NGFs)

Neurotrophins and their receptors regulate cell proliferation, differentiation, and cell death of normal human kidney and neuronal cells (Sariola, 1991; Alpers, 1993; Durbeek, 1993; Donovan, 1994). In addition, these factors have been implicated in the pathology of WT (Thomson, 1989; Donovan, 1994).

The p75 low-affinity nerve growth factor receptor has been identified in epithelial WT cells in culture (Thomson, 1989), and it has been demonstrated that high-affinity trk receptors are expressed in various histological tumor components in a series of 10 WTs (Donovan, 1994). The level of p75 expression was high in areas mainly consisting of blastema and decreased in regions with epithelial differentiation. The trk A and B receptors were primarily found within stromal components, whereas the trk C and trk C' (truncated) receptors were present within epithelial structures (Donovan, 1994). Eggert et al (2001) found that high levels of trkBfull mRNA expression in WT is associated with worse outcome. Conversely, low levels of trkBtrunc mRNA expression are associated with favorable outcome. Cells that express functional, full-length trkB may be susceptible to proliferation and/or survival signaling that leads to WT progression.

1.7.4.8. Glial cell line derived neurotrophic factor (GDNF)

GDNF is a potent proliferation and survival factor of dopaminergic neurons. Séguier-Lipszyc et al (2001) demonstrated the presence of GDNF in the epithelial cells of the most differentiated neuroectodermal tube and in the endothelial cells in 20 chemotherapeutically treated WT and in normal kidney both at the protein and mRNA level. Expression of GDNF was proportional to proliferation and inversely proportional to apoptosis. Also, its co-receptors GFR- α 1 and - α 2 mRNA were found in the investigated WT. In summary, the NGFs appear to play a role in WT development and could also act as a prognostic indicator in clinical WT.

1.7.5. Cell adhesion molecules and extracellular matrix proteins (ECM)

Cell-matrix interactions play an important role in normal renal development as well as in tumor invasion and metastasis and these interactions are mediated by adhesion receptors. Several families of cell surface receptors, for example cadherins, selectins, the neural N-CAM, integrins and CD44, govern these adhesive interactions (Zoller, 1995).

1.7.5.1. Integrins and ECM

Peringa et al (1994) examined the expression of integrin subunits $\alpha 1$ - $\alpha 6$, $\beta 1$ and $\beta 4$, and of ECM proteins fibronectin, laminin and collagen I and IV, in 20 frozen WT samples and in 5 fetal and 2 adult kidneys. Their distribution in tumor epithelium and mesenchyme showed strong similarities with that in their fetal counterparts. In contrast, the expression patterns in tumor blastema differed strongly from the fetal blastema. Undifferentiated WT blastema was characterized by expression of $\alpha 3$ and $\alpha 6$ integrin chains in the absence of ECM proteins. Tumor blastema with epithelial commitment was demonstrated to have an increased expression of $\alpha 3$ and $\alpha 6$, the appearance of $\alpha 2$ integrin and production of laminin. Tumor blastema with mesenchymal commitment showed loss of $\alpha 3$ and $\alpha 6$, and expression of $\alpha 1$, $\alpha 4$, and $\alpha 5$ (Peringa, 1994). It is speculated that the inability of the undifferentiated blastema to produce ECM proteins is related to its relatively high metastatic potential when compared with epithelium and mesenchyme.

1.7.5.2. Tenascin

Tenascin is a ECM protein residing prominently in the stroma of several epithelial malignancies. Its role in tumor spread is unclear, but it may facilitate epithelial cell migration during embryogenesis and carcinogenesis, by reducing rather than increasing cell matrix interactions (Chiquet-Ehrismann, 1986). A human sarcomatoid WT cell line growing in nude mice induced a strong mouse stromal response with increased deposition of mouse tenascin (Talts, 1993).

1.7.5.2. Hyaluronic acid (HA)

Hyaluronic acid (HA) is a prominent component of the ECM of WT (Hopwood, 1978; Roth, 1988). In these studies that was markedly elevated in the serum and urine of some patients with WT. Stained WT tissues with HA- binding protein demonstrated a prominent blastemal staining. This staining was markedly reduced when sections were predigested with hyaluronidase. HA may serve as a biochemical marker for diagnosing and monitoring tumor recurrence; an immunodiagnostic test (e.g. radioimmunoassay) is currently under development. The major advantage of such a test is that it will circumvent the cumbersome and time-consuming steps inherent to a cell culture bioassay. More studies are needed to determine its prognostic value as the published studies so far did not reach conclusive results.

1.7.6. Nuclear acid content

Flow cytometric analysis of DNA ploidy has been used successfully to predict patient survival in a number of malignancies (Gustafson, 1982; Kokal, 1986). There have been a few studies investigating the relationship between DNA ploidy and survival in WT (Tanaka, 1983; Rainwater, 1987). Tanaka and associates (1983), using static cytofluorometric analysis of neoplasms from 8 patients, determined that one WT was DNA diploid, and 7 possessed a combination of diploid and tetraploid DNA patterns. In this study patient survival and DNA

ploidy were not correlated. Douglass et al (1986) found a striking correlation between hyperdiploid tumor cells, histological subtype of anaplastic WT and poor prognosis. In contrast to Tanaka, but in agreement with Douglass, Oppedal et al (1988) found distinctly aneuploid tumors with polyploid cells and the tendency of aneuploid tumors to be associated with early tumor related death. In 30 patients having tumors with a favorable histology and 4 with anaplastic variants of WT, Layfield et al (1989) found no statistical significant relationship between DNA-ploidy and patient survival. Anaplastic tumors, however, were more likely to be aneuploid or tetraploid than diploid in this series. Falkmer (1992) studied the methodology and potential problems of applying flow cytometry and static cytometry techniques for the measurements of nuclear DNA content (ploidy). According to this study, many discrepancies among published series were documented of having a methodological origin. Since advantages and disadvantages of both flow cytometry and static cytometry outweighed each other, Falkmer recommended that the two measurements should be combined if applied for prognostic purposes (Falkmer, 1992). Currently, tumor DNA flow cytometry is not employed as a routine diagnostic technique, as its additional value is not substantiated.

1.7.7. Nuclear morphometry

Partin et al (1990) studied nuclear morphometry of 9 WT patients with favorable histological disease with the Hopkins Morphometry system. He found that nuclear roundness, ellipticity, convexity and bending energy provide useful information that aid in predicting which children with WT will respond to therapy. The results of this preliminary study should be confirmed in a larger number of WT patients with the use of multivariate analysis, as other parameters may influence prognosis. On the other hand, this technique is time-consuming and therefore considered less suitable for clinical applications.

1.7.8. N-myc expression

The myc family of cellular oncogenes has three well-characterized members (C-myc, N-myc, and L-myc) as well as several additional members (Zimmerman, 1986). N-myc is characteristic of several tumors which originate from primitive cell lineages. This is consistent with the view that enhanced N-myc expression could be involved in the development of at least some of these tumors. WT exhibited greatly enhanced levels of N-myc expression, in the absence of gene amplification. Also, a high expression level of N-myc is associated with markedly diminished C-myc expression (Zimmerman, 1986; Nisen, 1986). In situ hybridization confirmed that the high levels of N-myc RNA were predominantly present in the blastemal elements of WT (Shaw, 1988). The attempts to detect N-myc protein by immunological methods have been unsuccessful, probably due to the extremely short half life of the polypeptide (Nisen, 1986) compared to the mRNA. Thus, mRNA detection as a measurement of N-myc may prove to be more practical than protein detection for diagnostic purposes, but is generally less applicable in the clinical setting.

1.7.9. Total plasma renin

Carachi et al (1987) has demonstrated abnormally high levels of total plasma renin in most of WT. The increase in total renin was due to increased levels of inactive renin (prorenin), rather than of the active renin. These high levels decreased to normal after

operation and chemotherapeutical intervention. Tsuchida et al (1990) determined plasma total renin level by radioimmunoassay (RIA) with monoclonal antibodies specific for both active and inactive renin (Yokomori, 1988). Lindop et al (1984; 1990) demonstrated immunoreactive renin in 10 of 19 WT and renin-specific mRNA (1.6 Kb in length) by Northern blot analysis, similar to the mRNA detected in normal kidney tissue and in kidney with renal artery stenosis. In nephroblastomas, the cells which contain immunoreactive renin are typically perivascular in distribution (Leckie, 1989).

1.7.10. Drug resistance markers

Multidrug resistant-associated Permeability-glycoprotein (P-gp) is a 170-kDa transmembrane glycoprotein that is involved in the cellular redistribution of drugs in the cytoplasm (Simon, 1994). Untreated and classical WT patients had no P-gp overexpression, except in areas of tubular differentiation associated with normal kidney tissue (Volm, 1995; Re, 1997). On the other hand, Sola and his associate (1994) detected P-gp in 12 non-treated WT with monoclonal antibody 17F9. WT patients treated with actinomycin D and vincristine showed increased P-gp expression (Re, 1997). However, the variation in methodology and use of different antibodies may have prohibited to obtain conclusive results (Simon, 1994; Re, 1997; Sola, 1994).

Drug resistance marker DNA Topoisomerase II α (Topo II α). Granzen et al (2001) reported that relatively low levels of Topo II α (marker of proliferation) were found in both blastemal and stromal components of treated as compared to untreated WT. These findings in accordance with a study of Volm et al (1995).

Drug resistance marker DNA Glutathione S-Transferase- π (GST- π) was frequently expressed in 57% of epithelial compartments of preoperatively treated WT, but was not demonstrated in stromal elements and in untreated tumors. This may result in the involvement of GST- π in the development of drug resistance in WT (Granzen, 2001).

1.7.11. Other potential tumor markers

PAL-E is an anti-endothelial cell antibody which was found not only restricted to the endothelium, but also specifically stained blastemal cells in WT, whereas mesenchymal and tubular components of WT were negative for this region (Schlingemann, 1985). Because of its specific blastemal staining it may serve as tumor marker. *BLA-1* antibody usually stained cell surfaces of all WT-containing blastemal components (Tarnowski, 1994). *PAX8*, a human paired box gene, may have a role in WT. Poleev (1992) found by RNAase protection analysis that *PAX8* is strongly expressed in all five investigated tumors as compared to its expression in adult human kidneys. Thus, its expression warrants further studies in WT. *CA 125* and *TPA* were detected in the conditioned culture media of a WT cell line, and the levels of CA 125 in the serum and the total tumor load of human heterotransplants into nude mice appeared to be correlated well (Ishiwata, 1991). These markers might therefore be suitable for detection of recurrence after surgery; however no data are available on their specificity and sensitivity. Some other markers appear to be of interest, but clinical studies in WT have not been performed yet. Grb-7 (growth factor receptor bound) was identified as a signaling protein which has a basic function in development of the epithelium of the kidney (Leavey, 1998). Fibrillin-1 cDNA was found to modulate events related to early organogenesis and possibly vascularization of the rat kidney (Kanwar, 1998). Also, the RET proto-oncogen plays a role in development of the mouse enteric nervous system and in kidney organogenesis (Attie-Bitach,

1998). TGF- β 1 mRNA is detectable in the metanephric blastema. Epithelial tubules derived from metanephric blastema show low levels of TGF- β 2 transcripts, and no TGF- β 3 expression is observed (Schmid, 1991). Telomerase is a ribonucleoprotein enzyme associated with cellular immortality (Kim, 1994; Shay, 1996). The pattern of the RNA component of human telomerase (hTR) expression in WT was strikingly similar to its expression during normal renal development and in nephrogenic rests. Thus, the pattern of hTR expression in WT and its precursor lesions may represent the aspect of the persistent fetal phenotype which predisposes to the development of malignancy (Yashima, 1998). Differential expression of the Heat Shock Protein 70 (HSP70) in the blastemal and epithelial compartments of nephroblastoma, using real time RT-PCR, may be of prognostic value in the pretreated patient (Efferth, 2001).

In addition, several genetic loci have been implicated in WT by LOH studies, or by the presence of germline translocations; these include 7p, 11q, 16q, 22q and loss of 4q (Wilmore, 1994; Radice, 1995; Steenman, 1997; Klamt, 1998). A significant association was noted between gain of 1q and increased risk of tumor relapse in patients with favorable histology of WT as detected by comparative genomic hybridization (CGH) (Hing, 2001).

1.8. Conclusions and scope of the thesis

As discussed in this chapter, the need to identify reliable prognostic tissue markers for WT has been highlighted. Unfortunately, the markers identified as yet fail to provide consistent predictive information regarding the clinical outcome of WT. There is a need to identify prognostic markers that would help in defining the biological aggressiveness of WT and determine the choice of therapy. During the course of this project, the research was focused on increasing our knowledge of the expression of various markers and their localization in the tumor and on the selection of markers that might be of prognostic value. All studies made use of paraffin-embedded WT specimens. The immuno-histochemical defined expression level was correlated with prognosis.

Other investigators studied the expression of both WT-1 and EGR-1 genes in WT mainly at the mRNA level. Chapter II describes the prognostic value of WT-1 and EGR-1 expression in treated WT patients studied at the protein level.

The prognostic value of apoptosis-associated markers (Bcl-2, Bax, and BCL-X) is discussed in chapter III. Preoperative chemotherapy of WT leads to tumor reduction by decreased proliferation and increased apoptosis. The expression level of apoptosis-associated markers in untreated WT tumor patients might predict response on preoperative chemotherapeutic treatment.

It is widely accepted that cell kinetic data are of prognostic value in many tumors. Because such data are relatively scarce for human WT, we investigated cell growth and cell death-associated markers in human treated WT specimens. The study described in chapter IV was performed to evaluate the expression and the prognostic significance of the proliferation markers MIB-1 (Ki-67) and cell cycle inhibitor p27^{kip1} in chemotherapeutically pretreated Wilms' tumor.

The involvement of CD44 in tumor progression, more specifically in the metastatic process, prompted us to investigate its prognostic value in WT. Chapter V describes the prognostic value of expression of CD44-standard and some its alternatively spliced variants in specimens of treated WT.

Chapter VI, VII, studied the expression of several growth factors with angiogenic activity and their correlation with prognosis. Insight into expression of these factors may add to understanding the role of angiogenesis in WT carcinogenesis. Chapter VI describes the expression of TGF- α , EGF-R and *c-erb* B-2, whereas chapter VII describes the expression of VEGF and its receptor Flt-1 and their relation with microvessel density (MVD) in WT.

The final chapter (VIII) comprises a brief discussion about the outcome of the studies described in this thesis. We made a first attempt to evaluate the significance of the results found in a preoperatively untreated group of patients compared with that obtained in studying preoperatively treated WT patients. Finally, new directions and perspectives for future research in the field of WT prognosis are presented.

CHAPTER 2

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 mRNA level. The present study evaluates the prognostic value of WT-1 and EGR-1 in 61 Wilms' tumor 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 nephroblastoma. 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 (Beckwith, 1994). At present, the prediction of outcome is based mainly on the histology and stage at the time of resection (Williams, 1985). Despite remarkable advances in elucidating molecular events involved in the pathogenesis of Wilms' tumor, an independent prognosticator has not been identified as yet (Wen, 1997).

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 which 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% (Slater 1992; Mannens 1988). The WT-1 protein has been implicated in many processes, such as proliferation, differentiation, and apoptosis (Kerr, 1994). Comparison of the amino acid sequence of the zinc fingers of WT-1 with the amino acid sequences of other zinc fingers proteins revealed a high degree of similarity of WT-1 and early growth response genes such as EGR-1 (Gessler, 1992). 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 (Jamieson, 1989).

The results of a number of transient transfection studies demonstrated that WT-1 represses transcription of promoters responsive to EGR-1 (Sukhatme, 1988; Dey, 1994). 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 pattern 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, 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 and E stained slides were reviewed by an experienced pediatric pathologist (J.C.D.H.) to assess the stage according to the TNM classification (Delemarre, 1996). 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 Cruze, CA). The specificities and characteristics of these antibodies have been published elsewhere (Gessler, 1992; Jamieson, 1989). The peroxidase-anti-peroxidase technique was used. Serial sections (5 μ m) 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% 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, Unicorn, 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 was done 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-anti-mouse antibody for monoclonal antibody or goat antirabbit for the polyclonal antibody and then rinsed with PBS containing 0.1% Tween. The peroxidase-anti-peroxidase (PAP) 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 x 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 analysed in total. Tissues 1-3 (see Fig. 6) originate 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 left kidney (WT-16), respectively. Morphologically, all six tissues contained the blastemal and stromal component, whereas in tissue 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 10mM Tris (pH 7.4), 150mM

NaCl (Sigma), 1% Triton x100 (Merck, Darmstadt, Germany), 1% deoxycholate (Sigma), 0.1% SDS (life Technologies), 5mM EDTA (Merck) and protease inhibitors (1mM phenyl-methyl-sulphonyl fluoride, 1 mM aprotinin, 50mg/Liter leupeptin, 1mM benzamidine and 1mg/Liter pepstatin, all from Sigma). The samples were spun at 35,000X g at 4°C for 10 min. The protein content of the supernatant was measured photometrically using the Bio-Rad, protein assay (Bio-rad, München, Germany). The proteins were transferred to a SDS-polyacrylamide gel, and electrophoresis was performed in 10x diluted tray buffer for 2 h. The gel was blotted to a 0.45µm cellulose nitrate membrane (Schleicher & Schuell, Dassel, Germany). Pre-stained 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 30s.

RESULTS

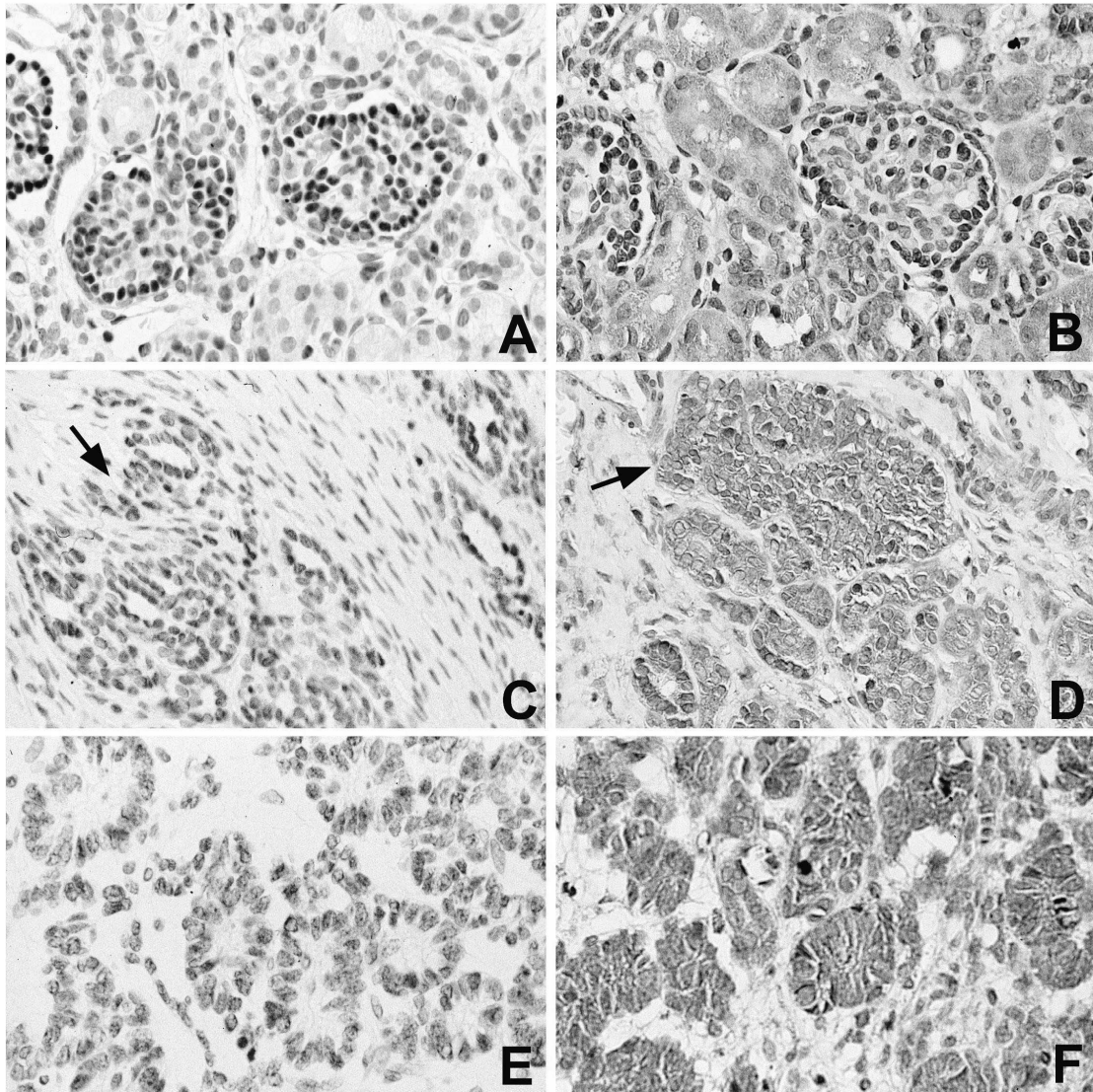
Clinicopathological findings. The T-stage distribution was T₁ in 21, T₂ in 20 and T₃ in 20 patients. Clinical progression occurred in 14 patients (23%). Twenty-five patients (41%) had large amounts of blastema. The patient's distribution was 29 (48%) females and 32 (53%) males. The mean overall follow-up period was 5.7 years, and the mean age at operation was 4.2 years. Eight patients (13%) died from their tumor. 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 (Table 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).

Table 1
Relationship between pT stage and blastemal expression
Data are presented as n, with percentages in parentheses

Score	WT-1*			EGR-1*		
	T1	T2	T3	T1	T2	T3
< 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%)

* P < 0.05 (Pearson χ^2 test)

**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 blastema (D) and epithelial compartments (F).

Arrows indicate stained blastemal cells in C and D, respectively. Slides were counterstained with Hematoxyline (X400).

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 component 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).

Table 2

Relationship between pT stage and epithelial expression
Data are presented as n, with percentages in parentheses

Score	WT-1*			EGR-1		
	T1	T2	T3	T1	T2	T3
< 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%)

Data are presented as numbers, percentages between brackets.

* 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

		EGR-1*			
	Score	< 10%	10-25%	25-50%	>50%
WT-1*	<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%)	-

* P < 0.05 (Pearson χ^2 test)

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' 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).

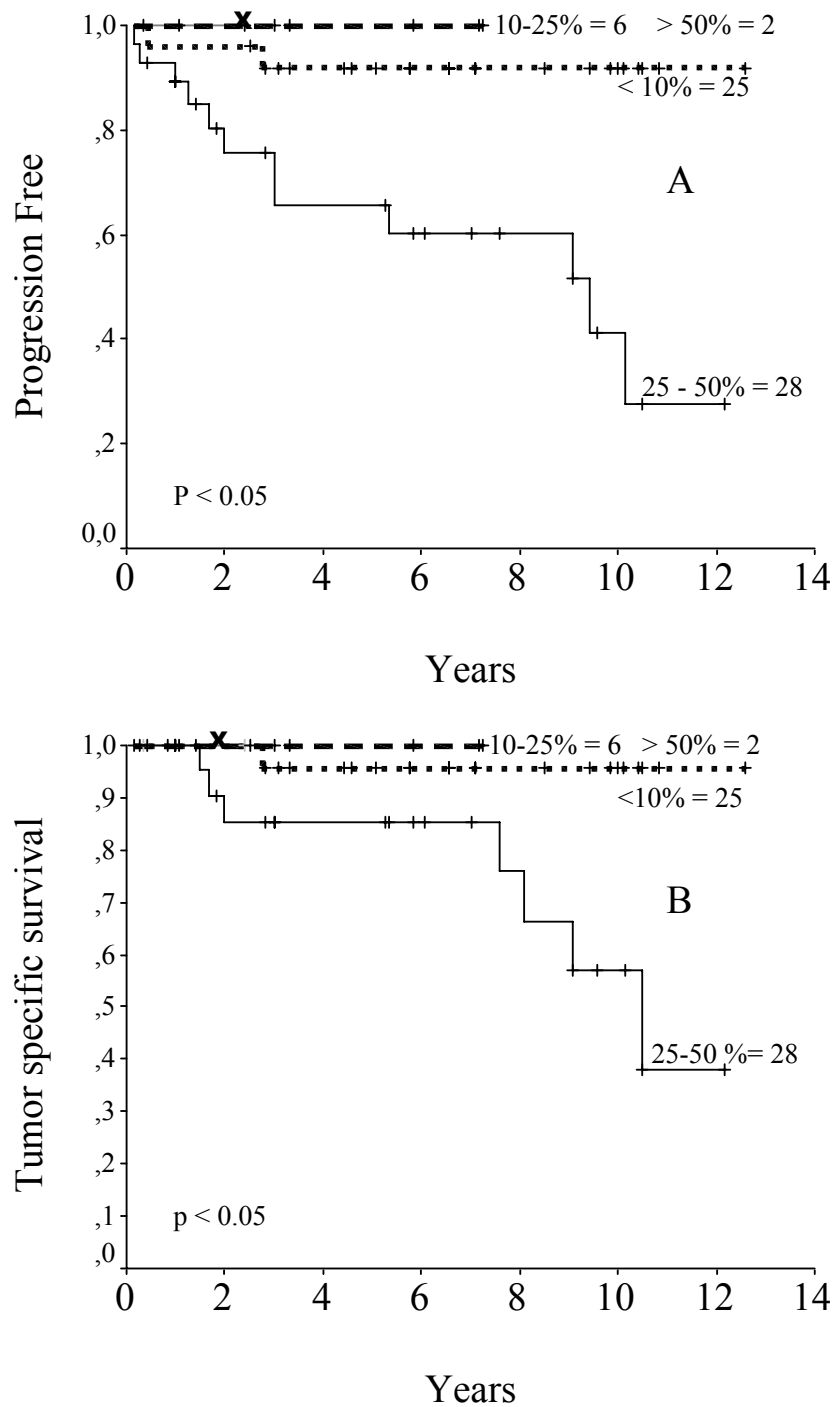
Table 4
Univariate analysis of prognostic markers

Variable	Outcome parameter							
	Blastema				Epithelium			
	Clinical progression		Tumor specific survival		Clinical progression		Tumor specific survival	
	X ²	P*	X ²	P*	X ²	P*	X ²	P*
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

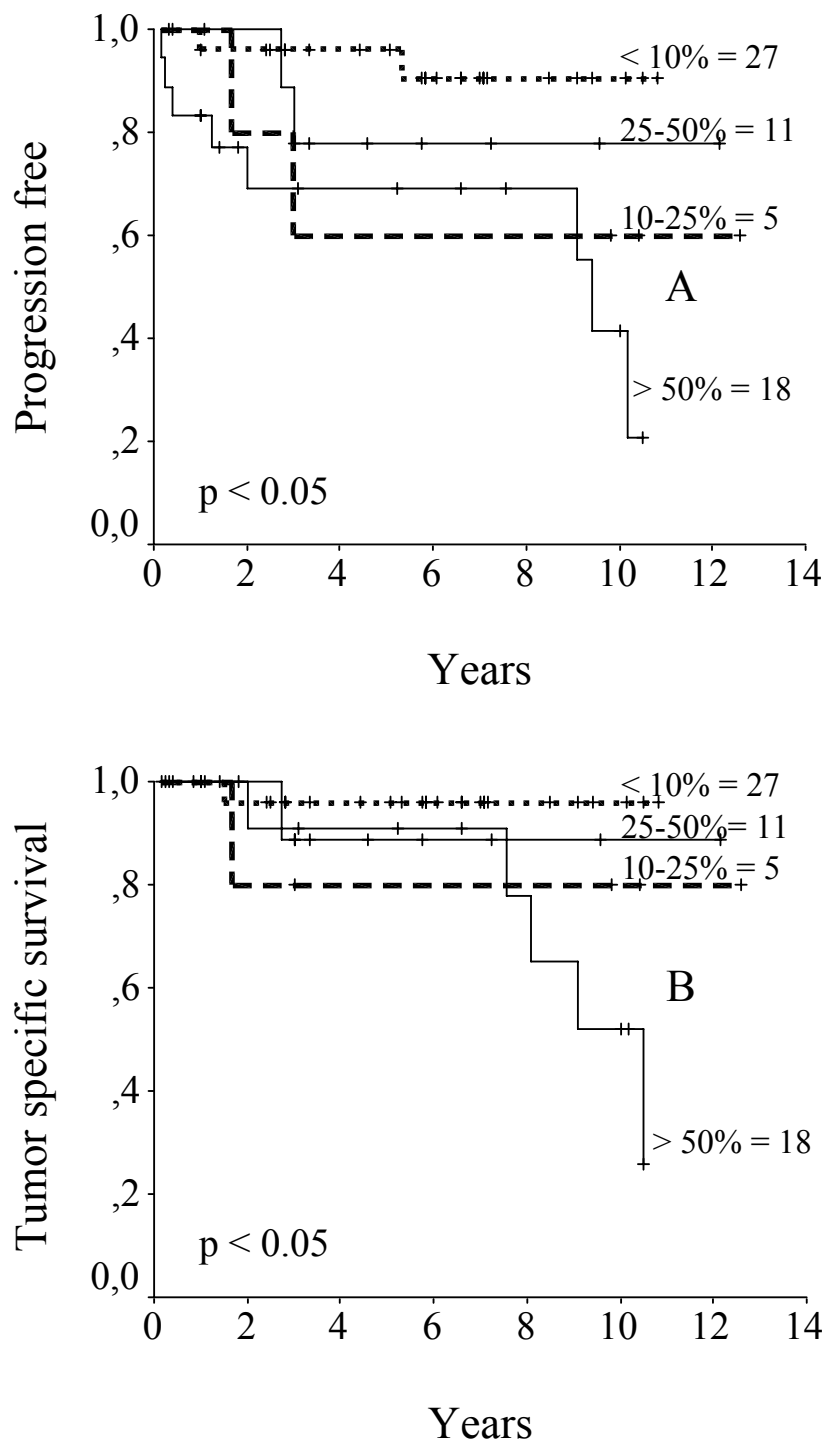
*Log-rank test for trend

Table 5
Results of Cox's multi-regression analysis

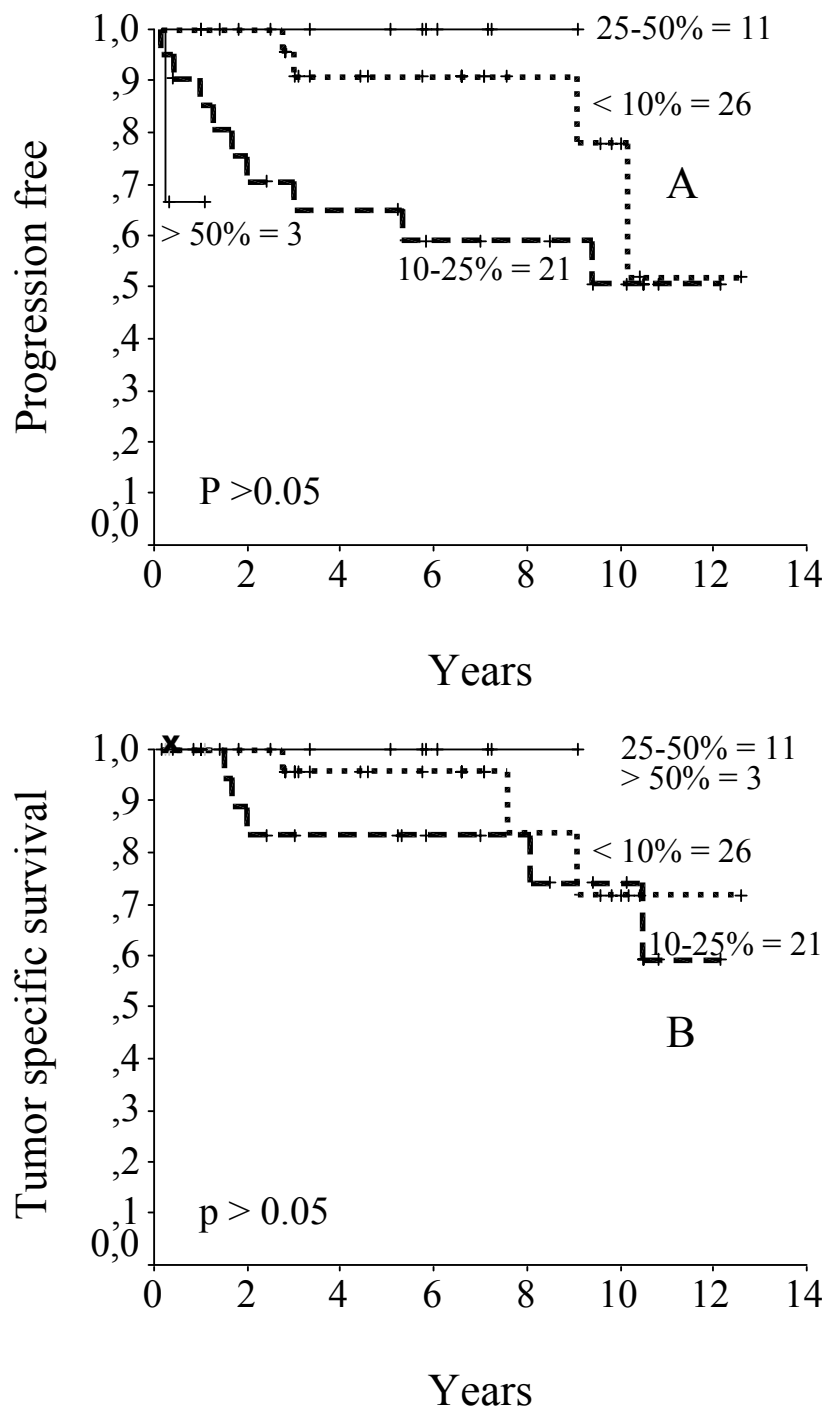
Outcome parameters	Variable	Hazard Ratio	95% Confidence Limit	P value
Clinical progression	WT-1 (blastema)	9.2	2.0- 42.2	0.004
	Stage	5.2	1.7- 15.9	0.003
Tumor specific survival	WT-1 (blastema)	7.4	0.91- 60.3	0.06

**Fig. 2**

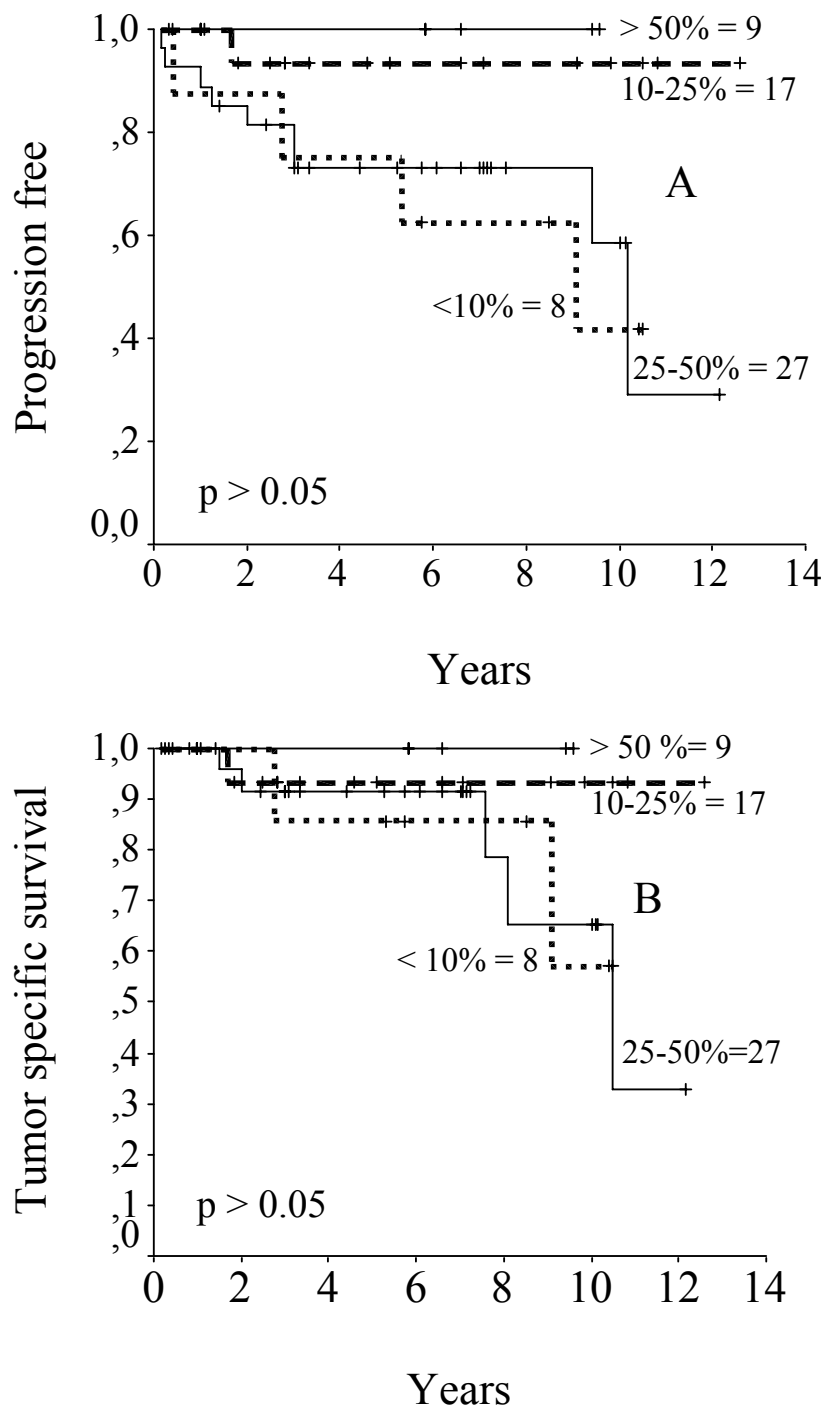
Kaplan-Meier curves show a significant relationship between blastemal WT-1 expression and clinical progression (A) and survival (B). Censored patients are indicated by tic marks along their lines. The end of follow-up of group >50% is indicated by an X. Number of patients per group are shown.

**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. Number of patients per group are shown.

**Fig.4**

Kaplan-Meier curves show a non-significant 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 X. Number of patients per group are shown.

**Fig.5**

Kaplan-Meier curves show a non-significant relationship between epithelial EGR-1 expression and clinical progression (A) and survival (B). Censored patients are indicated by tic marks along their lines. Number of patients per group are 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 component, whereas in tissue 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.

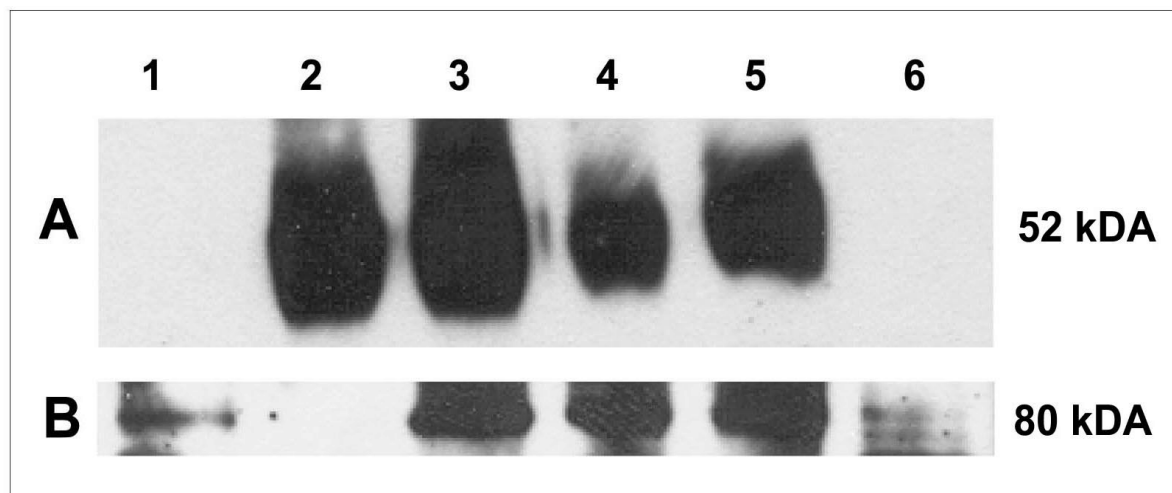


Fig. 6. Immunoblot analysis of WT-1 (A) and EGR-1 (B) proteins in nephroblastoma tissues derived of six human xenograft models with approximate indication of size markers

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 mRNA level and was compared with clinical, histological, and molecular features (Gerald, 1992; Zhuang, 1997; Scharnhorst, 2000). 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 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 (Rackley, 1995). Because, normal kidney tissue was present in almost all sections, this could serve as an internal control for the immuno-staining 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 (Pritchard-Jones, 1990;1991). 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 with 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 using Northern blot analysis (Gerald, 1992; Pritchard-Jones, 1990; Miwa, 1992). Epithelial differentiation in tumors was accompanied by diffuse WT-1 expression (Fig. 1E).

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 (Mundlos, 1993). 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 (Hastie, 1993; Rauscher, 1993). On the other hand, the WT-1 gene was found to be overexpressed in leukemia (Menssen, 1995), and ovarian cancer (Bruening, 1993), 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 (BPH) than in normal prostatic tissues, but no changes were seen in prostatic adenocarcinoma (Dong, 1997). In addition, altered expression of WT-1 gene was demonstrated in human breast cancer (Silberstein, 1997). 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 (Haber, 1993) or in the presence of modulating factors (Rauscher, 1993). A correlation exists between the levels of WT-1 expression and poor prognosis in human hematopoietic malignancies (Inoue, 1994; Bergmann, 1997).

Very recent studies, using RNA slot blot analysis, showed expression of EGR-1 during murine nephrogenesis and overexpression in some clinical Wilms' tumor (Scharnhorst, 2000; Rackley, 1995). In the present study, at the protein level EGR-1 was expressed in the three components of Wilms' tumor (blastema, epithelial, 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 (Calogero, 1996). 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 (Huang, 1997; Levin, 1995), whereas EGR-1 is overexpressed in prostate cancer (Eid, 1998). 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 (Mohn, 1991; Ahmed, 1996). 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 (Gerald, 1992; Madden, 1991).

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.

ACKNOWLEDGMENTS

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CHAPTER 3

The Prognostic Significance of Apoptosis-associated Proteins Bcl-2, Bax and Bcl-X in Clinical Nephroblastoma

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SUMMARY

Apoptotic cell death represents an important mechanism for the precise regulation of cell numbers in normal tissues. Various apoptosis associated regulatory proteins, such as Bcl-2, Bax and Bcl-X, may contribute to the rate of apoptosis in neoplasia. The present study was performed to evaluate the prognostic value of these molecules in a group of 61 Wilms' tumours of chemotherapeutically pre-treated patients using an immunohistochemical approach. Generally, Bcl-2, Bax and for Bcl-X_{S/L} were expressed in the blastemal and epithelial components of Wilms' tumour. Immunoreactive blastema cells were found in 53 %, 41 % and 38 % of tumours for Bcl-2, Bax and for Bcl-X_{S/L}, respectively. An increased expression of Bcl-2 was observed in the blastemal component of increasing pathological stages. In contrast, a gradual decline of Bax expression was observed in the blastemal component of tumours with increasing pathological stages. Also blastemal for Bcl-X_{S/L} expression decreased with stage. Univariate analysis showed that blastemal Bcl-2 expression and the Bcl-2/Bax ratio were indicative for clinical progression, whereas epithelial staining was of no prognostic value. Multivariate analysis showed that blastemal Bcl-2 expression is an independent prognostic marker for clinical progression besides stage. These findings demonstrate that alterations of the Bcl-2/Bax balance may influence the clinical outcome of Wilms' tumour patients by deregulation of programmed cell death.

INTRODUCTION

Apoptosis, a genetically programmed mechanism of eliminating cells in response to a variety of stimuli, is a widespread phenomenon occurring at different stages of morphogenesis, growth and development as well as in normal turnover of adult tissues (Reed, 1994). Loss of apoptotic response in tumour cells may be one of the mechanisms involved in malignant progression and resistance to chemotherapy (Arends, 1991). Studies have shown that several proteins are involved in the regulation of apoptosis. Abnormalities in the expression of these proteins may contribute to tumorigenesis by altering the balance between cell growth and cell death (Vaux, 1993). A number of cellular and viral homologues of Bcl-2 have been identified. Beyond Bcl-2 this family of proteins includes Bcl-X, and Mcl-1 which act as blockers of apoptosis, whereas others such as Bax and Bak appear to promote apoptosis (Oltvai, 1993).

Structurally, Bcl-2 is an integral membrane protein localised to membranes of the nucleus and endoplasmic reticulum. The protein product of the Bax gene, which has 21% homology to the Bcl-2 protein, can form heterodimers with Bcl-2 leading to abrogation of its ability to suppress apoptosis (Oltvai, 1993). In its long splice variant (Bcl-X_L), Bcl-X is another potent antagonist of apoptosis, whereas the short splice variant (Bcl-X_S) promotes cell death. Recent studies demonstrated that Bcl-X_L is mainly involved in the mechanism of resistance to chemotherapeutic agents and radiation (Datta, 1995). An abnormal pattern of Bcl-2, Bax and Bcl-X expression has been demonstrated in several human malignancies (McDonnell, 1992; Gazzaniga, 1996; Krajewska, 1996a,b; Xie, 1998).

Wilms' tumour, or nephroblastoma, is a paediatric malignancy of the kidney and one of the most common solid tumours in children (Beckwith, 1994). Preoperative chemotherapy as treatment for Wilms' tumour has increasingly been more used in Europe by International Society of Paediatric Oncology (SIOP) which is different from the protocol used in the USA by the National Wilms' Tumour Study (NWTS) (D'Angio, 1983). Despite the remarkable response to chemotherapy, 5% to 10% of tumours are fatal due to metastases and/or the occurrence of drug resistance (Green, 1994). Since it appears that programmed cell death is a crucial event in normal kidney development one may expect oncogenic events to result from defects in the pathways of renal cell death (Coles, 1993). Therefore, the prognostic value of Bcl-2, Bax and for Bcl-X_{S/L} were investigated in specimens of nephroblastoma patients which were chemotherapeutically treated prior to surgery. Having access to this type of material derived from a substantial number of patients with a good stage distribution, the aim of our study was to find factors which could predict the clinical outcome of these patients after being treated by chemotherapy and surgery.

MATERIALS AND METHODS

Patients:

During the period 1987-1999, 61 patients with nephroblastoma were treated by neo-adjuvant chemotherapy and subsequent tumour nephrectomy. Following treatment 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.

Sample selection:

All nephrectomy specimens were fixed in 10% buffered formalin and embedded in paraffin. The hematoxylin and eosin stained slides were reviewed by an experienced paediatric pathologist (JCDH). The tumour stage (pT stage) was assigned according to adaptation of the SIOP trial protocol established in the SIOP meeting in Stockholm in 1994 (Boccon-Gibod, 1998). Among the paraffin blocks available of individual patients those containing tissue with the three different cell types of Wilms' tumour (blastema, epithelial and stromal) were selected. In addition, adjacent normal kidney tissue was taken from each patient.

Antibodies:

The following primary antibodies were used: mouse monoclonal antibody against Bcl-2 (clone 124) (DAKO, A/S, Glostrup, Denmark) and rabbit anti-human Bax (P-19) and Bcl-X_{S/L} (S-18 against both the Bcl-X_L and Bcl-X_S) polyclonal antibodies from Santa Cruz, California, USA. The specificity and characteristics of these antibodies have been published elsewhere (Oltvai, 1993; Datta, 1995).

Immunohistochemistry:

The PAP (peroxidase-anti-peroxidase) technique was used. Serial sections (5 µm) from all samples were mounted on 3-aminopropyl-trietoxysilane (Sigma Co, St Louis, MO, USA) coated glass slides, which were incubated overnight in a 60°C incubator. After dewaxing in fresh xylene for 10 min and passage in 100% methanol for 10 min, the sections were rinsed in methanol containing 3% hydrogen peroxide for 20 min to block endogenous peroxidase activity. The slides were rinsed with distilled water. In order to enhance antigen exposure, the slides were microwaved at 700 W in 0.1 M citrate buffer at pH 6.0 for 15 min. After cooling and rinsing with PBS, the slides were placed in a Sequenza immunostaining system (Shandon, Unicorn, UK). Sections were incubated with 10% normal rabbit serum (prior to monoclonal antibody) or 10% normal goat serum (prior to polyclonal antibody) (DAKO). Incubation was done in PBS/5% bovine serum albumin (BSA) for 15 min and subsequently overnight with the primary antibody at 4°C. The antibodies were diluted in PBS/5%BSA at 1:50 for Bcl-2, 1:150 for Bax and 1:75 for Bcl-X_{S/L}. Subsequently, the slides were rinsed with PBS/Tween (0.1%) and incubated for 30 min with rabbit-anti-mouse antibody for monoclonal antibody or goat-anti-rabbit for polyclonal antibody (DAKO), followed by rinsing with PBS/Tween (0.1%). The PAP complex (DAKO) was diluted in PBS/5%BSA at 1:300 and incubated for 30 min followed by rinsing with PBS. The antigen-antibody binding was visualised 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 adjacent to the tumour served as positive control.

Immunostaining analysis:

The slides were examined at x25 magnification without knowledge of the clinical outcome of the patients. The amount of positive staining was assessed as the estimated percentage positively staining cells: <10% was considered negative, >10% was considered positive.

Statistical analysis:

Statistical analysis was performed using the SPSS 9 software package. The association between Bcl-2, Bax, Bcl-X_{S/L} expression and clinico-pathological features was analysed using χ^2 test. 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.

Protein extraction and Western blot analysis:

To further confirm the Bcl-X_{S/L} immunohistochemical data, Western analysis was performed with tissues from Wilms' tumour xenografts. 6 different xenograft tissues were analysed in total. Tissues 1-3 (cf. Figure 4) originate 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, WT-16) were from one individual patient, being a specimen of primary tumour in right kidney (WT-15), lymph node metastasis (WT-15LN), and primary tumour in left kidney (WT-16), respectively. Morphologically, all 6 tissues contained the blastemal and stromal component, whereas in tissue 1 and 3 epithelial cells were present as well. The frozen tissues were crushed in a liquid nitrogen chilled metal cylinder. The tissue homogenates were transferred to a lysis buffer consisting of 10mM TRIS (pH 7.4), 150 mM NaCl (Sigma), 1% triton x100 (Merck, Darmstadt, Germany), 1% deoxycholate (Sigma), 0.1% sodium dodecyl sulphate (SDS, Gibco BRL), 5mM EDTA (Merck) and protease inhibitors (1mM phenylmethyl-sulphonyl fluoride, 1 mM aprotinin, 50mg l⁻¹ leupeptin, 1mM benzamidine and 1mg l⁻¹ pepstatin, all from Sigma). The samples were spun at 35,000g at 4°C for 10 minutes. The protein content of the supernatant was measured photometrically using the bio-rad protein assay (Bio-rad, München, Germany). The proteins were transferred to a SDS-polyacrylamide gel and electrophoresis was performed in 10 times diluted tray buffer for 2 hours. The gel was blotted to a 0.45µm cellulose nitrate membrane (Scheicher & Schuell, Dassel, Germany). Pre-stained markers were used as size standards (Novex, San Diego, CA). The immunoblot was blocked for 1 hour with 5% dry milk (Sigma) in 0.1% Tween-20 (Sigma). The antibodies were diluted 1:1000 in 5% dry milk and applied overnight at 4°C. After rinsing with PBS/0.1% Tween, the blot was incubated with horseradish peroxidase labeled goat anti-rabbit (1:2000, DAKO) for 1 hour. Subsequently, a one minute incubation with a 1:1 mixture of luminol and oxidizing reagent (DuPont NEN, Chemiluminescence kit, Boston, MA) was performed. Excess reagent was removed by placing the blot on a piece of Whatmann paper. Finally, the antibodies were visualised by exposure of the blot to an X-ray film for 30 seconds.

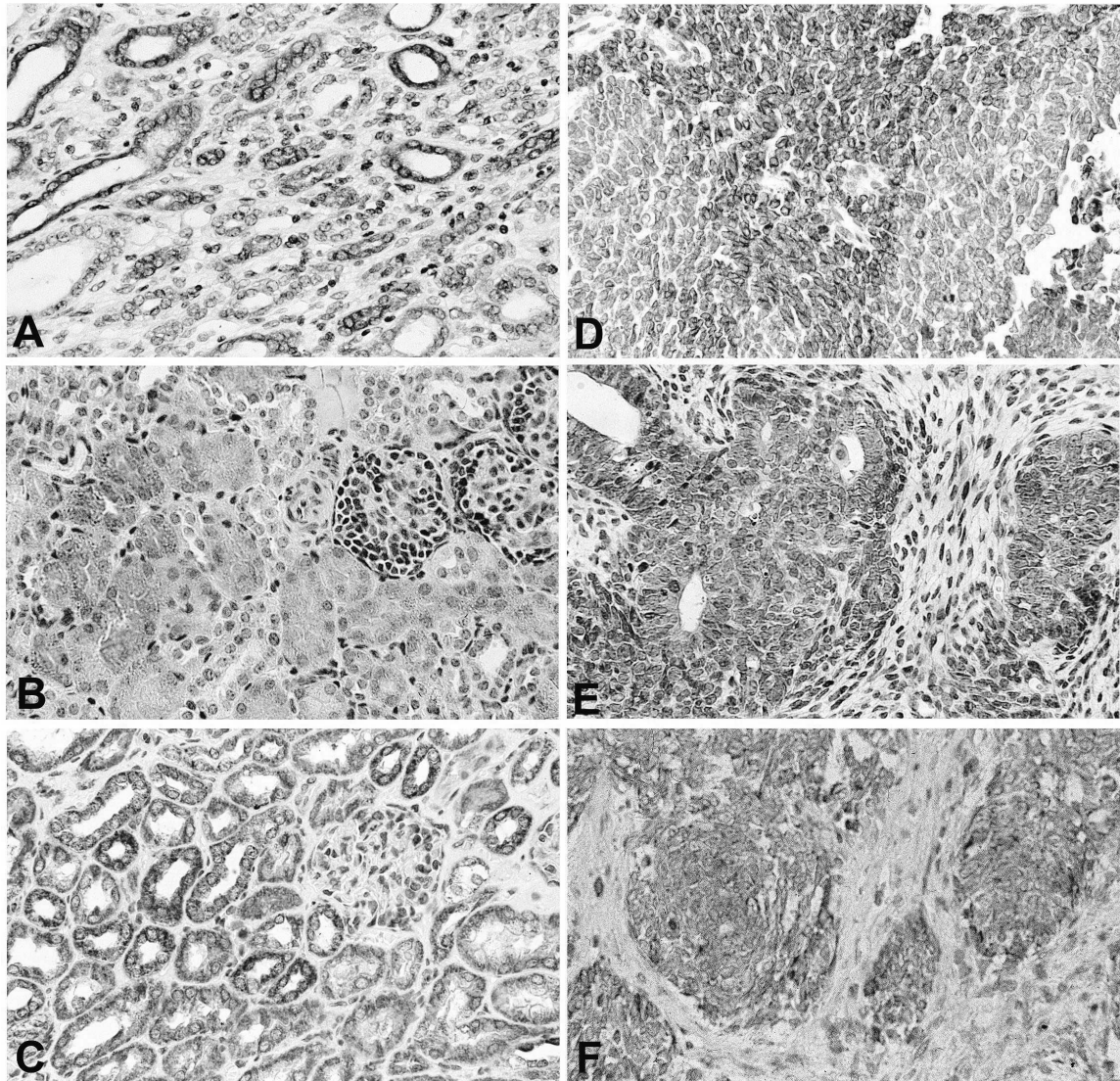
RESULTS

Clinico-pathological findings:

The tumour-stage distribution was T₁ in 21, T₂ in 20 and T₃ in 20 patients. There were 29 (48%) females and 32 (53%) males. The mean age at surgery was 4.2 years, and the mean overall follow-up period was 5.7 years. Clinical progression occurred in 14 patients (23%), and 8 patients (13%) died from their tumour. At the end of the follow-up period 53 patients were alive.

BCL-2, BAX, BCL-X_{S/L} expression in normal renal tissues:

Immunoreactivity for Bcl-2 was found in the Henle's loop, collecting ducts and parietal layer of Bowman's capsule of the normal kidney (Figure 1A). Immunoreactivity for Bax was found in the proximal and distal convoluted tubules and parietal layer of Bowman's capsule, but was not expressed in the collecting ducts (Figure 1B). Expression of Bcl-X_{S/L} was evident mainly in the collecting duct and to lesser extent in the proximal and distal convoluted tubules, whereas expression in the glomeruli was not found (Figure 1C).

**Fig 1**

Normal renal tissue sections and nephroblastoma tissues were immunostained for Bcl-2 (A,D); Bax (B,E); Bcl-X_{s/l} (C,F), respectively. Slides were counterstained with hematoxylin. Magnification 400x.

BCL-2 expression in Wilms' tumour tissues:

Bcl-2 positive (i.e. >10% stained) blastemal and epithelial cells were found in 32 (53%) and 33 (54%) of the Wilms' tumours studied, respectively (Table 1). Bcl-2 staining was mainly perinuclear, but some cytoplasmic staining was also found (Figure 1D). Some specimens showed expression of the majority of cancer cells, whereas in others only small areas of the tumour were found positive for the Bcl-2 protein. The majority of the infiltrating lymphocytes in the tumour stroma were strongly Bcl-2 positive, serving as internal control for the staining procedure. Bcl-2 expression gradually increased from T₁, T₂ to T₃ for both the blastemal and epithelial component. No statistically significant relationship between stage and Bcl-2 expression was found. The nephroblastoma sections showed an intense expression of the epithelium with little expression in the stromal component.

BAX expression in Wilms' tumour tissues:

Blastemal and epithelial expression of Bax was found in a similar percentage of Wilms' tumours (41 and 43% of cells, respectively) (Table 1). Bax staining was mainly cytoplasmic (Figure 1E) and the staining pattern of most of the specimens was weak to moderate. Bax expression in both blastema and epithelium gradually decreased from T₁ to T₃, but the trend was not statistically significant. The epithelial component of the tumour demonstrated diffuse expression of Bax.

Table 1
Relationship between pT stage and blastemal and epithelial expression

Stage	Bcl-2		Bax		Bcl-X _{S/L}	
	Blastema	Epithelial	Blastema	Epithelial	Blastema	Epithelial
T1	9 (15)	9 (15)	10 (16)	10 (16)	9 (15)	13 (21)
T2	11 (18)	12 (20)	9 (15)	10 (16)	8 (13)	13 (21)
T3	12 (20)	12 (20)	6 (10)	7 (11)	6 (10)	9 (15)
Total	32 (53)	33 (54)	25 (41)	27 (44)	23 (38)	35 (57)
P Value	>0.05		>0.05		>0.05	

Data are presented as numbers, percentages between brackets.

BCL-X_{S/L} expression in Wilms' tumour tissues:

Blastemal and epithelial expression of Bcl-X_{S/L} was found in 23 (38%) and 35 (57%) of the Wilms' tumour studied, respectively (Table 1). The percentage of tumours with Bcl-X_{S/L} positive blastema was generally lower than those staining positive for Bcl-2. The majority of Bcl-X_{S/L} positive tumours had very intense cytoplasmic staining pattern, showing a more widely distribution (Figure 1F). There was no statistically significant relationship between stage and Bcl-X_{S/L} expression.

Immunoblot analysis of tissue lysates of a panel of human Wilms' tumour xenografts identified the specificity of the Bcl-X_{S/L} antibody used for immunohistochemistry (Figure 4). Morphologically, all 6 tissues contained blastemal and stromal component, whereas in tissue 1 and 3 epithelial cells were present as well. The Bcl-X_L protein was found to have migrated as a doublet of proteins with an apparent molecular mass of 28-32 kDa. The Bcl-X_L was clearly detectable in all 6 samples analysed, particularly the 32kDa message. A clear 22 kDa band compatible with Bcl-X_S was detected in 3 samples positive for Bcl-X_L, whereas faint bands were found in the remaining two samples, of which one was devoid of Bcl-X_S (Figure 4).

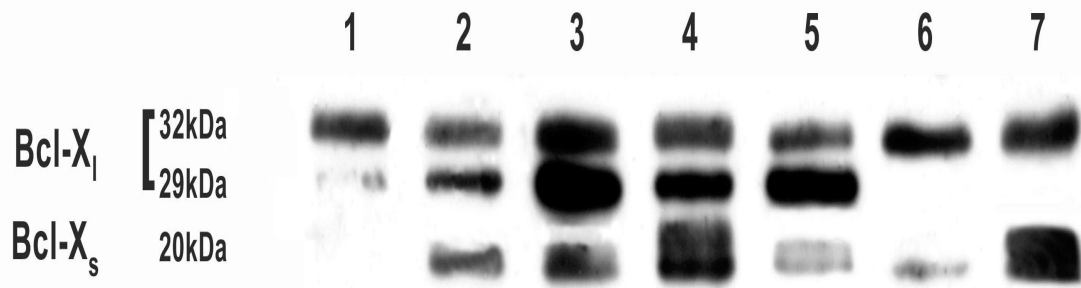


Fig. 4. Immunoblot analysis of Bcl-X_{S/L} proteins in nephroblastoma tissues derived of 6 human xenografts models (lanes 1-6) and normal kidney (lane 7) with approximate indication of size marker.

Prognostic value of BCL-2, BAX and BCL-X_{S/L}:

Univariate analysis using the logrank test showed a prognostic value of blastemal Bcl-2 expression for clinical progression (Table 2, Figure 2). The blastemal expression for both Bax and Bcl-X_{S/L} and epithelial expression of the 3 proteins did not show any prognostic value (Table 2). To test whether Bcl-2, Bax, and Bcl-X_{S/L} expression had any prognostic impact a multivariate Cox' regression analysis was done including the parameters pT stage, Bcl-2, Bax, and Bcl-X_{S/L} expression. PT stage was divided as pT1-2 vs. pT3. In that analysis, only the Bcl-2 blastemal expression could be identified as independent prognostic variable besides stage (data not shown).

Table 2

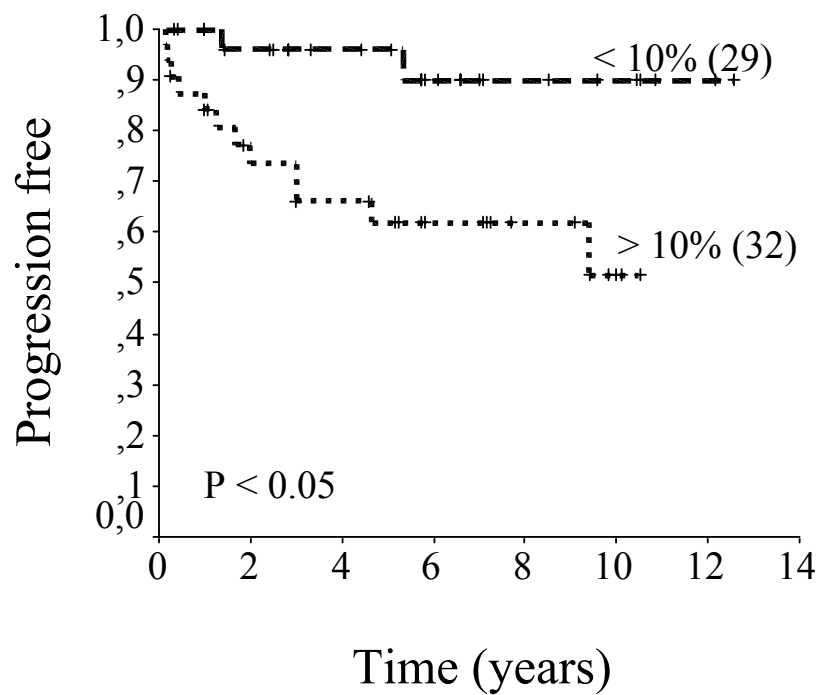
Univariate analysis of prognostic markers

Variable	Blastemal				Epithelial			
	Clinical progression		Tumor specific survival		Clinical progression		Tumor specific survival	
	X ²	P*	X ²	P*	X ²	P*	X ²	P*
Bcl-2	7.31	0.006	3.32	0.07	0.01	0.94	1.09	0.29
Bax	1.11	0.29	1.02	0.31	1.79	0.19	0.26	0.61
Bcl-X _{S/L}	0.52	0.47	1.22	0.27	1.44	0.23	0.63	0.43
Bcl-2/Bax	10.31	0.02	4.56	0.21	1.92	0.59	1.33	0.72

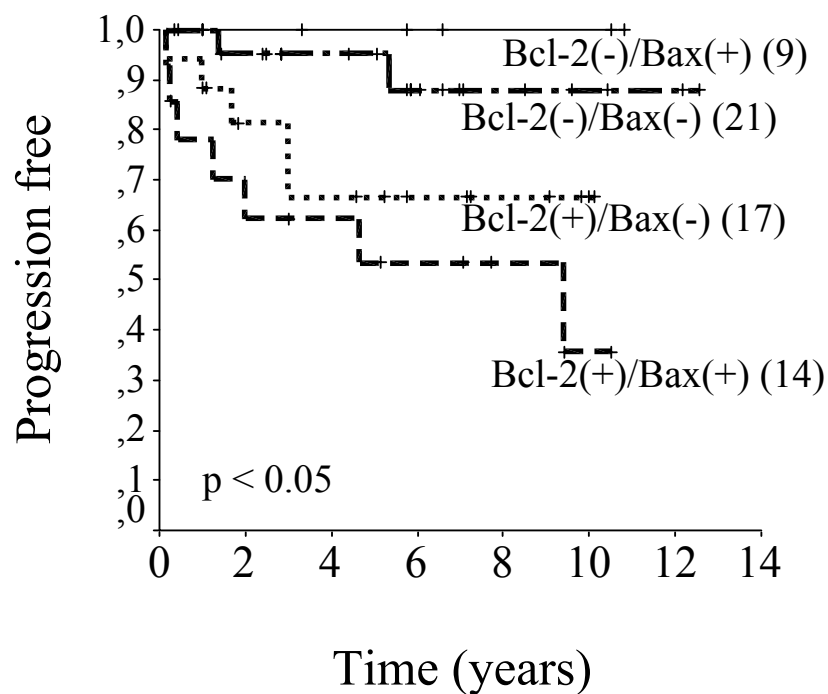
*logrank test

Prognostic value of the Bcl-2/Bax ratio.

By examining the Bcl-2 to Bax ratio versus outcome, 4 patients groups were identified. The Kaplan-Meier curves of Figure 3 show the prognostic influence of Bcl-2/Bax expression on the time to progression. Clearly, patients with high (>10%) expression of both Bcl-2 and Bax had a statistically significant poor prognosis compared to the 2 groups of patients with Bcl-2 negative expression (i.e. Bcl-2 and Bax negative or Bcl-2 negative/Bax positive) (Table 2, Figure 3). Generally, the data of Figure 3 is consistent with the notion that the patients with low Bcl-2/Bax ratio do better than those with high ratio.

**Fig.2**

Kaplan-Meier curves showing the relationship between blastemal Bcl-2 expression and clinical progression. Censored patients are indicated by a tic marks along their line. Number of patients per group is shown between brackets.

**Fig.3**

Kaplan-Meier curves showing the relationship between clinical progression and blastemal ratio of Bcl-2/Bax. Censored patients are indicated by a tic marks along their line. Number of patients per group is shown between brackets.

Bcl-2 and Bax were simultaneously expressed in 28% and 25% of blastemal and epithelial components of tumours, respectively.

DISCUSSION

Over the past few years, Bcl-2 has moved from being a molecule exclusively implicated in lymphoid translocation (14; 18) to being an important gene involved in key mechanisms in the regulation of cell death, by inhibiting apoptosis. Consequently, Bcl-2 is implicated in the process of multidrug resistance of cancer. For this reason, studying the prognostic value of Bcl-2 gene family in malignant processes is warranted. In the present study we have examined the expression of Bcl-2 and some of its related proteins, Bax and Bcl-X_{S/L}, to study its prognostic value in Wilms' tumour. All patients studied received chemotherapy before operation. It is realised that in the majority of patients having a good response upon chemotherapy, treatment did affect the cellular compartments of the Wilms' tumour, the blastema in particular. Consequently, this may influence the (Bcl-2, Bax, and Bcl-X_{S/L}) immunophenotype of the remaining tumour component. The present study did not allow comparative studies of the effect of treatment upon the expression of the apoptotic markers studied. The expression level of the various markers, however, reflects the status of the tumour after chemotherapy, regardless the occurrence of drug resistance. Therefore the final status of the tumour removed, may predict the clinical outcome of the patient, e.g. whether metastasis will occur.

Expression of Bcl-2, Bax and Bcl-X_{S/L} was found in normal kidney tissues, which were used as internal positive controls for the immunohistochemical reaction (Figure 1 A, B, and C) as well as for the western blot (Figure 4). In general, increased Bcl-2 expression was related to advanced disease. Reduction of Bax immunostaining in high stage tumours inconsistent with the more aggressive character of these tumours. Most clinically oriented studies on Bcl-2 expression have found a positive correlation with adverse prognosis in a variety of solid tumours, including non-small-cell lung carcinoma (Pezzella, 1993), nasopharyngeal carcinoma (Lu, 1993), prostatic carcinoma (McDonnel, 1992), and neuroblastoma (Castle, 1993). In contrast, Bcl-2 expression was correlated with favourable prognosis in tumours arising from epithelial as in stage II colon carcinoma (Sinicrope, 1995) and node positive treated breast cancer (Gasparini, 1995). However, in other studies of patients with renal cell tumours (Paraf, 1995), invasive transitional cell carcinoma of the bladder (Glick, 1996), and head and neck cancer (Drenning, 1998), such correlation was not found. A recent study failed to demonstrate prognostic Bcl-2 significance in Wilms' tumour because of a limited number of cases studied (Re, 1999). In another study, Tanaka (1999) showed that preoperative chemotherapy did not significantly influence either the occurrence of apoptosis or expression of Bcl-2 in a group of treated Wilms' tumour patients. In the present study, a prognostic value of blastemal Bcl-2 expression was found for clinical progression. The mechanism by which Bcl-2 contributes to the progression of nephroblastoma is unknown. Based on gene transfer experiments (Vaux, 1988) and studies of transgenic mouse models (Strasser, 1990), Bcl-2 functions by inhibiting cell death rather than affecting the rate of cell proliferation.

Information regarding the prognostic significance of Bax in human tumours is scarce. It has, however, been shown that reduced Bax expression correlates with poor prognosis in patients treated with chemotherapy for metastatic breast adenocarcinomas (Krajewski, 1995)

and in radiotherapeutic glottic squamous cell carcinomas (Xie, 1998). High levels of Bax expression correlates to a better outcome for patients with low-grade tumour of urinary bladder (Gazzaniga, 1996). Bax expression alone had no influences upon survival of stage I patients with radically resected non-small-cell lung cancer (Apolinario, 1997), however, a study of the Bax mRNA expression levels in nephroblastoma, was not indicative that its expression could have a role in the prognosis (Re, 1999). This was confirmed by the outcome of the present study showing an absence of any prognostic value of Bax. The ratio between the Bcl-2 and the Bax proteins has been described as a cellular rheostat determining the cellular response to apoptotic stimuli (Oltvai, 1993). Given the potential role of these proteins in malignant tumours, the prognostic significance of combined Bcl-2 and Bax expression has been studied by many authors. Gazzaniga (1996) demonstrated that the Bcl-2/Bax expression ratio had prognostic impact for disease progression in low-grade bladder tumours. Interestingly, in nephroblastoma Bcl-2/Bax expression had prognostic significance for the predication of clinical progression at univariate analysis.

The present data demonstrate that the level of Bcl-2 expression exceeds that of Bcl-X_{S/L}, although Bcl-X_{S/L} staining is extensive and intensive compared to Bcl-2. These results are not similar to those reported for untreated prostatic and gastric cancer, in which Bcl-X_{S/L} immunoreactivity was found in 100% and 85% of the tumours, respectively (Krajewska, 1996a,b). Bcl-X_{S/L} was expressed in 100% of untreated gliomas, but not related to the response to radiotherapy or chemotherapy (Rieger, 1998). In the present study, Western blot analysis demonstrated that the anti-apoptotic Bcl-X_L protein was the dominant isoform of the Bcl-X_{S/L} protein in experimental human nephroblastoma. This was in agreement with the outcome of studies of Re et al. suggesting the oncogenic potential of Bcl-X_L overexpression in the development of anaplastic Wilms' tumour (Re, 1999). Accordingly, reduction of Bcl-X_L expression may influence the outcome of patients treated with radiotherapy and/or chemotherapy (Krajewska, 1996b; Datta, 1995; Decaudin, 1997). On the basis of the present non-quantitative immunohistochemical data of various apoptosis-associated proteins no conclusion can be drawn with respect to the clinical behaviour of Wilms' tumors. Still, reduction of the expression of the anti-apoptotic Bcl-X_L protein, may contribute to enhanced sensitivity to radiotherapy/ chemotherapy. Indeed, cumulating in vitro data suggest that Bcl-X_L may play an important role in the susceptibility of tumour cells to chemotherapeutic agents and radiation. For this experimental study it seems that the immunohistochemical expression of one single marker will provide sufficient information on the chemosensitivity or radiosensitivity.

In conclusion, Bcl-2, Bax and Bcl-X_{S/L} were expressed in normal renal tissues and in nephroblastoma. Bcl-2 expression and the Bcl-2/Bax ratio showed prognostic value in the specimens of chemotherapeutically treated Wilms' tumour patients.

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CHAPTER 4

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 means of immuno-histochemical analysis of 62 Wilms' tumors. Patients were preoperatively treated by chemo-therapeutic 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%), the difference being statistically significant ($p < 0.01$). 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 prognosticator 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 (Beckwith, 1994). Currently, the prediction of outcome is based mainly on the histo-pathology and stage of disease at the time of resection (Williams, 1985). 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 (Wen, 1997).

Advances in cell cycle research have led to the identification of proteins responsible for the regulation of cell proliferation, which may be used as tumor marker. The Ki-67 monoclonal antibody has been developed and used in evaluating cellular proliferation rates of malignant tumors (Barbareschi, 1994; De Riese, 1993; Gerdes, 1993; Youssef, 1995). 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 (G₁, S, G₂ and M phases), but not in resting (G₀) cells (Cattoretti, 1992). The clinical value of proliferation markers for prognostication of nephroblastoma is still subject of debate (Benjamin, 1991; Khine, 1994; Delahunt, 1994).

The p27^{Kip1} protein is a cyclin dependent kinase inhibitor, affecting the G1/S traverse in response to extracellular signals (Coats, 1996; Polyak, 1994a,b). 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 (Sanchez-Beato, 1997). There is increasing evidence that low expression of p27^{Kip1} is highly indicative for disease progression in many tumor types (Cheville, 1998; Dellas, 1998; Florenes, 1998; Loda, 1997; Newcomb, 1999; Catzavelos, 1997; Tan, 1997; De Riese, 1991).

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 immunohisto-chemistry on paraffin embedded material.

MATERIALS AND METHODS

Patients:

During the period 1987-1999, 62 patients with nephroblastoma were treated by neo-adjuvant chemotherapy and subsequent tumor nephrectomy. Twenty-six patients (42%) were female, 36 patients (58%) were male. Patients were treated according to SIOP protocol 9 and some according to 93-01, receiving actinomycin D and vincristine. Following 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 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 (Boccon-Gibod, 1998). 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:

The following primary antibodies were used: mouse monoclonal antibodies against Ki-67 (MIB-1, Immunotech, Marseille, France) and against p27^{Kip1} (Clone 1B4, Novocastra Laboratories LTD, Newcastle, UK). The specificity and characteristics of these antibodies have been published elsewhere (Cattoretti, 1992; Polyak, 1994a,b).

Immunohistochemistry:

The PAP (peroxidase-anti-peroxidase) immunohistochemistry technique was used and applied to serial sections (5 µm) from all samples which were mounted on 3-aminopropyl-trietoxysilane (Sigma Chemical Co., St. Louis, Missouri, USA) 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 phosphate buffered saline (PBS) 5% bovine serum albumin (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-anti-mouse antibody. The PAP complex (DAKO) was diluted in PBS/5%BSA and incubated for 30 minutes, following 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 and quantification:

Two independent observers, using a standard light microscope with an x40 objective and equipped with an ocular grid, evaluated the slides. 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 1,000 tumor cells in 5 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 ≥ 5%. The cut-off value of 5% was statistically known to be the lowest value at which discrimination with a significant probability value was achieved. Based on previous studies, for p27^{Kip1} protein a cut-off value of 50% of the tumor cells was used (Loda, 1997; Newcomb, 1999).

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 Chi-square test. MIB-1 and p27^{Kip1} expression in normal kidney were studied using the Spearman rank correlation test since the data were not normally distributed. For analysis of survival data Kaplan-Meier curves were constructed and the logrank 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 seven 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

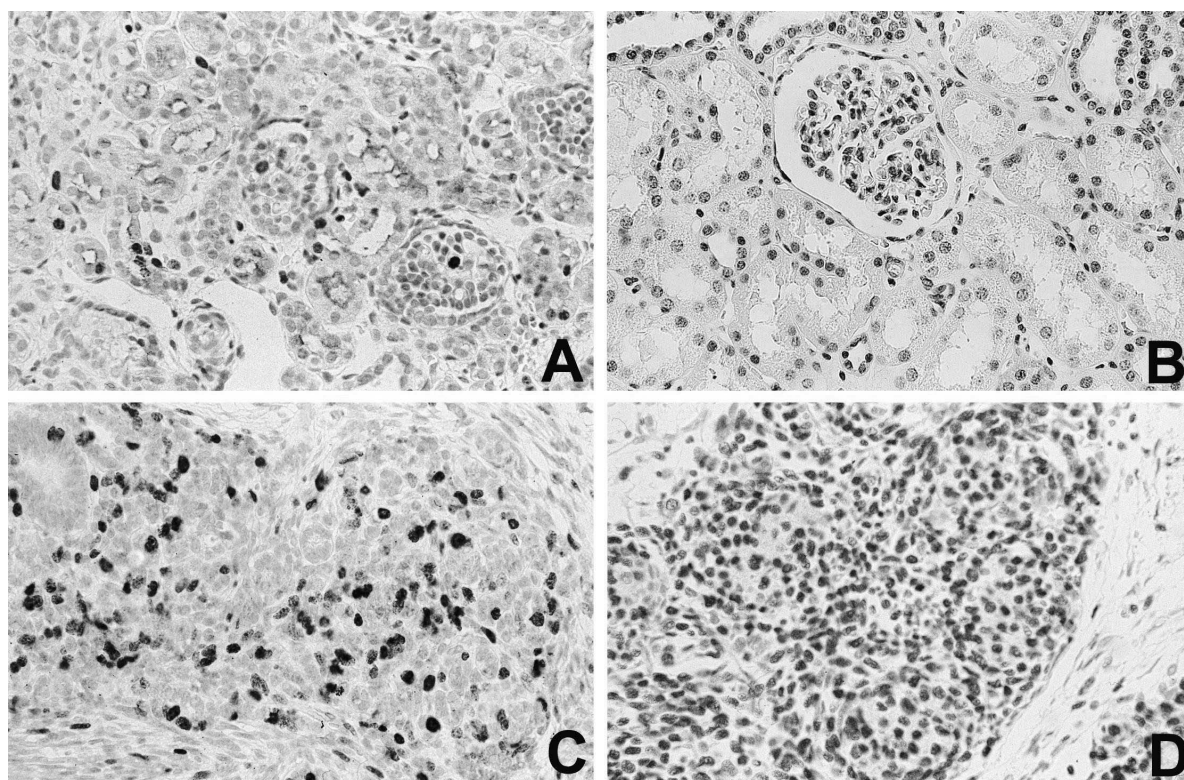
The proliferation index (PI) of normal renal tissue, i.e. the percentage of MIB-1 positive cells ranged between 0% and 3% (1.5 ± 1 ; mean $\pm$ SD) (Table 1; Fig. 1.A). In Wilms' tumors, immunostaining was localized in the blastemal and epithelial nuclei. The frequency of positive nuclei varied from specimen to specimen and among tumors having the same stage. In 19% of cases the highest PI were 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 to 42%), whereas for epithelial cells MIB-1 PI was $19.9\% \pm 18.6$ (range: 0 to 53%) (Table 2). In the lesions studied, the PI for the epithelium were significantly higher than those found for the blastema (Spearman rank correlation coefficient, $p < 0.01$). In 7/62 (11%) specimens, derived from tumors of various stages, no labeling with MIB-1 antibody was found. At a cut-off PI of 5%, MIB-1 positive blastemal and epithelial cells were found in respectively 38 (61%) and 41 (66%) of the Wilms' tumors studied. Neither for blastema, nor for the epithelium, a correlation between MIB-1 and pathological stage was found (Table 2).

P27^{Kip1} expression in Wilms' tumor tissues

The percentage of p27^{Kip1} positive cells in normal renal tissue ranged between 0% and 55% (21 ± 14.2) (Table 1; Fig. 1.B). In Wilms' tumors p27^{Kip1} was expressed in the blastemal and epithelial nuclei at variable levels, usually with a low background, and with a rather diffuse staining pattern (Fig. 1.D). 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 internal positive control. The mean percentage of blastemal p27^{Kip1} positive cells was identical to that of the epithelium being $55.1\% \pm 24$ and $59\% \pm 27$, respectively (Table 2). The p27^{Kip1} scores of blastemal and epithelial tumor cells showed a significant inverse correlation compared to 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 cut off value of 50%, p27^{Kip1} positivity for blastemal and epithelial cells was found in respectively 35 (57%) and 45 (73%) of the Wilms' tumors studied. Neither for blastema, nor for epithelium, a correlation between p27^{Kip1} and pathological stage was found (Table 1).

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

	Wilms' tumors tissues				Normal renal tissue	
	MIB-1		P27 ^{Kip1}		MIB-1	P27 ^{Kip1}
	Blastemal	Epithelial	Blastemal	Epithelial		
Mean $\pm$ SD	9.4 ± 10	19.9 ± 18.6	55.1 ± 24	$59\% \pm 27$	1.5 ± 1	21 ± 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 non-neoplastic and nephroblastoma tissues. MIB-1 antigen, respectively p27 expression in normal renal tissues (A and B) and nephroblastoma tissues (C and D) outlined. Slides were counterstained with hematoxylin. (magnification x400)

Table 2Relationship between pT stage and blastemal and epithelial expression of MIB-1 and p27^{KIP1}

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 value	>0.05		>0.05	

Data are presented as numbers of MIB-1 positive (cut-off value 5%) and p27^{KIP1} positive (cut-off value 50%) Wilms' tumors, percentages between brackets

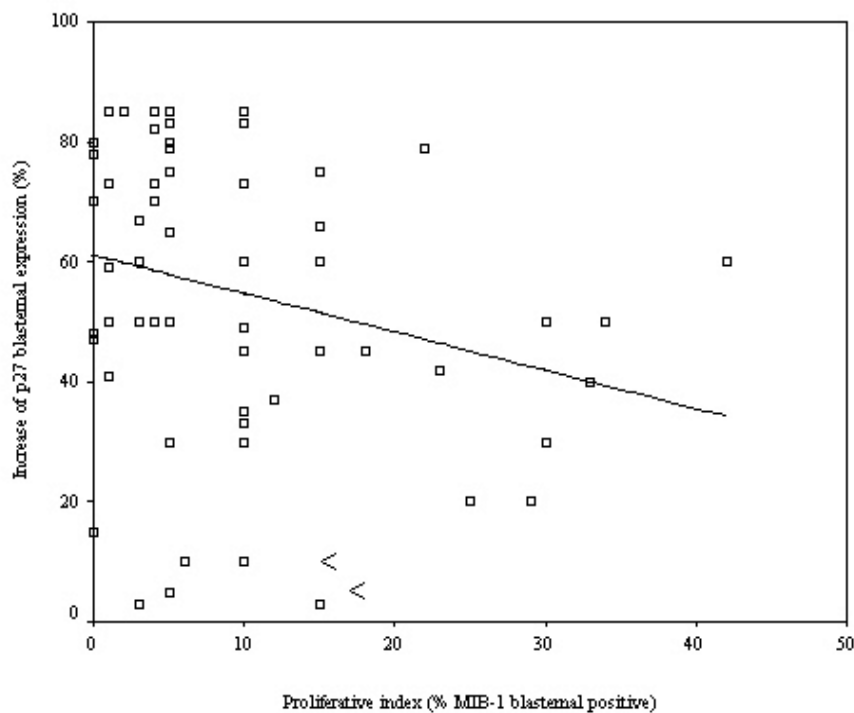


Fig. 2. Relationship between MIB-1 PI and p27^{KIP1} expression in nephroblastoma. Scatterplot of MIB-1 PI versus p27^{KIP1} expression with regression line showing a correlation of the two cell cycle regulators using Spearman's correlation coefficient. Arrows indicate a separate population of Wilms' tumor.

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 scatter-plot shown in Fig. 2 a separate population of Wilms' tumor becomes apparent having relatively low (< 20) percentage of both MIB-1 and p27^{Kip1} positive cells.

Prognostic value of MIB-1 and p27^{Kip1}

Univariate analysis, using the logrank test, showed prognostic significance of blastemal MIB-1 PI and p27^{Kip1} expression for clinical progression and tumor related death (Table 3, Fig. 3, 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' 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 vs pT3; for MIB-1 was classified as PI $< 5\%$ versus $> 5\%$; 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 variables for clinical progression besides stage (Table 4).

Table 3
Univariate analysis of prognostic markers

Variable	Blastemal				Epithelial			
	Clinical progression		Tumor specific survival		Clinical progression		Tumor specific survival	
	X ²	P*	X ²	P*	X ²	P*	X ²	P*
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

*logrank test

Table 4
Results of Cox's multi-regression analysis

Outcome parameters	Variable	Hazard Ratio	95% Confidence Limit	P value
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

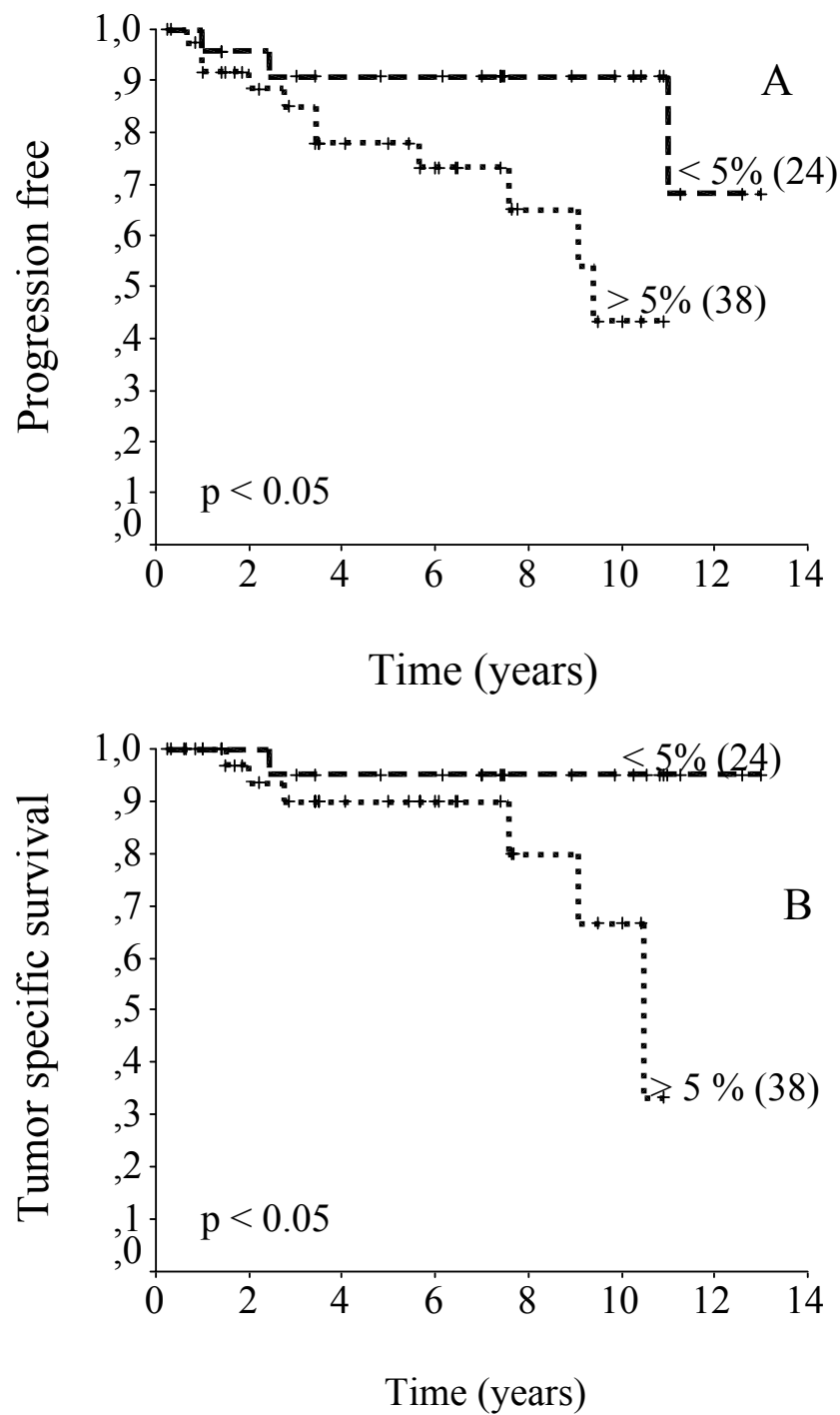
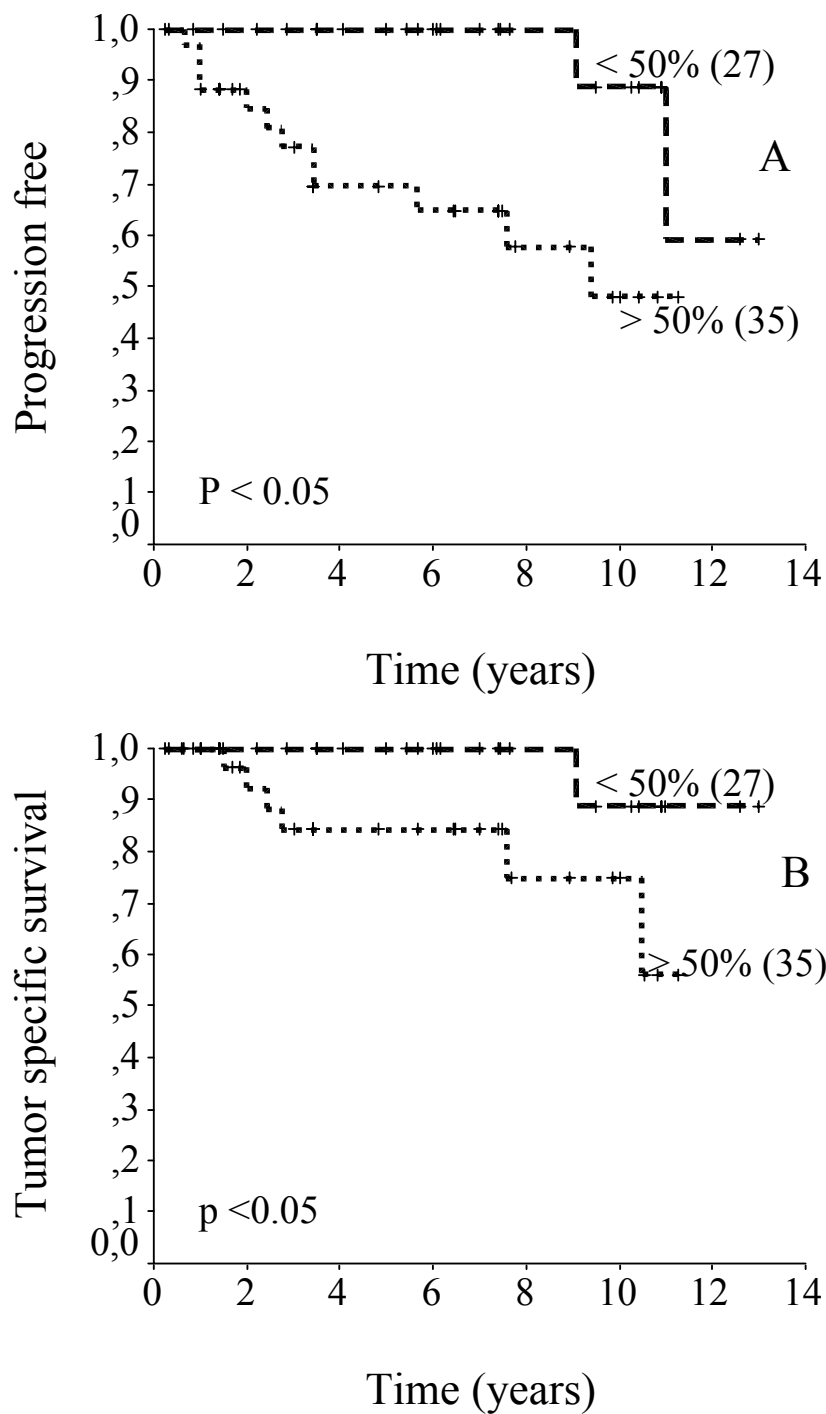


Fig.3

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 per group is shown between brackets.

**Fig.4**

Kaplan Meier curves showing a relationship between blastemal P27 expression and clinical progression (A) and survival (B), respectively. Censored patients are indicated by a tic marks along their line. Number of patients per group is shown between brackets.

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 proliferative index (PI) of human cancers. 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% to 5% of all DNA-synthesizing cells is microscopically detectable (De Riese, 1991). In recent years, MIB-1 (a true equivalent of Ki-67) has been proven to be the best proliferation marker for routine use in formaline fixed and paraffin embedded tissue sections (Barbarechi, 1994; Youssef, 1995; Rose, 1994). The present study was carried out 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 and prostatic cancer (Youssef, 1995; Hall, 1988; Simony, 1990; Brown, 1998; Shyr, 1999; Keshgegian, 1998), as well as in Wilms' tumor (Delahunt, 1994). 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 (1994). The lack of correlation between PI as measured with the 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 (Porschen, 1994).

Lack of MIB-1 staining has been observed in 11 percent of all tumors studied. This very low proliferative activity in nephroblastoma tissue has also been reported by other investigators (Khine, 1994). Also, the observation that tumor stroma shows less proliferative activity than blastemal and epithelial components (Mierau, 1987; Khine, 1994), 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 non-plastic tissues. However, the precise histogenetic relation between the blastema and the stroma is as yet, not conclusively established and need 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 cell cycle-dependent kinase inhibitor p27^{Kip1} expression has been described in a number of human tumors (Steeg, 1997). In this report, we show that high levels of the p27^{Kip1} are associated with poor survival in nephroblastoma. This is contradictory to most observations of other tumors studied, in which a low p27^{Kip1} is associated with poor prognosis, e.g. in colorectal, epithelial ovarian, breast, gastric, Barrett's, esophageal, non-small-cell lung and prostatic carcinomas (Loda, 1997; Newcomb, 1999; Catzavelos, 1997; Tan, 1997; Mori, 1997; Singh, 1998; Esposito, 1997; Yang, 1998; Vis, 2000; 2002). Our finding in WT is, however, consistent with previous evidence that p27^{Kip1} is often expressed at relatively high levels in human cancer cell lines which are further characterized by increased the expression of cyclin D1 or cyclin E (Ciaparrone, 1998; Sgambato, 1996). Also, high levels of p27^{Kip1} are associated with poor survival in invasive cervical carcinoma (Dellas, 1998).

High proliferative activity, as assessed by immunohistochemistry Ki-67, 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 (Sanchez-Beato, 1997; Jordan, 1998; Lioyd, 1997). In contrast, studies on colorectal and breast cancer showing no correlation between tumor cell proliferation and p27^{Kip1} have been reported (Loda, 1997; Tan, 1997). 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} proteins contribute to, or reflect, cell proliferation by alteration of the kinetic behavior in primary treated Wilms' tumor. Since 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' tumors. 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 this small subset of 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. The findings may not necessarily be identical in untreated Wilms' tumors.

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' tumour, the blastemal component in particular. In the mean time we have been able to perform a similar immuno-histochemical study on material derived from patients that did not receive any therapy prior to 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 group described in the present manuscript. Because most of the patients responded well upon chemotherapeutical treatment, it was not to our surprise to observe that proliferation was affected and MIB-1 scores were lower in the pre-treatment group, whereas the reverse 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 chemotherapeutical 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 dynamics 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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CHAPTER 5

Expression and Prognostic value of CD44 isoforms in Nephroblastoma (Wilms Tumor)

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ABSTRACT

Purpose: CD44 is a group of transmembranous glycoproteins formed by alternative splicing of a single messenger RNA. An abnormal pattern of CD44 expression has been demonstrated in several human malignancies. We evaluate the prognostic value of standard CD44 (CD44s) and some of its isoforms in treating clinical Wilms tumor.

Materials and methods: The immunohistochemical expression of CD44 isoforms was studied in paraffin material of 61 clinical Wilms tumor. Patients were treated preoperatively with chemotherapy and mean followup was 5.7 years.

Results: Generally CD44s, CD44v5 and CD44v10 were expressed in the normal kidney tissues and at variable levels in the 3 cell types of Wilms tumor (blastemal, epithelial and stromal). No CD44v6 expression was found neither in normal kidney or in tumor tissue. CD44s, CD44v5 and CD44v10 epithelial expression gradually decreased from stage T1 to T3. By contrast the percentage of CD44 positive cells in the blastemal component significantly increased from T1 to T3. CD44 immunoreactive blastema cells were found in 62%, 44% and 41% for CD44s, CD44v5 and CD44v10, respectively. Blastemal expression of CD44s and CD44v5 statistically significantly correlated with clinicopathological stage. Univariate and multivariate analyses showed that blastemal CD44v5 expression was indicative of poor prognosis.

Conclusions: Increased expression of CD44v5 in the blastemal part of Wilms tumor correlated with tumor stage, clinical progression and tumor related death. Therefore, blastemal CD44v5 expression may be of value in identifying patients with a high propensity for distant metastases. These patients might benefit from adjuvant chemotherapy and/ or radiotherapy.

INTRODUCTION

Wilms tumor is a pediatric malignancy of the kidney and one of the most common solid tumors in children (Beckwith, 1994). Despite remarkable improvement in treatment, recurrence develops in approximately 20% of patients with stage II or III disease, in more than 50% of those with stage IV disease and in patients with tumors with unfavorable histological characteristics (Williams, 1985). The biological behavior of Wilms tumor can be predicted based on the histopathological findings and staging. However, attempts to identify additional and possibly more effective independent prognosticators have not been successful to date (Wen, 1997).

Cell-cell and cell-extracellular matrix interactions are fundamental to many biological processes including cell growth, differentiation and motility. Several families of cell surface receptors, such as cadherins, selectins, integrins, members of the Ig superfamily and CD44, govern these adhesive interactions (Zoller, 1995). CD44 is a family of type I transmembrane glycoproteins that is expressed in a variety of cells including those of epithelial, mesenchymal, and hematopoietic origin. CD44 is involved in a number of important biological processes, including lymphocyte homing and activation, hematopoiesis, angiogenesis, survival from apoptosis, and possibly tumor metastasis (Koopman, 1993; Trochon, 1996; Ayroldi, 1995; Herrlich, 1993a,b).

The human CD44 gene is composed of 19 exons, 9 of which are variably expressed due to alternative splicing of the messenger RNA (mRNA). Standard CD44 (CD44s) is composed of exons 1-5 and 15-19 (s1-s10). The variant forms contain 1 or more of exons 6-14 (v2-v10, v1 is not expressed in humans) which are positioned between exon 5 and 15. In this way, numerous CD44 variants can potentially be generated (Secreaton, 1992). The most widely expressed CD44 molecule is the 85 to 90 kDa. CD44s. Alternative splicing of variant exons may generate CD44 isoforms of molecular weight ranging from 90 to greater than 220 kDa. with a different capacity to bind hyaluronic acid (Bartolazzi, 1995). An interesting aspect of CD44 is its presence in cancer cells. With tumor progression, strict splice control seems to be lost or changed in many human as well as rodent tumors. Cleavage varies with tumor origin (Herrlich, 1993a). Recent studies have indicated that expression of CD44 isoforms may be a good predictor of the prognosis of various cancers (Stauder, 1995; Favort, 1993; Speiser, 1997). It has been suggested that expression of certain CD44 splice variants could be used for cancer screening, detection or staging (Matsamura, 1992). Involvement of CD44 in cell migration and tumor progression prompted us to investigate the prognostic value of CD44 isoforms in nephroblastoma for parameters, metastatic progression and patient survival.

MATERIALS AND METHODS

Patients. From 1987-1999, 61 patients with nephroblastoma were treated with neo-adjuvant chemotherapy according the successive protocols of the Société internationale d'Oncologie Pédiatrique and subsequent tumor nephrectomy. After treatment patients were followed regularly and all data on diagnosis, treatment and followup were stored in a data base. Clinical progression was defined as histologically or cytologically proven local recurrence or the 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 hematoxylin and eosin stained slides were reviewed by an experienced pediatric pathologist to assess stage according to the TNM classification (Boccon-Gibod, 1998). Tumor samples containing the 3 different cell types of Wilms tumor were selected from the tissue blocks available from individual patients. In addition, adjacent normal kidney tissue was taken from each patient.

Patient demographics. Our study included 29 (48%) females and 32 (53%) males. T-stage distribution was T1 in 21, T2 in 20 and T3 in 20 patients. Clinical progression occurred in 14 patients (23%). Mean overall followup was 5.7 years (range 0.3 to 14.4). Mean patient age at operation was 4.2 years. At the end of follow-up 53 patients were alive while 8 (13%) died of tumor.

Immunohistochemistry. The primary mouse monoclonal antibodies used were CD44s (clone SFF-2), CD44v5 (clone VFF-8), CD44v6 (clone VFF-18) and CD44v10 (clone VFF-14). The specificity and characteristics of these antibodies have been described previously (Herrlich, 1993; Secreton, 1992). 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, Missouri) coated glass slides and subsequently incubated overnight at 60°C. To enhance antigen exposure slides were microwaved at 700 W in 0.1 M citrate buffer at pH 6.0 for 15 minute. Sections were incubated with 10% normal rabbit serum (DAKO A/S, Glostrup, Denmark) in phosphate buffered saline 5% bovine serum albumin for 15 minute and subsequently with the monoclonal antibody at 4°C overnight. Antibodies were diluted in phosphate buffered saline 5% bovine serum albumin at 1:100. After being incubated with rabbit-anti-mouse antibody, the peroxidase antiperoxidase complex (DAKO) was diluted in phosphate buffered saline 5% bovine serum albumin and incubated for 30 minutes, following which antigen-antibody binding was visualized with diaminobenzidine tetrahydrochloride dihydrate (Fluka, Neu-Ulm, Germany). In all staining sessions a high CD44 expressing tissue of the human prostate cancer xenograft (PC82) served as positive control and a specimen of normal kidney tissue which staining negatively for all CD44 isoforms served as negative control. In addition, staining of patient tissue was controlled by replacing the primary antibody by phosphate buffered saline 5% bovine serum albumin.

Immunostaining analysis (Quantification). Slides were studied at x25 magnification without knowledge of clinical outcome. Semiquantitative evaluation was performed by 1 of us (T.H.vd.K.), according to a consensus scoring protocol. The amount of positive staining was assessed as the estimated percentage of positively stained cells in both tumor compartments (blastema and epithelium, and less than 10% was considered negative and 10 % or greater was considered positive (Ghanem, 2000; Ioachim, 1999).

CD44 mRNA expression. Expression of CD44 mRNA was studied in tumor samples from 6 well characterized human Wilms tumor xenografts, 1 xenograft derived cell culture and, as a control, the prostate cancer xenograft PC82. Tumor samples were snap-frozen in liquid nitrogen chilled isopentane (Merck, Darmstadt, Germany) and stored at -80°C until use. Total RNA of snap-frozen tumor samples was prepared by the guanidine isothiocyanate / cesium chloride centrifugation method using the RNazol-B kit (Campro, Veenendaal, The Netherlands) according to manufacturer protocol. First strand complementary DNA (cDNA) was produced with 1 μ g of isolated RNA using random hexamer primers and Moloney-murine leukemia virus reverse transcriptase in the presence of deoxynucleotide- triphosphate and ribonuclease inhibitor (Perkin Elmer Cetus, Norwalk, connecticut) in a final volume of 10 μ L. The primers used were (P1+) Sense 5'-GACACTATTGCTTCAATGCTTCAGC and (P1-) antisense 5'-GATGCCAAGATGATCAGCCATTCTGCAAT. These primers should

amplify both CD44s cDNA and all possible splice variants. Integrity of RNA samples was determined by reverse transcriptase polymerase chain reaction (RT-PCR) analysis with primers specific for the glyceraldehyde-3-phosphate dehydrogenase gene: sense (exon 4) 5'-ACGGGAAGCTTGTGATCAATGG and anti-sense (exon 7/8) 5'-GGCAGTGATGGCATGGACTGTGGTCATG. Amplification of cDNA was done using superTaq DNA polymerase (Sphaero, HT biotechnology, United Kingdom) in a thermal cycler (Perkin Elmer Cetus). The polymerase chain reaction mix was heated to 94°C for 4 minute followed by 40 cycles of 1 minute at 94°C, 1 minute at 60°C and 1 minute at 72°C. After the last cycle the tubes were kept at 72°C for 10 minutes. Polymerase chain reaction products were separated on a 1% agarose gel in the presence of ethidium bromide and analyzed under ultraviolet light.

Statistical analysis. Statistical analysis was performed with the SPSS 8.02 software package (SPSS, Chicago, Illinois). The association between CD44 expression and clinicopathological features was analysed using Chi-square tests. 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

CD44 expression in Wilms tumor tissues.

In figure 1 membranous immunostaining of normal renal tubular structures is shown for CD44s, CD44v5 and CD44v10, respectively. CD44v6 was not expressed in normal kidney tissues. CD44s, CD44v5 and CD44v10 immunoreactive blastemal cells (greater than 10% positively stained cells) were found in 38 (62%), 27 (44%) and 25 (41%), of Wilms tumor, respectively (table 1). CD44 immunoreactive tumor epithelium cells (greater than 10% positively stained cells) were found in 47 (77%), 37 (61%) and 25 (41%) for CD44s, CD44v5 and CD44v10, respectively (table 2). Immunostaining was generally membranous. Blastemal expression of CD44v5 and CD44s statistically significantly correlated with clinicopathological stage of disease (table 1). The lymphocytes that were present in most tissue sections showed strong immunoreactivity for CD44s and could serve as a positive internal control. (fig. 2, B). Occasionally CD44v5 and CD44v10 immunoreactive lymphocytes were seen as well. The stromal component of tumors showed some non-specific staining. CD44v6 expression was not found.

Table 1.
Relationship between pT stage and blastemal expression

Stage	No. CD44s(%)	No. CD44v5(%)	No. CD44v10(%)
T1	8 (13)	4 (7)	7 (12)
T2	13 (21)	10 (16)	8 (13)
T3	17 (28)	13 (21)	10 (16)
Total	38 (62)	27 (44)	25 (41)
p Value	<0.001	<0.01	>0.05

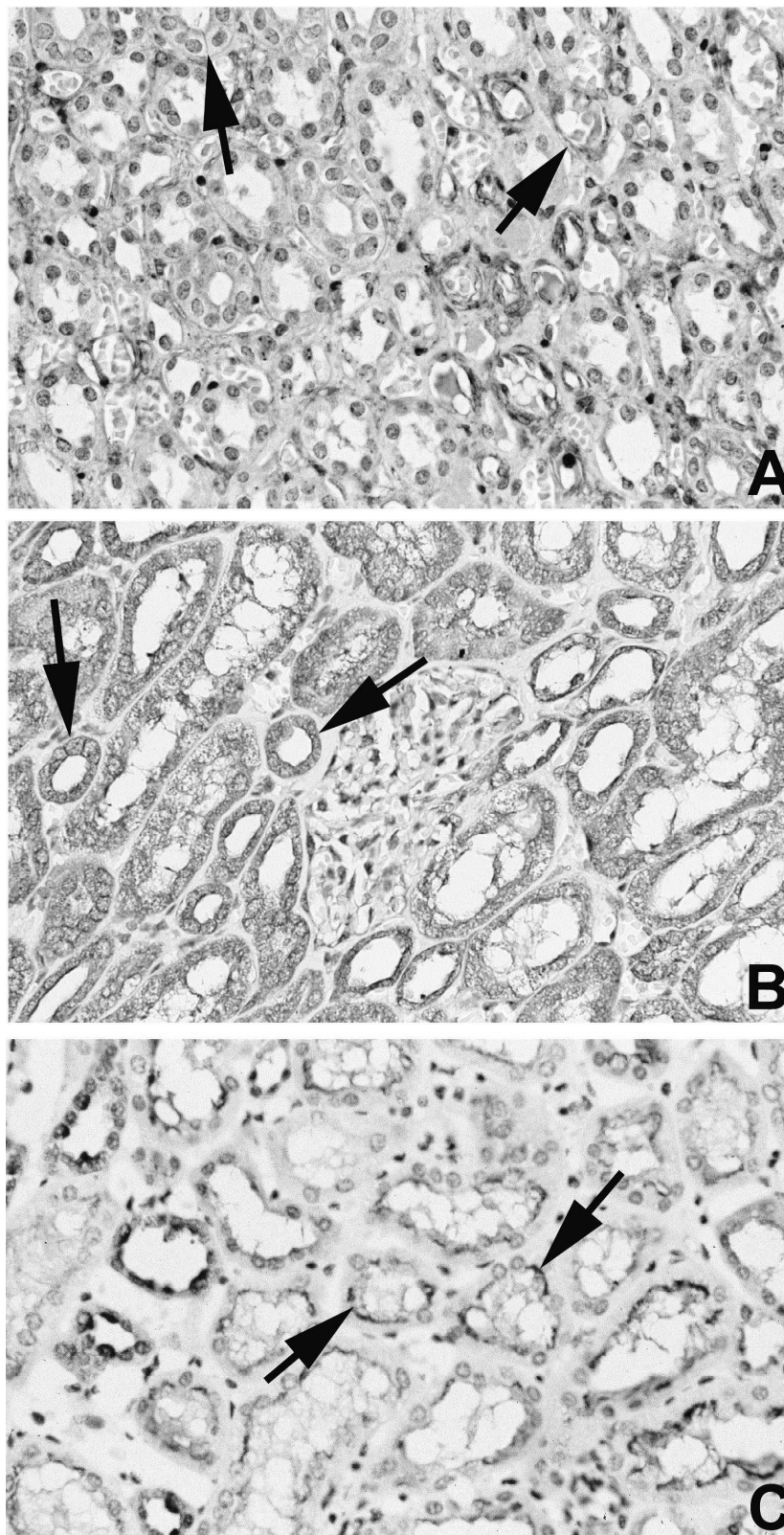
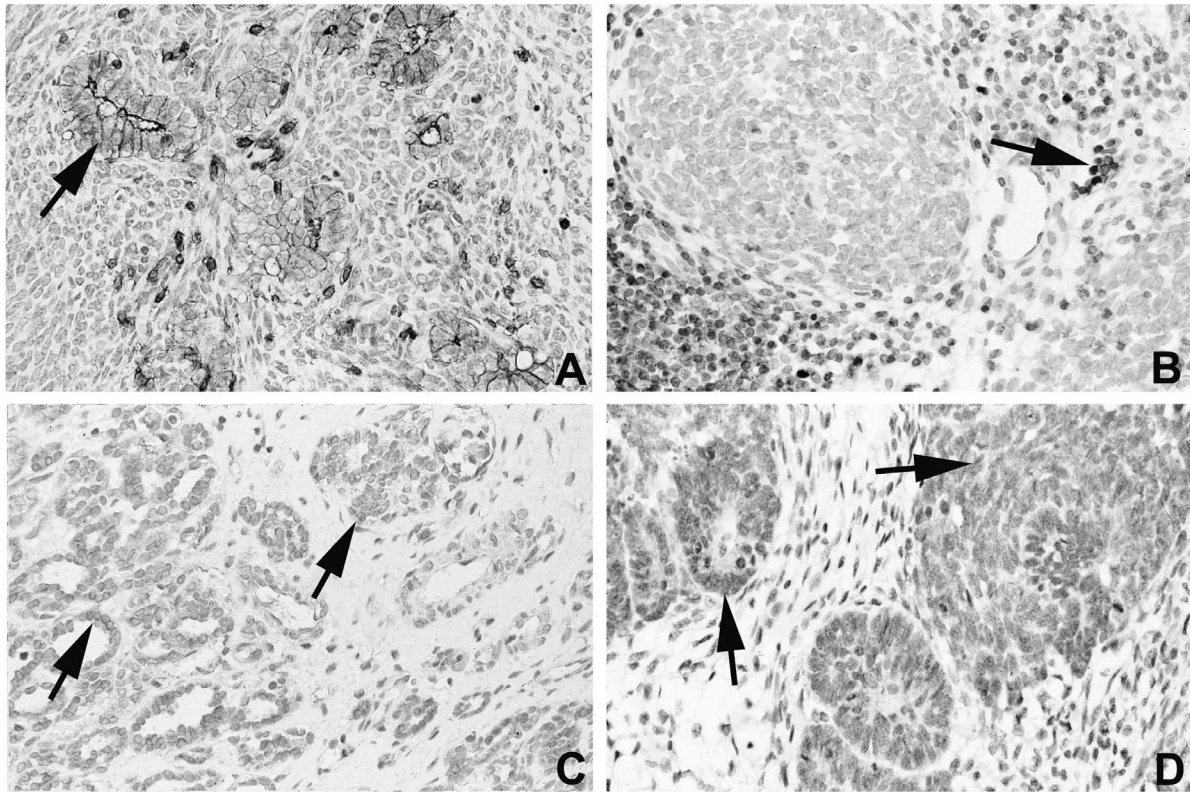


Fig1
CD44 immunohistochemistry of renal tissues. A, CD44s. B, CD44v5. C, CD44 v10. Arrows indicate membranous staining of renal tubules. H&E, reduced from X400

**Fig. 2**

CD44 immunohistochemistry of Wilms' tumor. A, CD44s blastemal expression. Arrows indicate membranous staining of blastema.

B, CD44s blastemal without expression with lymphocytes strongly immunoreactive with CD44s (arrows). C, CD44v5 blastemal and epithelial expression with strong labeling. Membranous staining of blastema and epithelium in C and D is indicated by arrows.

H&E, reduced from X 400

CD44 mRNA expression was determined in 6 Wilms tumor xenografts and 1 xenograft derived cell culture by means of RT-PCR analysis (fig. 3). It is noteworthy that a 200 bp fragment representing CD44s was found in all samples with variable levels of expression. Of the samples (including the cell culture) 4 showed an expression equal to the PC-82 prostate xenograft control sample, representing a tissue with high CD44 expression at RNA as well as protein level. Beyond CD44s some of the samples showed 1 or more CD44 splice variants at relatively low expression level. All samples gave an equally strong signal with glyceraldehyde-3-phosphate dehydrogenase primers (data not shown). Using the same antibodies as applied to patient materials, expression of CD44 in the experimental tissues at the protein (immuno-histochemical) level correlated with the patterns of expression at the RNA level.

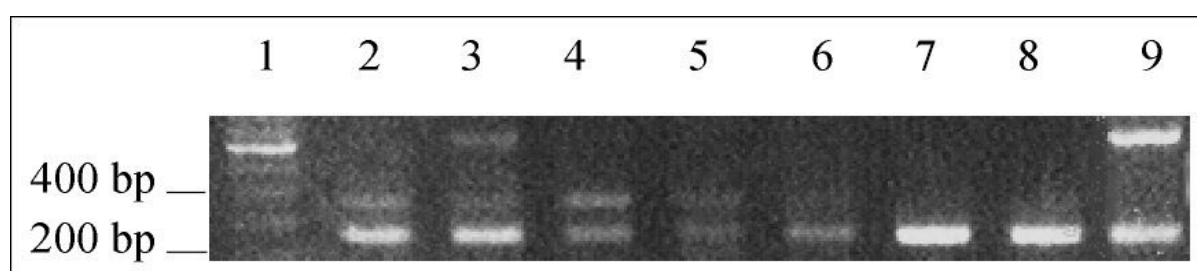


Fig. 3. CD44mRNA expression of CD44 transcripts in 7 human Wilms tumor xenografts (lanes 2-7) and 1 xenograft-derived cell culture (lane 8) was analysed by RT-PCR. RNA of human prostatic carcinoma xenograft tissue (PC-82) served as positive control (lane 9). Ladder markers are in lane 1. The 200 bp band represents CD44s and all other bands are splice variants.

Table 2 Relationship between pT stage and epithelial expression			
Stage	No. CD44s(%)	No. CD44v5(%)	No. CD44v10(%)
T1	19 (31)	16 (26)	11 (18)
T2	16 (26)	12 (20)	7 (12)
T3	12 (20)	9 (15)	7 (12)
Total	47 (77)	37 (61)	25 (41)
p Value	>0.05	>0.05	>0.05

Analysis of prognostic factors.

Because CD44 expression has been shown to be associated with the metastatic process, the predictive value of CD44 was also evaluated for the risk of clinical progression. Kaplan-Meier curves were constructed to investigate the possible correlation between CD44 expression in blastemal and epithelial component, and the probability of progression and tumor specific survival. Univariate analysis using the log-rank test showed a correlation between blastemal CD44v5 expression and clinical progression as well as tumor specific survival (table 3). Of 34 patients with a CD44v5 positive Wilms tumor 19 had clinical progression and died of tumor with a CD44v5 negative Wilms tumor whereas 3 of 27 had progression and/or died. In contrast to the CD44v5 variant, blastemal and epithelial

expression of CD44s and CD44v10 did not show prognostic value (table 3). The relationship between blastemal expression of CD44s, CD44v5 and CD44v10 and the 2 outcome parameters (clinical progression and tumor specific survival) are shown in the Kaplan-Meier curves (figs. 4 to 6, respectively). No correlation was found between epithelial expression of the several CD44 isoforms and the outcome parameters.

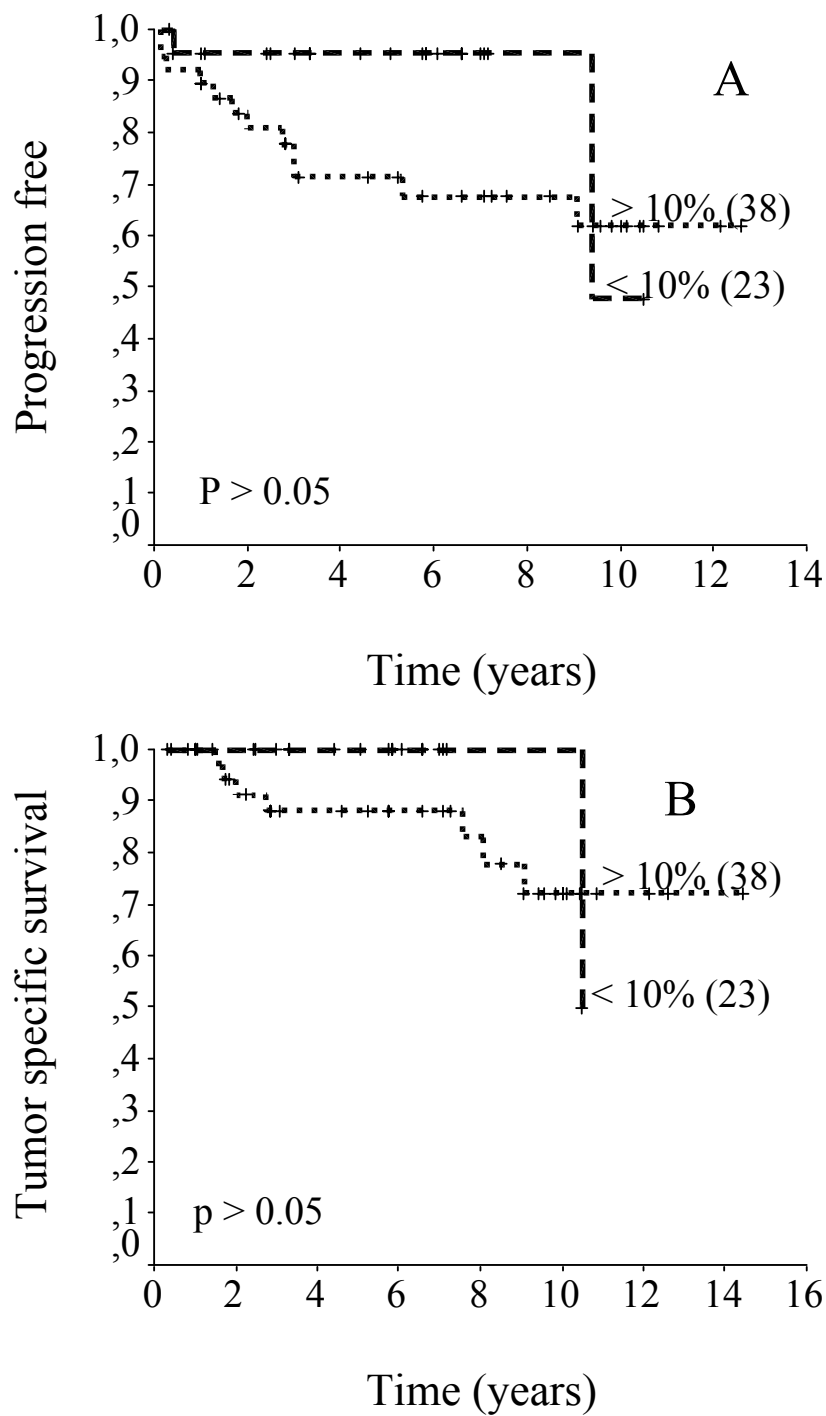
To test whether CD44 expression has any prognostic impact, a multivariate Cox's regression analysis was done including the parameters pT stage, and CD44 expression. pT-stage was divided as: pT1-2 versus pT3. Blastemal expression of CD44v5 was demonstrated as being an independent predictor of clinical progression (table 4). There was no independent marker predicting statistically significant tumor specific survival.

Table 3
Univariate analysis of prognostic markers

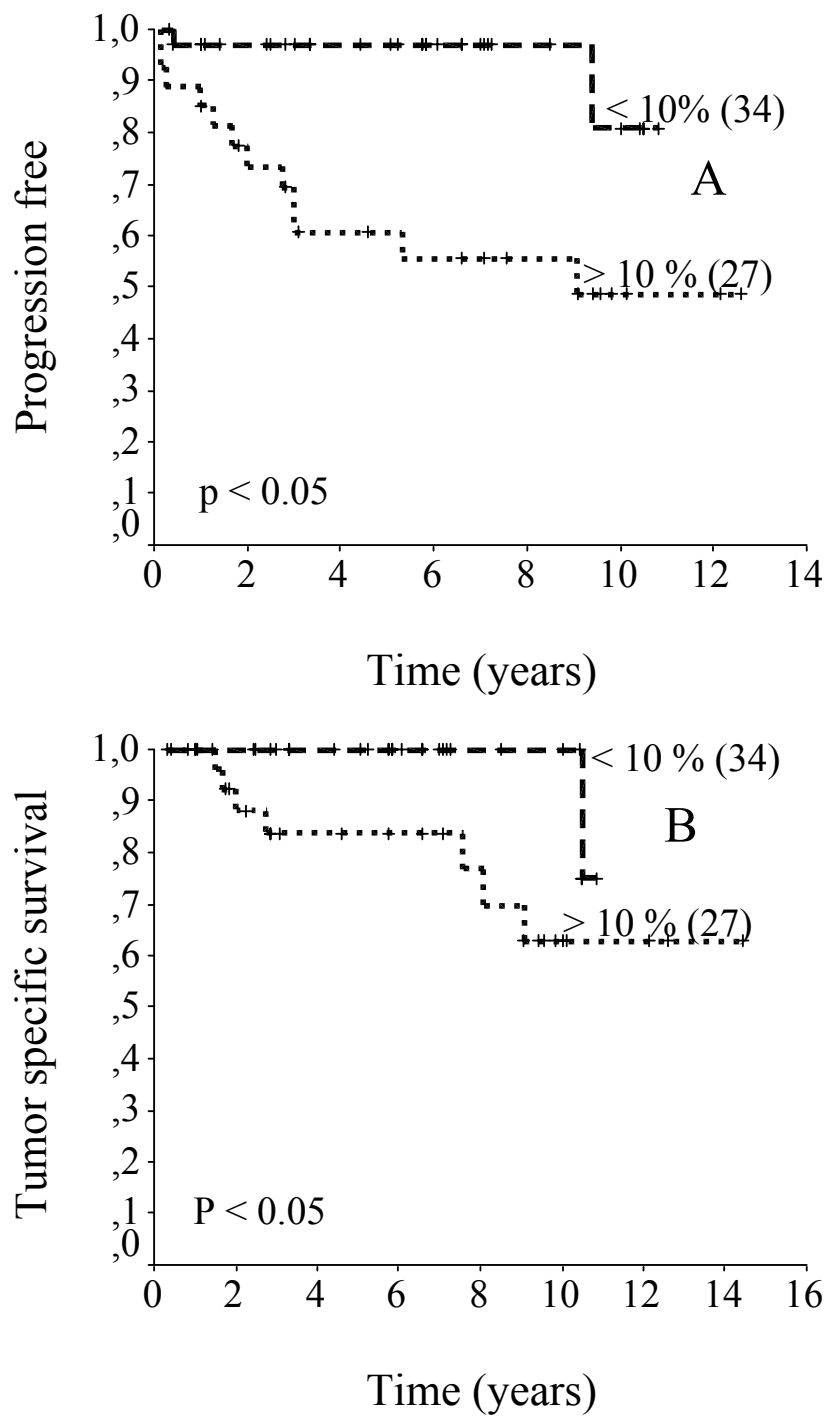
Variable		Outcome parameter			
		Clinical progression		Tumor specific survival	
		Chi-Square	p Value (Log rank)	Chi-Square	p Value (Log rank)
Blastema	CD44s	2.68	0.12	0.76	0.38
	CD44v5	10.08	0.001	4.15	0.04
Epithelial	CD44s	1.7	0.19	2.15	0.14
	CD44v5	0.39	0.53	0.13	0.72
	CD44v10	1.04	0.31	0.05	0.83

Table 4
Results of Cox's multi-regression analysis of CD44v5 (blastema)

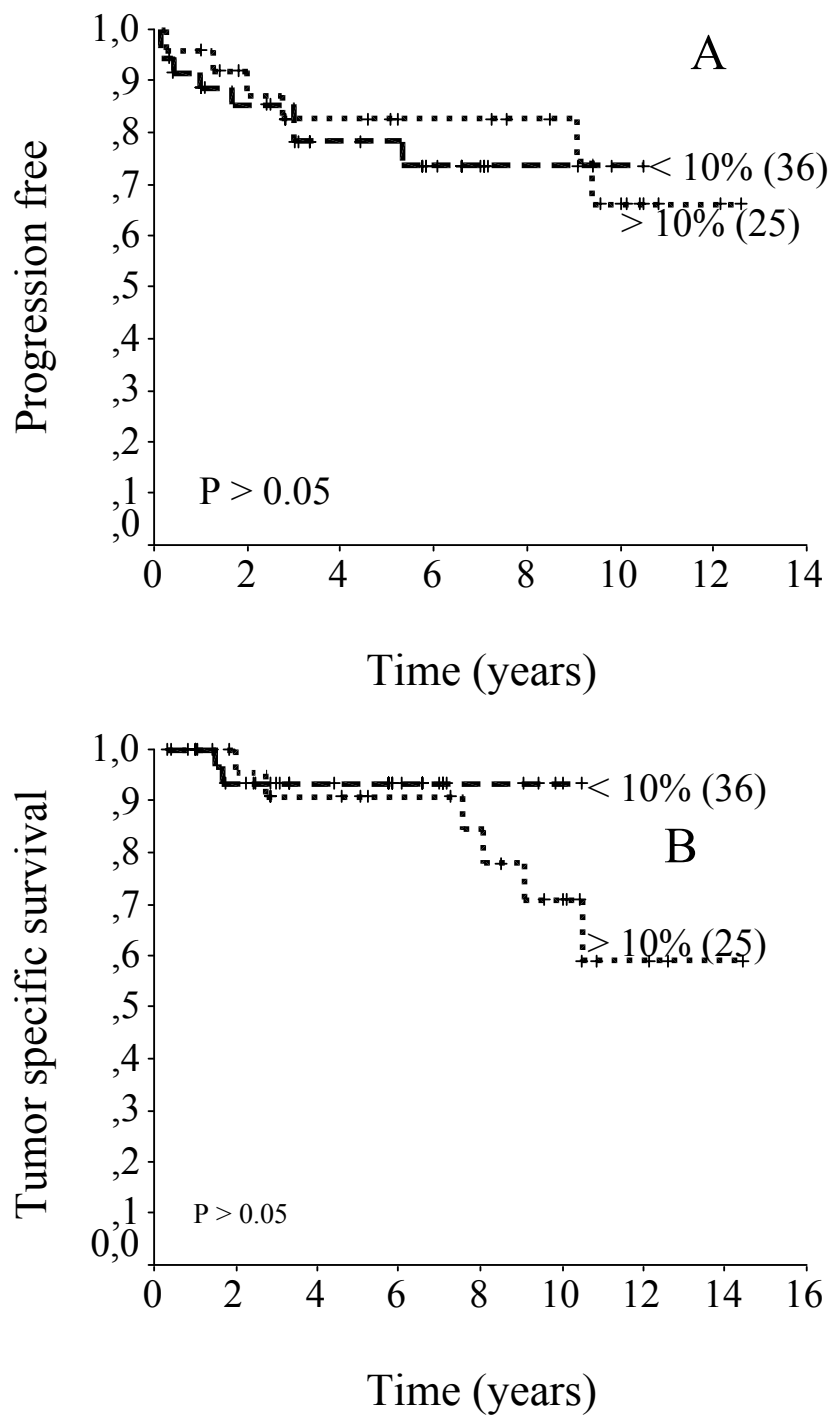
Clinical progression		
	Hazards ratio	7.8
	95% CI	1.7-43.7
	p Value	0.007
Tumor specific survival		
	Hazards ratio	6.8
	95% CI	1.1-55.1
	p-Value	0.08

**Fig.4**

Kaplan-Meier curves show a non-significant relationship among clinical progression, tumor specific survival and blastemal expression of CD44s, respectively. Censored patients are indicated by a tic marks. Number of patients per group is shown between parentheses.

**Fig.5**

Kaplan-Meier curves show a significant relationship among clinical progression, tumor specific survival and blastemal expression of CD44v5, respectively. Censored patients are indicated by tic marks. Number of patient per group is shown between parentheses.

**Fig.6**

Kaplan-Meier curves show a non-significant relationship between clinical progression, tumor specific survival and blastemal expression of CD44v10, respectively. Censored patients are indicated by tic marks. Number of patient per group is shown between parentheses.

DISCUSSION

A number of functions attributed to CD44 may have a role in tumor biology (Tarin, 1993) and, therefore studying the prognostic value of CD44 molecules in malignant processes is warranted. Our study was performed to evaluate whether the expression of CD44 isoforms is a prognostic factor in nephroblastoma evaluate in a substantial number of patients with long-term followup. All patients received chemotherapy before nephrectomy. CD44 isoforms were detected in many normal tissues at mRNA and protein levels (Fox, 1993). Immunohistochemical staining of CD44s, CD44v5 and CD44v10 in normal renal tissues showed membranous staining of almost all tubular structures, mainly at the basolateral side of the renal tubular epithelium facing the interstitium (fig. 1). This staining suggests that CD44 may have a role in cell-cell or cell-extracellular matrix interactions in the normal renal tubular compartment, possibly by retention of the polysaccharide hyaluronan and/or other extracellular matrix molecules (Terpe, 1996a). Staining of normal kidney tissues was used as internal control for the immunohistochemical procedure. CD44v6 was not detectable in the normal kidney tissues studied. Using immunohistochemical and RT-PCR analyses of fetal and adult kidney, others have demonstrated complete absence of exons 6v (Herrlich, 1993; Terpe, 1996). In our study we initially detected CD44s expression at the RNA level in a series of human Wilms tumor xenograft tissues (fig. 3), demonstrating that these tumors do in fact produce these molecules. Our results could be confirmed at the protein level by immunohistochemistry, making use of the same variant specific antibodies applied to patient materials studied.

The 3 components of Wilms tumor (blastema, epithelial and stromal) expressed CD44s, CD44v5 and CD44v10 at the protein level. Most tumors studied expressed multiple CD44 isoforms (fig. 2), and the predominant isoform on tumor cells was CD44s (table 1, 2). Various associations of CD44 isoforms expression with clinico-pathological features have been reported. Studies on CD44 expression in clinical tumors have demonstrated a positive correlation with adverse prognosis in nonHodgkin lymphoma, cervical cancer, colorectal cancer and gastric adenocarcinoma (Stauder, 1995; Speiser, 1997; Mulder, 1994; Terpe, 1994b). In other studies such correlations were not found in ovarian cancer and transitional cell bladder carcinoma, (Cannistra, 1995; Hong, 1995) or CD44 expression correlated with favorable prognosis in neuroblastoma and squamocellular tumors (Favort, 1993; Salmi, 1993). With respect to CD44 expression in breast cancer prognosis, various studies have shown contradictory results (Kaufmann, 1995; Friedrichs, 1995). Therefore, it appears that the biological role of CD44 molecules is not identical for all organs and various tumors. This finding also applies to the prognostic value of CD44 in renal cell cancer (Kan, 1995; Paradis, 1999). Univariate and multivariate analyses revealed that blastemal CD44v5 protein expression in pretreated patients was associated with poor prognosis. Thus, CD44v5 should be considered a marker for poor prognosis, independent of stage. This marker may indicate that the blastemal component of Wilms' tumor is associated with increased aggressiveness of the tumor with a higher tendency for clinical progression, that is metastasis (Bruce-Beckwith, 1996).

All patients received chemotherapy preoperatively and the majority had a good response. This treatment regimen may have affected the cellular components of Wilms tumor tissue, blastema in particular, and may also have influenced the CD44 immunophenotype of the remaining tumor. However, in a pilot study of tumor tissues derived from patients receiving no chemotherapy preoperatively similar CD44 staining results were obtained, suggesting that chemotherapy did not significantly affect the expression of CD44 (variants)

and, thus, the predictive value of the CD44 markers we studied.

Several studies have indicated that increased expression of the CD44 gene is associated with metastatic disease. Moreover, transfection of CD44v6 in a non-metastatic CD44 negative cell line led to metastatic behavior (Gunthert, 1991). Consequently, it has been stated that CD44v6 is causally related to the metastatic process. However, in our study Wilms tumor did not express CD44v6. The correlation of CD44v5 expression with Wilms tumor prognosis may suggest that variant CD44 molecules containing exon v5 may have a role in Wilms' tumor progression.

CONCLUSIONS

Our study may provide evidence for the role of CD44 splice variants in nephroblastoma tumor biology and for their use as prognostic markers. Immunohistochemical assessment of CD44v5, which can be performed reliably on paraffin embedded Wilms tumor, yields a possible prognosticator that might be used to identify patients with a high propensity for disease progression. If confirmed in other, larger series, these patients might benefit from adjuvant chemotherapy and/ or radiotherapy.

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CHAPTER 6

Expression and Prognostic value of Epidermal Growth Factor Receptor, Transforming Growth Factor- α and c-*erb* B-2 in Nephroblastoma

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ABSTRACT

Background: Wilms tumor is one of the most common solid tumors in children. A transforming growth factor- α (TGF- α)/ epidermal growth factor receptor (EGF-R) autocrine loop plays an important role in tumor growth. Abnormal expression of TGF- α , EGF-R and *c-erb* B-2 has been demonstrated in several human malignancies.

Methods: The immunohistochemical expression of TGF- α , EGF-R and *c-erb* B-2 was studied in paraffin material of 62 clinical Wilms tumors. Patients had a mean follow-up of 5.7 years.

Results: Generally, TGF- α , EGF-R and *c-erb* B-2 were expressed in tissue of the normal kidney and at variable levels in the three cell types of Wilms tumor, i.e., blastemal, epithelial, and stromal cells. Immunoreactive blastema cells were found in 48%, 44% and 34% of tumors for TGF- α , EGF-R, and *c-erb* B-2, respectively. It was found that TGF- α , EGF-R, and *c-erb* B-2 blastemal and epithelial expression gradually increased from T1 to T3. The blastemal expression of TGF- α was statistically significantly correlated with clinico-pathological stages. Both univariate and multivariate analysis showed that blastemal TGF- α expression was indicative for clinical progression, but neither blastemal TGF- α , nor EGF-R and *c-erb* B-2 expression correlated with patients survival. Epithelial staining was of no prognostic value. The simultaneous expression of TGF- α /EGF-R was indicative for clinical progression at univariate level.

Conclusions: Increased expression of TGF- α in the blastemal part of Wilms tumor correlated with tumor classification and clinical progression. These findings suggest that significant expression of TGF- α and EGF-R may play a role in promoting transformation and/or proliferation of Wilms tumor, perhaps by an autocrine mechanism. Therefore, their expression may be of value in identifying patients at high risk of tumor recurrence.

INTRODUCTION

Wilms tumor is a pediatric malignancy of the kidney and one of the most common solid tumors in children (Beckwith, 1994). Despite the remarkable response to chemotherapy, 5%-10% of tumors are fatal because of occurrence of metastases and drug resistance (Groot-Loonen, 1990). Predicting the prognostic significance of Wilms tumor may be difficult, which may be because of either the efficacy of salvage chemotherapy or the limited number of the patients studied. One of the reasons for the extremely malignant potential of nephroblastoma is its different oncogenetic background in comparison with other cancers. Experimental evidence has been obtained that a variety of growth factors influence the biologic character of various neoplasms especially the malignant potential. It is well documented that Wilms tumor tissue expresses high levels of insulin-like growth-factor II (IGF-II). Previous studies on the role of IGF and its receptors in nephroblastoma did not lead to conclusive results about their prognostic value, however (Little, 1987; Reeve, 1985). Accordingly, the effects of other growth factors on human Wilms tumor should be investigated to better understand its biologic characteristics as well as to possibly improve treatment results.

Among various growth factors, the family of epidermal growth factor (EGF) polypeptides comprises together with their cell membrane receptor [e.g., EGF receptor (EGF-R)], one of the biologically most pluripotent system of autocrine/paracrine acting growth factor. These factors are non tissue-specific growth factors produced and acting locally in various tissues (Mroczkowski, 1989). The EGF receptor mediates the biological signal not only of EGF, but also of other related peptide growth factors, such as transforming growth factor- α (TGF- α) (Derynck, 1988). Binding of the ligand to the EGF receptor triggers a cascade of events, which ultimately leads to growth regulation of a variety of epithelial and mesenchymal cell types. This suggests that TGF- α and EGF-R may play an important role in the proliferation of tumors (Carpenter, 1990). Accordingly, the expression of EGF-R may be indicative for the malignant potential of tumors. Recent studies have indicated the significant role of TGF- α and EGF-R in the progression of various malignancies, such as bladder, breast, lung and gastrointestinal cancer (Neal, 1985; Klijn, 1994; Tateishi, 1990; Yasui, 1988). Research on the autocrine or paracrine system of various growth factors has further supported the implications of the involvement of TGF- α and EGF-R in carcinogenesis (Sporn, 1985).

The *c-erb B-2* gene (HER-2/neu) is a receptor, located on chromosome 17q21, encoding a 185- kilodalton transmembrane glycoprotein with tyrosine kinase activity. This protein shows extensive structural similarity to, but is distinct from, the EGF-R. However, it does not need EGF to be activated (Semba, 1985). In vitro cell transformation studies demonstrated a contribution to the promotion of the malignant phenotype (Hudziac, 1987). *c-erb B-2* overexpression also appears to be associated with poor prognosis in ovarian and lung carcinomas (Slamon, 1989; Kern, 1990).

In the current study, the expression pattern of TGF- α , EGF-R, and *c-erb B-2* was studied immunohistochemically in Wilms tumor tissues and the prognostic value was determined in patients treated preoperatively by chemotherapy and radical nephrectomy.

MATERIALS AND METHODS

Patients

During the period 1987-2000, 62 patients with nephroblastoma were treated by neo-adjuvant chemotherapy and subsequent 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 due to direct effect of metastases.

Sample selection

All nephrectomy specimens were fixed in 10% buffered formalin and embedded in paraffin. The hematoxylin and eosin stained slides were reviewed by an experienced pediatric pathologist (JCDH). The tumor classification was determined according to the International Society of Paediatric Oncology (SIOP) in Stockholm pTNM classification (Delemarre, 1996). Among the tissue blocks available from 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.

Antibodies

The following primary antibodies were used: mouse monoclonal antibodies TGF- α (Clone 213-4.4; Oncogene Research Products, Darmstadt, Germany), *c-erb* B-2 (clone 3B5, Dr. M. van de Vijver, Dutch Cancer Institute, Amsterdam, The Netherlands), and rabbit polyclonal antibody against EGF-R (1005; Santa Cruz Biotechnology, Santa Cruz, CA).

Immunohistochemistry

An immunohistochemical approach was used, based on the peroxidase-anti-peroxidase (PAP) technique. Serial sections (5 μ m) from all samples were mounted on 3-aminopropyl-trietoxysilane (Sigma Chemical Co., St. Louis, MO) coated glass slides, which were incubated overnight, in a 60°C incubator. After dewaxing in fresh xylene for 10 minutes and rehydration in 100% methanol for 10 minutes, the sections were rinsed in methanol containing 3% hydrogen peroxide for 20 minutes to block endogenous peroxidase activity. The slides were rinsed with distilled water. To enhance antigen exposure, We microwaved the slides at 700 W in 0.1 M citrate buffer at pH 6.0 for 15 minutes. After cooling and rinsing with phosphate buffered saline (PBS), the slides were placed in a Sequenza immunostaining system (Shandon, Unicorn, UK). Sections were incubated with 10% normal rabbit serum for use of monoclonal antibody or 10% normal goat serum for application of polyclonal antibody (DAKO, A/S, Glostrup, Denmark). Incubation was conducted in PBS/5% bovine serum albumin (BSA) for 15 minutes and subsequently overnight with the primary antibody at 4°C. The antibodies were diluted in PBS/5%BSA at 1:50 for TGF- α and 1:100 for EGF-R. Subsequently, the slides were rinsed with PBS/Tween (0.1%) and incubated for 30 min with rabbit-anti-mouse antibody for monoclonal antibody or goat-anti-rabbit for polyclonal antibody (DAKO), followed by rinsing with PBS/Tween (0.1%). The PAP complex (DAKO) was diluted in PBS/5%BSA at 1:300 and incubated for 30 minutes followed by rinsing with PBS. The antigen-antibody binding was visualized with diaminobenzidine tetrahydrochloride dihydrate (DAB, Fluka, Neu-Ulm, Germany) as chromogen. The sections were lightly counterstained with hematoxylin. For *c-erb* B-2, incubation with primary antibody was done without antigen retrieval at room temperature at dilution 1: 10 PBS/5%BSA for 1 hour. 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. In

addition, adult human skin, human salivary gland, and breast adenocarcinoma served as positive control for TGF- α , EGF-R and *c-erb B-2*, respectively.

Immunostaining analysis (quantification)

Immunostained slides were examined at x25 magnification without knowledge of the clinical outcome of the patients. We scored TGF- α staining semiquantitatively based on intensity and distribution of cytoplasmic staining as follows: absent, weak (10% of cells) or present (> 10% of cells). The expression of EGF-R was evaluated as positive, when staining was observed in either the cell membrane or cytoplasm. The intensity of EGF-R staining was assessed as follows: weak or absent staining (< 10% of cells), intermediate (10- 25 % of cells), focally strong (25-50%), and strong (>50% of cells). Similarly, *c-erb B-2* staining scored based on the percentage of positive cells in a particular area. For the purpose of statistical analysis TGF- α , EGF-R and *c-erbB-2* staining specimens were regarded as positive when the percentage of positive cells was greater than 10%.

Statistical analysis

Statistical analysis was performed using the SPSS 9 software package. The association between TGF- α , EGF-R, and *c-erb B-2* expression and clinicopathological features was analyzed using the chi-square test. 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* values less than 0.05 considered statistically significant.

RESULTS

Clinico-pathological findings

The T classification was T1 in 22, T2 in 19 and T3 in 21 patients. The patient's distribution was 26 (42%) females and 36 (58%) males. The mean overall follow-up period was 5.7 years, and the mean age at operation was 4.7 years. 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.

TGF- α , EGF-R and *c-erb B-2* expression in normal renal tissues

We found that TGF- α showed focal immunoreactivity in a few distal tubules mainly the collecting ducts and glomeruli (Fig. 1A). Epidermal growth factor receptor immunohistochemistry of normal kidney showed a very intense staining of proximal and distal convoluted tubules and collecting ducts, as well as in the parietal layer of Bowman's capsule, whereas it was entirely absent in glomerular visceral epithelial cells (Fig. 1B). *c-erb B-2* immunohistochemistry of normal kidney showed an intense positivity along the cell membranes of the collecting ducts and weak staining of proximal and distal convoluted tubules, whereas *c-erb B-2* was entirely absent from glomeruli (Fig. 1C).

TGF- α , EGF-R and *c-erb B-2* Expression in Wilms tumor Tissues

Transforming growth factor- α immunostaining was cytoplasmic. As could be expected, EGF-R and *c-erb B-2* immunostaining was present mainly in the membranes of tumor cells and to a minor extent in the cytoplasm (Fig. 1, D-F). Immunostaining of TGF- α was mainly focal, whereas EGF-R and *c-erbB-2* showed diffuse staining. Epithelial differentiation in tumors was accompanied by diffuse expression. There was variability in the intensity of staining in the same tumor component and among the tumors of similar stage classification.

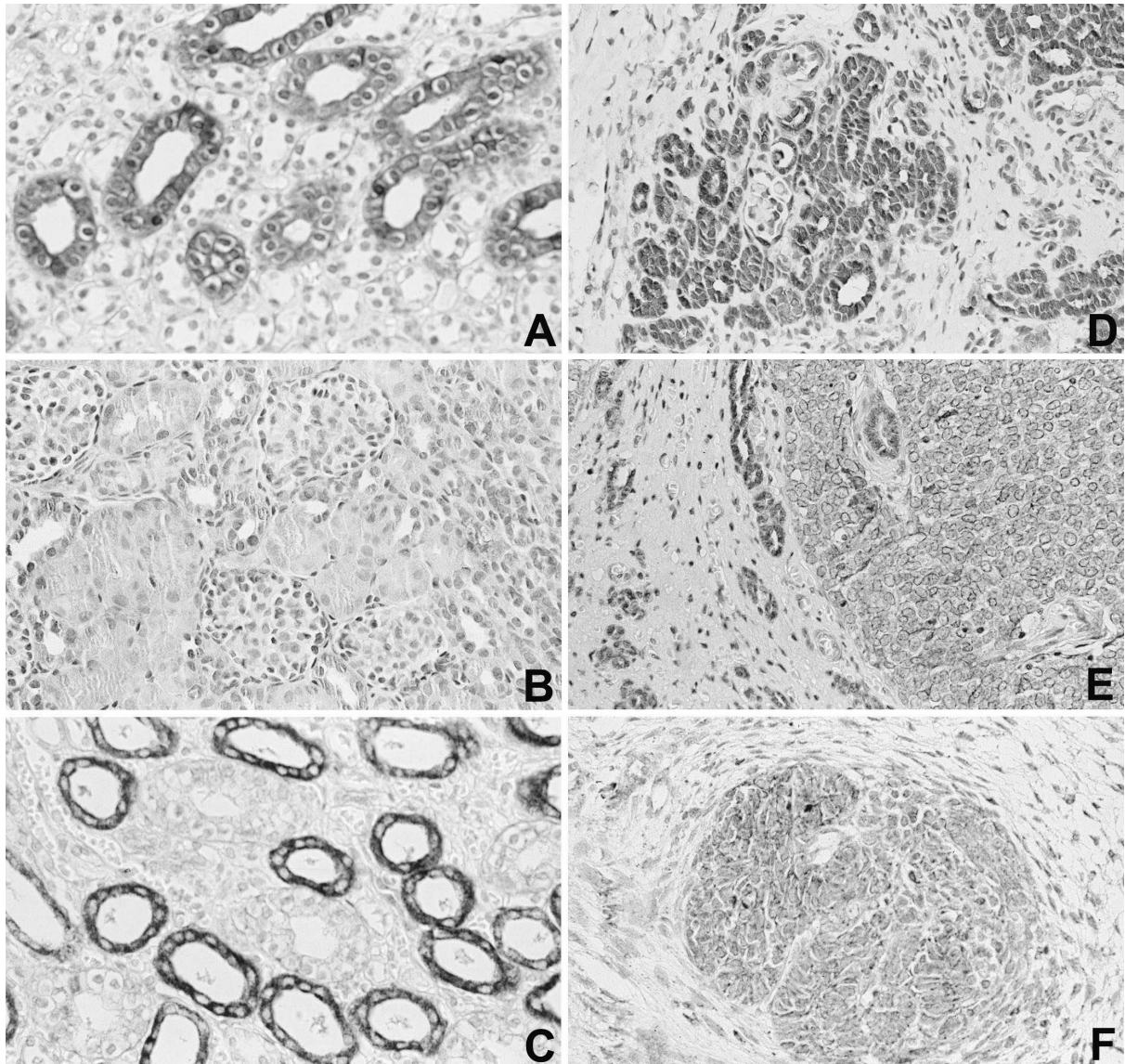


Fig.1
Immunohistochemistry of normal kidney (A-C) and nephroblastoma tissues (D-F) for transforming growth factor- α (A and D), epidermal growth factor receptor (B and E) and c-erb B-2 (C and F), respectively. Slides were counterstained with hematoxylin. Orig. magnification X400

Immunostaining was generally heterogeneous. Expression of TGF- α was also demonstrated in tumor blood vessels. Transforming growth factor- α , EGF-R, and *c-erb B-2* immunoreactive blastemal cells were found in 48%, 44 and 34% of tumors, respectively. Transforming growth factor- α , EGF-R and *c-erb B-2* immunoreactive epithelial cells were found in 50%, 60% and 53% of tumors, respectively. The blastemal expression of TGF- α correlated with clinicopathological stage, which was not the case for the epithelial expression (Table 1 and 2). Neither blastemal nor epithelial EGF-R or *c-erb B-2* protein expression correlated with clinicopathological stage (Table 1 and 2).

Table 1
Relationship between pT stage and blastemal expression

Stage	TGF- α ^a	EGF-R	c-erbB-2
T1	6 (10)	7 (11)	5 (8)
T2	9 (15)	7 (11)	7 (11)
T3	15 (24)	13 (21)	9 (15)
Total	30 (48)	27 (44)	21 (34)
p Value	<0.05	>0.05	>0.05

TGF- α : transforming growth factor- α ; EGF-R: epidermal growth factor receptor

^aChi-square test= 8.4; P= 0.015

Data are presented as numbers, percentages between brackets.

Table 2
Relationship between pT stage and epithelial expression

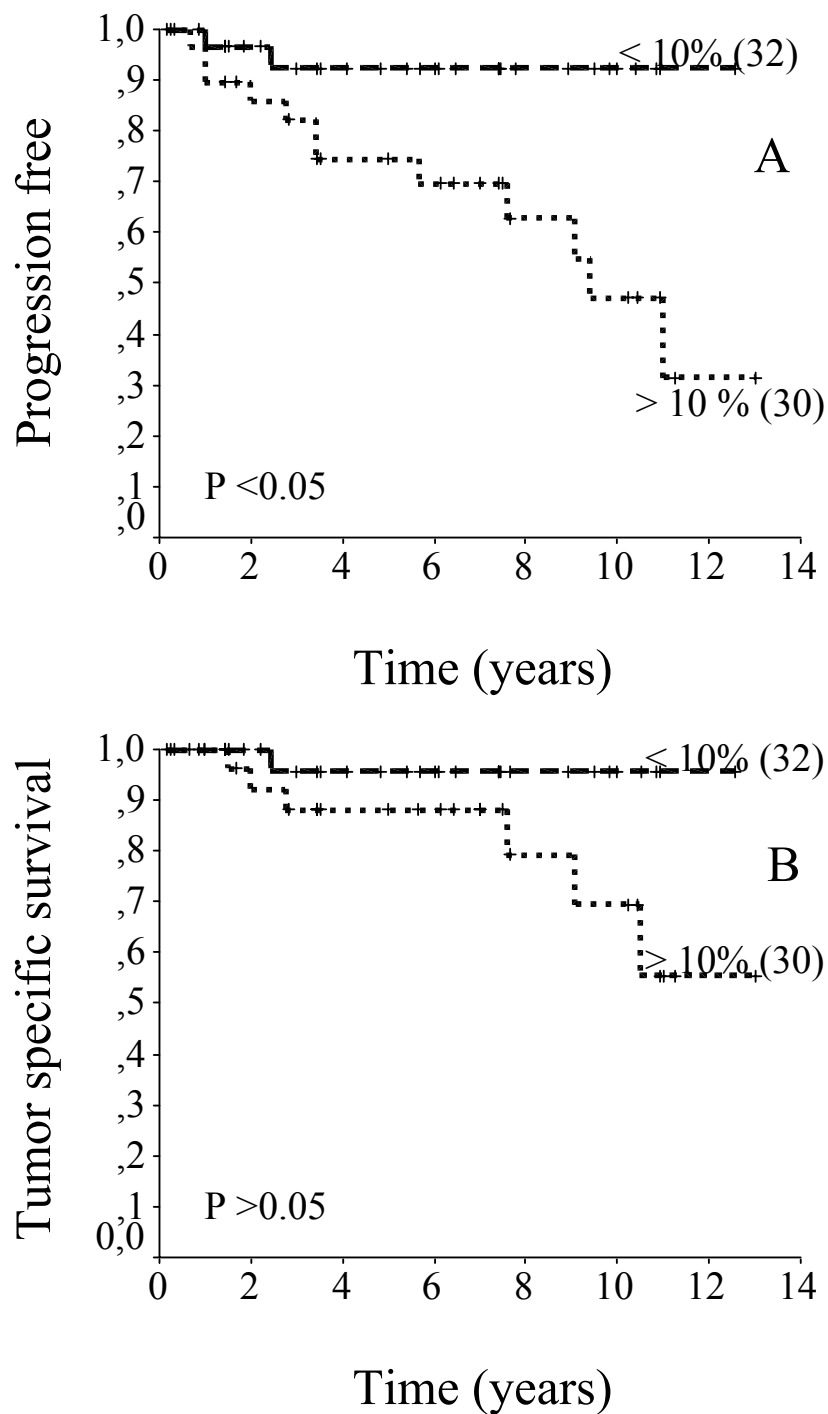
Stage	TGF- α	EGF-R	c-erbB2
T1	9 (15)	10 (16)	8 (13)
T2	9 (15)	13 (21)	11 (18)
T3	13 (21)	14 (23)	14 (23)
Total	31 (50)	37 (60)	33 (53)
p Value	>0.05	>0.05	>0.05

TGF- α : transforming growth factor- α ; EGF-R: epidermal growth factor receptor

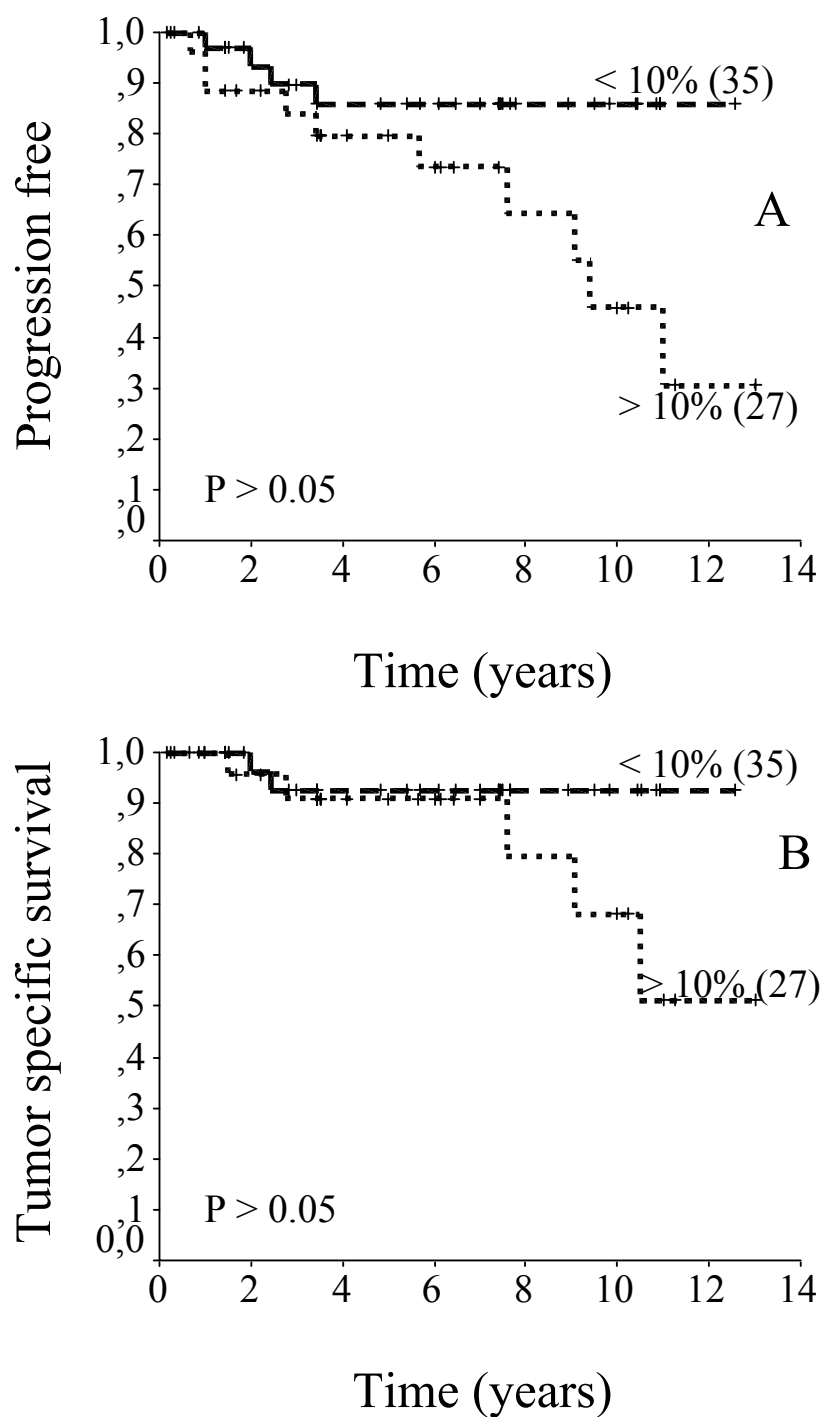
Data are presented as numbers, percentages between brackets.

Prognostic value of TGF- α , EGF-R and c-erbB2

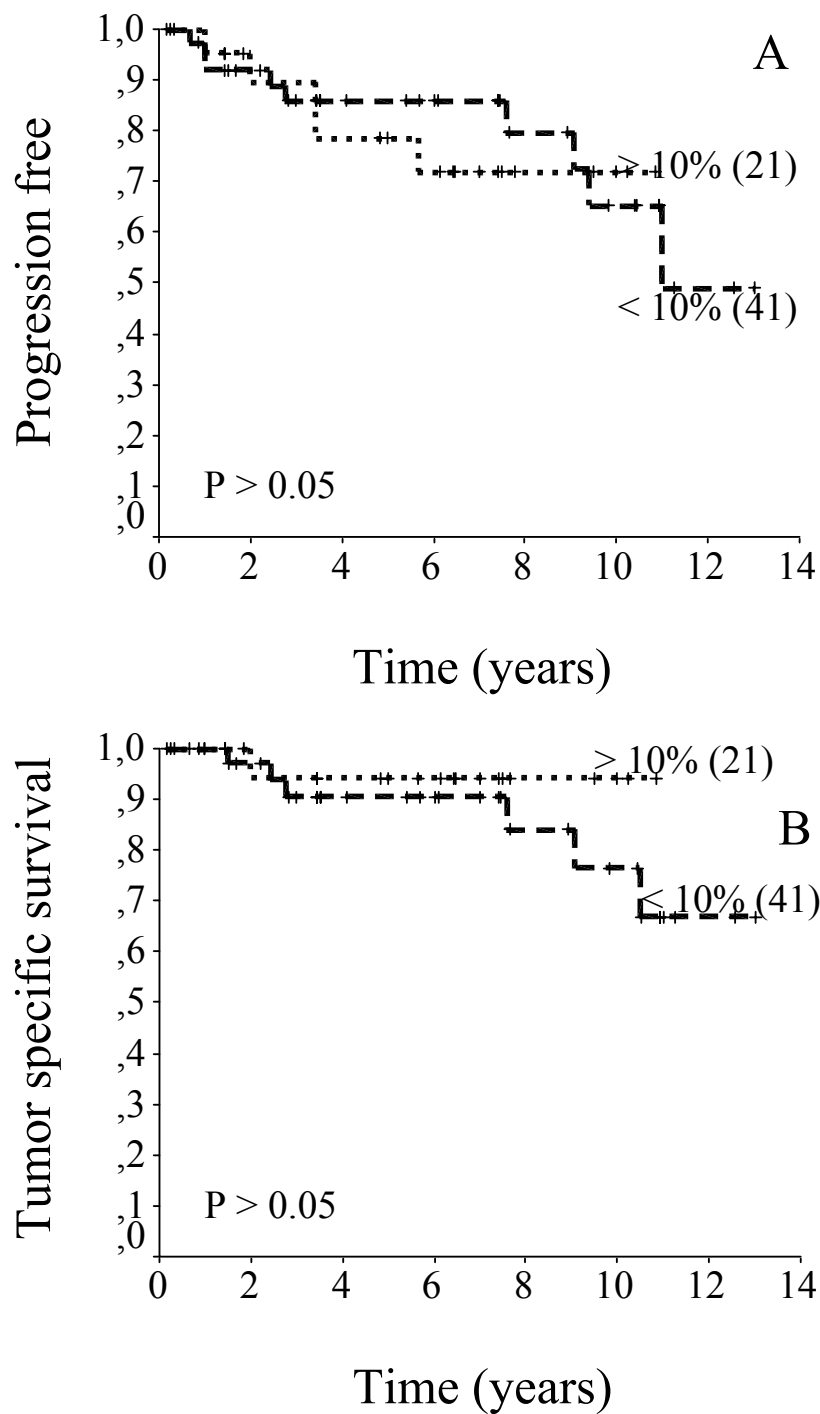
Univariate analysis, using the log-rank test, showed a prognostic value of blastemal TGF- α expression for clinical progression (Table 3; Fig. 2). Neither blastemal expression of both EGF-R and *c-erb B-2* (Figs. 3 and 4) nor epithelial expression of the three proteins studied showed any prognostic value (Table 3). To test whether TGF- α , EGF-R and *c-erb B-2* expression has any prognostic impact, we conducted a multivariate Cox's regression analysis including the parameters pT stage and TGF- α , EGF-R, and *c-erb B-2* expression. PT classification was divided as pT1-2 versus pT3. Blastemal expression of TGF- α has a hazard ratio of 5.6, indicating an independent marker for poor prognosis for clinical progression. Biologically the factors in the current study interact with each other, which prompted investigators to further investigate coexpression of these factors. By combining the dichotomizes TGF- α and EGF-R expression (TGF- α /EGF-R), four patients groups could be

**Fig.2**

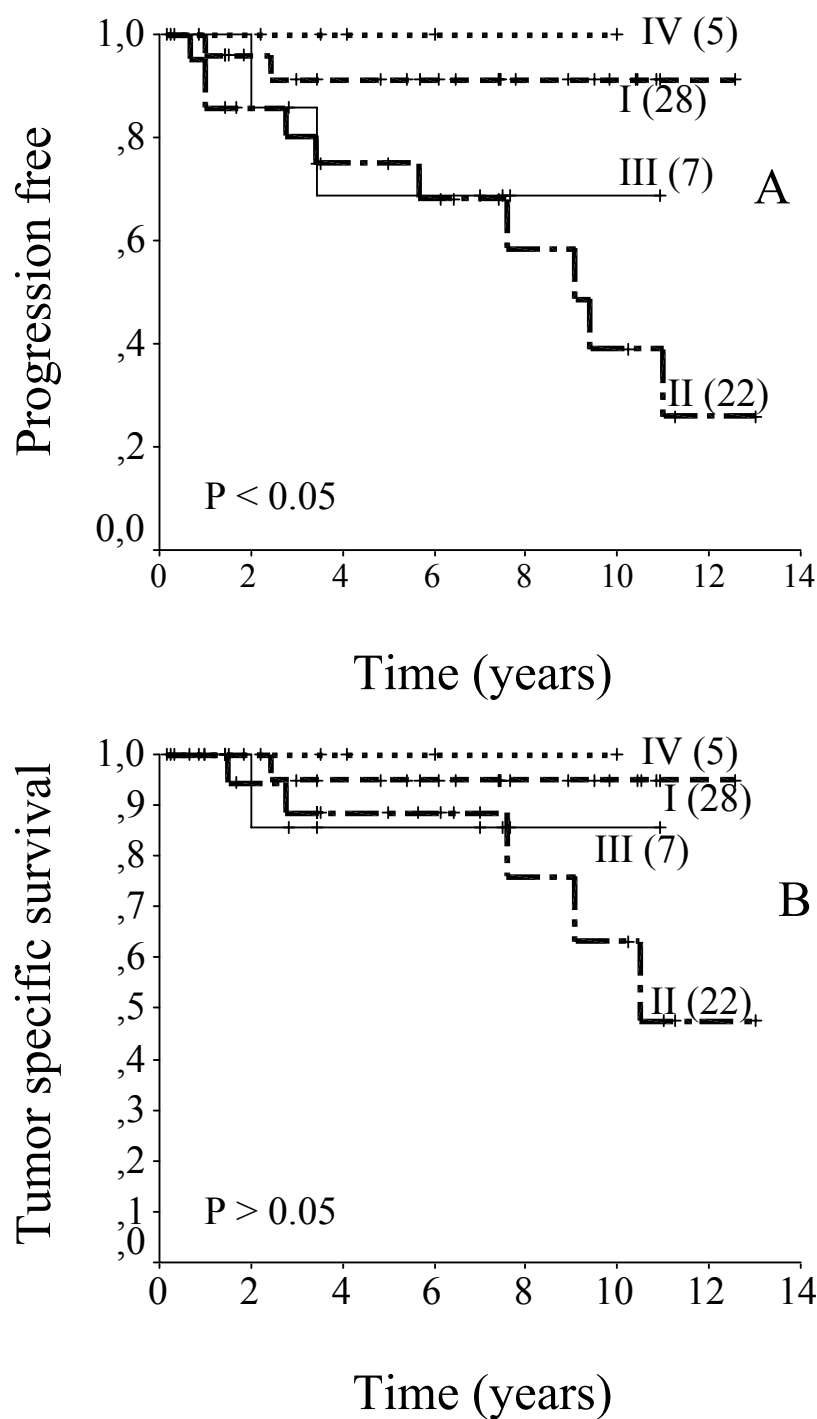
Kaplan-Meier curves showing a relation between blastemal transforming growth factor-Alpha and clinical progression (A) and survival (B), respectively. Censored patients are indicated with tic marks along their line. Number of patients per group is shown in parentheses.

**Fig.3**

Kaplan-Meier curves showing a relation between blastemal epidermal growth factor receptor expression and clinical progression (A) and survival (B), respectively. Censored patients are indicated by a tic marks along their line. Number of patients per group is shown in parentheses.

**Fig.4**

Kaplan-Meier curves showing a relation between blastemal *c-erb* B-2 expression and clinical progression (A) and survival, respectively. Censored patients are indicated by tic marks along their line. Number of patients per group is shown parentheses.

**Fig.5**

Kaplan-Meier curves showing the relation between blastemal combination of transforming growth factor-Alpha (TGF)/epidermal growth factor receptor(EGF-R) and clinical progression (A), tumor specific survival (B) respectively. Group I [TGF-alpha (-)/EGR-R (-)]; Group II [(TGF-alpha (+)/EGF-R (+)]; Group III [TGF-alpha (-)/EGF-R (+)]; Group IV [TGF-alpha (+)/EGF-R (-)].Censored patients are indicated by tic marks along their line. Number of patients per group is shown parentheses.

constructed. The Kaplan-Meier curves show the prognostic influence of TGF- α /EGF-R expression on the parameter time to progression. Clearly, two separate prognostic groups can be recognized: patients with no or low (<10%) expression of both antigens (TGF- α and EGF-R negative) or with high (>10%) expression (TGF- α and EGF-R positive) had a statistically significant prognosis compared with the other two groups (TGF- α positive-EGF-R negative or TGF- α negative-EGF-R positive; Fig. 5; Table 3). Transforming growth factor- α and EGF-R positive blastema and epithelium was found in 35% and 40% of patients tumors, respectively. The other combination did not show any significant improvement of predicting clinical outcome of patients.

Table 3
Univariate analysis of prognostic markers

Variable	Blastemal				Epithelial			
	Clinical progression		Tumor specific survival		Clinical progression		Tumor specific survival	
	X ²	P ^a	X ²	P ^a	X ²	P ^a	X ²	P ^a
TGF- α	6.54	0.01^b	3.23	0.07	0.28	0.59	1.38	0.24
EGF-R	3.76	0.05	2.11	0.14	2.05	0.15	2.92	0.09
c-erbB2	0.1	0.75	0.78	0.38	1.49	0.22	0.25	0.62
TGF- α / EGF-R	8.27	0.04^b	4.19	0.24	3.32	0.35	3.73	0.29
TGF- α / c-erbB2	6.68	0.08	4.13	0.25	1.94	0.58	1.32	0.72
EGF-R/ c-erbB2	5.31	0.15	6.67	0.08	3.15	0.37	3.42	0.33

TGF- α : transforming growth factor- α ; EGF-R: epidermal growth factor receptor

^alog-rank test; ^bStatistically significant

DISCUSSION

Wilms tumor is a malignant disease well known for its unpredictable course and tendency of tumor recurrence or metastasizing, sometimes years after primary treatment. The biological behavior of Wilms tumor is difficult to prognosticate on the basis of histopathological findings only. The current study was conducted to investigate whether the expression of TGF- α , EGF-R, and c-erb B-2 at the protein level has a prognostic value in specimens of clinical nephroblastoma, using paraffin-embedded tissue sections. All patients received chemotherapy before tumor nephrectomy. The clear expression of TGF- α , EGF-R, and c-erb B-2 in normal kidney, which was present in almost all stained tissue sections, could serve as an internal control for the immunostaining procedure.

Transforming growth factor- α is a single-chain polypeptide growth factor involved in the regulation of normal and malignant cell growth (Derynck, 1988). A TGF- α /EGF-R autocrine loop plays an important role in tumor growth. Therefore, overexpression of TGF- α is possibly an important factor in tumorigenesis (Di Marco, 1989). Some studies did report on a correlation between enhanced TGF- α levels in the primary tumor and decreased survival (Reinartz, 1994; Iihara, 1993; Yonemura, 1992; Yamanaka, 1993). However, lower TGF- α expression may predict a poor prognosis in renal cell carcinoma (RCC) (Hofmockel, 1997). In contrast, other investigators (Wen, 1996) failed to demonstrate a significant correlation between tumor levels of TGF- α and survival at all. In the current study of clinical nephroblastoma, expression of TGF- α in the blastemal tumor component predicts a poor

prognosis. Expression of TGF- α was also demonstrated in tumor blood vessels. TGF- α is well recognized as a potent angiogenic factor. It is able to induce neurovascularization in the hamster cheek pouch at low concentrations and induce vascular growth in a tracheal model (Schreiber, 1986; Schraufnagel, 1992). Expression of TGF- α in tumor vessels may therefore provide a possible mechanism of vascular invasion and subsequent dissemination of tumors.

Studies of various malignancies demonstrated an increased expression of EGF-R in breast carcinoma, gastric carcinoma, and RCC (Klijn, 1994; Sugiyama, 1989; Mydlo, 1989). Epidermal growth factor receptor expression has been reported to be of prognostic value for predicting the survival in endometrial carcinoma and bladder carcinoma (Khalifa, 1994; Lipponen, 1994). The expression of EGF-R protein in breast carcinoma has been correlated with both a poor prognosis and a lack of response to endocrine therapy. Moreover, it has been reported to be a factor associated with increased metastatic potential in breast carcinoma (Klijn, 1994; Fox, 1994). The value of EGF-R as an independent predictor of survival in RCC, prostatic carcinoma, non small cell lung carcinoma, cervical carcinoma, and head and neck carcinoma is still controversial (Lager, 1994; Prewett, 1996; Rusch, 1997; Kimmig, 1997; Rubin, 1998). In the current study of clinical nephroblastoma, a correlation between EGF-R expression and prognosis could not be demonstrated.

We demonstrated *c-erb B-2* protein expression in all renal tubular structure, which was in agreement with the outcome of earlier studies (Freeman, 1989; Rotter, 1992). These findings suggest that *c-erb B-2* protein is a normal membrane constituent of normal epithelial renal tissue. The inverse relationship between TGF- α and *c-erb B-2* receptor proteins has been reported in the female genital tract (Wang, 1992) and in adult human skin (Marguire, 1989). However, this relationship is not evident in normal renal tissues. *c-erb B-2* was first identified as an oncogene related to tumorigenesis in rats exposed to a teratogenic agent (Padhy, 1982). *c-erb B-2* oncogene activation is related to prognosis in tumors of hormone sensitive tissues, such as those of the breast and ovary (Slamon, 1989; Singleton, 1992). Its prognostic significance in cancer of urinary bladder carcinoma and prostate is still controversial (Sato, 1992; Lipponen, 1993; Veltri, 1994; Berner, 1993). In the current study, *c-erb B-2* has no prognostic impact on the clinical outcome of patients. Therefore, the expression of *c-erb B-2* may not play an important role in the aggressive behavior of Wilms tumor. As described above, the rates of TGF- α , EGF-R, and *c-erb B-2* expression was different among various cancers; generally, their expression rates seem to be higher in advanced stage cancer rather than in early stage disease.

Because specific binding of growth factors to their receptors is a prerequisite for their biological action, co-expression in the same tissue is essential for eliciting their specific actions. The interaction between ligands and EGF-R results in auto-tyrosine phosphorylation of EGF-R, leading to activation of the Janus Kinase (JAK2) and mitogen-activated protein (MAP) kinases. These, in turn regulate gene expression and promote cell growth. Transforming growth factor- α and EGF-R are expressed by proliferating and differentiating cells (Fukuyama, 1991). The autocrine loop formed by TGF- α and EGF-R has prognostic implication in the case of RCC (Prewett, 1996) and esophageal carcinoma (Iihara, 1993). In previous studies of TGF- α and EGF-R coexpression in patients with head and neck carcinoma, disparate results have been reported (Wen, 1996; Issing, 1996). In the current study, the combined expression patterns of TGF- α and EGF-R in Wilms tumor were indicative for poor prognosis. This finding suggests that interaction between TGF- α and EGF-R may play a role in promoting transformation and/or proliferation of Wilms tumor by an autocrine mechanism. Expression of TGF- α also may prevent drug-induced apoptosis by activating EGF-R and triggering anti-apoptotic pathways, leading to drug resistance.

Furthermore, co-expression of *c-erb* B-2 oncoprotein and EGF-R predicts risk for tumor spread in breast carcinoma (Osaki, 1992), a finding that was not reported for urinary bladder carcinoma (Lipponen, 1993). In the current study, no correlation was found between the expression of the *c-erb* B-2 and the EGF-R status. These results suggest that the expression of both gene products was independent and unrelated in Wilms tumor.

In an additional study of a group of patients receiving no chemotherapy before to surgery, similar results were obtained (Ghanem M, Van der Kwast TH, Sudaryo M, Mathoera R, Nijman J, Molenaar V, et al., unpublished results). This suggests that chemotherapeutic treatment did not significantly affect the predictive value of the markers studied. Moreover, more conclusive results may be obtained with increased numbers of patients included. This particularly applies to the TGF- α marker in relation to tumor specific survival and to the EGF-R marker in relation to both progression as well as tumor specific survival.

In conclusion, the members of the EGF-family of polypeptide growth factor and their receptors studied are expressed to a variable extent in all Wilms tumor investigated. The autocrine loop formed by TGF- α and EGF-R seems to play a possible role in the progression of Wilms tumors. Therefore, expression of both TGF- α and EGF-R may be of value in identifying patients at high risk of tumor recurrence and in guiding therapy. Such therapy may include fusion proteins or immunotoxins against TGF- α and EGF-R using toxins elaborated by *Pseudomonas* or diphtheria species (Philips, 1994) or monoclonal antibodies against EGF-R, such as those used in patients with breast carcinoma (Baselga, 1994).

CHAPTER 7

Expression and Prognostic Relevance of Vascular Endothelial Growth Factor (VEGF) and its receptor (Flt-1) in Nephroblastoma

Mazen A. Ghanem, Gert J. van Steenbrugge, Mondastri K. Sudaryo, Rejiv B. Mathoera, Rien J. M. Nijman, Theo H. van der Kwast

ABSTRACT

Aims: To investigate the prognostic relevance of vascular endothelial growth factor (VEGF) and its receptor Flt-1 in nephroblastoma and whether tumor microvessel density (MVD) immunoreactivity, determined by the CD31 antigen, is related to the expression of VEGF and Flt-1.

Methods: The expression of VEGF and Flt-1 and MVD were investigated by means of immunohistochemical analysis in 62 Wilms's tumours. Patients were treated preoperatively with chemotherapy and had a mean follow-up of 5.7 years.

Results: In general, VEGF and Flt-1 were expressed in normal kidney parenchyma and to a variable extent in the three main components of Wilms's tumour, namely: the blastemal, epithelial, and stromal cells. In tumor tissue, 52% and 47% of blastemal cells were positive for VEGF and Flt-1, respectively. A non-significant correlation was found between the expression of VEGF and Flt-1 in blastemal and epithelial cells and the clinicopathological stages. MVD was significantly higher in VEGF and Flt-1 positive tumours than in VEGF and Flt-1 negative tumours. Univariate analysis showed that the expression of VEGF, and Flt-1 in blastemal cells was indicative of clinical progression and tumour specific survival. In addition, MVD expression was indicative of clinical progression. Epithelial staining was of no prognostic value. In a multivariate analysis, VEGF protein expression by blastema cells was an independent prognostic marker for clinical progression.

Conclusions: These results indicate that VEGF and Flt-1 protein expression are closely related to MVD and seem to be an important predictor for poor prognosis in treated patients with Wilms's tumour. Therefore, the expression of these molecules in primary Wilms's tumour may be useful in identifying those patients at high risk of tumor recurrence and in guiding antiangiogenic therapy.

INTRODUCTION

Wilms's tumour is a paediatric malignancy of the kidney and one of the most common solid tumours in children (Beckwith, 1994). Despite the remarkable response to chemotherapy, 5%-10% of tumours are fatal because of the occurrence of metastases and drug resistance (Groot-Loonen, 1990). Therefore, it is important to find markers that can predict recurrence or the development of metastases so that we can screen for high risk, early stage patients, who may need preventive chemotherapy or other adjuvant treatment. In solid tumours, it has been suggested that angiogenesis plays an important role in tumor progression and the spreading of metastasis through the bloodstream (Senger, 1993). In fact, tumour growth beyond 1-2 mm is strictly dependent on angiogenesis (Folkman, 1990).

It has been reported that several growth factors with angiogenic activity are produced by solid tumours (Folkman, 1995). Vascular endothelial growth factor (VEGF) is an angiogenic factor that is highly specific for endothelium, and also functions as a vascular permeability factor (Senger, 1983). VEGF has been identified during normal growth and development in the kidney, and it has been found in genitourinary neoplasms (Breier, 1992; Sowter, 1997). The Fms-like tyrosine kinase (Flt) is a transmembrane receptor of the tyrosine kinase family, which has been identified as a receptor for VEGF (De Vries, 1992; Shibuya, 1990). Recently, the expression of VEGF receptors has been detected in several types of non-endothelial cells, such as melanoma cell lines, some leukaemic cell lines, and retinal progenitor cells, suggesting that VEGF receptors are not restricted to endothelial cells (Gitay-Goren, 1993; Shibuya, 1995; Yang, 1996). Previous studies indicated that VEGF and its receptor Flt-1 play an important role in tumour metastasis, and are associated with poor prognosis in clinical human tumours (Dvorak, 1995; Xie, 1999; Takanami, 1997). It has also been reported that tumour angiogenesis, as reflected by microvessels density (MVD), may provide prognostic information in carcinomas of the breast, the uterine cervix, and the ovary, in addition to a variety of other malignancies (Wiggins, 1995; Hollingsworth, 1995; Fontanini, 1995; Gasparini, 1993).

With regard to Wilms's tumour, one study using a murine model reported the overexpression of VEGF in a highly metastatic Wilms' tumor line (Kayton, 1999). However, the clinical relevance of this animal study has not yet been demonstrated. In our present study, the prognostic relevance of VEGF and its receptor Flt-1 and the association of their expression with MVD were determined in patients with Wilms's tumour who were treated by preoperative chemotherapy and radical nephrectomy.

MATERIALS AND METHODS

Patients:

During the period 1987-1999, 62 patients with nephroblastoma were treated by neoadjuvant chemotherapy and subsequent tumour 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 confirmed local recurrence or the appearance of distant metastases. Tumor death was defined as death resulting from the direct effect of metastases.

Sample selection:

All nephrectomy specimens were fixed in 10% buffered formalin and sections routinely obtained from the most vital appearing tumour areas were embedded in paraffin

wax. The haematoxylin and eosin stained slides were reviewed by an experienced paediatric pathologist. The tumor stage was assessed according to the SIOP trial protocol established in the SIOP meeting in Stockholm in 1994 (Boccon-Gibod, 1998). Among the tissue blocks available from individual patients, tumour samples containing the three different cell types of Wilms's tumour were selected. In addition, adjacent normal kidney tissue was taken from each patient.

Antibodies:

The following primary antibodies were used: rabbit antihuman VEGF and Flt-1 polyclonal antibodies (A-20 and C-17, respectively) from Santa Cruz, Santa Cruz, California, USA and mouse monoclonal antibody against CD31, (clone JC/70A; Dako A/S, Glostrup, Denmark). The specificity and characteristic of these antibodies have been published elsewhere (Senger, 1993; Gitay-Goren, 1993; Shibuya, 1995).

Immunohistochemical staining procedures:

Rabbit polyclonal antibodies against VEGF and Flt-1. The PAP (peroxidase-anti-peroxidase) immunohistochemistry technique was used and applied to serial sections (5 µm) from all samples, which were mounted on 3-aminopropyl-trietoxysilane (Sigma CO, St. Louis, Missouri, USA) coated glass slides, and which were subsequently incubated overnight at 60°C. For antigen retrieval, the slides were microwaved at 700W in 0.1M citrate buffer at pH 6.0 for 15 minutes. Sections were incubated with 10% normal goat serum (Dako A/S) in phosphate buffered saline (PBS)/5% bovine serum albumin (BSA) for 15 min. Subsequently, the sections were incubated with the primary antibody for one hour at 37°C in a humid chamber. The antibody was diluted in PBS/5%BSA at a 1:75 dilution for VEGF. After being incubated with goat antirabbit antiserum, the PAP complex (Dako) was diluted in PBS/5%BSA and incubated for 30 minutes, after which antigen antibody binding was visualised with diaminobenzidine tetrahydrochloride dihydrate (DAB; Fluka, Neu-Ulm, Germany). Negative controls were included by replacing the primary antibody with IgG fraction of normal rabbit serum. Normal kidney tissue, which was present in most tissue specimens, served as positive control.

Immunohistochemical staining with rabbit polyclonal antibody to Flt-1 was performed in the same way as that for VEGF, except that microwave treatment was omitted (Xie, 1999). The dilution of antibody for Flt-1 was 1/30.

Monoclonal antibody to CD31. Serial sections of the same specimens immuno-stained for VEGF and Flt-1 were used. The immunostaining procedure was similar to that of VEGF, with the exception of microwave treatment, which was performed by digestion with 0.1% trypsin for 10 min at 37°C. After rinsing with cooled PBS, slides were incubated with 10% normal rabbit serum for 15 min (Dako A/S). Subsequently, slides were incubated with the primary antibody CD31 (Dako A/S) for one hour at 37°C in a humid chamber. The antibody was diluted 1:10 in PBS/5%BSA. After being incubated with rabbit antimouse immunoglobulin (Dako A/S), the PAP complex was diluted in PBS/5%BSA and incubated for 30 minutes, after which antigen antibody binding was visualised with DAB (Fluka).

Immunostaining analysis (quantification):

The slides were examined at x25 magnification without knowledge of the clinical outcome of the patients. The proportion of tumour staining for VEGF and Flt-1 staining was graded on an arbitrary scale of 0-4: 0, no positive tumor cells; 1+, < 10% positive; 2+, 10-25% positive; 3+, 25-50% positive; and 4+, > 50% positive. The specimens were regarded as positive when the percentage of positive cells was > 10%.

Microvessel staining and counting:

Immunohistochemical staining for the endothelial marker CD31 was carried out to evaluate the MVD in tumour tissues. The number of CD31-positive vessels was recorded by counting any positively stained endothelial cells or endothelial cell clusters as a single, countable microvessels in a x250 field (x25 objective and x10 ocular; equivalent to 0.64 mm^2 /x 250 field) in the five areas with the highest vascular density. The mean of five counts was used as the MVD for each case. MVD was performed by two investigators (GM and MRB), neither of whom had knowledge of the clinical outcomes or clinicopathological features or expressions of VEGF. MVD was not only analysed as a continuous variable, but also as a dichotomous variable, defining < 15 microvessels/field (0.64 mm^2) as less vascularised and >15 /field as highly vascularised. The results of the CD31 counts were expressed as a mean (SD).

Statistical analysis:

Statistical analysis was performed using the SPSS 9.0 software package. The association between VEGF and Flt-1 expression and clinicopathological features was analysed using Pearson's χ^2 test. VEGF and Flt-1 immunostaining and its association with CD31 counts were analysed by the paired sample *t*-test and the ANOVA test. For the analysis of survival data, Kaplan-Meier curves were constructed and the logrank test for trend was performed. Multivariate analysis was performed using Cox's proportional hazards model, with $p < 0.05$ being considered significant.

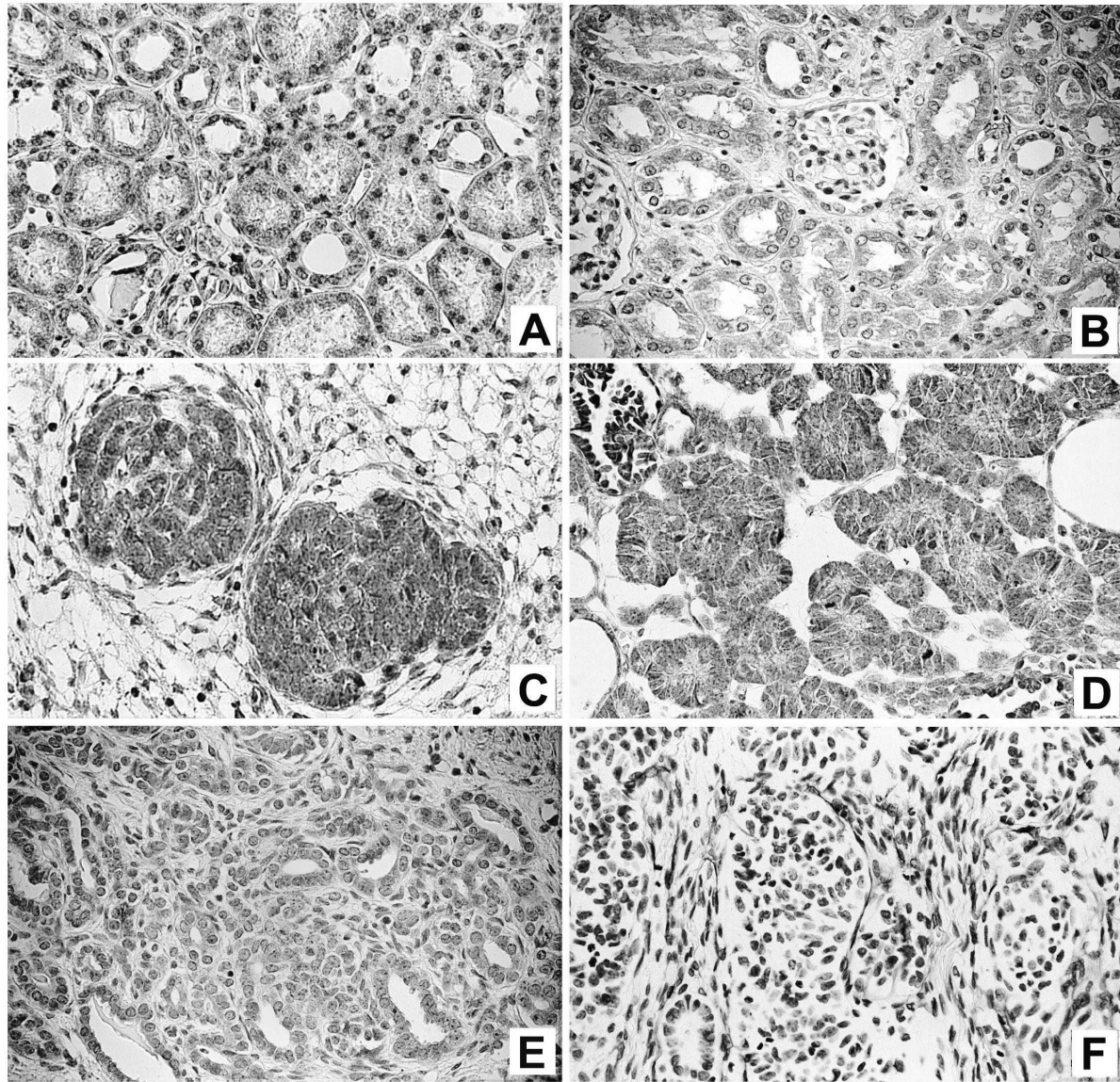
RESULTS

Clinicopathological findings

Twenty six of the patient were girls and 36 were boys. The stage distribution was pT1 in 22, pT2 in 19, and pT3 in 21 patients. All the tumour studied were of the classical triphasic type. Three of the patients had low risk tumours, whereas 59 had an intermediate risk tumours. The mean overall follow-up period was 5.7 years, and the mean age at surgery was 4.7 years. Clinical progression occurred in 14 patients and seven patients died from their tumor. At the end of the follow-up period, 55 patients were alive.

Expression of VEGF and its receptor Flt-1 in Wilms's tumour tissues

Immunoreactivity for VEGF and Flt-1 was found in most renal tubular structures, whereas staining was faint or absent in glomeruli (fig 1A, B). VEGF and Flt-1 immunoreactive blastemal cells were found in 32 and 29 of the patients with Wilms' tumour, respectively. VEGF and Flt-1 immunoreactive tumour epithelial cells were found in 38 and 35 of the patients, respectively. Immunostaining was mainly localised to the cytoplasm of both the blastemal and epithelial cells (fig 1C-E). Most sections stained more intensely for Flt-1 than for VEGF. In addition, VEGF and Flt-1 staining was seen in the stromal component of all the tumour studied. Tumour cells with relatively strong staining for Flt-1 were seen more often in the peripheral than in the central zone of the tumour (result not shown). Absent or faint VEGF staining was seen on some endothelial cells. Although increased expression of VEGF and Flt-1 was seen in both the blastemal and epithelial component of the tumours with increasing pathological stages (T1 to T3), this difference was not significant (table 1). Intertumoral variation was limited. Epithelial differentiation in tumours was accompanied by diffuse expression (fig 1D, E).

**Fig. 1**

Immunohistochemical staining for VEGF and Flt-1 in normal renal tissues (A,B) and in nephroblastoma tissues (C-E). VEGF (A) and Flt-1 (B) were detected in the tubular structures of normal renal tissue. VEGF and Flt-1 were mainly identified in the cytoplasm of the blastemal component (C and E respectively) and in the epithelium of nephroblastoma tissue (D and E respectively). Nephroblastoma tissue was stained with CD31 (F), detecting microvessels which are seen in the stromal tissue. (magnification X400).

Table 1

Relation between pT stage and blastemal and epithelial cell expression

Stage	VEGF		Flt-1	
	Blastemal	Epithelial	Blastemal	Epithelial
T1	9 (15)	10 (16)	8 (13)	8 (13)
T2	9 (15)	13 (21)	8 (13)	12 (19)
T3	14 (23)	15 (24)	13 (21)	15 (24)
Total	32 (52)	38 (61)	29 (47)	35 (57)
p Value	>0.05		>0.05	

Data are presented as numbers, with percentages in parenthesis
 VEGF, vascular endothelial growth factor

MVD and its correlation with VEGF and Flt-1 expression

Microvessels were defined by the presence of CD31 stained capillaries or small clusters of CD31 positive cells (fig 1F). The microvessel count ranged from 0 to 50 with a mean of 20.5 (SD, 14.8). The number of vessels counted in tumours with VEGF and Flt-1 blastemal and epithelial positive cells was significantly greater than that found in the negative tumour (table 2). The high vascularisation foci occurred most frequently within the tumour stroma. An increase in the microvessel count was seen in both the blastemal and epithelial component of tumour with increasing pathological stages (data not shown). However, some patients had a high MVD despite being VEGF and Flt-1 negative and some patients had a low MVD although they were VEGF and Flt-1 positive.

Table 2

Correlation between MVD and the expression of VEGF and Flt-1

	Cell		MVD	
	component	Expression	Mean (SD)	p value
VEGF	Blastema	Negative	11.5 ± 10.1	
		Positive	29.5 ± 12.9	< 0.0005*
	Epithelial	Negative	11.5 ± 12.9	
		Positive	26.7 ± 13.2	< 0.0005*
Flt-1	Blastema	Negative	13.8 ± 12.2	
		Positive	28.7 ± 13.3	< 0.0005*
	Epithelial	Negative	14.2 ± 12.4	
		Positive	25.9 ± 14.3	< 0.003*
VEGF and Flt-1	Blastema	Both negative	10.3 ± 9.6	
		Both positive	31.1 ± 12.6	< 0.0005°
		Either VEGF or Flt-1 positive	22.1 ± 12.6	
	Epithelial	Both negative	9.8 ± 9.5	
		Both positive	27.3 ± 13.4	< 0.0005°
		Either VEGF or Flt-1 positive	21.9 ± 15.2	

* Paired sample *t*-test; ° Anova test

Prognostic value of VEGF and Flt-1 molecules

Univariate analysis using the logrank test for trend showed a prognostic value of blastemal cell VEGF and Flt-1 expression for clinical progression and tumour related death (table 3; figs 2, 3). However, the expression of VEGF and Flt-1 by epithelial cells had no prognostic value (table 3), whereas MVD had prognostic value for clinical progression but not for tumour related death (fig 4). To test whether VEGF, Flt-1, and MVD had prognostic impact, a multivariate Cox' regression analysis was performed using the parameters pT stage, and VEGF, Flt-1, and MVD expression. The parameters that were not dichotomic were dichotomised as follows: pT1-2 versus pT3; immunoreactive score for VEGF and Flt-1 <10% versus >10%, and for MVD <15 microvessels/field (0.64mm²) versus >15/field. In that analysis, the expression of VEGF by blastemal cells had a hazard ratio of 7.1, indicating that it was a predictor of poor prognosis for clinical progression. There was no independent marker to predict tumor specific survival.

Table 3
Univariate analysis of prognostic markers

Variable	Blastemal cells				Epithelial cells			
	Clinical progression		Tumor specific survival		Clinical progression		Tumor specific survival	
	χ^2	P Value	χ^2	p Value	χ^2	p Value	χ^2	p Value
VEGF	12.8	0.0003	6.7	0.001	0.01	0.91	0.58	0.45
Flt-1	6.25	0.01	5.2	0.02	0.18	0.68	0.07	0.79

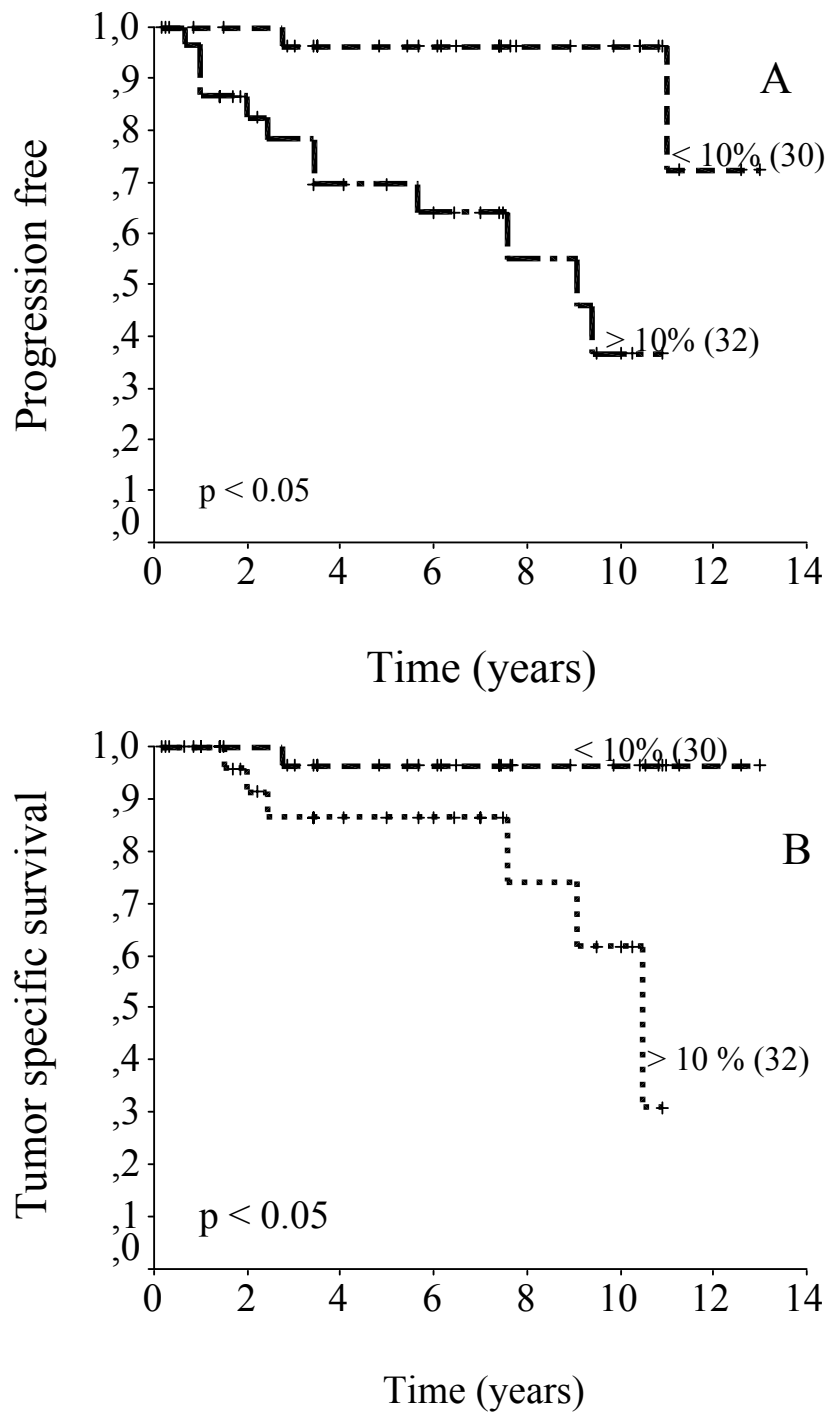
P-value were estimated using the logrank test; VEGF, vascular endothelial growth factor

DISCUSSION

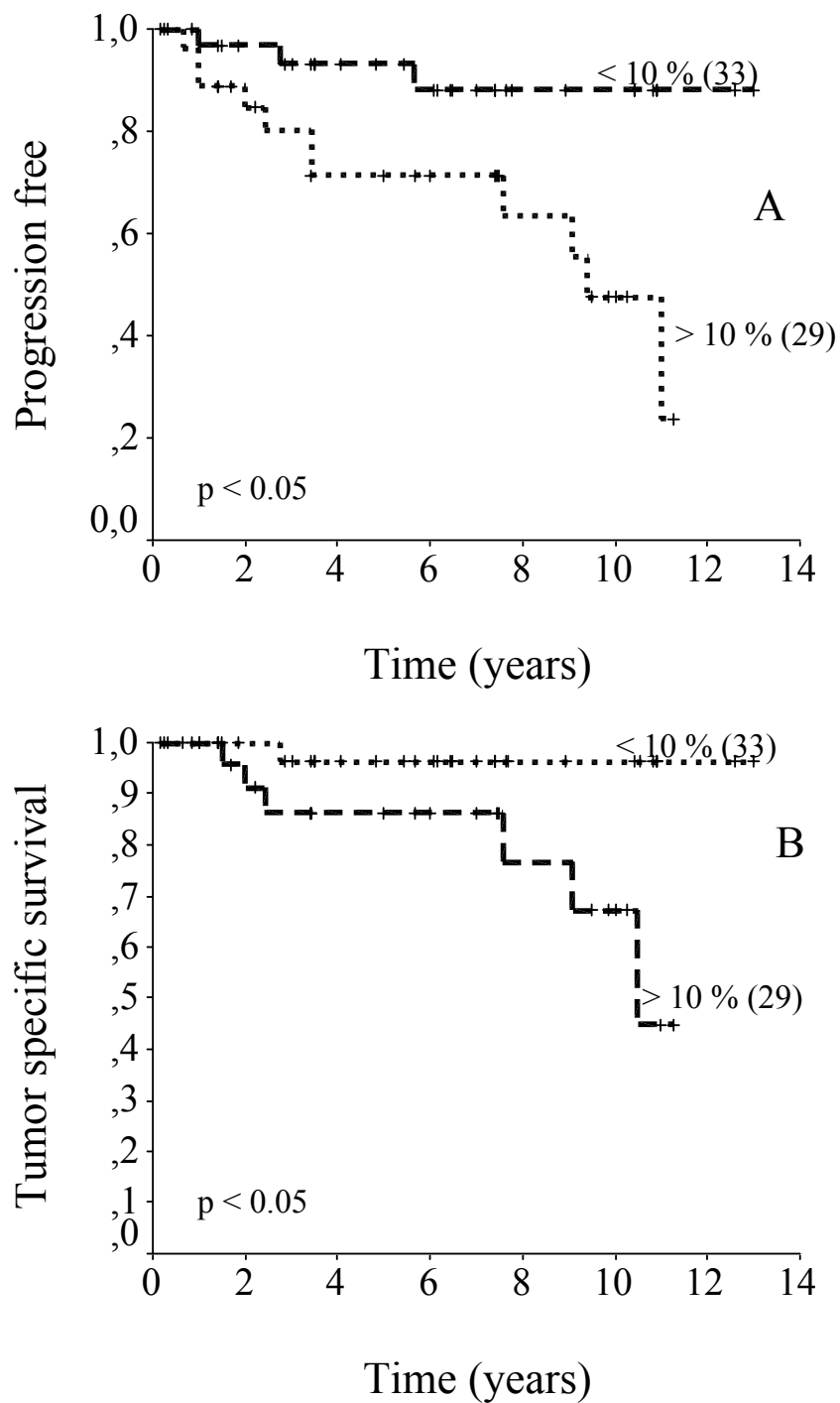
Angiogenesis is essential for tumour growth and metastatic activity and is regulated by a variety of angiogenic molecules. Recently, several angiogenic factors have been identified, and VEGF is thought to be one such factor (Breier, 1992). Our present study was carried out to investigate whether the expression of VEGF proteins and its receptor (Flt-1) has prognostic value in specimens of nephroblastoma, using paraffin wax embedded tissue sections. All the patients received chemotherapy before nephrectomy. The expression of VEGF and its receptor (Flt-1) in normal kidney, which was present in almost all stained tissue sections, served as an internal control for the immunostaining procedure.

VEGF is a multifunctional cytokine that acts in a highly specific way as a mitogen on endothelial cells (Senger, 1993;1983). VEGF is overexpressed in several malignant tumors (Dvorak, 1995). An association between VEGF expression in the primary tumour and poor prognosis has been found in colorectal carcinoma, node negative breast cancer, and early stage ovarian cancer. An increase in the serum concentration of VEGF was also shown to be a predictor of metastasis in hepatocellular carcinoma and gastrointestinal tumours (Ishigami, 1998; Gasparini, 1997; Paley, 1997; Jinno, 1998; Brown, 1993). In contrast, a recent study by Kawauchi et al. (1999) could not show a correlation between VEGF expression and either MVD or prognosis in synovial sarcoma. With respect to VEGF expression and gastric cancer prognosis, contradictory results are reported (Tanigawa, 1997; Maeda, 1996).

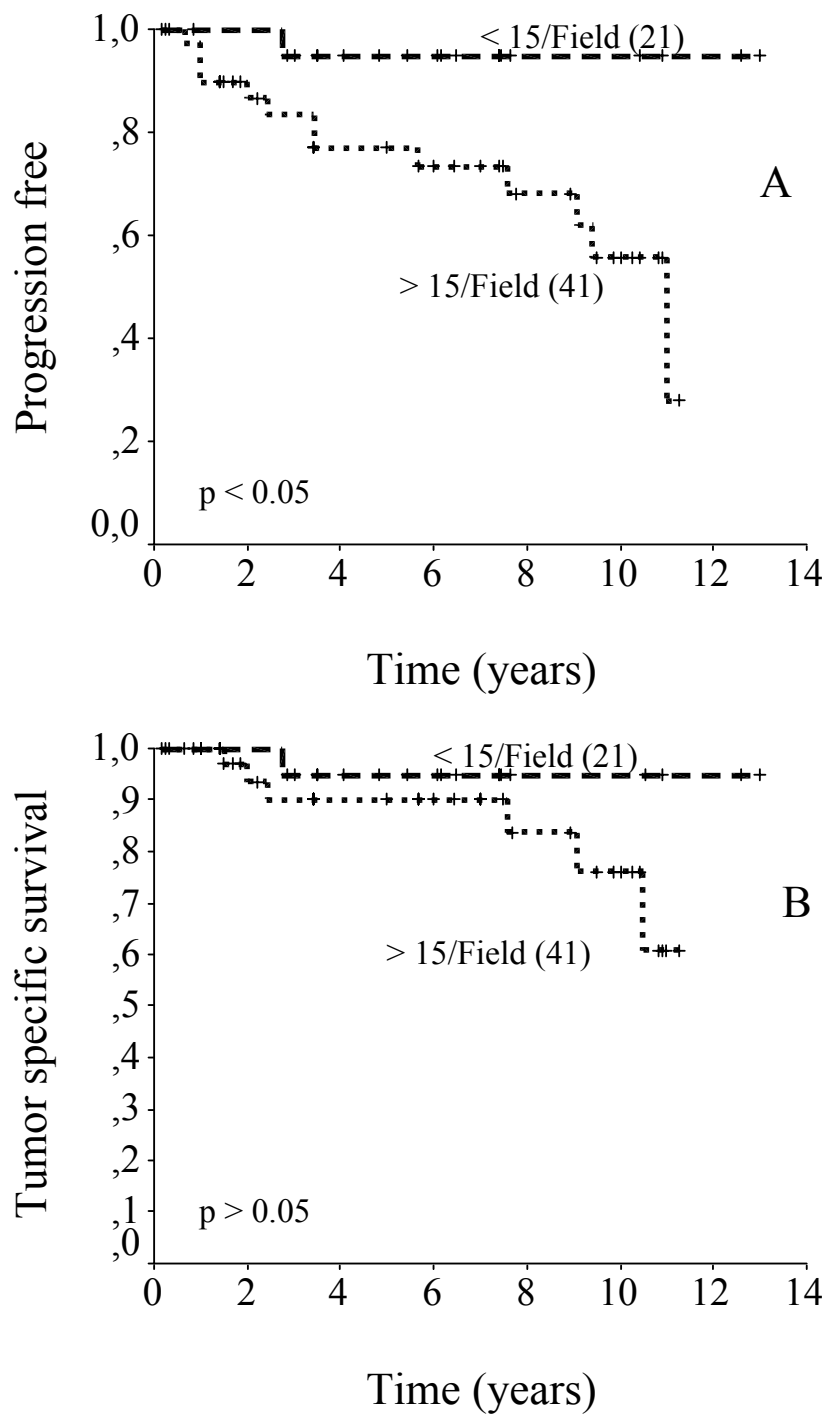
A few studies have reported an association between Flt-1 receptor and poor prognosis in human tumours (Xie, 1999; Takanami, 1997). In our present study, we have shown that the

**Fig.2**

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

**Fig.3**

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

**Fig.4**

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

expression of VEGF and Flt-1 by blastemal cells in primary Wilms's tumour correlates with poor prognosis for patients with nephroblastoma. This is in line with the view that the blastemal component of Wilms' tumour is associated with increased aggressiveness of the tumour, with a higher tendency of clinical progression; that is, metastatic ability (Boccon-Gibod, 1998). Chemotherapy is known to affect the cellular compartments of the Wilms's tumour-the blastemal component in particular. Having access to this type of material derived from a considerable number of patients with a good stage distribution, the aim of our study was to determine those factors that could predict the clinical outcome of patients after chemotherapy and surgery. We were able to perform a similar immunohistochemical study on material derived from a limited number of patients with Wilms's tumour who did not receive chemotherapy before surgery. Preliminary results of VEGF and Flt-1 staining in some of these tumours showed that overall scores of the blastemal and epithelial cells are higher, but that these patients have a similar clinical outcome to those in the pretreatment group described in here.

Our immunohistochemical study also revealed that high MVD correlated positively with the degree of VEGF expression and poor prognosis for patients with nephroblastoma. Recently, Skoldenberg et al (2001) confirmed the significant impact of high MVD in Wilms's tumour on survival and also found that the serum concentration of VEGF in these patients was three times higher than that seen in controls. The significant correlation between MVD and VEGF expression seen in our study suggests that VEGF is one of the major molecules responsible for neoangiogenesis in nephroblastoma, although the angiogenic potential is believed to be regulated by a balance between various types of angiogenic molecules and angiostatic substances. An association between VEGF expression and MVD has also been reported in carcinomas of the lung, stomach, breast, and vulva (Fontanini, 1995; Maeda, 1996; Toi, 1995; Obermair, 1996). A high MVD may promote metastatic disease by exposing tumours to a greater endothelial surface area, thus increasing the likelihood of haematogenous dissemination (Dvorak, 1995). In addition, the production of VEGF may enhance the metastatic capability of a tumour in several other ways (Pepper, 1991; Mandriota, 1995).

Although the reciprocal role of VEGF in tumour growth and chemotherapeutic efficacy remains to be elucidated, our data support the independent prognostic value of blastemal cell VEGF expression, which has also been documented for patients with oral squamous cell carcinoma treated with chemotherapy (Maeda, 1998). However, Albo et al (1994) have shown a possible correlation between tumour MVD and chemosensitivity- they found that tumours with a higher MVD are more sensitive to chemotherapy. Because the delivery of the chemotherapeutic reagents to the tumour is largely dependent on the blood supply, VEGF induced neovascularization may in turn favourably affect the response to systemic chemotherapy (Kaya, 2000).

The finding that an enhanced vascular supply stimulated by VEGF induces an increased risk of metastasis suggests that antiangiogenic treatments could be a potential adjuvant therapeutic strategy for the prevention of metastasis after the resection of the tumour. In experimental animal studies, anti-VEGF antibody (A4.6.1) successfully suppressed both primary tumour growth and metastasis in a murine model of anaplastic Wilms's tumour (Rowe, 2000). These results, together with the results of our clinical study, suggest that antiangiogenesis treatments, such as ligands that antagonise the interaction of VEGF with its receptors or antibodies directed against VEGF and its receptors, may prove useful in the management of Wilms's tumour (Mori, 1995; Morisita, 1995).

In conclusion, the results of our present study have shown that the expression of VEGF and its receptor (Flt-1) in primary Wilms' tumour correlates with an increase in MVD

in the tumour tissue and poor prognosis for patients with nephroblastoma. These findings suggest that VEGF secreted by nephroblastoma cells elicits neoangiogenesis. If these results are confirmed in additional studies, VEGF, Flt-1, and MVD could emerge as additional parameters for predicting the outcome of patients with nephroblastoma. VEGF and its receptor Flt-1 may also be considered as potential targets of an antiangiogenic therapeutic strategy.

CHAPTER 8

GENERAL DISCUSSION

General Discussion

Knowledge of prognostic factors is important for several reasons: (1) to assess the prognosis of the individual patient; (2) to optimize the intensity of treatment based on the prognosis; (3) to improve follow-up of patients during tumor progression; (4) to stratify patients for better planning of trials and (5) to better analyze the results of clinical trials (De Voogt, 1989).

During the last decade several studies have been carried out aiming at identifying clinically relevant prognostic factors for WT patients. Chapter I (general introduction) provides a literature review on most of the prognostic tissue markers, in addition to histopathology and tumor stage, that have been studied in WT. For almost all reviewed markers, except for the histopathology and tumor stage, conflicting results have been published. A large number of studies are impaired by methodological shortcomings like: small number of samples studied, heterogeneous patient population with regard to stage distribution or treatment, inappropriate statistics, such as absence of multivariate analysis.

Another well-accepted reason for these conflicting results is the histological nature of nephroblastoma, which consists of three different histological elements (blastemal, epithelial, and stromal). The classical (triphasic) nephroblastoma consists of all the three components, but biphasic and monophasic nephroblastomas are not rare at all. Therefore, the potential heterogeneous character of WT may lead to inconsistent results in the concerning studies. Another, more basic problem in the application of prognostic tumor markers is the use of different staging systems. Generally, when only few patients were included, stage compression was performed, i.e. a number of stages were combined and subsequently the accuracy may fall dramatically. The TNM system is the most suitable one for the present studies because this system did not undergo changes with time and has become time proven. A disadvantage of staging systems that have changed in time is that for study purposes generally a central review of clinical and pathological data is required.

In the papers presented in this thesis, we have searched for parameters of prognostic relevance for clinical WT. In general our survey encompasses the study of a broad panel of tumor markers linked with the biological behavior of WT. Some of these tumor markers e.g. EGR-1, CD44, *cerbB-2* and VEGF and its receptor have not been studied in WT before. As proliferation markers have been proven to be of prognostic significance in other tumors, we also intended to study the prognostic impact of the proliferation marker MIB-1 in WT. Similarly, factors of the EGF-family of growth factors have been shown to be important in other tumors. The expression of these genes was studied at the protein level in WT samples using an immuno-histochemical approach.

Initially, we have evaluated the prognostic significance of all tumor markers in WT patients treated preoperatively with chemotherapy, followed by the identification of these tumor markers in untreated WT patients. The latter was done to assess for each marker studied whether or not the treatment affected its expression and in addition to confirm or disprove whether these markers continued to be of prognostic significance even after patients received chemotherapy. Extended data concerning time relapse to progression and tumor related death were available. In the present study, the unequivocal expression of all these markers (except CD44v6) in normal kidney tissue, present in almost all sections, served as an internal control for the immunostaining procedure.

One of the functionally interesting markers, which was easily detectable in WT tissues, is the protein product of the tumor suppressor gene WT-1. Besides many studies of WT expression at the RNA and DNA level, this is, to our knowledge, the first study of WT

expression at protein level, making use of the commercially available polyclonal antibody 588. The protein product of WT-1 binds to the same DNA sequences as the protein product of the EGR-1 gene. A relationship between WT-1 and the transcription factor, EGR-1 expression was demonstrated in clinical nephroblastoma (Scharnhorst, 2000). Blastemal WT-1 and EGR-1 expression are related to prognosis (Chapter II).

The expression of the apoptosis inhibitor Bcl-2 was of unfavorable prognostic value in patients treated preoperatively with chemotherapeutics (Chapter III). Bcl-2 expression was elevated in advanced tumors, suggesting that Bcl-2 expression is related to tumor progression. Tumor remaining after effective chemotherapeutic intervention is thought to be chemoresistant, which may be associated with an increased expression Bcl-2. A combined Bcl-2/Bax expression was of prognostic value predicting clinical progression. Western blot analysis demonstrated that the anti-apoptotic Bcl-X_L protein was the dominant isoform of the Bcl-X_{S/L} protein in experimental human nephroblastoma. The reduced expression of the anti-apoptotic Bcl-X_L protein may contribute to enhanced sensitivity to radiotherapy and/or chemotherapy.

Cell kinetic aspects are often associated with the malignant potential of neoplasm. The availability of monoclonal antibody MIB-1 (Ki-67) has facilitated the detection and assessment of proliferative activity. The p27^{Kip1} protein is conspicuously present in cells undergoing terminal differentiation. The present study (Chapter IV) clearly demonstrates the prognostic value of both MIB-1 and p27^{Kip1} expression in the preoperatively treated WT patients.

The biological role of CD44 molecules is not identical for all organs and various tumors. However, enhanced expression of the cellular adhesion molecule CD44v5 in preoperatively treated WT patients was of independent prognostic value in the prediction clinical progression. Since it was the first published study on CD44 expression in clinical Wilms' tumor, comparable data are not available (Chapter V).

Chapter VI, VII shed light on the putative angiogenesis mechanism of WT and the potential metastatic activity of this tumor, which is regulated by a variety of angiogenic molecules. Blastemal VEGF, Flt-1, TGF- α and systematic MVD expressions are associated with prognosis, and a significant correlation was found between level of VEGF and Flt-1 and MVD expression. Our findings suggest that WT tissue secrete angiogenic factors that activate neovascularisation around the tumors. As a consequence, specific antiangiogenic therapies may be a potential adjuvant therapeutic strategy for suppression of tumor growth and prevention of metastasis after resection of tumor.

Univariate analysis of the pre-treated group of patients showed that blastemal WT-1, EGR-1, MIB-1, p27^{Kip1}, CD44v5, VEGF and Flt-1 were indicative for clinical progression as well survival, whereas blastemal Bcl-2 and TGF- α were correlated with clinical progression only (Table 1). It is, however, not practical to test all these markers on WT separately. Therefore in multivariate analysis we tried to determine which of the markers studied has the strongest prognostic value in addition to histopathological analysis and staging. In a multivariate analysis of the group of preoperatively treated patients, only p27^{Kip1} protein expression of the blastemal tissue component proved to be the best independent prognostic marker for clinical progression, beside stage.

Immuno-histochemical expression of the previously studied tumor markers in non-treated nephroblastoma

The SIOP (Société Internationale d'Oncologie Pédiatrique) approach is different from that of the NWTs (National Wilms' Tumor Study) in that it does not involve preoperative treatment. NWTs investigators believe that preoperative treatment may compromise histological evaluation of the tumor, as well as it may obscure tumor staging. Thereby, such approach may compromise the ability to optimize treatment regimens for the individual patient (D'Angio, 1983). Available information on the impact of chemotherapy on the expression pattern of tumor markers in clinical WT is limited. Therefore, we performed a preliminary study with untreated WT, in order to evaluate the expression of the previously studied tumor markers in treated clinical WT. Specimens of surgically removed untreated tumors were kindly provided by Prof. Dr. Molenaar V.M., Department of Pathology, University Hospital Groningen. Specimens were obtained from 43 untreated patients with a median follow-up period of 8 years. The stage distribution (NWTs protocol) in this group was stage 1 in 22, stage 2 in 5, stage 3 in 11, stage 4 in 4 and stage 5 in 1 patient. Clinical progression occurred in 7 patients and 2 patients died from their tumor. The small number of cases in each stage precluded the establishment of clear-cut relationship between tumor markers and clinical follow-up. The main goal of the evaluation of this group of patients was to investigate the expression patterns of the various markers in comparison to those studied in the chemotherapeutically pretreated group of patients, as reported in the chapters of this thesis.

In general, the data of the preoperatively untreated group demonstrate that the expression of most of the tumor markers has the same trend as in the treated group. The level of expression (intensity) or proportion of positive cells was higher for most of the markers in the untreated as compared to the treated WT. In the untreated series, the association between expression of prognostic markers and the surgical stage could not be shown. This is probably mainly due to the disproportionate stage distribution of the cases in this small group of patients, as 22 of the 43 cases (51%) were stage I. In this series, no significant correlation between any tumor marker and survival could be demonstrated, as only 2 (5%) patients died of disease. Because of the difference between SIOP and NWTs protocols, stage-for-stage outcome comparison between these two studies could not be performed (Bracken, 1982).

The expression of both Bcl-2 and Bax were similar to those reported for the preoperatively treated group. Remarkably, in the untreated series Bcl-X expression was found in 85% of the tumors. This percentage of Bcl-X was relatively high in comparison to the preoperatively treated group, in which blastemal Bcl-X immunoreactivity was found in only 38% of the tumors. It is tempting to speculate, however, that immuno-histochemical expression of the single marker, Bcl-X, might be a useful parameter to predict chemo- or radiosensitivity of tumors at the start of therapy.

The mean percentage of blastemal and epithelial components of WT was higher in the MIB-1 expression in untreated as compared to the treated group of patients. The contrary applies to p27^{Kip1} expression, i.e. its expression level was lower in untreated as compared to treated group. These observations confirmed previous studies correlating proliferating cell nuclear antigen (PCNA) labeling scores with the effect of therapy in WT (Hall, 1990). In this study it was shown that chemotherapeutic manipulation of tissues resulted in decreased PCNA staining in WT cell cultures exposed to dexamethasone and cyclosporin A (Kurki, 1986). Generally, chemotherapy of WT leads in general to a selection of tumor cells, which are not in cell cycle, because it knocks out the proliferative cohort of cells. On the other hand, chemotherapeutical treatment may also lead to an enhanced apoptotic response of tumor cells.

Such a response is determined by the expression of various apoptotic proteins which either promote (Bax, Bcl-X_S) or block (Bcl-2, Bcl-X_L) the potency of tumor cells to undergo apoptosis. Strong expression of markers that block the apoptotic process lead to drug resistance (Decaudin, 1997)

With regard to angiogenesis, TGF- α , VEGF and Flt-1 expression in tumor cells was significantly correlated with the MVD in untreated WT. In general, in these patients the overall scores of the expression levels of TGF- α , VEGF and Flt-1 in the blastemal and epithelial components were higher, but these patients had a similar clinical outcome compared to that in patients of the pretreatment group. These results indicate that in untreated WT counting of microvessels in the tumor shows correlation with other angiogenic factors.

We analyzed the prognostic value of the various markers studied, both in the treated and untreated group of patients. Univariate analysis of the untreated group showed that blastemal WT-1, MIB-1, p27^{Kip1}, TGF- α and VEGF were indicative for clinical progression (Table 1). In multivariate analysis of the patient data, the tumor suppressor gene product, WT-1, expressed by blastemal tissue, was the only independent prognostic marker for clinical progression. This is in contrast to the preoperatively treated group, in which blastemal p27^{Kip1} was the strongest independent prognostic marker for clinical progression. This difference may be attributed to the effect of chemotherapy, but the small number of untreated cases and their skewed distribution of T stage or over representation of stage I patients may also have influenced this outcome.

Table 1: Univariate analysis of all tumor markers studied in the blastemal component in both treated and untreated groups of WT patients

Outcome parameter	Clinical progression		Tumor related survival	
	Treated	Untreated	Treated	Untreated
	P*	P*	P*	P*
WT-1	+	ND	+	ND
EGR-1	+	-	+	-
Bcl-2	+	-	-	-
Bax	-	-	-	-
Bcl-X	-	-	-	-
Bcl-2/Bax	+	-	-	-
MIB-1	+	+	+	-
P27 ^{Kip1}	+	+	+	-
CD44s	-	-	-	-
CD44v5	+	+	+	-
CD44v10	-	+	-	-
TGF- α	+	+	-	-
EGF-R	-	-	-	-
c-erb B2	-	-	-	-
TGF- α / EGF-R	+	-	-	-
TGF- α /c-erb B2	-	-	-	-
EGF-R/c-erb B2	-	-	-	-
VEGF	+	+	+	-
Flt-1	+	-	+	-

P*: Log-rank test for trend

-: Not statically significant

+: Statistically significant

ND: Not determined

The here described prognostic impact of WT-1 expression at the protein level was recently confirmed by a study based on cDNA microarray analysis. A total of 15 favorable histology untreated WT was studied using microarrays containing 19,968 cDNAs. A total of 267 cDNAs were significantly overexpressed (at least 3-fold) in all of the tumors compared to non-cancerous kidney and among these were known WT-related genes such as IGF II and WT-1 (Takahashi, 2002)

In summary, our data suggest that the use of a simple immunostaining technique for the detection of one or more of the most relevant tumor marker proteins may add to the decision-taking of the clinician with regard to therapy. WT patients might benefit from the evaluation of these markers to more precisely estimate the need for adjuvant chemotherapy and/or radiotherapy. Most factors studied were expressed in the blastemal as well as in the epithelial compartment of WT. In contrast to the expression in the blastema, none of the epithelium-localized factors had any prognostic value, however. Apparently, blastemal-bound expression of particular markers has a much stronger prognostic impact than their expression in the epithelium. Interestingly enough, a recent study of platelet-derived growth factor (PDGF) and its receptor demonstrated that this growth factor and its receptor were exclusively expressed in the epithelial compartment (Ghanem et al, unpublished results). Univariate analysis showed that epithelial PDGF-AA and PDGF- α receptor expression were indicative for both clinical progression and tumor specific survival. Patients positive for PDGF-AA and PDGF- α receptor had a longer survival than those negative for both molecules. In multivariate analysis, epithelial PDGF- α protein expression was an independent prognostic marker for clinical progression as well as tumor specific survival.

Concluding remarks and future perspectives

The precise prognostic value of the here described tumor markers has to be further investigated in larger prospective multicenter studies with longer follow-up periods. The difference in chemotherapeutic protocols and their effects on the expression of tissue markers must be taken into consideration for future studies. Further detailed kinetic studies on chemotherapeutical sensitivity of WT could only be performed in well characterized animal models representing clinical WT. Examples of such models are the human xenografts developed in athymic nude mice at our institution (Wen, 1997). These model systems represent different stages of disease and have different origins (i.e. primary tumor or metastasis) and however, they allow chemotherapeutical manipulation. In addition, expression of potential prognostic factors, such as those presently studied in clinical WT, can be investigated under conditions of chemotherapeutical treatment.

It is of importance to determine which angiogenic factor predominantly mediates angiogenesis as this could offer novel opportunities for therapeutic intervention of the disease. Taking in to account the role of apoptosis- and proliferation-based markers, the factors responsible for induction of angiogenesis are worth to investigate in more detail. Our data seem to indicate that the vasculature of WT plays an important role in tumor progression. Therefore, it is warranted to address this aspect in animal models research, as well.

An unbiased survey of gene expression in WT, using oligonucleotide microarrays emphasizes the benefits of using standardized methods of microarray-based studies. A recent technological advance was made by the development of tissue microarrays, allowing the rapid immunohistochemical analysis of a large number of potential prognostic markers on a large set of tumor samples. Application of this technique might have led to the investigation of the prognostic impact of a much larger set of markers than those reported in this thesis. A

disadvantage of the tissue microarray technique is the limited amount of tumor tissue that can be investigated. Particularly for heterogeneous tumors like WT this could lead to a lack of sensitivity to detect potentially clinically relevant markers. As shown before, expression of a given marker in the blastemal component of WT might have a different impact as compared to its expression in the stromal or epithelial component. This problem can –to some extent- be circumvented by microdissection of the different components of a WT followed by their incorporation as separate spots on the microarray (Li, 2002). However, this is a time consuming process and in our patient material often the foci of blastema were very small, hampering the above-mentioned approach.

Another promising approach is the technology of cDNA microarray studying the expression of large numbers of genes at the mRNA level, exploring the new world of the genome with DNA microarray (Brown and Botstein, 1999). After extraction of mRNA from a number of WT and its hybridisation on a cDNA array containing a large number of genes the level of expression of a multitude of genes in WT can be investigated. It may be anticipated that this technique will discover prognostic markers for WT additional to those described in this thesis. On the other hand, mRNA expression levels do not necessarily correlate directly with protein levels and a number of potentially important markers may be missed by this approach. cDNA microarray analysis of WT is now also yielding important information on expression of molecules with a role in chemotherapy resistance, like the recently reported value of Topoisomerase II- α and stathmin (Takahashi, 2002).

Future approaches in WT research needs to be multidisciplinary, cytogenetic as well as molecular-genetic, optimally combining the specific points of view obtained so far and the technical potentials of the contributing disciplines.

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SUMMARY

SUMMARY

Wilms' tumor (WT) is the most common solid tumor in children, accounting for 85% of all childhood kidney cancers. The clinical decision making of WT patients treatment currently depends on tumor stage and histopathology. Nevertheless, it is very difficult to predict the clinical outcome on an individual basis. This thesis describes clinical studies on the application of prognostic tissue markers known to be of potential prognostic significance in other malignancies, with the aim to identify WT patients with a high propensity to develop disease progression. These patients might benefit from the optimal treatment by chemotherapy and / or radiotherapy.

The introductory chapter (1) reviews most of the prognosticators that have been studied in WT: tumor stage, tumor histopathology, Wilms' tumor genes (WT1, WT2, WT3, p53), proliferation by counting mitotic figures and expression of proliferation associated proteins (PCNA, Ki-67), growth factors (IGF-I and II, EGF, PDGF, bFGF, VEGF, NGFs, Midkine), cell adhesion molecules and extracellular matrix proteins (integrins, tenascin, hyaluronic acid), nuclear DNA content, nuclear morphometry, N-myc, total plasma renin, apoptosis, Bcl-2 and other possible markers. Despite a large number of published studies no prognosticators, except for tumor histopathology and stage, were identified and are being used clinically. This is partially due to the heterogeneous nature of the tumor

Chapter 2 describes the expression of both WT1 and early growth response-1 (EGR1) proteins, which were demonstrated having a distinct interrelationship. Both proteins are expressed in the blastemal and epithelial component of WT, whereas stromal tissue was negative for WT-1. Our study demonstrated a relationship between WT-1 and EGR-1 expression in clinical nephroblastoma. It seems that the immuno-histochemical expression of these two molecules will provide sufficient information about the interrelationship between WT-1 and EGR-1 in nephroblastoma. Both blastemal WT-1 and EGR-1 expression are related to prognosis.

Apoptosis is a crucial event in normal kidney development. Loss of apoptotic response in tumor cells may be one of the mechanisms involved in malignant progression and resistance to chemotherapy. Therefore, the prognostic value of apoptosis-associated proteins (Bcl-2, Bax and Bcl-X) was studied in the same set of tumor samples studied for WT-1 and EGR-1 (chapter 3). Neither Bcl-2, nor Bax or Bcl-X were correlated with tumor stage. Only Bcl-2 was correlated to prognosis. Also, Bcl-X expression provides information on the chemosensitivity or radiosensitivity of WT.

Tumor growth is the net result of tumor cell divisions (proliferation) and cell death. The study in chapter 4 was performed to evaluate the expression and the prognostic significance of the tumor proliferation marker MIB-1 (Ki-67) and cell cycle inhibitor p27^{kip1}. As expected a strong inverse correlation was found between the blastemal expression of the two markers. The low mean percentage of MIB-1 and the high mean percentage of p27^{kip1} were sufficiently informative about the defined growth fraction after initiation of chemotherapy. The results support the conclusion that MIB-1 and p27^{kip1} are relevant markers for assessing the proliferative activity and tumor cell dynamic of WT.

The involvement of CD44 isoforms in several human tumors and its putative role in tumor progression prompted us to investigate its prognostic value in WT. The expression patterns of CD44 isoforms (CD44s, CD44v5, CD44v6 and CD44v10) were studied in clinical WT samples. We conclude from the results presented in chapter 5 that CD44 expression increased from low to high stages. Increased expression of two types of CD44 (CD44s and CD44v5) was correlated with tumor stage, but only CD44v5 was correlated to clinical

progression and survival. CD44s and CD44v10 were of no additional prognostic value. Neither in normal kidney nor in tumor tissue any CD44v6 expression was found.

Chapters 6, 7 deal with the expression of several growth factors with angiogenic activity. Chapter 6 describes the expression of TGF- α , EGF-R and c-erbB-2 in treated WT. The significant expression of TGF- α and EGF-R suggested their roles in proliferation of WT, perhaps by an autocrine mechanism. Blastemal TGF- α expression was indicative for clinical progression.

Chapter 7 describes that expression of VEGF and its receptor Flt-1 are closely related with microvessel density (MVD) and their expression in the blastemal component seems to be an important predictor for poor prognosis. The level of MVD was also indicative for clinical progression.

All the previous work has been established in patients preoperatively treated with chemotherapy. Chapter 8 discusses the expression and prognostic significance of all the previously studied markers in a group of preoperatively untreated patient with nephroblastoma using a similar immuno-histo-chemical approach. The results demonstrated that expression of the apoptosis-associated markers, tumor proliferation marker MIB-1 (Ki-67) and cell cycle inhibitor p27^{kip1}, were affected by chemotherapy. Similarly to tumors from pretreated patients, blastemal WT-1, MIB-1, p27^{kip1}, TGF- α and VEGF expression in untreated patients was correlated with clinical progression. In a multivariate analysis, WT-1 protein expression was an independent prognostic marker for clinical progression. On the other hand a multivariate analysis of all previously studied tumor markers in the preoperatively treated group demonstrated that p27^{kip1} blastemal expression was an independent prognostic markers for clinical progression.

In the general discussion recommendations are given about future research on prognostic tissue markers in WT. In conclusion, expression of any factor in blastema, being the most malignant component of WT, has potentially better predictive value for poor prognosis than expression profiles in the epithelial component. Our findings provide evidence for a role of various molecules in nephroblastoma tumor biology and for their ability to be applied as prognostic markers in treated and untreated WT patients.

SAMENVATTING

SAMENVATTING

Wilms' tumor is de meest voorkomende solide tumor bij kinderen, welke verantwoordelijk is voor 85% van alle niertumoren op de kinderleeftijd. De klinische besluitvorming ten aanzien van de behandeling van patiënten met een Wilms' tumor wordt thans bepaald door de uitgebreidheid van de tumor, het tumorstadium, en de histopathologische kenmerken van de tumor. Desondanks blijkt het moeilijk om bij patiëntjes met een Wilms' tumor het klinische beloop op individuele basis te voorspellen. Dit proefschrift beschrijft een aantal klinisch-pathologische studies over de toepassing van enkele moleculaire merkstoffen, waarmee mogelijk een betere voorspelling over het klinische gedrag en beloop van Wilms' tumoren gedaan kan worden. Het belangrijkste doel van deze onderzoeken was het identificeren van die patiënten met een Wilms' tumor die een duidelijk verhoogd risico lopen op een ongunstig klinisch beloop. Studie in trial verband van een dergelijke geselecteerde patiënten-populatie zou mogelijk kunnen leiden tot een optimalere behandeling met cytostatica en/of bestraling.

Het inleidende hoofdstuk (1) geeft een overzicht van de moleculaire merkstoffen en histopathologische kenmerken met prognostische betekenis voor Wilms' tumoren die tot dusver bestudeerd zijn naast de conventionele prognostische parameters zoals tumorstadium en histopathologische kenmerken: de "Wilms' tumorgenen" WT-1, WT-2, WT-3, p53, proliferatieve activiteit zoals bepaald door het tellen van mitosefiguren of door expressie van proliferatie geassocieerde eiwitten (PCNA, Ki-67), groeifactoren (IGF-I en II, EGF, PDGF, bFGF, VEGF, NGF's, Midkine), celadhesie moleculen en extracellulaire matrix eiwitten (integrines, tenascine, hyaluronzuur), DNA inhoud en morfometrie van de celkern, apoptose, bcl-2, N-myc expressie en andere mogelijke merkstoffen. Ondanks een groot aantal gepubliceerde studies werden geen klinisch relevante prognostische factoren naast de gebruikelijke histopathologische tumorkenmerken aangetoond. Een belangrijke oorzaak voor het falen om nieuwe, op moleculaire markers gebaseerde prognostische parameters te ontdekken is mogelijk de wisselende cellulaire samenstelling van Wilms' tumoren. Deze tumoren bestaan namelijk uit wisselende hoeveelheden blastoom, epitheliaal (klierbuisweefsel) en stroomaal weefsel.

Hoofdstuk 2 beschrijft de expressie van zowel WT-1 en vroege groeirespons-1 (EGR-1) eiwitten op weefsel niveau, welke twee eiwitten een opvallende onderlinge relatie in expressie patroon vertonen. Beide eiwitten kunnen worden aangetroffen in de blastoom en epitheelcomponent van Wilms' tumoren, terwijl deze ontbreken in de stroma-component. Er werd een verband gevonden tussen de mate van expressie van WT-1 en EGR1 in onze serie van Wilms' tumoren. Bovendien bleek de mate van expressie van WT-1 en EGR-1 in de blastoom component van Wilms' tumoren van prognostische betekenis te zijn.

Apoptose is essentieel voor de normale ontwikkeling van de nier. Verlies van het vermogen tot een apoptotische respons op in- of uitwendige prikkels door tumorcellen zou een van de mechanismen kunnen zijn, die leiden tot tumor progressie en resistentie tegen chemotherapie. Om deze reden werd de prognostische betekenis van aan- of afwezigheid van apoptose geassocieerde eiwitten (bcl-2, bax en bcl-X) onderzocht in dezelfde serie tumoren, die gebruikt was voor onderzoek naar WT-1 en EGR-1 expressie. Noch bcl-2, noch bax of bcl-X expressie toonden een correlatie met tumorstadium. Uitsluitend de expressie van bcl-2 was gecorreleerd met de prognose van de patiënt. Ook suggereerden de resultaten, dat bcl-X expressie informatie verschaft over de mate van therapie resistentie van Wilms' tumoren.

De groei van een tumor is het netto resultaat van tumorceldelingen (proliferatie) en celdood. Het onderzoek, beschreven in hoofdstuk 4 werd uitgevoerd om een schatting te

maken van het aantal tumorcellen in celcyclus zoals bepaald door expressie van de proliferatie marker MIB-1, ook wel genoemd Ki-67. Ook de prognostische waarde van deze proliferatie marker en van de cel cyclus remmer p27^{kip} werd onderzocht. Zoals verwacht kon worden werd een duidelijk omgekeerde relatie gezien tussen de percentages positieve cellen voor beide markers in het blastoom van de Wilms' tumoren. Het gemiddeld lage percentage van MIB-1 positieve tumorcellen en het gemiddeld hoge percentage van p27^{kip} tumorcellen leken te passen bij het groeiremmende effect van de gegeven chemotherapie bij deze patiënten. De resultaten ondersteunen het gezichtspunt, dat MIB-1 en p27^{kip} relevante markers zijn om de proliferatie en groei van Wilms' tumoren in te schatten.

Wegens de aangetoonde betrokkenheid van isoformen van het cel-adhesiemolecuul CD44 bij het bepalen van het biologisch gedrag van verschillende andere menselijke maligne tumoren en hun vermeende rol bij tumor progressie werd die mogelijke rol van deze moleculen bij Wilms' tumoren verder onderzocht. In hoofdstuk 5 is het expressiepatroon van de CD44 isoformen, CD44s, CD44v5, CD44v6 en CD44v10 in een serie Wilms' tumoren beschreven. Geconcludeerd werd, dat bij hoger tumorstadium de CD44 expressie toeneemt. Ook correleerden de toegenomen expressie van de isoformen CD44s en CD44v5 met het tumorstadium, maar uitsluitend de mate van expressie van CD44v5 toonde een significante correlatie met progressie en overleving. In tegenstelling hiermee had de mate van CD44s en CD44v10 expressie geen prognostische betekenis. Overigens werd noch in de normale nier, noch in Wilms' tumorweefsel enige CD44v6 expressie gevonden.

De hoofdstukken 6 en 7 behandelen de aanwezigheid van verschillende groeifactoren met angiogene activiteit. Hoofdstuk 6 beschrijft de expressie van TGF- α , haar receptor, EGF-R en c-erbB-2 in Wilms' tumoren van patiënten die behandeld waren met chemotherapie. De duidelijke aanwezigheid van zowel TGF- α als EGF-R suggereert een belangrijke rol van deze autocriene loop bij de groei van Wilms' tumoren. Juist de mate van expressie van TGF- α in het blastoom van Wilms' tumoren was gerelateerd aan klinische progressie.

Hoofdstuk 7 beschrijft de bevinding, dat aanwezigheid van VEGF en de daarmee corresponderende receptor Flt-1 gepaard gingen met de aanwezigheid van talrijke kleine bloedvaatjes (microvessel density). Bovendien bleek hun expressie een belangrijke voorspeller voor slechte prognose. Ook had de mate van microvessel density een voorspellende waarde voor klinische progressie van patiënten met een Wilms' tumor.

De resultaten van al het hiervoor beschreven onderzoek was verkregen met tumorweefsel van patiënten, die voor operatie behandeld waren met chemotherapie. Hoofdstuk 8 bespreekt de mate van expressie en de prognostische betekenis van alle tevoren bestudeerde markers getest op tumorweefsel verkregen van een groep van pre-operatief onbehandelde Wilms' tumorpatiënten. Hierbij werden dezelfde immunohistochemische technieken gebruikt. Vergelijking van de in dit onderzoek verkregen resultaten met de eerdere resultaten toonde aan dat expressie van apoptose geassocieerde moleculaire merkstoffen, de proliferatiemarkers MIB-1 en de celcyclus remmer p27^{kip}, TGF- α en VEGF beïnvloed werden door voorafgaande chemotherapie. Verder bleek, dat net zoals bij de behandelde patiënten de expressie door blastoom van WT-1, MIB-1, p27^{kip}, TGF- α en VEGF in de onbehandelde patiënten correleerde met klinische progressie. Bij multivariatie-analyse toonde uitsluitend de expressie van WT-1 nog een toegevoegde prognostische betekenis voor klinische progressie. Aan de andere kant bleek bij multivariatie-analyse van alle tevoren bestudeerde prognostische markers in de behandelde groep uitsluitend het percentage p27^{kip} positieve tumorcellen van het blastoom een onafhankelijke prognostische parameter voor klinische progressie te zijn.

In de algemene discussie van dit proefschrift worden enkele aanbevelingen gedaan voor het verrichten van toekomstig onderzoek op het gebied van moleculaire weefsel merkstoffen bij Wilms' tumoren. Op grond van alle door ons verkregen resultaten lijkt het verstandiger om dit toekomstig onderzoek vooral te richten op de expressie van moleculaire merkstoffen in de meest maligne component van Wilms' tumoren, te weten het blastoom, aangezien de expressie van moleculaire merkstoffen door deze component de meeste prognostische betekenis had. Onze bevindingen verschaffen bewijs voor de rol van een aantal verschillende moleculen ten aanzien van het biologisch gedrag van Wilms' tumoren en de mogelijkheid deze te benutten als prognostische markers voor zowel behandelde als niet behandelde patiënten.

ARABIC SUMMARY

الملخص العربي

إن نتائج علاج مرض ويلمز بالأطفال قد تقدمت كثيرا في الحقتين الأخيرتين. وبالرغم من هذا التقدم الملحوظ في طرق العلاج المختلفة إلا أن نسبة ارتداد الورم حوالي من 20 % في المرحلة الثانية و الثالثة و 50 % أو أكثر في المرحلة الرابعة لانتشار الورم. وإن العوامل المسؤولة عن نمو و انتشار الورم غير معروفة لأنه من الصعب عزل و ذرع الخلايا السرطانية المختلفة من الورم و دراستها. كما يتميز هذا الورم بالتركيب الجيني المعقد. و الهدف من هذا البحث هو دراسة طرق نسخ دلائل ورم الكلى بالأطفال (ورم ويلمز) و مستقبلاتها في النماذج المعملية و الحالات الإكلينيكية و دورها في نمو و انتشار الورم. و تمت دراسة هذا البحث على عدد 61 مريضا بورم ويلمز . هذه المجموعة من المرضى تمت معالجتها كيميائية قبل استئصال الكلى جذريا جراحيا. و أيضا تمت دراسة هذا البحث على عدد 42 مريضا حيث هذه المجموعة من المرضى لم تتم معالجتها كيميائية قبل استئصال الكلى جذريا جراحيا. و قد أظهرت هذه الدراسة أهمية تواجد نسيج البلاستيما بالنظر عن المكونات النسيجية الأخرى في هذا المرض. و كما يمكن استعمال المقايضة المناعية النسيجية الكيميائية لعينات مرض ورم ويلمز التي يمكن من خلالها إثبات دلائل أورام مختلفة التي تساعدنا على معرفة تشخيص المرض كذلك الذين لديهم استعداد لظهور أورام ثانوية بعد العملية الجراحية الجذرية للورم و كما يمكن توضيح ما إذا كان هؤلاء المرضى يستفيدون من العلاج الكيماوي من عدمه.

LIST OF PUBLICATIONS

ACKNOWLEDGEMENTS

CURRICULUM VITAE

LIST OF PUBLICATIONS

Mazen A. Ghanem, Theo H. van der Kwast, Jan C. den Hollander, Mondastri K. Sudaryo, Monique H. A. Oomen, Marinus A. Noordzij, Marry M. van den Heuvel, Shoukri M. Nassef, Rien J. M. Nijman, and Gert J. van Steenbrugge. Expression and prognostic value of Wilms' tumor 1 and early growth response 1 proteins in nephroblastoma. *Clin. Cancer Res.*, 6:4265-4271, 2000.

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Mazen A. Ghanem, Dik J. Kok, Katja P. Wolffentbittel. Evaluation, treatment and outcome of children with major blunt renal trauma. *Submitted*

Mazen A. Ghanem, Katja P. Wolffenbittel, Ann de Vylder, Rien J.M. Nijman. Long term bladder dysfunction and renal function in boys with posterior urethral valves on the basis of urodynamic findings. *Submitted*

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- 5- Training in Andrology Unit, Erasmus M.C., Rotterdam, May until from July, 2000 and 14th of October until 8th of November, 2002 (Prof. Dr. R.F.A. Weber, Dr. G.R. Dohle).

Prize

Winner of the International scientific award during the 3rd world Congress of Urological Research, October, 1999, Paris, France

APPENDIX
COLOR FIGURES

CHAPTER 1:

Figure 1 (page 15)

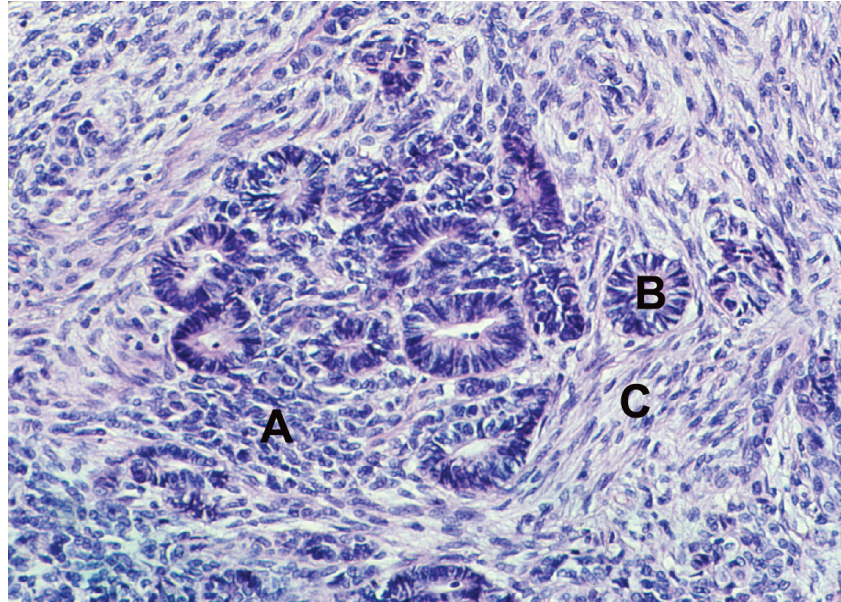
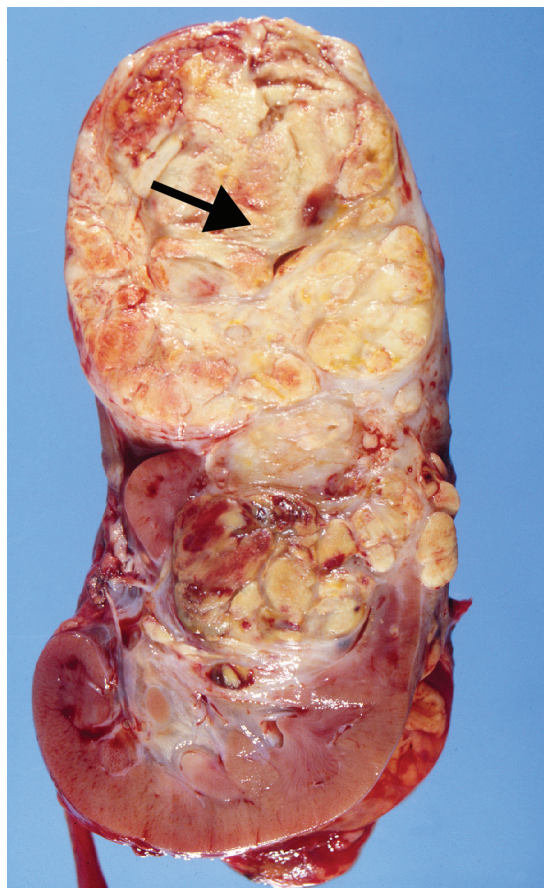
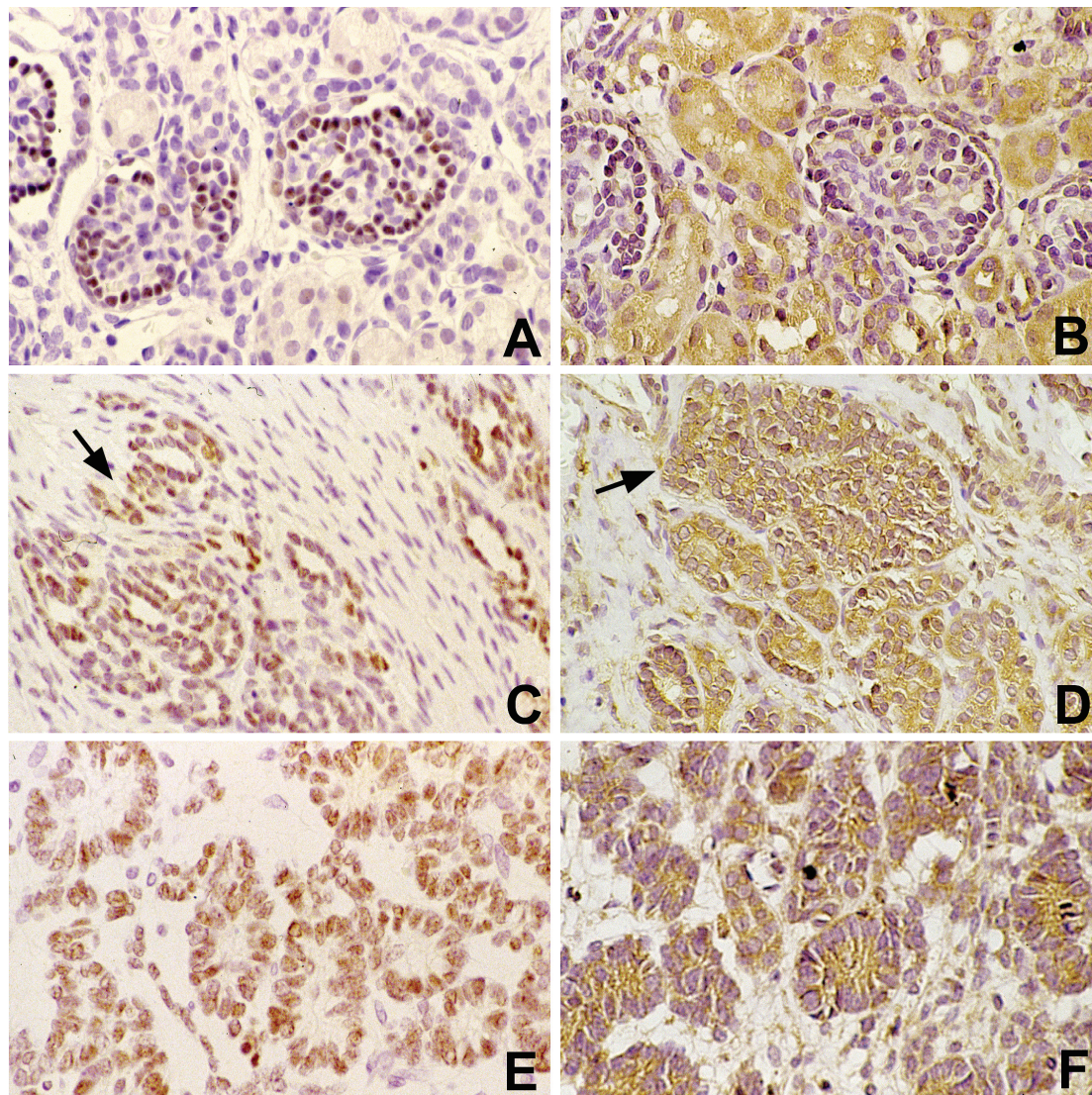


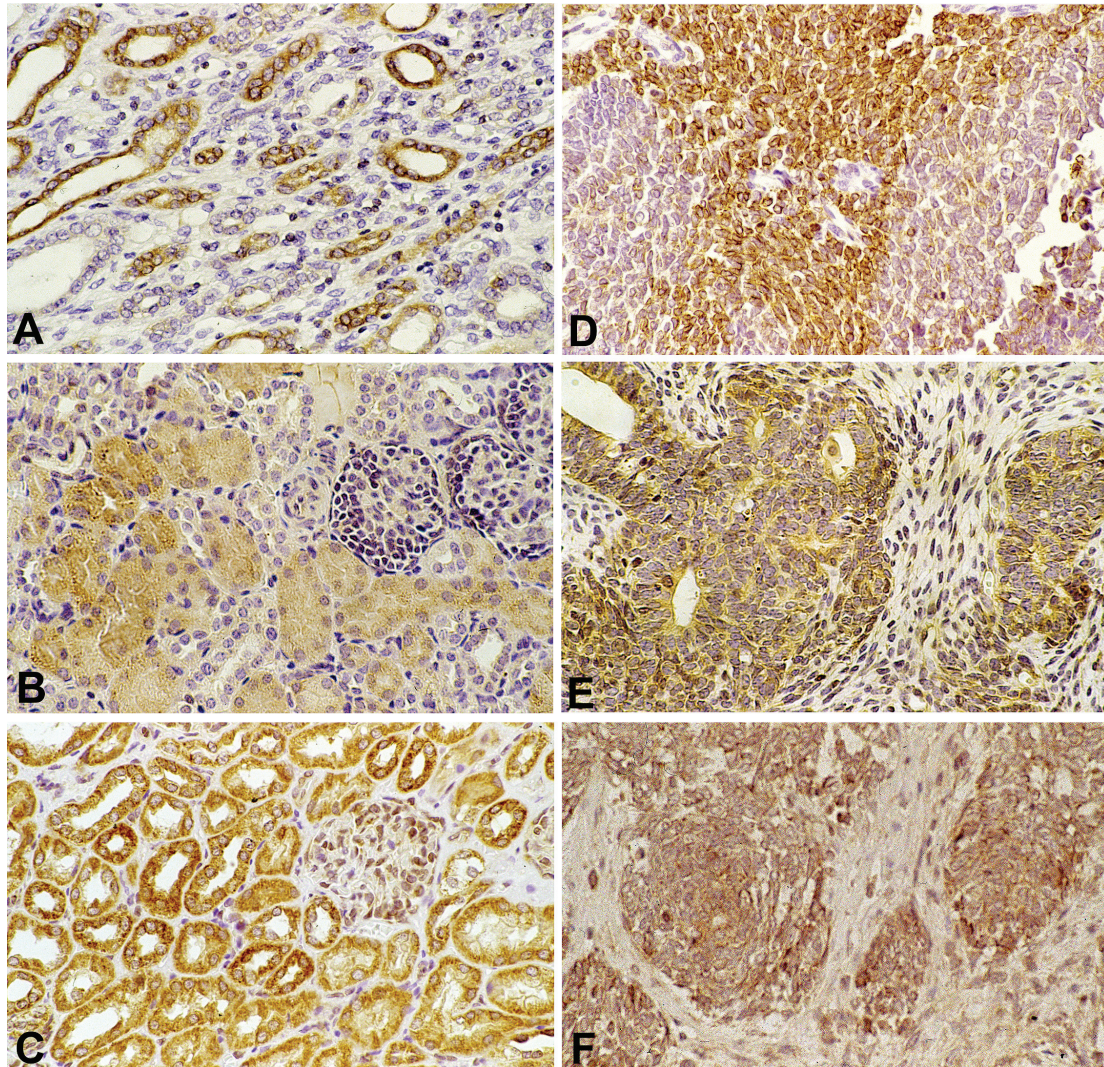
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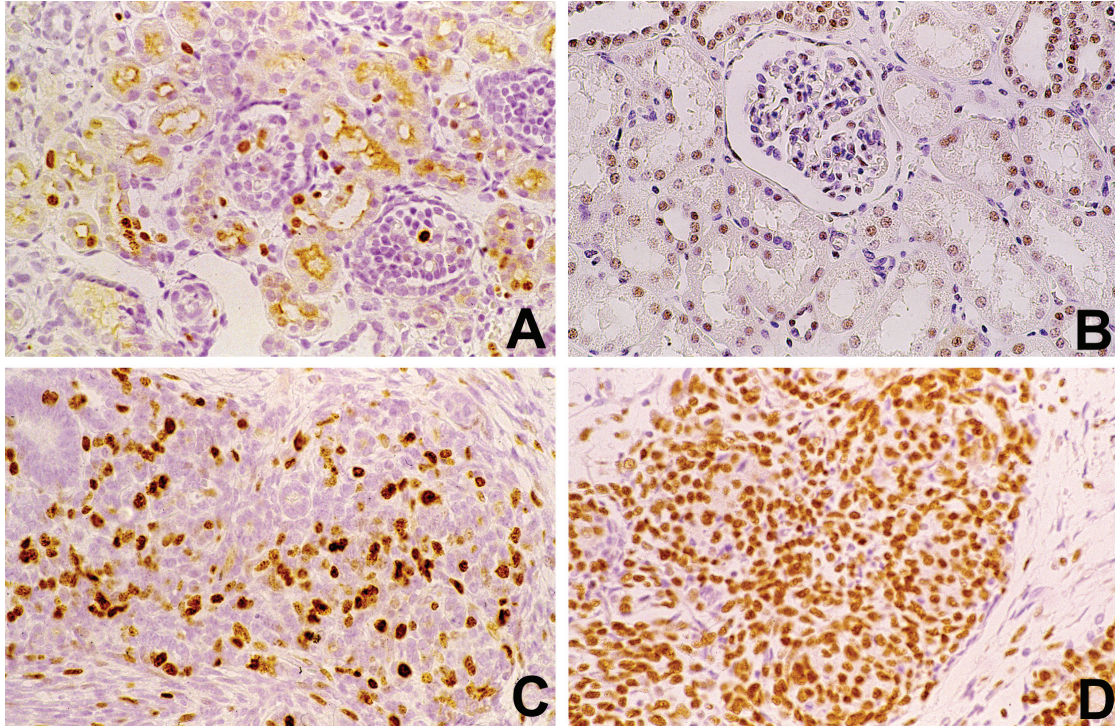


*CHAPTER 2:***Figure 1 (page 36)**

CHAPTER 3:

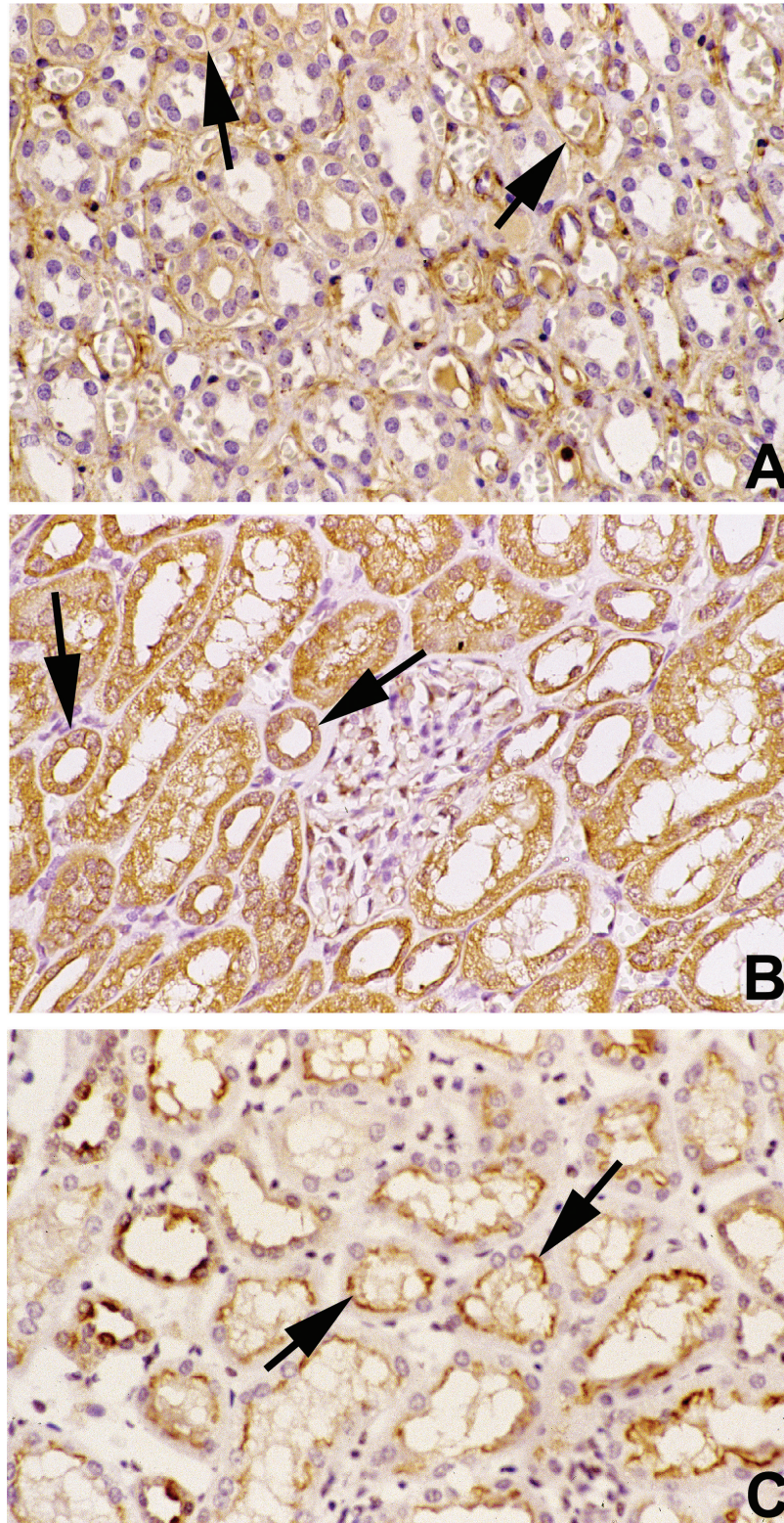
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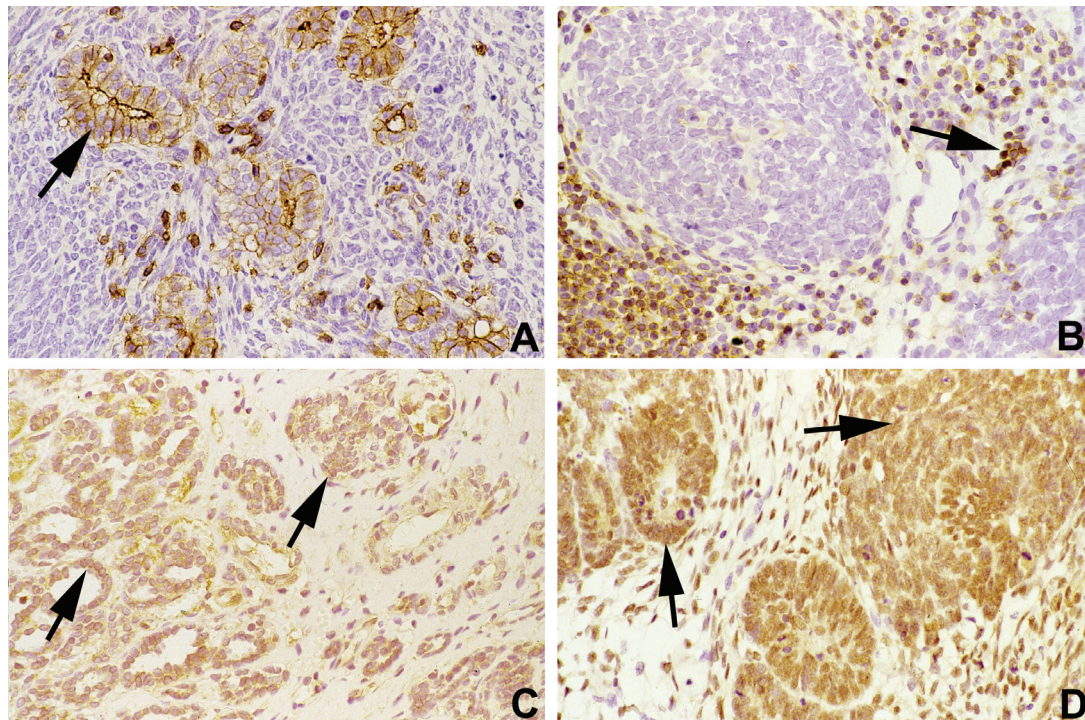


*CHAPTER 4:***Figure 1 (page 64)**

CHAPTER 5:

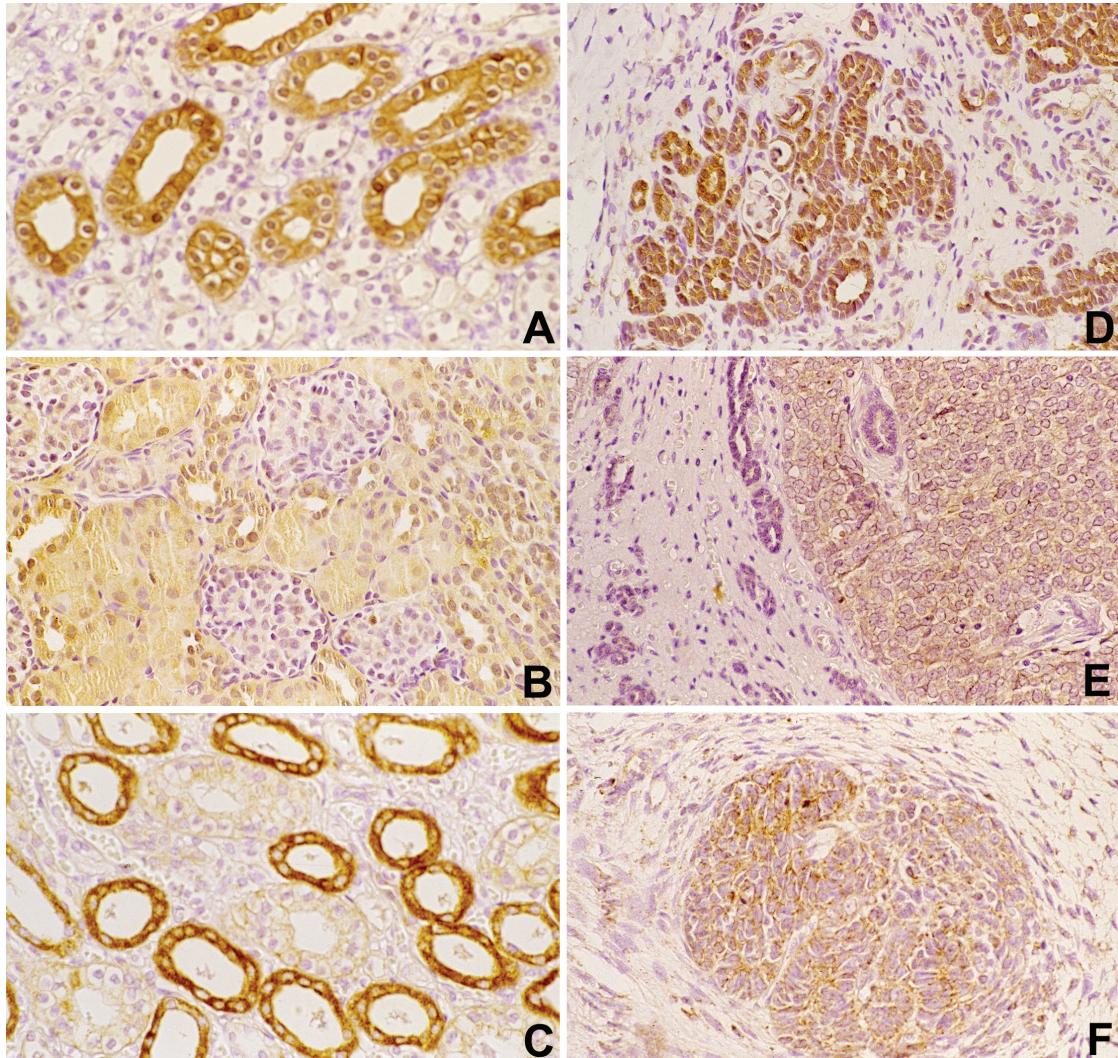
Figure 1 (page 78)



*CHAPTER 5:***Figure 2 (page 79)**

CHAPTER 6:

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*CHAPTER 7:***Figure 1 (page 106)**