American Journal of Pathology, Vol. 148, No. 5, May 1996

Immunohistochemical Detection of p53 in Wilms' Tumors Correlates with Unfavorable Outcome

Chitra Lahoti,* Paul Thorner,*
David Malkin,† and Herman Yeger*

From the Departments of Pathology and Oncology,† The Hospital for Sick Children, University of Toronto, Toronto, Canada*

The role of p53 in the pathogenesis and progression of Wilms' tumors is only partly understood. Although p53 mutations were initially reported only in anaplastic Wilms' tumors, we had reported that, of two of twenty-one cases that had a p53 mutation, one tumor showed no evidence of anaplasia. To determine the significance of p53 expression in all clinical stages of Wilms' tumor, twenty-eight cases were analyzed for p53 immunoreactivity. Paraffin sections were immunolabeled with two different monoclonal antibodies, recognizing both mutant and wild-type p53. Fifteen of sixteen tumors in the recurrent/metastatic group and three of twelve tumors in the nonmetastatic/nonrecurrent group showed p53 immunopositivity. Only one of three positive tumors in the latter group showed moderate to strong positivity, whereas twelve of sixteen metastatic/recurrent tumors revealed a similar degree of p53 positivity. The positivity was stronger in the metastasis/recurrences as compared with the corresponding primary tumor. Western blot analysis revealed p53 expression in all of the Wilms' tumors tested, suggesting its involvement in the development of Wilms' tumors. Single-strand conformation polymorphism analysis performed on twenty-three of these tumors revealed p53 mutations in four of fourteen recurrent/metastatic tumors and none in the nonmetastatic/nonrecurrent group. Our results show that, whereas 60% of cases were immunopositive for p53 protein, mutations were detected in only 16% of tumors, indicating that wild-type p53 protein is retained in the other tumors. We conclude that p53 immunopositivity strongly correlates with recurrence/metastasis in Wilms' tumors. Furthermore, the accumulation of p53 in these tumors is not only due to mutations but may also involve stabilization of normal p53 with other proteins. (Am J Pathol 1996, 148:1577–1589)

Wilms' tumor is one of the common solid tumors of childhood with an incidence of approximately 1 in 10,000 children. It is highly responsive to chemotherapy and carries an overall good prognosis. However, 10 to 15% of tumors are metastatic at presentation or recur/metastasize after initial therapy.[1] At present, histological evidence of anaplasia or loss of heterozygosity for chromosome 16q[2] are two indicators of poor prognosis associated with significantly lower relapse-free and overall survivals. Presently, the histological assessment of anaplasia has been documented in only approximately 4% of cases and is not present in all of the tumors that recur or metastasize.

Loss of wild-type p53 or aberrant expression of mutant p53 are indicative of progression events and serve as an unfavorable prognostic marker in a wide variety of tumors.[3] Mutations of p53 have been detected in more than 50% of human cancers[3] whereas overexpression of p53 detected immunohistochemically in cancers of breast, colon, lung, or brain is associated with tumor progression and unfavorable prognosis.[3–6] This overexpression is thought to be due to either stabilization of mutant p53 or stabilization of wild-type p53 by interaction with other proteins that prevents its degradation. These events can lead to loss of the function of p53 as a transcription factor involved in the regulation of the cell cycle. Normally, in response to DNA injury, cellular levels of wild-type p53 increase and lead to arrest before G1/S transition in the cell cycle.[7] In these cells, p53 activates transcription of the p21/*cip*1 gene, which encodes the p21 protein that binds to multiple cyclin-dependent kinases and blocks the activation of cy-

Supported by the National Cancer Institute of Canada with funds from the Canadian Cancer Society.

Accepted for publication January 15, 1996.

Address reprint requests to Dr. Herman Yeger, Department of Pathology, The Hospital for Sick Children 555 University Avenue, Toronto, Ontario M5G 1X8 Canada.

clin/*cdk* complexes leading to G1 arrest.[8] p53 also participates in DNA repair by activating repair genes including GADD45 and PCNA; alternatively, p53 participates in the initiation of apoptosis by interacting with other gene products such as Bax,[9] Bcl-2,[10] and Mdm2.[11] Expression of wild-type p53 has been reported in frozen sections of rapidly dividing tissues such as the basal layer of squamous epithelium[12] and cervical mucosa.[13] p53 is involved both in tumorigenesis and in progression of a cancer, as loss of wild-type p53 allows cells with damaged DNA to divide without DNA repair, resulting in the generation of a cell population with altered genetic functions.

Although the status of p53 in Wilms' tumors has been investigated, its significance is not clear. An earlier study by Lemoine et al[14] found p53 immunopositivity in 100% of Wilms' tumors as detected by immunohistochemistry on frozen sections using antibody PAb240. This antibody recognizes both mutant and denatured wild-type epitopes on p53. Recent studies from other laboratories and our own have shown that p53 mutations are uncommon in Wilms' tumors.[15–17] Thus, most Wilms' tumors likely possess only wild-type p53. In Wilms' tumors, inactivation and retention of wild-type p53 may be due to its interaction with other proteins, thus circumventing normal functions of p53 and allowing the malignant cells with altered DNA to divide. WT1, the Wilms' tumor suppressor gene[18,19] encodes a 50-kd protein that has been shown to complex with p53 protein *in vitro*.[20] In this complex, p53 and WT1 modulate each other's functions; in the absence of p53, WT1 acts as a transcriptional activator of the EGR1 gene, whereas in the presence of p53, WT1 induces repression of EGR1. WT1 itself potentiates the transcriptional activation of p53.[20] Maheshwaran et al[20] also found p53-WT1 complexes in three Wilms' tumors analyzed. To understand the role of p53 in the progression of Wilms' tumors, we analyzed a series of cases for p53 immunopositivity and p53 mutations. Although the presence of mutations correlated closely with metastasis or recurrence, a substantial fraction of Wilms' tumors showed p53 immunopositivity without demonstrable p53 mutations and displayed a similar poor outcome.

Materials and Methods

Patients and Tissues

Samples of Wilms' tumor (both formalin-fixed tumor blocks and snap-frozen tumor stored at −70°C) from 28 patients were obtained from the Pathology Department at The Hospital for Sick Children, Toronto, between 1983 and 1993. In the tissue bank, tumors were assigned WiT numbers in order of presentation; those listed represent the 28 cases with sufficient material for the study. Cases were chosen with adequate follow-up, especially for the nonmetastatic/nonrecurrent group. Pretreatment tumor tissue was available in all of these cases in the form of either excised tumor (11 cases) or biopsy from the primary tumor (17 cases). Clinical information including age, staging, therapy, and outcome as well as histological type are given in Table 1. The first group included 12 cases with no metastasis/recurrences. In the first group, 9 patients had been followed up for more than 6 years and only 3 patients had a follow-up of only 4 years. All of the patients in this group are alive and free of the disease. The second group included 16 patients with metastasis/recurrences (indicating more aggressive tumors); 15 of these patients developed metastases/recurrences at a later time, whereas 1 (WiT49) presented with lung metastases at the time of initial diagnosis. As shown in Table 1, 5 patients in the second group expired due to extensive spread of the tumor.

The majority of these tumors had classical triphasic histology, showing epithelial, mesenchymal, and blastemal components in varying proportions. The epithelial component consisted of tubules and glomeruloid structures whereas the mesenchymal components consisted of heterologous and/or homologous elements. The homologous elements were present in all of the cases, consisting of a fibroblastic stroma. Heterologous elements were present in 11 primary tumors and included squamous, neural, chondroid, and frequently rhabdomyoblastic differentiation. Anaplasia was noted in 6 primary tumors, 5 belonging to the recurrent/metastatic group and 1 in the nonmetastatic category. In 2 cases, anaplasia was documented only in metastasis/recurrences and not in the primary tumor. Anaplasia was present in three patients who died of the disease.

Cell Lines

Five different cell lines with known p53 and WT1 status served as controls for both immunohistochemistry and Western blot analysis. K562 and OCIM2 (kindly supplied by Dr. S. Benchimol, Ontario Cancer Institute) were leukemic cell lines. K562 carries a p53 mutation that results in loss of p53 protein expression but does express wild-type WT1. OCIM2 is mutant for p53 but expresses p53 protein. The status of WT1 is not known for OCIM2. The SV40-immortalized putative podocytic parent cell line 56A1[21] was kindly provided by Dr. J-D. Sraer (Tenon Hospital, Paris,

Table 1. *Clinical Data on Wilms' Tumors*

Sample	Age at diagnosis (months)	Sex	Histopathology	Stage	Metastases	Recurrence	Treatment	Follow-up (years)	Outcome
WiT1	48	M	FH	III	–	–	Chemo	12	A/NED
WiT10	84	M	Anaplasia	III	–	–	Chemo/rad	11	A/NED
WiT12	22	F	FH	V*	–	–	Chemo	11	A/NED
WiT13	24	F	FH	III	–	–	Chemo	11	A/NED
WiT24	43	M	FH	III	–	–	Chemo	10	A/NED
WiT26	23	F	FH	II	–	–	Chemo	9	A/NED
WiT32	06	F	FH	III	–	–	Chemo	8	A/NED
WiT52	04	F	FH	I	–	–	Chemo	6	A/NED
WiT72	72	F	FH	I	–	–	Chemo	4	A/NED
WiT75	12	M	FH	V*	–	–	Chemo		
WiT76	02	M	FH	I	–	–	Chemo	4	A/NED
WiT81	08	M	FH	I	–	–	Chemo	4	A/NED
WiT21	48	F	Anaplasia	V	Lungs	+	Chemo/rad	DOD	
WiT28	60	M	FH	V*	–	+	Chemo	11	A/NED
WiT34	60	F	Anaplasia	IV	Multiple	–	Chemo/rad		
WiT41	24	F	Anaplasia	IV	Lungs	–	Chemo/rad	7	A/NED
WiT47	86	F	FH	I	–	+	Chemo/rad	6	A/NED
WiT49	24	F	Anaplasia	IV	Multiple	+	Chemo/rad	DOD	
WiT59	26	M	FH	III	–	+	Chemo/rad	6	A/NED
WiT67	24	M	FH	IV	Testis	–	Chemo/rad	5	A/NED
WiT73	117	F	FH	I	Lungs	–	Chemo	5	A/NED
WiT78	16	F	FH	I	Liver	–	Chemo	4	A/NED
WiT96	72	F	FH	I	Lung	+	Chemo/rad	DOD	
WiT100	36	M	Anaplasia	IV	Multiple	–	Chemo/rad	DOD	
WiT102	72	F	Anaplasia	III	Lungs×3	–	Chemo/rad	3	A
WiT105	60	M	Anaplasia	IV	Multiple	–	Chemo/rad	DOD	
WiT110	120	M	FH	III	–	+	Chemo/rad	2	A/NED
WiT112	72	F	Anaplasia	III	+	+	Chemo/rad	2	A/NED

FH, favorable histology; chemo/rad, chemotherapy/radiation; A, alive; NED, no evidence of disease; DOD, dead of disease.
*Indicates patients with bilateral Wilms' tumors.

France) whereas the 56A1E14 cell line, which was co-transfected with CMV-cDNA WT1 construct (+17 amino acids, +KTS isoform of WT1) and pSV*neo*, was generously donated by Dr. C. Campbell (Cleveland Clinic Research Institute). The 56A1 cell line expresses very low levels of WT1 (H. Yeger, unpublished data). The cell lines were maintained in Dulbecco's minimal essential medium F12 supplemented with 10% fetal bovine serum. 56A1E14 cultures were further supplemented with 400 μg/ml G418 to maintain selection under neomycin resistance.

Immunohistochemistry for p53

Tumor tissue and cell line pellets were fixed in 10% formalin, embedded in paraffin, and sectioned. For each case, sections were selected for immunostaining from at least two representative areas of the tumor. In five cases, sections were stained from all of the tumor blocks processed (range of 6 to 13). Besides performing immunostaining on the resected tumor, we immunostained the original tumor biopsy (wherever available) to investigate the possibility of p53 immunopositivity secondary to treatment effect.

Immunostaining for p53 was performed with an indirect immunoperoxidase method and enhanced by an antigen retrieval technique by microwaving in 0.01 mol/L sodium citrate, pH 6.0. This technique was standardized for the microwave in our laboratory. Endogenous peroxidase was blocked with 0.3% H_2O_2. The sections were first blocked in 1% normal goat serum and then incubated overnight at 4°C with two different p53 antibodies, DO-7 (1:100) and 1801 (1:60; (Novocastra Laboratories). Both antibodies recognize amino-terminal epitopes and detect both wild-type and mutant p53. These antibodies were compared for their staining pattern and to identify an antibody that performed well using microwaving for antigen retrieval. The slides were then washed three times with wash buffer (30 mmol/L Tris, pH 7.5, 150 mmol/L NaCl, 1% bovine serum albumin, 0.05% Triton X-100), incubated for 30 minutes at room temperature with biotinylated goat anti-mouse IgG (Molecular Probes, Eugene, OR) diluted 1:250 in antibody diluting buffer (Dimensions Lab), washed three times as above, and incubated with avidin-peroxidase (Vector Laboratories, Burlingame, CA) diluted 1:1000 in diluting buffer for 30 minutes at room temperature, followed by final washes in Tris-

buffered saline. Bound antibody complex was visualized by reaction in 3,3′-diaminobenzidine substrate (Vector) and counterstained with Mayer's hematoxylin. The immunohistochemically stained sections were assessed independently by two pathologists (P. Thorner and C. Lahoti) and H. Yeger and scored both for number of positive cells and for the intensity of immunopositivity. The stained slides were scored blindly by the authors (P. Thorner and H. Yeger) without prior information about stage and clinical outcome and subsequently reviewed blindly by C. Lahoti. The correlation between positive and negative cases was 100% among the authors, although minor differences in grading of immunopositivity in a few cases were resolved by jointly viewing the patterns. The numbers of immunopositive cells in sections were assigned as extensive when 76 to 100% of tumor cells were positive, frequent when 50 to 75% of cells were positive, and restricted when less than 50% of cells were positive. A case was categorized as negative when none or only a few (<5) cells on the whole slide showed a weak staining or no positive cells were present. The intensity of immunopositive staining was graded as strong when the nuclei showed a dark brown color, moderate when the color was intermediate golden brown, and weak when the nuclei were light brown. Cytoplasmic positivity was considered only when the cytoplasm showed a brown granular positivity. Incubations without the primary antibody served as a negative control. No positive staining was noted in the controls.

p53 Mutation Analysis

DNA was extracted by a standard phenol chloroform extraction method from 23 tumor samples kept frozen at −70°C. Twelve primary nonmetastatic/nonrecurrent tumors and eleven metastatic/recurrent tumors were screened for p53 mutations by single-strand conformation polymorphism (SSCP) analysis.[15] Nine sets of primers were generated to amplify exons 2 and 4 through 11 of the p53 gene. These primers have been published previously.[21,22] Polymerase chain reaction (PCR) was carried out for each of the above exons in a 50-μl total volume in an automated thermocycler (Perkin-Elmer Corp., Norwalk, CT). The reaction conditions were as follows: 250 ng of DNA, 50 mmol/L Tris-HCl, pH 8.6, with 1.5 mmol/L $MgCl_2$, 0.2 mmol/L of each dNTP, 250 ng of each primer, 0.1 μl of [^{32}P]dCTP (3000 μCi/ml), and 2.5 U of *Taq* polymerase (Cetus Laboratory, Emeryville, CA). PCR conditions were as follows: 85°C for 10 minutes followed by 35 cycles at 94°C (45 seconds), 55°C (45 seconds), and 72°C (45 seconds). The reaction was terminated with a 7-minute incubation at 72°C followed by 3 minutes at 94°C. To the PCR product, an equal volume of loading buffer (95% formamide, 20 mmol/L EDTA, 0.05% bromphenol blue, and 0.05% xylene cyanol) was added. The samples were then loaded onto acrylamide-TBE nondenaturing gels after denaturation at 85°C for 5 minutes. Different concentrations of acrylamide and glycerol were used depending on the exon fragment being analyzed (4.5 to 9% acrylamide and 2 to 10% glycerol). The period of electrophoresis depended on the size of exon and the gel concentration of acrylamide. The gels were dried and exposed to Kodak X-Omat film with an intensifying screen at −70°C for 4 to 72 hours. Each fragment was run in two or three electrophoretic conditions depending on the specific exon being analyzed. Each gel run included known mutant p53 samples and a sample of wild-type p53. DNA samples determined to be abnormal by detectable band shifts on SSCP were reamplified with the SSCP primers encompassing the abnormal region. The PCR product was purified, subcloned directly into T-tailed pBSK+ bluescript vector and sequenced in both directions by Sanger's method with the Sequenase 2.0 kit (U.S. Biochemicals/Amersham, Cleveland, OH).

Western Blot Analysis

To corroborate the results of immunohistochemistry, p53 protein analysis was performed by Western blot technique using the above cell lines as well as on selected Wilms' tumors for which frozen tumor tissue was available. Pellets from cultured cell lines and small amounts of tumor tissue frozen at −70°C were lysed with 1 ml of RIPA buffer (150 mmol/L NaCl, 50 mmol/L Tris, pH 8.0, 1% Nonidet P-40, and 0.5 mmol/L Pefabloc SC (Boehringer Mannheim, Indianapolis, IN). This cell suspension was gently vortexed every 15 minutes for approximately 1 to 2 hours and then centrifuged at high speed for 10 minutes. The supernatant was collected in a separate tube, and quantification of proteins was performed using the Bradford method. Supernatant equivalent to 50 μg of protein for normal and tumor tissues and 10 to 25 μg for cell lines was mixed with an equal amount of sample buffer (2% sodium dodecyl sulfate, 20% glycerol, 62.5 mmol/L Tris-HCl, pH 6.8, 0.005% bromphenol blue, and 2% β-mercaptoethanol) and denatured by boiling for 5 minutes. The samples were then loaded on 10% acrylamide gels and run for 1 hour in a Bio-Rad minigel system. The proteins were then transferred to Immobilon-P (Millipore, Bedford, MA) by semi-dry electrophoresis. The membranes were then blocked overnight in 10% skim milk in Tris-buffered saline with 0.1%

Tween 20 followed by incubation with a mixture of two primary anti-p53 antibodies, DO-1 (1:1000; Oncogene Science) and 1801 (1:1000) for 1 hour. After three washes in 1% skim milk in Tris-buffered saline with 0.1% Tween 20, the membranes were incubated with anti-mouse IgG-alkaline-phosphatase Fab fragments (1:1000; Boehringer Mannheim) for 1 hour. After three washes with 1% skim milk solution, the final wash was performed in 0.065 mol/L amino methyl propanol buffer, pH 9.5, for 20 minutes. The membranes were then incubated in Lumiphos (Boehringer Mannheim) and exposed to Kodak X-Omat film for 2 hours to overnight.

Results

Immunohistochemical Staining for p53 in Fetal Kidney

Wilms' tumor arises from the nephrogenic blastema in the developing fetal kidney. We therefore first examined human fetal kidney sections for p53 staining. Immunostaining for p53 on paraffin sections of human fetal kidney revealed no p53 expression in the blastema or developing tubules and glomeruli (not shown). However, on Western blot analysis, p53 protein was present in low but detectable levels (see lane 5 in Figure 3), indicating that, in developing kidney, wild-type p53 is expressed at low levels not detectable in paraffin sections.

p53 Immunostaining in Cell Lines

To assess p53 protein expression in cell lines with known mutational status of p53, immunostaining was performed on cell lines K562, OCIM2, 56A1, and 56A1E14. K562 showed no p53 staining whereas OCIM2 showed a heterogeneous staining pattern with the majority of cells showing moderate staining and some weak to strongly positive cells (not shown). Both 56A1 and 56A1E14 were uniformly immunopositive for p53. However, the positivity was stronger in the latter.

p53 Immunostaining in Wilms' Tumors

Both antibodies, 1801 and D0–7, showed positivity in the same tumors, although the 1801 antibody generally yielded a weaker signal. It was noted that in the case of WiT47 the 1801 antibody showed strong cytoplasmic immunopositivity, not seen with D0–7. The results of p53 immunostaining among the two groups of Wilms' tumors, metastatic/recurrent and nonmetastatic/nonrecurrent, indicated a significant expression of p53 in the former group as summarized in Tables 2 and 3. In general, p53 immunostaining in most Wilms' tumors was nuclear and not restricted to any particular cell type. Positivity was noted in tubules, blastema, and anaplastic cells, although anaplastic cells were not always positive (Figure 1A). The stroma was invariably negative with both antibodies. Heterologous elements such as squamoid and chondroid elements were negative. The rhabdoid cells in one case were negative for p53 staining, whereas two other cases showed some weak cytoplasmic staining. As shown in Table 2, nine of twelve cases in the nonmetastatic/nonrecurrent category were negative. Two cases showed only weak positivity whereas one had focal strong positivity. This latter case showed predominant tubular differentiation with focal anaplasia. The tumor showed strong positivity both in anaplastic cells and in adjacent non-anaplastic tumor tubules.

Table 2 shows that the majority of 16 primary tumors and their metastases/recurrences were immunopositive for p53. Due to heterogeneity in the degree of immunopositivity, tumors were graded based on both the proportion of positive cells as well as the intensity of positivity. The intensity of staining as well as the number of positive cells both increased in the metastases/recurrences as compared with the primary tumor. In the primary tumor of WiT49, only a few scattered anaplastic cells were strongly positive for p53 (Figure 1B) whereas its metastasis showed areas of extensive strong positivity intermixed with areas of positive and negative cells (Figure 1C). Cytoplasmic positivity for p53 was noted in 3 tumors using the 1801 antibody; in 2 cases it was very focal, and 1 of the primary tumors showed moderate to strong cytoplasmic positivity and weak nuclear positivity with DO-7 antibody (Figure 1D). The recurrent tumor of this latter case, however, showed weak to moderate nuclear positivity only. Histologically, this tumor showed predominantly epithelial differentiation surrounded by myxoid mesenchymal tissue.

In the metastatic/recurrent tumors, both increased numbers of immunopositive cells and stronger immunopositivity was observed in comparison with the nonmetastatic group, which showed an overall weak and restricted immunopositivity in intensity and number of p53-immunopositive cells.

Heterogeneity in p53 Immunostaining

In the majority of the primary as well as the metastases/recurrences, variation in intensity of p53 immu-

Table 2. *Results of p53 Immunostaining in Wilms' Tumors*

WiT	Intensity		Extent of immunopositivity	
	Primary	Rec/met	Primary	Rec/met
1			–	
10	Strong		F/hom	
12	Weak		Rare	
13				
24				
26				
32				
52	Weak		Rare	
72				
75				
76				
81				
21	Weak	Weak-strong*	<50%	>50%
28	Moderate	Moderate-strong	>50%	>50%
34	Moderate	Moderate-strong	>50%	>50%
41	Strong	NA	F/hom	NA
47	Moderate-strong†	Moderate-strong	>50%	>50%
49	Moderate-strong	Moderate-strong	Rare	>50%
59	Weak-moderate	Weak	<50%	<50%
67	Weak-moderate	Weak-moderate	<50%	>50%
73	Weak-moderate	Weak-strong	>50%	>50%
78	Negative	Weak	Negative	>50%
96	Weak-moderate	Weak-moderate‡	<50%	>50%
100	Strong	Strong	>50%	>50%
102	Weak-moderate	Moderate	>50%	>50%
105	Strong	NA	>50%	NA
110	Weak-moderate	NA	<50%	NA
112	Weak	Moderate	>50%	>50%

F/hom, focal area of homogeneous positivity; rec/met, recurrences/metastases; NA, radiological evidence of metastases, no tissue taken.
*Few cells strongly positive.
†Predominantly cytoplasmic staining.
‡Few cells moderately positive.

nopositivity as well as in the number of positive cells was noted in different areas of the tumor (Figure 1A). This variation in p53 immunostaining was present not only in the same section but also among different areas of tumor. One primary tumor had only a few strongly p53-positive tumor nodules located at the periphery of the tumor whereas the main tumor showed rare positive cells. This patient later developed lung metastasis. The primary tumor of another patient (WiT102) revealed heterogeneous weak to moderate positivity, whereas the metastasis showed moderate to strongly positive areas (Figure 1, E and F) adjacent to a p53-immunonegative nodule. This patient developed two more lung metastases that were also positive. WiT41 was an interesting case in which the initial two sections immunostained for p53 were negative. As the patient had lung metastases, all of the blocks from the primary tumor were immunostained, revealing strong p53 immunopositivity in two blocks where there was a focus of anaplasia (Figure 1G). To further investigate the problem of heterogeneity, in addition to staining at least two blocks on all cases, all of the available tumor blocks from four cases of metastatic/recurrent Wilms' and two cases of nonmetastatic Wilms' tumors were immunostained for p53. The nonmetastatic/nonrecurrent cases were included to verify their true negativity. The results in these six cases are shown in Table 4. Among the metastatic/recurrent category, WiT49 and WiT102 showed very few cells positive in the primary tumor although the metastases/recurrences were markedly increased in the number of immunopositive cells and in intensity. WiT102 showed significant heterogeneity in the original sections of both

Figure 1. *Immunoperoxidase labeling of Wilms' tumors with the DO-7 anti-p53 monoclonal* (**A** *to* **C** *and* **F** *to* **G**) *and the 1801 monoclonal* (**D**) *antibodies, counterstained with hematoxylin.* **A:** *WiT49 metastasis showing strong nuclear positivity in the tubules and some anaplastic cells. Note that not all of the anaplastic cells are positive for p53. Magnification, ×225.* **B:** *Primary tumor, WiT49 showing focal strong positivity in the blastema. ×90.* **C:** *Metastasis of WiT49 showing strong heterogeneous staining for p53. Note the heterogeneity in tubular staining as well as in the blastema with diffusely staining areas adjacent to areas with positive and negative cells. ×90.* **D:** *Cytoplasmic positivity for p53 in WiT47. In many of these cells the positivity appears to concentrate around the nucleus. ×225.* **E:** *Primary tumor, WiT102 showing heterogeneous weak to moderate p53 immunostaining. ×225.* **F:** *Metastasis of WiT102 showing moderate to strong p53 positivity with a significant increase in number of positive cells as compared with the primary tumor. ×225.* **G:** *Primary tumor WiT41 showing a strongly p53-positive area adjacent to a totally negative area. The positive area shows features of anaplasia whereas the negative area has a histology of classical Wilms' tumor. ×90.*

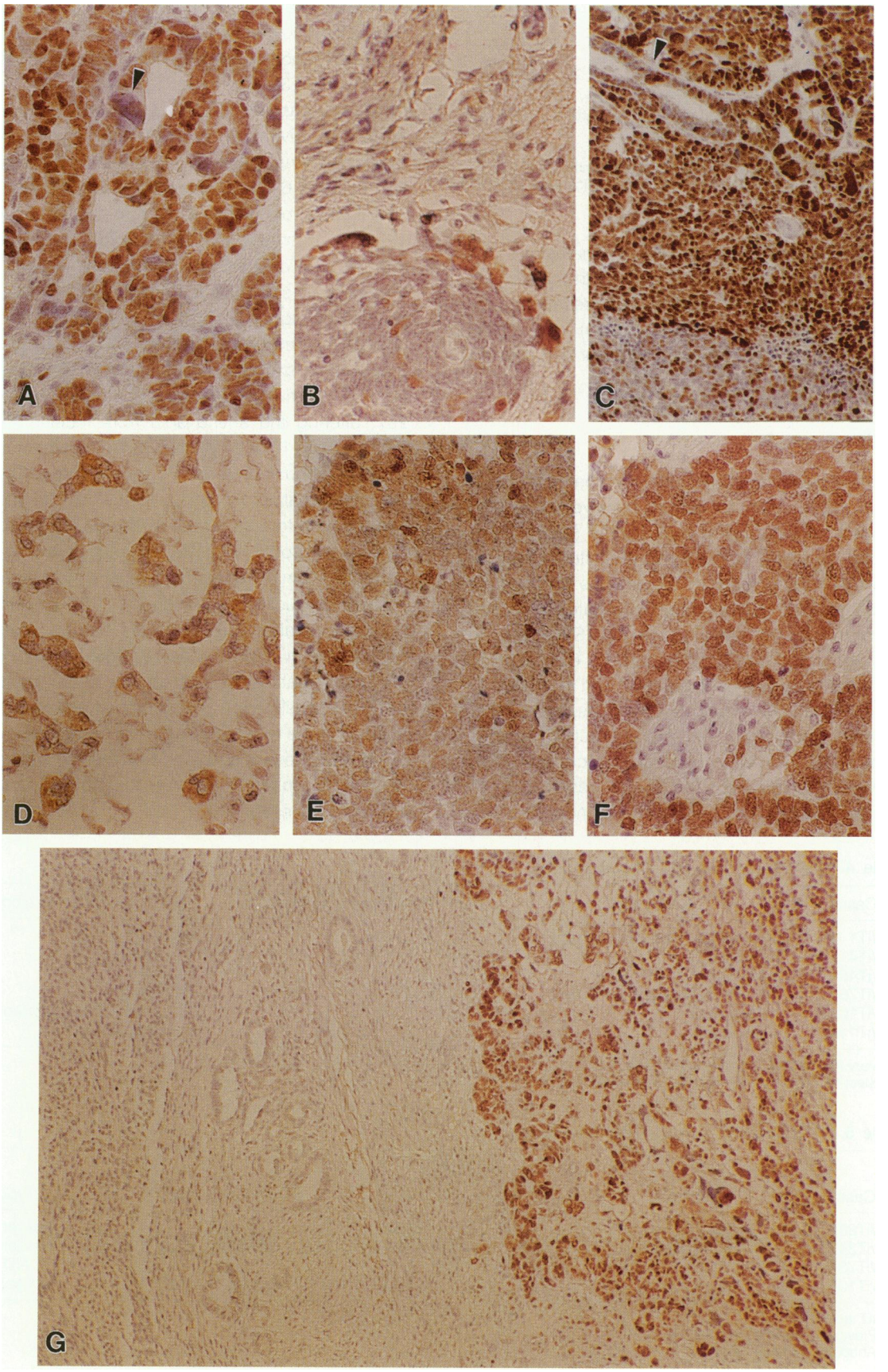
A
B
C
D
E
F
G

Table 3. *Summary of p53 Immunostaining in Primary Wilms' Tumors*

Primary tumor	Percent positive*
Nonrecurrent/Nonmetastatic (3/12 cases)	25%
Recurrent/Metastatic (15/16 cases)	94%

*Difference between nonrecurrent/nonmetastatic and recurrent/metastatic cases is statistically significant by χ^2 test, $P < 0.01$.

the primary tumor as well as the metastasis. The results of the immunostaining on both the pretreatment biopsy and the post-treatment resected specimen (in areas of viable tumor cells) were similar, indicating that chemotherapy did not alter the p53 immunopositivity.

SSCP Analysis

An earlier study by Malkin et al[15] revealed mutations in 2 of 21 (9.5%) Wilms' tumors; however, p53 protein analysis was not performed in this study. We included 12 cases from the original study and analyzed more cases for p53 mutations to determine whether p53 immunopositivity correlates with possible mutations in the p53 gene. The SSCP analysis was performed on 23 Wilms' tumors, examining the p53 coding sequence in exons 2 and 4 through 11. Mutations in p53 were found in 4 cases, all belonging to the metastatic/recurrent group (Table 5), whereas none of the 12 nonmetastatic/nonrecurrent tumors showed any mutations. In 3 of 4 cases, both the primary tumor and the recurrence/metastasis showed similar results. In WiT73, the primary tumor had an intronic mutation whereas the recurrence was negative for p53 mutations. Anaplasia was present in 2 cases, WiT34 and WiT105, of which WiT34 was focal whereas WiT105 was diffusely anaplastic. WiT28 had favorable histology showing features of classical Wilms' tumor. WiT34 contains a true mutation in exon 6 represented by a single base pair substitution from G to A in the first position of codon 199, thus changing the amino acid from glycine to arginine. Besides this true mutation, WiT34 also had a base pair substitution from A to G in the third position of codon 213 in exon 6, which did not change the amino acid arginine and thus represents a polymorphism. Exon 7 mutation in WiT28 showed a transition from T to A in the second position of codon 257, altering the amino acid from leucine to glutamine. Exon 10 of WiT105 revealed a point mutation at a CpG dinucleotide at codon 342 (Figure 2). This nonsense mutation converts cytosine to thymidine and arginine to a stop codon. The p53 immunopositivity was strong in this tumor and, thus, the stop codon in exon 10 does not appear to interfere with protein production, although the p53 protein appears to be altered. This patient died of extensive metastases in a short period of time, indicating the aggressive nature of this tumor. The intron 5 mutation in WiT73 consisted of an insertion of an extra nucleotide, G in the mid-region of intron 5 (396 base pairs away from the last nucleotide of exon 5). The exact effect of this insertion on the message and protein is still under investigation.

Table 4. *Heterogeneity in p53 Immunopositivity*

Case	Category	Number of blocks examined	Number of positive blocks
WiT1	Nonmetastatic/nonrecurrent	13	All negative
WiT41	Metastatic	12	12 positive
WiT49	Metastatic	11	All positive*
WiT76	Nonmetastatic/nonrecurrent	14	All negative
WiT102	Metastatic	11	All positive, heterogeneous
WiT112	Recurrent	10	Focally positive†

*Very few strongly positive tumor cells.
†Most of the tumor was necrotic with few foci of viable cells.

Table 5. *p53 Mutation Analysis in Wilms' Tumors*

Case	Histology	Mutation		Amino acid switch	Type
		Exon	Codon		
WiT28*	Classical	7	257	Leu→Gln	Missense
WiT34*	Anaplastic*	6	199	Gly→Arg	Missense
WiT73	Classical	Intron 5			ND
WiT105	Anaplastic	10	342	Arg→stop codon	Nonsense

ND, not determined.
*Previously reported in Malkin et al.[12]
†Anaplasia present focally.

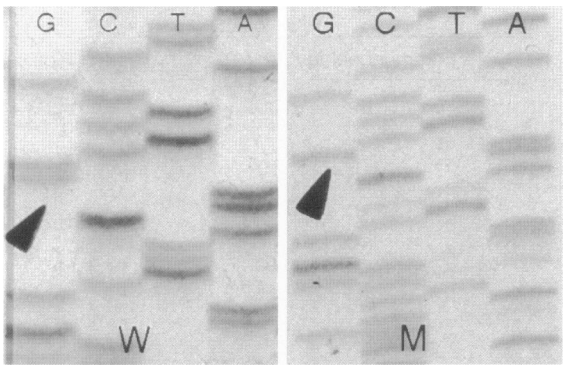

Figure 2. *SSCP and sequence analysis were performed on WiT105. Sequence analysis of exon 10 of WiT105 showing a GC deletion at codon 342* (arrow). Lane M, *WiT105;* lane W, *normal exon 10* (arrow *showing normal sequence of codon 342). Wild-type sequence for codons 340 to 343 is CTA CAA GGC TCT and, for mutant p53, is CTA CAA GCT CTC.*

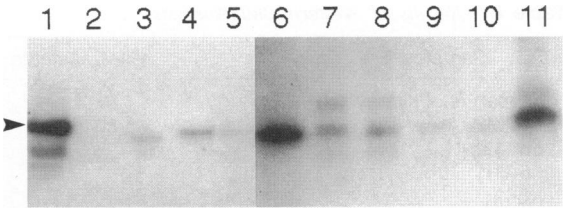

Figure 3. *Western blot analysis for p53 protein in a series of Wilms' tumors and cell lines in comparison with normal fetal kidney.* Lanes 1, *56A1E14;* lane 2, *K562;* lane 3, *WiT112m;* lane 4, *WiT47;* lane 5, *human fetal kidney;* lane 6, *OCIM2;* lane 7, *WiT1;* lane 8, *WiT13;* lane 9, *WiT24;* lane 10, *WiT28r;* lane 11, *WiT34m. Note that K562* (lane 2) *is a leukemic cell line with no p53 expression whereas OCIM2* ((lane 6) *is another leukemic cell line that expresses large amounts of mutant p53 protein. In contrast, the SV40-transformed cell line 56A1E14* (lane 1) *has retained large amounts of wild-type p53 protein. A small amount of p53 is expressed in human fetal kidney. WiT1, WiT13, and WiT24 are nonmetastatic/nonrecurrent Wilms' tumors whereas WiT112m, WiT47, WiT28r, and WiT34m are metastatic/recurrent tumors. In this analysis, only WiT34m* (lane 11) *expressed large amounts of mutant p53 protein.*

The intensity of p53 immunostaining in tumors harboring mutations, WiT28, WiT34, and WiT105, was moderate to strong, whereas in WiT73, it was weak to moderate. The p53 immunostaining was present not only in blastema and tubules but also in the anaplastic cells. In WiT105, the immunostaining was strong in almost all of the cells including the anaplastic cells. WiT28, WiT34, and WiT73 showed a heterogeneous staining pattern with frequent to extensive positive cells intermixed with negative cells. The recurrence of WiT28 exhibited the same mutation as in the primary tumor. However, the number of p53-immunopositive cells as well as the intensity of staining was greater in the recurrence.

Western Blot Analysis

Western blot analysis revealed a band of approximately 53 kd corresponding to p53 protein in human fetal kidney as well as in all of the Wilms' tumors and cell lines (Figure 3). Human fetal kidney showed a weak band indicating that fetal kidney expresses low levels of p53. K562, a leukemic cell line with mutant p53, was negative for p53 protein. The amount of p53 present in each tumor and cell line shown in Table 6 is calculated relative to the amount present in human fetal kidney. Podocytic cell lines 56A1 and 56A1E14 retain large amounts of p53 by virtue of forming complexes with large T antigen. All of the tumors had more p53 protein than human fetal kidney. Although the cell lines showed a close correlation between immunostaining and Western blot results, a similar correlation between the amount of p53 detected by Western blot analysis and p53 immunostaining among the tumor samples was not noted.

Discussion

The results of p53 immunostaining in our study indicate that most of the Wilms' tumors (94%) that are immunopositive for p53 carry a higher risk of metastasis or recurrence in contrast to the majority of p53-negative primary tumors, which did not metastasize or recur. The strength of immunopositive staining was also greater in the former group than in most cases of positive nonmetastatic tumors. In 10 of our cases, the metastases/recurrences were more strongly immunopositive for p53 than their primary tumor, either due to increased numbers of p53-positive cells or to an increase in the intensity of staining. Thus, p53 immunopositivity correlates well with the aggressiveness of Wilms' tumor.

The study by Lemoine et al[14] suggested that p53 immunopositivity in Wilms' tumors indicated expression of wild-type p53 as subsequent reports on mutational analysis demonstrated p53 mutations in only 2 of 21 Wilms' tumors,[15] no mutations in 38 Wilms' tumors analyzed by Waber et al,[16] three mutations in 66 tumors analyzed by Takeuchi et al,[17] and p53 mutations in only the anaplastic Wilms' tumors described by Bardeesy et al.[22] The results of our study also show the presence of p53 protein in all of the Wilms' tumors and human fetal kidney examined by Western blot analysis. Although, in general, no good correlation was found between p53 immunopositivity and Western blot analysis of tumor tissues, in cases like WiT34m, possessing a mutant p53 protein, extensive moderate to strong immunopositivity was indeed matched by a relatively high level of p53 protein detectable by Western blot analysis. In general, immunostaining for p53 revealed either a weak or undetectable reaction in the majority of nonmeta-

Table 6. *Results of Western Blot Analysis*

Samples	Clinical status	Immunonology	Western blot*
Human fetal kidney		–	1
OCIM2		+++	10-fold
56A1E14		++++	100-fold
56A1		++++	100-fold
K562		–	
WiT1	Nonmetastatic/nonrecurrent	–	4-fold
WiT13	Nonmetastatic/nonrecurrent	–	4-fold
WiT24	Nonmetastatic/nonrecurrent	–	1.5-fold
WiT28r	Recurrent	+++	1.5-fold
WiT34m	Metastatic	+++	20-fold
WiT47	Recurrent	+++†	3.5-fold
WiT112m	Metastatic/recurrent	+++	2-fold

*The amount of p53 shown in the tumors is relative to the amount of p53 in human fetal kidney.
†Cytoplasmic positivity.

static tumors as well as in human fetal kidney. A plausible explanation for this discrepancy is that in these tumors wild-type p53 protein, which has a short half-life (20 minutes), is present in low levels that are not detectable by immunostaining on paraffin sections. In most of the recurrent/metastatic tumors, p53 protein may interact with another gene product that stabilizes it or p53 is mutated and has a long half-life (up to 24 hours). In these tumors, p53 protein would be readily detected by immunostaining on formalin-fixed tissue as well as on Western blot analysis; nevertheless, in a few of these tumors, the amount of p53 detected by Western blot was less than that noted on immunostaining. Protein degradation in tumor tissue used for Western blot analysis secondary to necrosis or improper protein preservation might account for this discrepancy. Cell lines and human fetal kidney showed a better correlation between the above two techniques.

The retention of wild-type p53 has been observed in other cancers, for example, in hepatocellular carcinoma, in which wild-type p53 binds to the HBV gene product, Hbx, present in the cytoplasm, and blocks the entry of p53 into the nucleus.[23] Stabilized wild-type p53 has also been detected in colon carcinoma,[24] breast carcinoma,[25] and cell lines from thyroid cancers.[26] Taken together, the studies of Lemoine et al[14] and ours suggest that the presence of wild-type p53 in Wilms' tumors could play a role in Wilms' tumorigenesis and/or malignant progression.

In general, p53 staining was nuclear. Two cases from the metastatic/recurrent group showed focal cytoplasmic positivity, and in one case from the same group, strong cytoplasmic staining was noted with antibody 1801, probably representing inactivated and retained wild-type p53. In past studies, cytoplasmic positivity had been ignored as false positive. Recent studies have confirmed the cytoplasmic expression of p53 not only in tumors[4,25] but also in normal tissues.[27] In fact, in both inflammatory breast and colon carcinomas, the presence of cytoplasmic p53 may represent an independent prognostic indicator.[4,25] p53 mutations in these cases were rare, indicating that retention of wild-type p53 in the cytoplasm likely led to its inactivation. Pezzella et al[27] hypothesize that cytoplasmic p53, which is often seen in a perinuclear location, is bound to $p34^{cdc2}$, a cyclin-dependent kinase, which is present in perinuclear centrosomes. In our case with diffuse cytoplasmic positivity, mutational analysis was negative for p53 mutation. The presence of a band corresponding to p53 on Western blot analysis indicated that cytoplasmic p53 was not merely a staining artifact but most likely altered wild-type p53.

p53 mutations were detected in only four cases, all of which belonged to the metastatic/recurrent group. Two of these tumors had anaplasia, whereas the other two tumors showed no evidence of anaplasia. This observation is in contrast to that reported by Bardeesy et al,[22] which indicated that mutations were detected only in anaplastic Wilms' tumors. One of our anaplastic tumors as well as one case in Bardeesy's report had a similar nonsense mutation in exon 10 that creates a stop codon. Takeuchi et al[17] also reported one case with a mutation in exon 10 that changed glutamine to arginine. Exon 10 has not been considered a hot spot for mutations; in fact, only 4% of all reported p53 mutations occur in exon 10.[3] This region of the gene is involved in oligomerization of p53 protein into dimers and tetramers, promoting its DNA-binding function. Thus loss of oligomerization will lead to loss of transcriptional function of p53 by affecting its DNA-binding ability. In two of our cases, the mutations were in the DNA-binding region itself. In our study and that of Bardeesy et al[22] and Takeuchi et al,[17] most mutations were found in the conserved domains. However, given that the independent observations of exon 10

mutations in Wilms' tumors constitute a significant fraction of all p53 mutations in Wilms' tumors, exon 10 should be examined more critically in Wilms' tumors.

Bardeesy et al[28] further noted that in the majority of their cases mutations were restricted to the anaplastic population, although in one case the mutation became homozygous in the anaplastic cells, indicating that progression to anaplasia is associated with clonal expansion of cells with p53 mutations. There was marked attenuation of apoptosis in the anaplastic population as compared with non-anaplastic cells, indicating that these cells are additionally selected to survive and proliferate. Most of the tumors were negative for p53 mutations but were immunopositive for p53. This discrepancy may indicate that 1) other p53 mutations may exist in the intronic regions not examined in this study (in fact, one of our cases had a mutation in intron 5), 2) the tumor sample processed for SSCP did not contain the mutant p53 cell population, or 3) the tumor overexpresses or retains wild-type p53.

In general, Wilms' tumors are large tumors and very heterogeneous with varying patterns of differentiation and proliferation in the same tumor. A sample from one area may not represent the entire tumor. In one of our cases, only 2 of 19 tumor sections had anaplasia and were strongly immunopositive for p53 whereas the remaining 17 blocks of the tumor were negative. Interestingly, in the two p53-immunopositive sections that contained anaplastic cells, only the anaplastic cells were strongly positive for p53. Tumor heterogeneity was also seen in a large number of our primary as well as the metastatic/recurrent tumors. In another case (WiT96), the entire primary tumor section was negative to weakly positive for p53 except for a few foci of tumor cells at the periphery invading the capsule. The patient later presented with lung metastasis. In such cases, most of the tumor sample will probably be negative for p53 mutations. These observations indicate that, in large tumors exhibiting heterogeneous morphology like Wilms' tumor, the results of immunohistochemistry and molecular analysis on a single tumor area may not be representative of the entire tumor. Therefore, for diagnostic and especially prognostic purposes, we recommend that p53 immunostaining should be performed on multiple blocks and in particular on areas exhibiting anaplasia or an invasive phenotype.

Finally, for Wilms' tumors with significant p53 immunopositivity and no indication of p53 mutations, the retention and inactivation of p53 can be due to its complexing with proteins, eg, WT1, MDM-2, and Bcl-2. These proteins are known to interact with p53 *in vitro* and modulate its function. As discussed previously, WT1 complexes with p53 *in vitro* and *in vivo*.[19] As only 10 to 16% of Wilms' tumors possess a mutation in WT1[29] and expression of WT1 is evident in most tumors,[19] it is possible that WT1 protein, both normal and altered, may complex with p53. It is relevant, therefore, that accumulated p53 protein can be detected in the majority of Wilms' tumors.[14] MDM2, an oncogene product, inhibits the ability of p53 to stimulate transcription by binding to a region that nearly coincides with the p53 acidic activation domain, thus concealing the activation domain of p53 from cellular transcription machinery.[11] p53 also interacts with Bcl-2 in the apoptotic pathway. Ryan et al[10] noted loss of G1 arrest function of p53 when Bcl-2 was present, and c-*myc* and *bcl-2* genes cooperate to inhibit p53 functions by retaining it in cytoplasm during the critical period of G1. Recent studies by Anderson et al[30] demonstrated that gliomas with wild-type p53 overexpress *bcl-2*, indicating the possible inactivation of wild-type p53 by Bcl-2. Preliminary results of Bcl-2 immunostaining on our tumors revealed significant Bcl-2 immunopositivity in all Wilms' tumors, suggesting its role in maintaining the tumor cell population. However, most of the tumors with wild-type p53 expressed a higher amount of Bcl-2 as compared with the ones with p53 mutations (manuscript in preparation). Our preliminary studies suggest that in Wilms' tumors Bcl-2 appears to play a similar role in tumor development by inhibiting wild-type p53 from participating in the process of apoptosis and in turn permitting unrestricted tumor cell proliferation.

In conclusion, the results of our study identify p53 immunopositivity as a strong prognostic indicator in Wilms' tumor. In addition to contributing to malignant progression, wild-type p53 may also be involved in earlier events of Wilms' tumorigenesis. It is relevant that a similar suggestion has recently been proposed for another embryonal tumor, neuroblastoma.[31]

Acknowledgments

We thank Dr. J-D. Sraer for kindly providing the 56A1 cell line, Dr. S. Benchimol for kindly providing the cell lines K562 and OCIM2, and Dr. C. Campbell for the generous gift of the 56A1E14 transfected cell line. We also thank E. Sexsmith for technical advice and M. Starr for assistance with photography.

References

1. Coppes MJ: The management and biological behavior of Wilms' tumor. Int Rev Exp Pathol 1994, 35:149–176
2. Grundy PE, Telzerow PE, Breslow N, Moksness J, Huff V, Paterson MC: Loss of heterozygosity for chromosome 16q and 1p in Wilms' tumors predicts an adverse outcome. Cancer Res 1994, 54:2331–2333
3. Greenblatt MS, Bennett WP, Hollstein M, Harris CC: Mutations of p53 tumor suppressor gene: clues to cancer etiology and molecular pathogenesis. Cancer Res 1994, 54:4855–4878
4. Bosani S, Viale G, Bossi P, Maggioni M, Coggi G, Murray JJ, Lee AKC: Cytoplasmic accumulation of p53 protein: an independent prognostic indicator in colorectal adenocarcinoma. J Natl Cancer Inst 1994, 86: 681–687
5. Thor AD, Moore DH II, Edgerton SM, Kawaski ES, Reihsaus E, Lynch HT, Marcus JN, Schwartz L, Chen L-C, Mayall BH, Smith HS: Accumulation of p53 tumor suppressor gene protein: an independent marker of prognosis in breast cancers. J Natl Cancer Inst 1992, 84: 845–855
6. Quinlan DC, Davidson AG, Summers CL, Warden HE, Doshi HM: Accumulation of p53 protein correlates with poor prognosis in human lung cancer. Cancer Res 1992, 52:4828–4831
7. Kastan MB, Zhan Q, El-Deiry WS, Carrier F, Jacks T, Walsh WV, Plunkett BS, Vogelstein B, Fornace AJ: A mammalian cell cycle check point pathway utilizing p53 and GADD45 is defective in ataxia-telangiectasia. Cell 1992, 71:587–591
8. Harper JW, Adami GR, Wei N, Keyomarsi SJ, Elledge SJ: The p21 *cdk*-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. Cell 1993, 75:805–816
9. Miyashita T, Reed JC: Tumor suppressor p53 is a direct transcriptional activator of the human *bax* gene. Cell 1995, 80:293–299
10. Ryan JJ, Prochownik E, Gottleib CA, Apel IJ, Merino R, Nunez G, Clarke MF: c-*myc* and *bcl-2* modulate p53 function by altering p53 subcellular trafficking during the cell cycle. Proc Natl Acad Sci USA 1994, 91:5878–5882
11. Oliner JD, Pietenpol JA, Thiagalingam S, Gyuris J, Kinzler KW, Vogelstein B: Oncoprotein mdm2 conceals the activation domain of tumor suppressor p53. Nature 1993, 362:857–860
12. Spandau DA: Distinct conformations of p53 are observed at different stages of keratinocyte differentiation. Oncogene, 1994, 9:1861–1868
13. Jeffers MD, Richmond J, Farquharson M, McNicol AM: p53 immunoreactivity in cervical intraepithelial neoplasia and non-neoplastic cervical squamous epithelium. J Clin Pathol 1994, 47:1073–1076
14. Lemoine NR, Hughes CM, Cowell JK: Aberrant expression of the tumor suppressor gene p53 is very frequent in Wilms' tumor. J Pathol 1992, 168:237–242
15. Malkin D, Sexsmith E, Yeger H, Williams BRG, Coppes MJ: Mutations of p53 tumor suppressor gene occur infrequently in Wilms' tumor. Cancer Res 1993, 54: 2077–2079
16. Waber PG, Chen J, Nisen PD: Infrequency of *ras*, WT1 or Rb gene alterations in Wilms' tumors. Cancer 1993, 72:3733–3738
17. Takeuchi S, Bartram CR, Ludwig R, Royer-Pokora B, Schneider S, Imamura J, Koeffler HP: mutations in p53 in Wilms' tumors. Modern Pathol 1995, 8:483–487
18. Call KM, Glaser T, Ito CY, Buckler AJ, Pelletier J, Haber DA, Rose EA, Kral A, Yeger H, Lewis WH, Jones C, Housman DE: Isolation and characterization of a Zinc finger polypeptide gene at the human chromosome 11 Wilms' tumor locus. Cell 1990, 60: 509–520
19. Yeger H, Cullinane C, Flenniken A, Chilton-MacNeill S, Campbell C, Huang A, Bonetta L, Thorner P, Williams BRG: Coordinate expression of Wilms' tumor genes correlates with Wilms' tumor phenotypes. Cell Growth Differ 1992, 3:855–864
20. Maheshwaren S, Park S, Benard A, Morris JF, Rauscher F III, Hill DE, Haber DA: Physical and functional interactions between WT1 and p53. Proc Natl Acad Sci USA 1993, 90:5100–5104
21. Delarue F, Virone A, Hagege J, Lacave R, Peraldi M-N, Adida C, Rondeau, Feunteun J, Sraer J-D: Stable cell line of T-SV40 immortalized human glomerular visceral epithelial cells. Kidney Int 1991, 40:906–912
22. Bardeesy N, Falkoff D, Petruzzi MJ, Nowak N, Zabel B, Adam M, Aguiar MC, Grundy P, Shows T, Pelletier J: Anaplastic Wilms' tumor, a sub-type displaying poor prognosis, harbours p53 gene mutations. Nat Genet 1994, 7:91–97
23. Ueda H, Ullrich SJ, Gangeni D, Kappel CA, Ngo L, Feitelson MA, Jay G: Functional inactivation but not structural mutations of p53 causes liver cancer. Nat Genet 1995, 9:41–47
24. Cripps KJ, Purdie CA, Carder PJ, White S, Komine K, Bird CC, Wyllie AH: A study of stabilization of p53 protein *versus* point mutations in colorectal cancers. Oncogene 1994, 9:2739–2743
25. Moll UM, Riou G, Levine AJ: Two distinct mechanisms alter p53 in breast cancer: mutation and nuclear exclusion. Proc Natl Acad Sci USA 1992, 89: 7262–7266
26. Wyllie FS, Haughton MF, Blaydes JP, Schlumberger M, Wynford-Thomas D: Evasion of p53 mediated growth control occurs by three alternative mechanisms in transformed thyroid epithelial cells. Oncogene 1995, 10:49–59
27. Pezella F, Micklem K, Turley H, Pulford K, Jones M, Kocialkowski S, Delia D, Aiello A, Bicknell R, Smith K, Harris L, Gattner KC, Mason DY: Antibody for detecting p53 protein by immunohistochemistry in normal tissues. J Clin Pathol 1994, 47:592–96
28. Bardeesy N, Beckwith JB, Pelletier J: Clonal expansion and attenuated apoptosis in Wilms' tumors are associ-

ated with p53 gene mutations. Cancer Res 1995, 55: 251–219
29. Pelletier J: Molecular genetics of Wilms' tumor: insights into normal and abnormal renal development. Can J Oncol 1994, 4:262–272
30. Anderson LM, Castleberg RL, Harsh GR IV, Louis DN, Hanson JW: Human gliomas with wild type p53 express *bcl*-2. Cancer Res 1995, 55:999–1001
31. Moll UM, LaQuaglia M, Benard J, Riou G: Wild-type p53 protein undergoes cytoplasmic sequestration in undifferentiated neuroblastomas but not in differentiated tumors. Proc Natl Acad Sci USA 1995, 92:4407–4411