Advances in Brief

Two Molecular Subgroups of Wilms' Tumors with or without *WT1* Mutations[1]

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Abstract

Purpose: **Wilms' tumors (WTs) exhibit more than one pattern of differentiation, each of which is associated with distinctive clinical features and treatment responses. Mutations in the *WT1* gene are found predominantly in WTs with stromal histology. To better understand the biological and clinical features in different WTs, we have analyzed WTs with and without *WT1* mutations for a set of parameters.**

Experimental Design: **Twenty-two new WTs were analyzed for *WT1* mutations by PCR single-strand conformational polymorphism. Five tumors with *WT1* mutations and six tumors without *WT1* mutations were studied for the presence of *WT1* transcripts and protein as well as for the expression of differentiation markers.**

Results: **Two new *WT1* mutations were identified in stromal-predominant tumors, and none were identified in the other histological subtypes. Tumors with *WT1* mutations expressed mutant messages, cytoplasmic truncated WT1 proteins, and muscle markers. In contrast, blastemal-predominant tumors without mutations showed nuclear WT1 protein staining. Both tumor types were positive for markers of early-induced mesenchyme and one marker of uninduced mesenchyme, but blastemal-predominant tumors also expressed cytokeratin, suggesting that these are further along the epithelial differentiation pathway.**

Conclusions: **Our data show that the two-hit inactivation of WT1 is operative in stromal-predominant WTs. Cells without functional nuclear WT1 protein start a faulty differentiation program. In contrast, blastemal-predominant tumors express wild-type WT1 and show early signs of epithelialization. The extensive rhabdomyomatous differentiation and the presence of *WT1* mutations may be used as a diagnostic tool to identify a tumor subtype that seems to respond poorly to chemotherapy. These studies provide a foundation for improvement in tumor classification and ultimately for the development of more individualized tumor treatments.**

Introduction

WT[3] or nephroblastoma is the most frequent renal tumor of childhood, affecting 1 in 10,000 children, usually before the age of 5 years (1). Histologically, it mimics various stages of nephrogenesis, indicating aberrant differentiation of a multipotential mesenchymal renal stem cell (2). Most tumors contain a mixture of undifferentiated blastemal cells, as well as differentiated epithelial and stromal elements. However, heterologous components such as striated and smooth muscle, cartilage, bone, or adipose tissues are observed in approximately 10% of WTs. Tumors with extensive rhabdomyogenesis have been called "fetal rhabdomyomatous type" and occur in younger children and are frequently bilateral (3, 4).

Nephrogenic rests, considered to be precursor lesions of WTs, are found in 30–44% of tumors (2). ILNRs, located deep within the parenchyma and indicating an earlier developmental disturbance, are associated with intralobar WTs containing heterologous elements. ILNR-like WTs are observed in patients with sporadic WT often associated with GUs, anirdia, or Denys-Drash syndrome. PLNRs occur at the periphery of the renal lobe later in renal developmental and are associated with perilobar WTs lacking heterologous elements. PLNR-like WTs are often found in patients with hemihypertrophy and Beckwith-Wiedemann syndrome. Recently, Ravenel *et al.* (5) identified LOI of *IGF2* as a valuable molecular tool for the classification of these two pathological subtypes. LOI and altered expression of *IGF2* were found mainly in PLNR-like tumors.

The *WT1* gene, a ZF transcription factor, was found to be mutated in 10–15% of sporadic WTs (for a review, see Ref. 6). Recently, we identified a high percentage of *WT1* germline mutations in stromal-predominant/ILNR-like tumors (7). Loss of the wild-type allele (LOH) was found in 7 of 13 analyzed cases, confirming Knudson's two-hit hypothesis for this subclass of WTs. All but one were nonsense mutations predicted to result in protein truncation. Only one missense mutation was found in a blastemal-predominant tumor, suggesting that muta-

Received 11/4/02; accepted 2/10/03.

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[1] Supported by the Elterninitiative "Kinderkrebsklinik e.V.," Düsseldorf.

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[3] The abbreviations used are: WT, Wilms' tumor; ILNR, intralobar rest; GU, genitourinary malformation; PLNR, perilobar rest; LOI, loss of imprinting; LOH, loss of heterozygosity; GST, glutathione *S*-transferase; RT-PCR, reverse transcription-PCR; EGFP, enhanced green fluorescent protein; ZF, zinc finger; aa, amino acid(s).

tions in *WT1* may play a minor role in the development of this subtype (7).

Alternative splicing of the *WT1* gene affecting exons 5 and 9 results in the synthesis of four major isoforms of the protein with different nucleic acid binding and transcriptional properties (8–10). The WT1 protein has many postulated functions both in regulating transcription and in posttranscriptional RNA processing (reviewed in Ref. 11). It is targeted to the nucleus by the presence of at least two nuclear localization signals, one lying within ZF I and one in ZF II and/or III (12). Reporter construct experiments have identified many putative *in vivo* target genes for both transcriptional activation and repression by WT1 (13, 14). However, significant variation has been found in these experiments, depending on the cellular context and the promoter used to express WT1 (15). At present, little is known about the *in vivo* situation. By analyzing *WT1* missense mutants, English and Licht (16) have found that activation and not repression by WT1 is critical for growth control. WT1 is predominantly expressed in the developing kidney, where it may play a role in mesenchymal to epithelial transition (17, 18). This hypothesis is supported by the findings that in WTs missing functional WT1, the mesenchymal cells can no longer differentiate into epithelia but can take on different fates, either stromal or myogenic (7, 19). During early nephrogenesis, a low level of WT1 is first detected in the uninduced mesenchyme and then increases dramatically during induction (20). Expression then becomes restricted to the posterior part of newly formed epithelium and is limited to the podocytes of the adult kidney. In tumors, WT1 is seen in the malignant counterparts of those elements that express the protein during normal development (that is, blastema and glomeruloid structures) but is absent from stromal cells (21).

To confirm and extend our previous observations that most stromal-predominant/ILNR-like tumors are caused by *WT1* mutations, we have studied more patients for mutations and examined these for the expression of a mutant *WT1* transcript and protein. Furthermore, we have studied the expression of a set of specific kidney development/differentiation markers as a first approach to identify a set of useful classification markers for WT.

Patients and Methods

Patients, Tissue, DNA, RNA, and Protein Isolation. Snap-frozen material from a total of 86 WTs was analyzed for *WT1* mutations [64 from a previously published study (7) and 22 reported here]. The parents of the patients were informed about the study and gave written consent. All patients were enrolled in the International Society of Pediatric Oncology SIOP9/GPOH study and received preoperative chemotherapy according to the SIOP9/GPOH protocol (22). Histological typing of the tumor specimens was determined after chemotherapy by two of us (I. L. and D. H). DNA, RNA, and protein were isolated simultaneously as described previously (23). Briefly, frozen tissue was finely ground with a mortar and pestol in liquid nitrogen and homogenized in 4 M guanidinium thiocyanate containing 0.5% sodium *N*-laurylsarcosine, 0.7% β-mercaptoethanol, and 25 mM EDTA (pH 7.0). The homogenate was loaded onto a cushion of 5.7 M CsCl and 0.1 M EDTA (pH 7.0; treated with diethyl pyrocarbonate), and RNA was pelleted overnight at 20,000 rpm. Proteins were recovered from the top part of the tube and dialyzed against 50% glycerol, 50 mM NaCl, 10 mM HEPES (pH 7.9), 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM DTT. DNA was isolated from the layer above the CsCl cushion, phenol-$CHCl_3$ extracted, and precipitated. The CsCl was removed from the centrifuge tube, and the RNA pellet at the bottom of the tube was rinsed with 70% ethanol and resuspended in H_2O.

Mutation Analysis of the *WT1* Gene. Of the 86 collected tumor specimens, 64 were previously analyzed for *WT1* mutations by PCR single-strand conformational polymorphism and direct sequencing (7). Here we have analyzed 22 additional patients with the same method.

Northern Blot. Total RNA (10 μg) was loaded on a 1% agarose gel in 4-morpholinepropanesulfonic acid buffer containing ethidium bromide and 0.22 M formaldehyde. Separation was at 30 V overnight. After photography, the gel was rinsed in H_2O for 5 min and transferred overnight in 10× SSC to gene screen membranes (New England Nuclear, Dupont). After transfer, the RNA was UV-cross-linked, and the filter was baked for 2 h at 80°C. Preparation of RNA, hybridization probes, and conditions were as described previously (24).

RT-PCR. For the identification of mutant *WT1* transcripts, different oligonucleotide primers were used: GW21 (5′-CCGCCTCACTCCTTCATC-3′) and GW25 (5′-ACCG-AGTACTGCTGCTCAC-3′); and Sus39 (5′-TCGCAATCA-GGGTTACAG-3′) and Sus40 (5′-GTGGGTCTTCAGGTG-GTC-3′); P1 and P4 were as described previously (25). Five μl of cDNA were amplified in a 50-μl PCR reaction containing 25 pmol of each primer, 200 μM each deoxynucleotide triphosphate, 1× Taq DNA polymerase buffer, and 1 unit of recombinant Taq polymerase (Life Technologies, Inc.). PCR cycle conditions were as follows: (*a*) for P1/P4, 3 min × 94°C; 30 cycles of 94°C × 1 min, 60°C × 2 min, and 72°C × 2 min; followed by a final elongation step at 72°C for 10 min; and (*b*) for GW21/25 and Sus 39/40, 3 min × 94°C; 30 cycles of 94°C × 1 min, 53°C × 1 min, and 72°C × 1 min; followed by a final elongation step at 72°C for 10 min.

Restriction Enzyme Digestion. Wild-type or mutant *WT1* alleles and transcripts were identified by digesting 15 μl of PCR products with enzymes specific for the mutant site. For digestion with *Bsi*EI (New England Biolabs), $MgCl_2$ was adjusted to 10 mM final concentration. Products were visualized on a 3% NuSieve-agarose gel stained with ethidium bromide.

Antibodies. For immunohistochemistry, the following antibodies were used in this study: anti-collagen I (COL-1; 1:5000; Sigma); anti-collagen IV (CIV 22; 1:50; DAKO); anti-cytokeratin (KL1; 1:10; Immunotech); anti-desmin (D33; 1:250; DAKO); anti-fibronectin (1:2000; DAKO); anti-LamA (4C7; 1:300; DAKO); anti-MyoD1 (5.8A; 1:10; DAKO); anti-myogenin (F5D; 1:30; DAKO); anti-SA (Sr-1; 1:10; DAKO); anti-vimentin (Vim3B4; 1:10; Roche Diagnostics); and anti-WT1 [C19 (1:500); WT-180 (1:100); Santa Cruz Biotechnology).

Immunostaining. Immunoperoxidase staining was performed using the Labeled StreptAvidin Biotin kit from DAKO according to the manufacturer's recommendations. After fixation in acetone or a 1:1 mixture of acetone and methanol for 10 min at –20°C and blocking of unspecific sites, sections were stained with the first antibody for 30 min followed by incubation

with the biotinylated secondary antibody and the streptavidin-peroxidase reagent. Negative controls consisted of consecutive tissue sections of each case in which the primary antibody was omitted.

Neutralization of WT1 Antibody. To demonstrate the specificity of the WT180 antibody in immunostaining, experiments were repeated after preincubation of the antibody with an excess of a purified WT1 protein for 1 h at room temperature. A fusion plasmid of GST and WT1 (26) was used for bacterial expression, and purification of the GST-tagged recombinant WT1 protein was performed using GSTrap columns from Amersham Pharmacia according to the manufacturer's recommendations.

Western Blot. Total protein extracts were separated on 10% SDS-PAGE and transferred to a nitrocellulose membrane (Schleicher & Schuell). The proteins were visualized using enhanced chemiluminescence (Amersham Pharmacia Biotech) as described by the manufacturer's recommendations. The membrane was sequentially probed with three primary antibodies: (*a*) WT180 (1:500; Santa Cruz Biotechnology); (*b*) WTC19 (1:1000; Santa Cruz Biotechnology); and (*c*) α-tubulin (1:500; Sigma).

Cell Culture and Transient Transfection. NIH3T3 and Cos-7 cells were cultured in DMEM (Biochrom) containing 10% FCS (Biochrom). For transfection, the cells were seeded at a density of $1–3 \times 10^5$ cells/coverslip and transfected according to the manufacturer's (Roche Applied Science) recommendations by using 3 μl of FuGENE 6 and 1 μg of plasmid DNA. The culture medium was changed after 16 h, and the cells were maintained for another 48 h to allow optimal expression of the fusion protein. The transfected cells were examined under a Zeiss Axiophot fluorescence microscope.

Plasmid Construction. By using different pairs of primers, three fragments were PCR amplified from a WT1 cDNA clone named CMV-WIT2F (kindly provided by Dr. Paul Baird, Australia) and cloned into pCRII from the TA cloning kit (Invitrogen). The primers used to generate fragments were as follows: (*a*) for the WT450 fragment, VS1a (5′-CGAATTCTATGGGCTCCGACGTGCGGGA-3′) and VS5a (5′-CAGTCGACTCAAAGCGCCAGCTGGAGTTT-3′); (*b*) for the WT380 fragment, VS1a and VS4a (5′-CAGTCGACTCAACCTGTATGTCTCCTTTG-3′); and (*c*) for WT220, VS1a and VS3a (5′-CAGTCGACTCACCT-CAGCAGCAAAGCCTG-3′). The amplicons were sequenced with IRD800-labeled M13 primers on a LICOR automatic sequencer to verify that no mutations had been introduced. All primers contained a start or a stop codon as well as *Eco*RI or *Sal*I restriction site that permitted subsequent directional in-frame cloning into pEGFP-C1 (Clontech), generating the following plasmids: (*a*) pEGFP-WT450, representing the wild-type WT1 isoform containing exon 5 and KTS; (*b*) pEGFP-WT380, representing a truncated WT1 form with a stop codon in exon 9; and (*c*) pEGFP-WT220, representing a truncated WT1 form with a stop codon in exon 3.

Results

Mutations in the *WT1* Gene. Here we report the analysis of 22 WTs (3 stromal-predominant tumors, 8 blastemal-predominant tumors, 5 tumors with triphasic histology, and 6 regressive tumors). Two new mutations were found in bilateral stromal-predominant tumors. One male patient (patient 9179) with genital abnormalities had a constitutional nonsense mutation in exon 8 (R362X) and one female (MD37) had a nonsense mutation in exon 6 (R295X) in the tumor. No blood DNA was available to test for a constitutional mutation.

Taken together with our previous study (7), we have now analyzed a total of 86 WT patients and found *WT1* mutations/deletions in 21 cases (24%). The mutation frequency was significantly different among the various histological subtypes: mutations were seen in 63% of tumors of stromal-predominant histology; 14% of tumors of triphasic histology; and 6% of tumors of blastemal-predominant histology. Of the 21 mutations, 17 were present in the germline, and 3 of these were deletions in patients with Wilms' tumor, aniridia, genital malformation, mental retardation syndrome. Six of 10 stromal tumors with *WT1* mutations showed LOH of 11p13 markers, and 1 tumor had a second somatic mutation.

These results confirm that the presence of *WT1* mutations correlates with the specific histological subtype of stromal-predominant or ILNR-like WTs.

Expression and Subcellular Localization of Mutant *WT1* Transcripts and Proteins in WTs. Using our previously described protocol (23), DNA, RNA, and protein were extracted simultaneously, allowing us to analyze the same material for *WT1* mutations as well as for RNA and protein expression. To examine whether tumors with *WT1* mutations express *WT1*, we first performed Northern blot analyses on WTs of different histology. As has been observed previously (21), tumors with blastemal-predominant histology expressed high levels of *WT1*, whereas stromal-predominant tumors expressed only low or undetectable levels of *WT1*. A Northern blot of different WTs probed for *WT1* and actin is shown in Fig. 1*A*. In the three stromal-predominant WTs 9274, 9177, and 9184, a *WT1* transcript with the highest level can be seen in 9274, where a smaller amount of RNA was loaded when compared with the actin signal. It was estimated that a mRNA can be detected by Northern blots if it comprises 0.001% of the total mRNA, corresponding to 50 copies/cell (27). Therefore, these stromal-predominant tumors contain an amount of RNA within the normal range of a low abundant message. From the right tumor of patient 9200, which shows no signal on the Northern blot (Fig. 1*A*) we have performed real-time RT-PCR to quantify *WT1* expression level. The expression was 178-fold lower than that in blastemal-predominant tumor 9168 (data not shown). Because tumor 9200 contains a mixture of cells, some of which most likely do not express *WT1*, we can deduce that this tumor contains about 5 copies or less of *WT1* RNA, which explains why, in this case, no transcript can be seen in Northern blots (Fig. 1*A*).

To study whether the detected *WT1* RNA corresponds to the mutant transcript, we performed RT-PCR and restriction enzyme digests. Four tumors were analyzed, and in all a mutant *WT1* transcript was detected (for tumor 9274, see Ref. 28). Fig. 1*B* shows an example for patient 9318, where a constitutional mutation (C>A in exon 7) causes the loss of a *Bsi*EI restriction site. The analysis was performed on tumor and blood DNA (DNA-PCR) and tumor RNA (RT-PCR) using two different

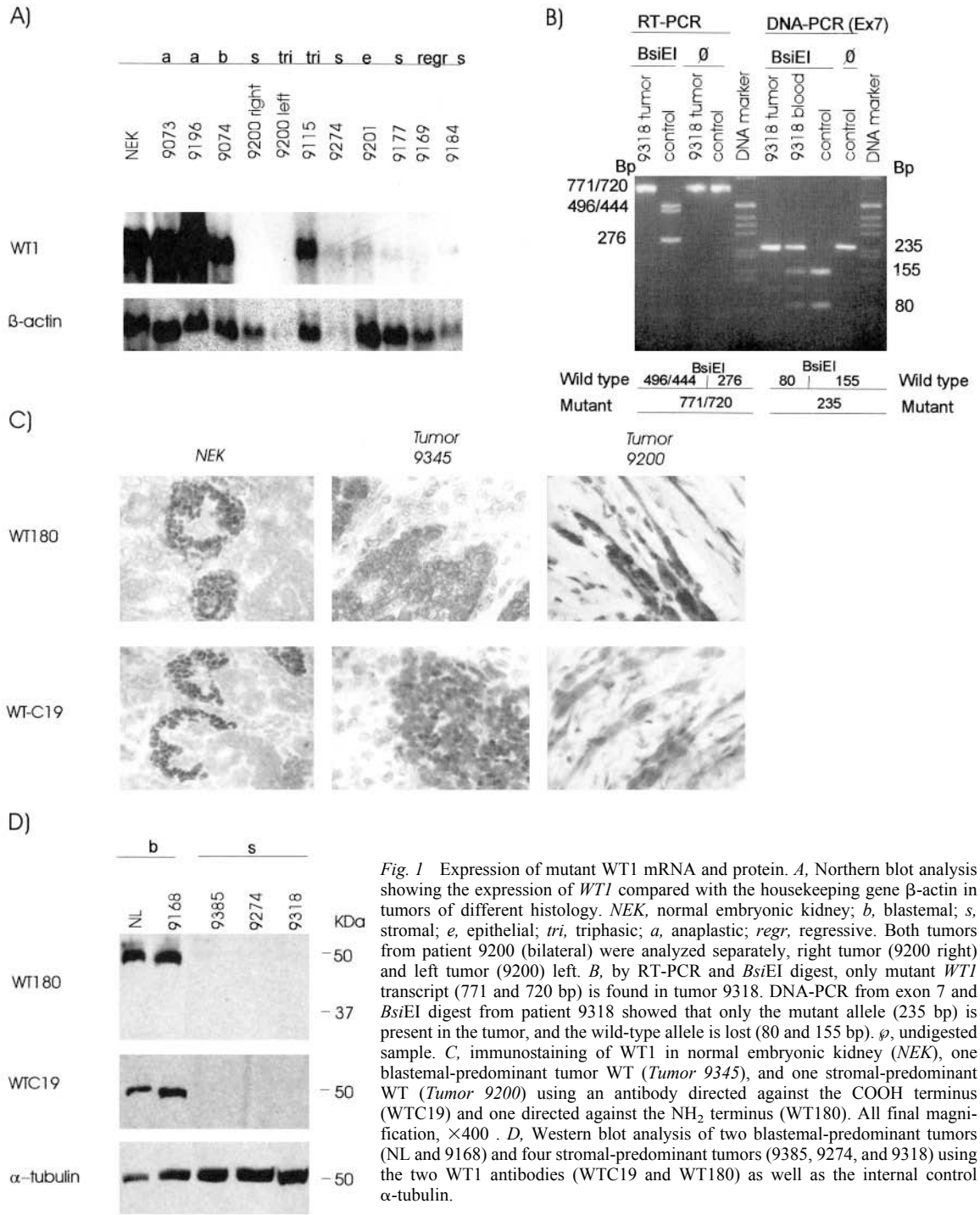

Fig. 1 Expression of mutant WT1 mRNA and protein. *A,* Northern blot analysis showing the expression of *WT1* compared with the housekeeping gene β-actin in tumors of different histology. *NEK,* normal embryonic kidney; *b,* blastemal; *s,* stromal; *e,* epithelial; *tri,* triphasic; *a,* anaplastic; *regr,* regressive. Both tumors from patient 9200 (bilateral) were analyzed separately, right tumor (9200 right) and left tumor (9200) left. *B,* by RT-PCR and *Bsi*EI digest, only mutant *WT1* transcript (771 and 720 bp) is found in tumor 9318. DNA-PCR from exon 7 and *Bsi*EI digest from patient 9318 showed that only the mutant allele (235 bp) is present in the tumor, and the wild-type allele is lost (80 and 155 bp). *φ*, undigested sample. *C,* immunostaining of WT1 in normal embryonic kidney (*NEK*), one blastemal-predominant tumor WT (*Tumor 9345*), and one stromal-predominant WT (*Tumor 9200*) using an antibody directed against the COOH terminus (WTC19) and one directed against the NH_2 terminus (WT180). All final magnification, ×400 . *D,* Western blot analysis of two blastemal-predominant tumors (NL and 9168) and four stromal-predominant tumors (9385, 9274, and 9318) using the two WT1 antibodies (WTC19 and WT180) as well as the internal control α-tubulin.

pairs of primers. As seen in Fig. 1*B, right*, the tumor DNA only shows the mutant allele resulting in a 235-bp undigested fragment of exon 7. Amplification of the cDNA and digestion with *Bsi*EI leads to an undigested 771- and 720-bp fragment, containing or lacking exon 5, respectively (Fig. 1*B, left*). This fragment represents the mutant transcript. A schematic drawing of the fragment sizes after digestion of the DNA-PCR and RT-PCR product is given below the figure.

Next we analyzed whether a truncated protein is synthesized from these mutant messages. By immunohistochemistry, we studied frozen sections for the presence of a wild-type protein using the WTC19 antibody directed against the COOH

Table 1 Expression of differentiation markers in two histological subclasses of WTs
A comparison to the differentiation state according to the kidney development database is shown.

	Kidney differentiation markers						Other markers			
	Cytokeratin	Vimentin	Laminin A	Collagen IV	Collagen I	Fibronectin	WT1	Desmin	Sarcomeric actin	Myogenin
The kidney development database (31)										
UMM	−	+	−	−	+	+	+	−	−	−
Early-induced MM	−	+	−	−	+	+	+	−	−	−
Condensed MM	+	Goes	+	+	−	−	+	−	−	−
Stroma	−	+	−	+	?	+	−	−	−	−
Stromal predominant WTs with *WT1* mutations										
9200	−	−	+	+	−	(+)	+	+	+	+
9614	−	−	+	+	−	(+)	+	+	+	+
9177	−	(+)	+	+	−	−	+	+	+	+
9385	−	−	+	+	−	(+)	+	+	+	+
9318	−	−	+	+	−	(+)	+	+	+	+
Blastemal predominant WTs without *WT1* mutations										
9345	(+)	+	+	+	−	(+)	+	−	−	−
9168	−	+	+	+	−	(+)	+	−	−	−
9545	(+)	+	+	+	−	(+)	+	−	−	−
9474	−	+	−	+	(+)	(+)	+	−	−	−
9497	(+)	(+)	+	+	−	(+)	+	−	−	−
9254	(+)	+	+	+	+	(+)	+	−	−	−

terminus and the presence of a truncated as well as a wild-type protein using the WT180 antibody against the NH_2 terminus. As described previously (17, 20) WT1 staining was restricted to the nucleus of podocytes and their precursors in normal embryonic kidney (Fig. 1*C, NEK*), demonstrating that both antibodies were specific. Although all six blastemal-predominant tumors showed nuclear staining with both antibodies (tumor 9345 in Fig. 1*C* and Table 1), no immunoreactivity was seen with the COOH-terminal antibody in stromal-predominant WTs (tumor 9200 in Fig. 1*C* and Table 1), demonstrating that no wild-type protein was synthesized. Using the NH_2-terminal antibody, all five cases with nonsense mutations had cytoplasmic staining in cells with a skeletal muscle morphology. In Fig. 1*C,* tumor 9200 shows a positive reaction with the WT180 antibody, and it can be seen that the cells, which were cut longitudinally, show the elongated morphology of muscle cells with small nuclei and staining of the cytoplasm. No reactivity was seen in the surrounding stroma. Most of these tumors had lost the wild-type allele, confirming that only a truncated mutant WT1 protein is synthesized. The specificity of the NH_2-terminal WT180 antibody was examined by the use of three different batches and by preabsorption with a purified GST-WT1 fusion protein containing aa 1–181 before incubation on tissue sections. This lead to a greatly reduced staining (data not shown).

Western blot analysis on total tumor protein extracts from two blastemal-predominant tumors (NL and 9168) and three stromal-predominant tumors (9385, 9274, and 9318) showed a full-length protein of M_r 52,000, with the WTC19 and WT180 antibodies only in the blastemal-predominant tumors (Fig. 1*D, b*). No wild-type or truncated WT1 protein could be detected with either antibody in the stromal-predominant tumors (Fig. 1*D, s*). This indicates that the amount of mutant WT1 protein is below the detection limit of the Western blot method, which is not suitable to detect a protein of low abundance (29), specifically if the cells used for protein extraction represent a mixture of WT1-expressing and non-WT1-expressing cells. In Fig. 1*C*, it can be seen that only a few cells in the stromal-predominant tumors are positive for WT1, further explaining why the truncated protein may not be visible in Western blot analysis.

Analysis of Subcellular Localization of Truncated WT1 Proteins in Cell Culture. To support our findings of a cytoplasmic truncated WT1 protein in tumors, we studied the subcellular localization of EGFP-WT1 fusion proteins of different length after transient transfection in NIH3T3 and Cos-7 cells. The full-length construct contained a cDNA encoding all 450 residues of the WT1 protein including exon 5 and KTS and an NH_2-terminal fusion of EGFP (EGFP-WT450). Two WT1 proteins truncated at aa residues 220 and 380 were constructed and designated EGFP-WT220 and EGFP-WT380, respectively (Fig. 2*A*). These corresponded to the naturally occurring mutations in patients 9184 and 9394. EGFP-WT220 lacked the entire ZF domain, whereas EGFP-WT380 lacked parts of ZF III and the entire ZF IV. The nuclear localization signal in ZF I was still present here. The constructs were verified by transient transfection in NIH3T3 cells and subsequent Western blot analysis (data not shown). In contrast to previous studies on subcellular localization of WT1 proteins (12, 30), we have directly visualized the EGFP-WT1 fusion proteins in native cells under a fluorescence microscope without fixation. Whereas the wild-type EGFP-WT450 fusion protein was restricted to the nucleus in both cell types, the deletion mutant EGFP-WT380 showed nuclear as well as cytoplasmic distribution (Fig. 2*B*). In contrast, the EGFP-WT220 fusion protein was mostly excluded from the nucleus in NIH3T3 cells and was present in the nucleus and the cytoplasm in Cos-7 cells. Fig. 2*C* summarizes the results.

Expression of Muscle Markers in Stromal-predominant WTs with *WT1* Mutations. Previously, four tumors with *WT1* mutations were studied for the expression of the myogenesis genes MyoD1, myosin heavy polypeptide (Myh3), and myogenin (MyoG) and found to be positive (19). To extend

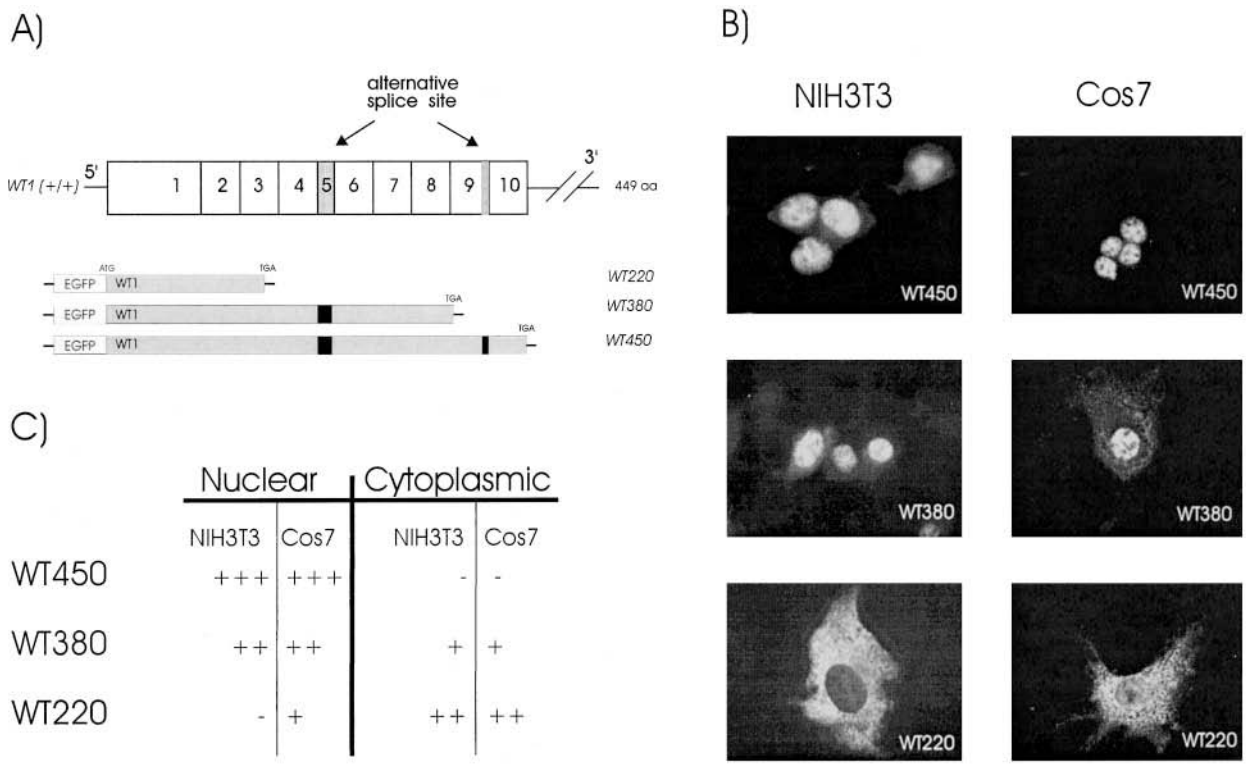

Fig. 2 Expression of EGFP-WT1 proteins in cell culture. *A,* schematic of the wild-type (WT450) and two truncated (WT220 and WT380) proteins. *B,* subcellular localization of EGFP-WT1 proteins in NIH3T3 and Cos-7 cells was investigated by direct visualization under a fluorescence microscope. *C,* summary of the observed cellular localization.

these observations, we have studied the expression of additional muscle differentiation markers in our five stromal-predominant tumors with *WT1* mutations. The muscle markers desmin, sarcomeric actin, MyoD1, and myogenin were present in the same cells that expressed the mutant WT1 protein but absent in the surrounding stroma. None of these markers were present in the blastemal clusters seen in the blastemal-predominant tumors (data not shown and summarized in Table 1).

Analysis of Kidney Differentiation Markers. To determine whether ILNR-like, stromal-predominant WTs with *WT1* mutations can be distinguished from PLNR-like, blastemal-predominant WTs by their differentiation stage, we have studied a set of markers. Markers for uninduced and early-induced mesenchyme were vimentin, collagen I, and fibronectin, and markers for condensed mesenchyme were cytokeratin, laminin A, and collagen IV (31). Using this set, we could show that cells in both tumor subtypes correspond to early-induced mesenchyme but express one marker of uninduced mesenchyme. The organoid structure of cell clusters in blastemal tumors and the beginning of cytokeratin expression demonstrates that this subclass is already one step further toward epithelial differentiation. Fig. 3 shows a representative example of one stromal-predominant tumor (tumor 9200) and one blastemal-predominant tumor (tumor 9345). Cytokeratin staining is negative in tumor 9200 and is seen only within blastemal clusters of tumor 9354. Vimentin staining is seen in stromal cells of both tumors and in blastemal clusters of tumor 9345, whereas muscle cells in tumor 9200 are negative (*cluster* of *blue-stained cells* in the *middle* of the picture). Staining for the extracellular matrix proteins collagen IV and fibronectin, as well as the membrane protein laminin A, surrounds the entire blastemal clusters in blastemal-predominant tumors, except in one case, where the individual tumor cells were positive (data not shown). In contrast, in stromal-predominant tumor 9200, each individual cell was surrounded by positive staining. Collagen I staining is negative in both tumors.

Table 1 summarizes the immunohistochemical results of five stromal-predominant tumors with *WT1* mutations and six blastemal-predominant WTs without *WT1* mutations as well as normal kidney specimens. Expression of markers was evaluated only in those cells that were positive for WT1 in both subclasses and compared with the expression of these markers as described in the kidney development database.[4]

Discussion

A major focus of this work was to establish diagnostic markers for the classification of clinically distinct subsets of WTs.

Our previous work and data presented in this report dem-

[4] http://www.ana.ed.ac.uk/anatomy/database.

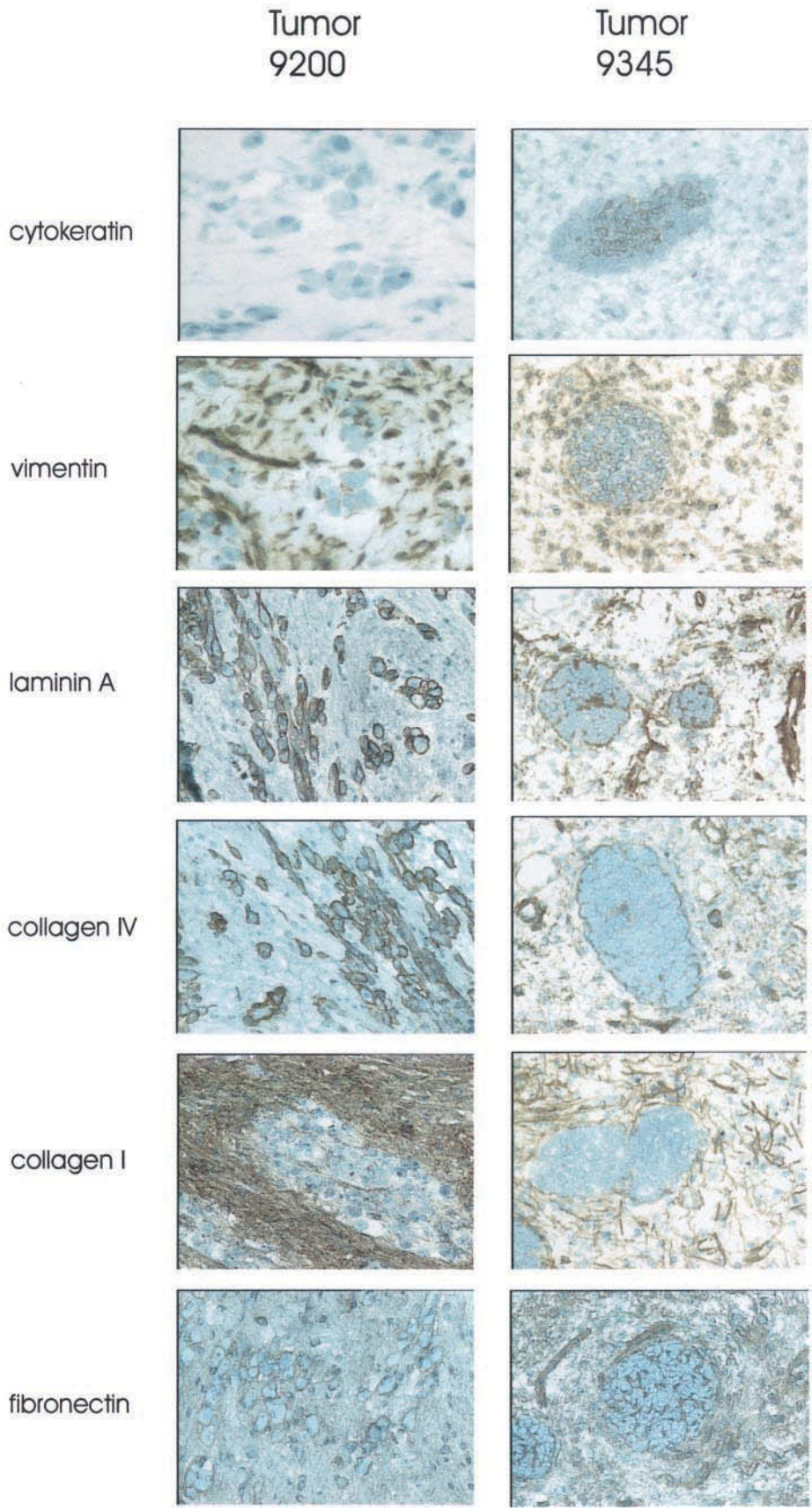

Fig. 3 Expression of differentiation markers. Immunostaining of cytokeratin, vimentin, laminin A, collagen IV, collagen I, and fibronectin in one stromal-predominant (9200) and one blastemal-predominant (9345) WT. All final magnification, ×400.

onstrate that *WT1* mutations occurred in a high precentage (63%) in one subset of WTs, the stromal-predominant or ILNR-like tumors. Importantly, 11 were present in the germline, and all but 1 were mutations resulting in protein truncation or loss of the entire gene (Ref. 7; data reported here). The germline mutations were found in three normal females, one normal male, and seven males with GUs of varying degrees. Interestingly, two males without GU had a tumor-specific mutation. Unilateral tumors developed in eight patients with germline mutations. An important clinical information from these data is that patients with germline mutations can present with unilateral tumors without associated abnormalities, and it should be kept in mind that these patients have a high risk for a contralateral tumor. Six of 10 (60%) stromal-predominant tumors had lost the wild-type DNA allele, and 1 tumor had a second somatic mutation, confirming Knudson's two-hit inactivation model for *WT1* in the development of this tumor subclass. In contrast, only one missense mutation in exon 2 was found in 16 blastemal-predominant WTs, suggesting that *WT1* mutations play a minor role in the development of this tumor subtype.

Mutant *WT1* mRNA was found in stromal-predominant WTs, and in cases where LOH occurred, only mutant transcripts were expressed. To our knowledge, only one other group found a mutant transcript in a tumor with a *WT1* alteration (32). In quantitative RT-PCR and Northern blot experiments, we have determined that the amount of the mutant transcript in different stromal-predominant tumors is between 5 and 50 copies/cell, which is in the normal range of low abundant messages (27). This could correspond to the amount found in early-induced cells, at a time when *WT1* expression starts. Blastemal-predominant WTs contain about 180-fold more *WT1* mRNA, which may correspond to the normal range in condensed, epithelialized cells.

In stromal-predominant WTs with a mutation, the WT1 protein could be detected by immunohistochemistry in individual cells only with an antibody that recognizes the NH_2 terminus, but not the COOH terminus, demonstrating that no intact wild-type protein was present. The mutant form was aberrantly localized in the cytoplasm, resulting in the absence of a functional WT1 protein in the nucleus. This was confirmed by analyzing the subcellular localization of different EGFP-WT1 mutants in unfixed, native cells. It is interesting that Englert *et al.* (30) found a WT1 protein truncated at aa 326 localized exclusively in the nucleus of Saos-2 and U2OS cells, two embryonal tumors not derived from kidney. Bruening *et al.* (12), who used the same cells as we did (NIH3T3 and Cos-7), also noted a cytoplasmic localization with some constructs. They have deduced that a second nuclear localization signal is present between aa 291 and 350, which is absent in our almost exclusively cytoplasmic construct WT220. Because all truncated proteins still contain the dimerization motif, it is possible that at a time where LOH has not yet occurred, the wild-type protein might be excluded from the nucleus by dimerization with the cytoplasmic truncated protein. This could be the first step in an abnormal differentation of early-induced cells. In contrast, blastemal clusters in blastemal-predominant tumors expressed wild-type WT1 protein in the nucleus. Therefore, one molecular distinction between these two subtypes of WTs is the presence of a nuclear wild-type WT1 protein in blastemal-predominant tumors and its absence in stromal-predominant tumors.

Interestingly, in stromal tumors, the truncated protein was restricted to cells with a rhabdomyomatous phenotype expressing desmin, MyoD, myogenin, and sarcomeric actin. It was described previously that tumors with *WT1* mutations may aberrantly differentiate into skeletal muscle (19). This suggests that the lack of nuclear WT1 protein in multipotential mesenchymal cells results in the failure of a proper epithelial differentiation and alternative differentiation programs; predominantly skeletal muscle as well as fat, cartilage, and bone are turned on. All these structures are not found during normal kidney differentiation and in blastemal-predominant tumors, suggesting that alternative programs are normally repressed. Whether WT1 is this repressor is not known. It should be noted, however, that all stromal-predominant tumors that we have studied were obtained after chemotherapy, and therefore rhabdomyomatous differentiation in response to chemotherapy cannot be excluded as has been proposed by Brisigotti *et al.* (33). However, we have studied several untreated stromal-predominant WTs for the presence of muscle differentiation by the expression of desmin and found them positive, suggesting that at least some muscle differentiation is present before chemotherapy.[5]

During early induction of kidney differentiation, vimentin, collagen I, and fibronectin are expressed, and at the stage of condensation, cytokeratin, laminin A, and collagen IV are expressed (31).[4] A switch from fibronectin to cytokeratin occurs at the stage of mesenchymal epithelial conversion. In stromal-predominant tumors, the cells expressing mutant WT1 and showing a rhabdomyomatous phenotype are at the differentiation stage of condensed cells except for the expression of fibronectin, which is normally turned off at that stage. Cells expressing wild-type WT1 in blastemal-predominant tumors are positive for markers of early-induced and condensed mesenchyme and may be one step further in epithelial differentiation because they start to express cytokeratin. Laminin A and collagen IV staining was not seen around each individual cell as in the case of stromal-predominant tumors but was seen surrounding a cluster of blastemal cells, suggesting that these may be further along the development to a more organoid structure. The expression of laminin A increases when epithelial cell polarization begins (34). It was shown that laminin A, collagen IV, and heparan sulfate proteoglycan first appear as spots on aggregating cells and then form a semicontinuous sheet on the surface of comma- and S-shaped bodies, suggesting a role in cell aggregation and later in epithelial differentiation (35). Forced expression of WT1 in mesenchymal NIH3T3 cells leads to up-regulation of E-cadherin, cytokeratin, heparan sulfate proteoglycan, and collagen IV, suggesting that WT1 is a positive regulator of epithelial differentiation (36). We have shown previously (37) that blastemal-predominant WTs expressing wild-type *WT1* are positive for *PAX-8*, which is turned on when epithelial differentiation begins, further supporting the notion that tumor cells expressing wild-type WT1 are at an early stage of epithelialization.

Taken together, these data show that two distinct subclasses of WTs can be distinguished on the basis of the presence or absence of *WT1* mutations and by their associated expression of specific differentiation markers. A similar subclassification was made on the basis of the presence or absence of LOI in PLNR-associated/blastemal-predominant WTs and ILNR-asso-

[5] V. Schumacher and B. Royer-Pokora, unpublished observations.

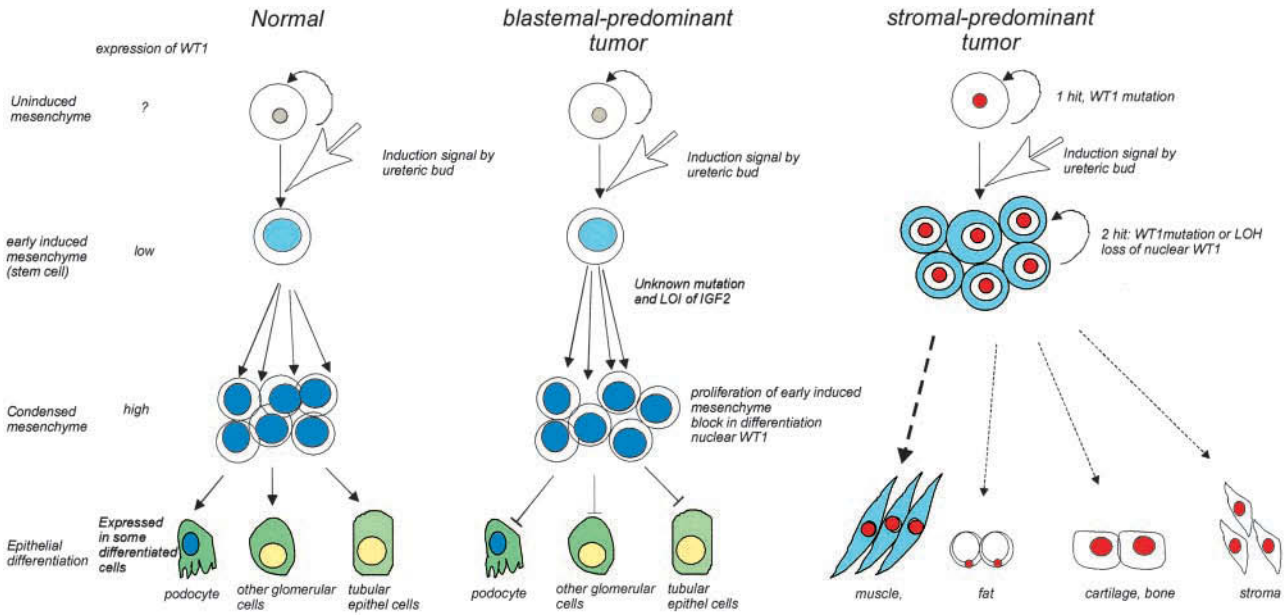

Fig. 4 Model for the development of two different subtypes of WT. *Left,* during normal kidney development, the *WT1* gene is turned on in early-induced mesenchyme and is needed for epithelial differentiation as well as induction of ureteric bud (41). Expression increases in condensed mesenchyme and is restricted to podocytes in adult kidney. *Middle,* in blastemal-predominant tumors, development proceeds normally until the stage of induced/condensed mesenchyme, where cells are arrested. *Right,* in the case of stromal-predominant tumors, the first *WT1* mutation is present in the germline or occurs very early in kidney development (cell with a *red nucleus, right*). After induction by the ureteric bud, the second allele of *WT1* is lost, creating a *WT1*−/− cell that randomly differentiates into other cell lineages.

ciated/stromal-predominant WTs by Ravenel *et al.* (5). This will be the basis to distinguish these two subtypes in future studies, rather than simply placing all WTs into one category. By doing so, it is expected that many of the previously unresolved inconsistencies might be resolved.

Based on the present data, we propose a model for the development of these two distinct subclasses of WTs (Fig. 4). Tumor cells in blastemal-predominant/PLNR-like WTs correspond to an early stage of induction/conversion to an epithelial phenotype. Cells arrested at the proliferative stage of early-induced mesenchyme are blocked from further differentiation, possibly by continuous expression of *WT1* and/or LOI of *IGF2*. In contrast, the first event leading to stromal-predominant/ILNR-like WTs is either a germline or early somatic truncation mutation in a multipotential kidney stem cell (Fig. 4, nucleus marked in *red*). This is followed by a second hit in a cell, most likely after induction by the ureteric bud, resulting in complete absence of a functional nuclear WT1 protein. Mesenchymal stem cells lacking WT1 fail to epithelialize and stochastically differentiate into other mesenchymal lineages. This ultimately leads to a heterogeneous tumor containing multiple cell types at the time of diagnosis. Microdissection of different cell types in two stromal-predominant tumors revealed LOH in all, indicating that this genetic event occurred in a multipotential stem cell early in tumor development (data not shown). Therefore, cells with a normal stromal appearance are also tumor cells, as has been described previously (38); however, they are negative for the WT1 protein. In summary, the two-hit inactivation of *WT1* is essential in stromal-predominant/ILNR-like WTs.

Of clinical importance is that stromal-predominant and fetal rhabdomyomatous tumors (which may be the same) respond poorly to chemotherapy, in contrast to most blastemal-predominant tumors (4, 22, 39, 40). Although patients with stromal-predominant tumors generally do not have a poor prognosis, these tumors tend to be very large at the time of diagnosis. Beckwith *et al.* (41) described the importance of differentiating between an aggressive tumor and a responsive tumor. Generally, tumors with a predominantly epithelial differentiation have a low degree of aggressiveness, but tumors presenting at an advanced stage may be resistant to therapy, and death rates may be higher than those for more aggressive tumors. None of the patients with a stromal-predominant tumor in the SIOP9/GPOH developed any relapse. Therefore, Weirich *et al.* (22) proposed to test a reduced or milder postoperative treatment in cases where tumors are completely resected, to avoid the unnecessary side effects of chemotherapy. Data from our work suggest that the presence of *WT1* mutations and extensive rhabdomyomatous histology could be used as diagnostic markers for nonresponding tumors. This will allow identification of patients who will benefit from changes in treatment protocols and possibly from the development of novel therapeutic approaches.

Acknowledgments

We thank all SIOP9/GPOH-participating clinicians for sending us tumor material. We thank Dr. Hans-Dieter Royer for critical reading of the manuscript.

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