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Correlation of germ-line mutations and two-hit inactivation of the *WT1* gene with Wilms tumors of stromal–predominant histology

(histopathology/truncation mutations/tumor suppressor gene/nephroblastoma/SSCP analysis)

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ABSTRACT **The *WT1* gene, located on chromosome 11p13, is mutated in a low number of Wilms tumors (WTs). Germ-line mutations in the *WT1* gene are found in patients with bilateral WT and/or associated genital tract malformations (GU). We have identified 19 hemizygous *WT1* gene mutations/deletions in 64 patient samples. The histology of the tumors with mutations was stromal–predominant in 13, triphasic in 3, blastemal–predominant in 1, and unknown in 2 cases. Thirteen of 21 patients with stromal–predominant tumors had *WT1* mutations and 10 of these were present in the germ line. Of the patients with germ-line alterations, six had GU and a unilateral tumor, two had a bilateral tumor and normal GU tracts, and two had a unilateral tumor and normal GU. Three mutations were tumor-specific and were found in patients with unilateral tumors without GU. These data demonstrate a correlation of *WT1* mutations with stromal–predominant histology, suggesting that a germ-line mutation in *WT1* predisposes to the development of tumors with this histology. Twelve mutations are nonsense mutations resulting in truncations at different positions in the WT1 protein and only two are missense mutations. Of the stromal–predominant tumors, 67% showed loss of heterozygosity, and in one tumor a different somatic mutation in addition to the germ-line mutation was identified. These data show that in a large proportion of a histopathologically distinct subset of WTs the classical two-hit inactivation model, with loss of a functional WT1 protein, is the underlying cause of tumor development.**

The *WT1* gene was isolated by positional cloning from chromosome 11p13 (1, 2) and encodes a transcription factor of the zinc finger (ZF) family. Loss of heterozygosity (LOH) studies showed that tumors frequently have lost markers from chromosome 11p. Subsequently, it was found that this loss is often limited to the region 11p15, where a second locus, *WT2*, involved in the development of Wilms tumor (WT) has been assumed to exist. Further LOH studies revealed loss of chromosome 16q in about 20% of WTs, suggesting that a third locus is located at this site. Susceptibility for the rare form of familial WT in several generations does not show linkage to either of these regions; therefore, another *WT* locus must exist in these cases (3–5). The *WT1* gene encodes four transcripts produced by alternative splicing (6, 7) and encodes a protein with a predicted size of 45–49 kDa. DNA binding to a GC-rich motif identical to the early growth response-binding site was demonstrated for the WT1 protein lacking splice II in the ZF (WT/−KTS), whereas the WT1 protein containing these amino acids (WT/+KTS) does not bind to this sequence (8). Recently it was shown that WT1/+KTS can also bind to a similar GC-rich DNA motif (9). More recent studies have established that the proline–glutamine rich amino terminus has transcriptional regulatory properties. It was shown that WT1 containing splice I (WT/+17aa) is a repressor, whereas WT1/−17aa can be either a repressor or activator depending on the architecture of the DNA binding site(s) under study (9–11).

Molecular analysis of WTs revealed that intragenic microdeletions/insertions and point mutations of one allele within the *WT1* gene are found in only 10–15% of WTs, suggesting that other alterations must contribute to Wilms tumorigenesis. Constitutional hemizygous deletions and mutations of the *WT1* gene have been demonstrated in most patients affected by WAGR (WT, aniridia, genitourinary anomalies, and mental retardation) and Denys–Drash syndrome, both predisposing to WT and urogenital malformations (GU) (12).

The WT1 protein is predominantly expressed in components of the urogenital system (fetal kidneys, the genital ridge, and the gonads), spleen, and mesothelial cells (13–16). The level of *WT1* expression in WTs varies greatly and can be correlated with tumor histology, with low levels in stromal–predominant tumors, and high levels in epithelial/blastemal–predominant tumors (13–20). *In situ* mRNA hybridization of tumors revealed that *WT1* is expressed in blastema and in immature epithelial structures but not in stroma or collecting duct derivatives or in more mature epithelial structures (15). A similar pattern is seen during normal nephrogenesis.

Pathogenetic heterogeneity in WTs may be due to the involvement of different genes in the different tumor types. However, WTs seen in Beckwith–Wiedeman syndrome (11p15) and WAGR patients (11p13) can show similar histology. According to Beckwith, two pathogenetic groupings can be classified by their association with nephrogenic rests, which according to their position and cellular components were termed intralobar nephrogenic rests (ILNR) and perilobar nephrogenic rests (PLNR) (21). These authors observed that ILNR-associated tumors have prominent stroma and PLNR-associated tumors are mainly composed of blastemal and epithelial elements, corresponding to later stages of development. ILNR are often found in tumors from patients with WAGR Denys–Drash syndrome and PLNRs in those from Beckwith–Wiedeman syndrome patients. Since ILNRs are found in tumors from patients with a clear 11p13 (*WT1*) involvement it was postulated that cells lacking *WT1* have a defect in epithelial cell differentiation resulting in stromal differentiation of the malignant clone (15, 22).

We analyzed the status of the entire *WT1* gene in 64 cases of WTs predominantly from patients with preoperative chemother-

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Abbreviations: LOH, loss of heterozygosity; WT, Wilms tumor; ZF, zinc finger; GU, genital tract malformations; RFLP, restriction fragment length polymorphism.

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apy and ascertained through the German SIOP9/GPO study (46). First we studied 32 consecutive cases, followed by patients selected for GU anomalies and bilateral tumors. When we noticed that most tumors with mutations had stromal–predominant histology we selected 11 more cases of this histological subtype; all patients had a unilateral tumor and normal GU. Of the 21 tumors/patients, 13 had *WT1* mutations/deletions and 10 of these were germ line. Six patients carrying germ-line mutations/deletions had GU anomalies and unilateral tumors, two had bilateral tumors, and two had a unilateral tumor and normal GU. Three mutations in patients with a unilateral tumor and normal GU were tumor-specific. A total of 15 *WT1* gene mutations and 4 deletions of the entire gene were identified in our series of 64 patients. Of the 15 *WT1* gene mutations, 12 were nonsense, 1 was a splice site, and 2 were missense mutations. In 7 of 11 cases where LOH analysis was performed the normal allele was lost.

MATERIALS AND METHODS

Patients. Of the 64 patients analyzed, 53 were registered in the nephroblastoma study trial SIOP9/GPO. Of the 64 patients, 9 were operated on without pretreatment, 52 patients received cytostatic preoperative treatment with vincristine and actinomycin D (23), and in 3 cases the status of pretreatment was unknown. The histological classification was known for 59 of the 64 cases analyzed. The subclassification of the standard tumors was as follows: 21 stromal–predominant (37%), 16 mixed triphasic (28%), 8 predominant–regressive (14%), 5 blastemal–predominant (9%), 3 epithelial–predominant (5.3%), and 2 without subclassification. In addition, four anaplastic tumors were analyzed. In the SIOP9/GPO study these numbers are: 14%, 38.2%, 36.2%, 7.7%, and 3.9%, respectively (24). The higher percentage of tumors with stromal–predominant histology is based on selection for these tumors. The mutation analysis was done on blood DNA in 8, on tumor metastases in 2, on tumor DNA in 41, and on blood/tumor DNA in 13 cases. All information on the patients, their clinical features, and the material and types of analysis performed is summarized in Table 1.

DNA/RNA Preparation from Tumors. Simultaneous extraction of high molecular weight DNA and RNA from frozen pulverized tumor tissues was performed as described (25). Constitutional DNA was isolated from the peripheral leukocytes of 21 patients as described (26).

PCR–SSCP Analysis. All *WT1* exonic sequences (exons 1–10) were amplified from genomic DNA by PCR and analyzed by SSCP. Exon 1 was analyzed in two overlapping fragments (1a and 1b) because of its large size. The sequences of the oligonucleotide primers for exons 1a, 1b, 2, 4, 6, 7, 8, 9, and 10 were as published (14, 27–30). New primers were designed for exons 3 and 5 (exon 3, 5′-GCTGTCTTCGGTTCTCTCTG-3′ and 5′-AGGACCCA-GACGCAGAGC-3′; exon 5, 5′-GGAATTCGGGGCTTGCA-GATCCATG-3′ and 5′-GGAATTCTCCTAACTCCTGCATT-GCCC-3′). Each PCR reaction contained 100–300 ng genomic DNA, 1× *Taq* DNA polymerase buffer [*ex2, ex4-ex10*: 10 mM Tris·HCl, pH 8.3/50 mM KCl/1.5 mM $MgCl_2$/0.01% gelatine; *ex1a*: 60 mM Tris·HCl, pH 8.5/50 mM KCl/15 mM $(NH_4)_2SO_4$/1.5 mM $MgCl_2$/0.02% gelatine plus 10% dimethyl sulfoxide (DMSO); *ex1b*: 60 mM Tris·HCl, pH 8.5/75 mM $(NH_4)_2SO_4$/7.5 mM $MgCl_2$ plus 10% DMSO], 25 pmol of each primer, 200 μM nucleotides, and 1 unit *Taq* polymerase in a total volume of 50 μl. Hot start PCR conditions were: 30 cycles at 94°C for 1 min, annealing for 1–2 min at 57°C–60°C, extension at 72°C for 1–2 min. For SSCP, 1–1.5-μl aliquots of the amplified product were added to 4–4.5 μl of 95% formamide, 10 mM EDTA. The DNA samples were denatured and run on 8% PAGE (29:1 or 49:1 acrylamide:bisacrylamide) containing 2% glycerol. Electrophoresis was performed in 1× TBE running buffer at 200 V and 10°C, 15°C, or 20°C for 4–7 hr and silver stained (Qiagen, Chatsworth, CA). Every sample was analyzed using two sets of electrophoresis conditions to maximize the sensitivity of the technique.

Direct Sequencing of PCR Products. Sequencing of the biotinylated PCR product was performed on streptavidin-coated magnetic Dynabeads as described by the manufacturer (Dynal). Direct sequencing was performed using the Sequenase version 2.0 sequencing kit (United States Biochemical) with T7 polymerase and [^{35}S]dATP according to the instructions of the manufacturer.

Analysis of Allele Loss. For studies on loss of heterozygosity, pairs of blood and tumor DNA from the same patient were analyzed by PCR in 13 cases with 4 polymorphic markers: restriction fragment length polymorphisms (RFLPs) in PR1 and PR4 will be described elsewhere, exon 7 (31), and a CA-repeat marker in the 3′ untranslated region (32). All PCR products were analyzed on 8 or 15% PAGE (29:1) containing 2% glycerol.

RESULTS

SSCP Analysis of Exons 1–10 of the *WT1* Gene. WTs were initially analyzed for gross alterations in the *WT1* gene by pulsed-field gel electrophoresis, Southern blot analysis, and reverse transcriptase–PCR. One homozygous deletion in a sporadic WT (*WT21*) of 200 kb spanning *WT1*, a constitutional deletion of 1300 kb in a patient with WT and GU (*HDWT2*, previously called *KJ*) and three hemizygous, constitutional submicroscopic deletions in WAGR patients were identified with pulsed-field gel electrophoresis and have been described (*ANS1*, *ANS2*, and *ANS3*) (33, 34). No alterations were seen in Southern blots and reverse transcription–PCR, where two overlapping fragments corresponding to the Pro/Gln-rich region and the four ZFs were amplified. Because no alterations were found with these methods we used the more sensitive SSCP method to search for single base pair mutations within the *WT1* gene (35). With this method all coding exons of the *WT1* gene, including exon 1, were analyzed in 54 WT samples, including tumor material from 2 WAGR and 2 metastasis patients and in 6 constitutional DNAs from WT patients. From these samples, 44 were unilateral sporadic, 8 unilateral with genitourinary abnormalities (GU), 5 bilateral, 2 WAGR, 2 metastasis, and 1 familial WT.

WT Patients with Associated Abnormalities. Ten patients with GU abnormalities were analyzed and two of these (*ANS2* and *ANS3*) were WAGR patients. In the remaining eight patients, mutations were found in four and all were germ line. WTs from patients with *WT1* mutations/deletions were of stromal–predominant histology in six cases, triphasic in one, and unknown in one. No mutations were found in the other four patients with SSCP and sequencing of exons 1a, 1b, 3, 6, 7, 8, 9, and 10. The other exons have not been sequenced.

Tumor DNA from patient *9184* showed only altered bands in SSCP of exon 1b and blood DNA was heterozygous for this alteration. Direct sequencing of tumor DNA showed a 7-bp deletion in exon 1b, resulting in a stop codon in exon 3. Blood DNA from patient *9595* showed a SSCP alteration in exon 6 and direct sequencing revealed a GT to GG change at position +2 in the conserved splice donor site in intron 6 (not shown). We have previously described patient *9274* with a germ-line mutation 1 nt 5′ of this mutation, affecting the same splice site in intron 6 and resulting in exon skipping (30). No tumor material from patient *9595* was available for RNA analysis, therefore, we could not determine the consequence of the splice site mutation. Blood DNA from patient *HDWT8* showed an altered band in exon 9. Direct sequencing revealed a C → T transition at codon 1546, creating a stop codon at this position. Tumor material was not available in this case. Tumor DNAs from two WAGR patients *ANS3* and *ANS2* were analyzed with SSCP during the course of this work and no alteration in the second allele could be identified.

Bilateral WTs. Five patients with bilateral WT were analyzed, the histology was stromal–predominant in three and mixed triphasic in two cases. None of these patients had GU anomalies. Mutations were found in four cases and all were present in the germ line. In three cases the tumor has lost the normal allele and therefore the wild-type WT1 protein should be absent. In one case, *HDWT7*, no tumor material was available for analysis.

Table 1. Summary of clinical and molecular features of all patients analyzed

Patient	Sex	Clinical features	Tumor	Stage/ histology	Preoperative treatment	DNA	PFGE	SSCP	Sequence analysis
			WT patients with associated abnormalities						
ANS1	m	Hypospadias, maldescended testis, aniridia	ul			c	+	−	−
HDWT2	m	Hypospadias, cryptorchidism	ul	II/ s	+	c	+	−	−
HDWT9	m	Hypospadia	ul		−	t	+	+	−
9139	f	Duplex kidney	ul	I/ s	+	t	−	+	+
9148	m	Hypospadias	ul	II/regr	+	t	−	+	−
9184	m	Unilateral testicular aplasia	ul	I/ s	+	t/c	+	+	ex 1b
9274	m	Hypospadias, cryptorchidism	ul	I/ s	+	t/c	−	+	ex/in 6
9391	m	Hypospadias	ul	IV/ tri	+	t	−	+	−
9595	m	Hypospadias, proteinuria	ul	I/ s	+	c	−	+	ex/in 6
ANS2-9115	m	Aniridia, cryptorchidism	ul	II/ tri	−	t/c	+	+	−
ANS3-9416	m	Maldescended testis, aniridia	ul	I/ s	+	t/c	+	+	−
HDWT8-9378	m	Maldescended testis, proteinuria	ul	I/ s	+	c	−	+	ex 9
			Bilateral WT						
HDWT7	f	Normal	bl	V/ tri	+	c	−	+	ex 7
9200	f	Normal	bl	V/ l:s, r:tri	+	t/c	−	+	ex 1b
9318	f	Normal	bl	V/ s	+	t/c	−	+	ex 7
9464	m	Normal	bl	V/ tri	+	t/c	−	+	ex 1a
9501	m	Normal	bl	V/ s	+	t	−	+	+
			WT patients with no associated abnormalities						
HDWT1	f	Proteinuria	ul	II/ stan	+	c	−	+	−
HDWT3	m	Normal	ul			c	−	+	−
HDWT4	m	Normal	ul	III/	+	met	+	+	−
HDWT5	f	Normal	ul	I/ e	−	t	+	+	−
HDWT6	f	Normal	ul	III/ stan	+	c	−	+	ex 7
HDWT10	m	Normal	ul	IV/ b	+	met	+	+	−
HDWT11	f	Normal	ul			t	−	+	+
5018	m	Normal	ul	I/ tri	+	t	+	+	−
5024	m	Normal	ul	I/ tgri	+	t	+	+	−
5302	f	Normal	ul	I/ anapl	+	t	−	+	−
9066	m	Normal	ul	III/ tri	+	t	+	+	−
9073	m	Normal	ul	II/ anapl	+	t	+	+	−
9074	m	Normal	ul	IV/ b	−	t	−	+	−
9094	f	Normal	ul	III/ b	+	t/c	+	+	ex 2
9098	m	Normal	ul	I/ tri	+	t	+	+	−
9112	m	Normal	ul	I/ tri	+	t	+	+	−
9125	f	Normal	ul	IV/ tri	+	t	+	+	−
9147	f	Normal	ul	IV/ regr	+	t	+	+	−
9165	f	Normal	ul	I/ tri	−	t	−	+	−
9168	f	Normal	ul	I/ b	−	t	+	+	−
9169	m	Normal	ul	IV/ negr	+	t	+	+	−
9174	m	Normal	ul	I/ tri	+	t	+	+	−
9177	m	Normal	ul	IA/ s	+	t/c	+	+	ex 6
9193	m	Normal	ul	II/ regr	+	t	−	+	−
9196	m	Normal	ul	II/anapl	+	t	−	+	−
9201	m	Normal	ul	I/ e	+	t	−	+	−
9206	f	Familial WT	ul	IV/ tri	+	t	−	+	−
9210	m	Normal	ul	II/ regr	+	t	−	+	−
9211	m	Normal	ul	III/ tri	−	t	+	+	−
9249	m	Normal	ul	IV/ tri	+	t	−	+	−
9258	f	Normal	ul	I/ regr	+	t	−	+	−
9288	m	Normal	ul	I/ s	+	t	−	+	−
9304	m	Normal	u	III/ regr	+	t	−	+	−
9310	m	Normal	ul	III/ b	−	t	−	+	−
9343	m	Normal	ul	IV/anapl	+	t	−	+	−
9358	m	Normal	ul	I/ s	+	t	−	+	+
9362	m	Normal	ul	I/ s	+	t	−	+	+
9385	m	Normal	ul	II/ s	+	t/c	−	+	ex 7
9394	f	Normal	ul	III/ s	+	t/c	−	+	ex 7
9415	f	Normal	ul	II/ e	−	t	−	+	−
9422	m	Normal	ul	IV/ s	+	t	−	+	+
9496	m	Normal	ul	I/ s	+	t	∓	+	+
9518	f	Normal	ul	II/ tri	+	t	−	+	−
9554	f	Normal	ul	II/ regr	+	t	+	+	−
9561	m	Normal	ul	II/ s	+	t/c	−	+	ex 7, 8
9572	m	Normal	ul	II/ s	+	t	−	+	+
9614	f	Normal	ul	III/ s	+	t/c	−	+	ex 5

This table presents a list of all patients, whether blood or tumor DNA was analyzed, the clinical details, preoperative treatment, and the methods used for analysis. m, male; f, female; ul, unilateral; bl, bilateral tumor; regr, regressive; tri, triphasic; s, stromal–predominant; b, blastemal–predominant; e, epithelial–predominant; anapl, anaplastic; stan, standard histology—no subclassification available; t, tumor; l, left; r, right, c, constitutiona;; PFGE, pulsed-field gel electrophoresis; met, metastasis—no primary tumor available; ex, exon; in, intron; sequence analysis; +, exons 1a, 1b, 3, 6, 7, 8, 9, 10 were sequenced. Patient numbers correspond to the SIOP study numbers.

Patient *HDWT7* was the twin of *HDWT6* who developed bilateral and unilateral WT, respectively. SSCP analysis of blood DNA showed an alteration in exon 7 (Fig. 1). Direct sequencing revealed a C → T transition, resulting in a stop codon in both patients. In patient *9200*, an alteration in exon 1b was found in blood DNA. No normal bands were seen in the DNAs isolated from the right and left tumor, demonstrating that both tumors have lost the normal *WT1* allele. Direct sequencing of tumor DNA showed a deletion of 26 bp, resulting in a stop codon in exon 2. Blood DNA from patient *9464* showed an alteration in exon 1a by SSCP. The tumor showed only the altered bands and direct sequencing revealed a 29-bp deletion creating a stop codon at position 89 in exon 1b. Analysis of the DNA from one of the two tumors from patient *9318* showed a slight shift in SSCP bands in

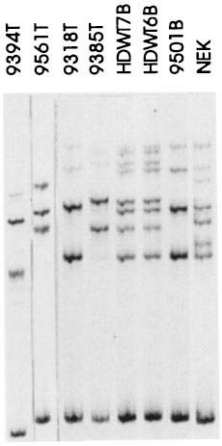

FIG. 1. SSCP analysis of exon 7 from patients *9394*, *9561*, *9318*, *9385*, *HDWT7*, and *HDWT6.* Only a slight shift can be seen in the upper bands in patient *9318* under the conditions used for this gel. NEK control DNA is heterozygous for the RFLP (alleles A and B) and patient *9501* is homozygous for allele A. Patients *HDWT7* and *HDWT6* are homozygous for allele A and the additional bands correspond to the mutation, which is identical to the mutation in patient *9385*. Lanes *9394*, *9561*, *9318*, and *9385* contain tumor DNA; only altered bands are seen.

exon 7 (Fig. 1). Direct sequencing of the tumor DNA revealed a C → A transversion, resulting in creation of a stop codon. The mutation is germ line and the tumor has lost the normal *WT1* allele.

WT Patients with No Associated Abnormalities. Of the 47 patients analyzed the histology was known in 44 cases: 11 stromal–predominant, 12 mixed triphasic, 8 epithelial or blastemal–predominant, 7 regressive, 4 anaplastic, and 2 standard with no further subclassification. Seven mutations were found, five in stromal–predominant tumors, one in a blastemal tumor, and in one case with standard histology. Four mutations were germ line and 3 were tumor-specific. In one tumor, a second, different, tumor-specific mutation was identified in addition to the germ-line mutation. Two of the mutations were missense and six nonsense.

In patient *9094*, SSCP analysis showed an alteration in blood DNA in exon 2 and the tumor was heterozygous. Direct sequencing of blood and tumor DNA revealed a C → T transition, changing Pro-181 to Ser in the Pro/Gln-rich region of the *WT1* gene. This patient had a germ-line missense mutation and the tumor retained one normal allele. SSCP analysis of tumor DNA from patient *9614* revealed an alteration in exon 5. To our knowledge, alterations in this alternative exon have never before been described. Sequence analysis revealed a G → C transversion changing a glycine to an alanine. This mutation is present in the germ line and the tumor has retained the wild-type allele. Tumor DNA from patient *9177* showed an altered band in exon 6. Subcloning of the PCR product and sequencing identified a 4-nt insertion changing the reading frame and resulting in a stop codon in exon 6. This mutation was not present in the germ line and the tumor had retained one normal allele.

SSCP analysis of tumor DNA from patient *9385* showed only altered bands in exon 7 (Fig. 1). Direct sequencing of tumor DNA revealed a C → T transition in exon 7, resulting in a stop codon. This mutation was not found in blood DNA and is therefore a somatic alteration. Using RFLP markers in the promoter region, the LOH of this genomic segment in tumor DNA could be identified, demonstrating that no normal *WT1* allele was present in the tumor. This mutation is identical to the germ-line mutation found in the twin patients *HDWT6* and *HDWT7*. SSCP of tumor DNA from patient *9394* showed only altered bands in exon 7 (see also altered sized double-stranded DNA at the bottom of the gel in Fig. 1). Direct sequencing of tumor DNA showed a deletion of 16 bp, resulting in a frameshift and a stop codon at position 380 in exon 9. The alteration was not present in blood DNA and was therefore a tumor-specific alteration with the loss of the normal *WT1* allele in the tumor.

SSCP analysis of tumor DNA from patient *9561* revealed an alteration in exon 8. Direct sequencing of tumor DNA showed a GA insertion resulting in a stop in exon 9. This alteration was not found in blood DNA. In addition, the tumor was homozygous for the rare allele B of the RFLP in exon 7 (Fig. 1). Only a very few patients/normal controls are homozygous for this allele (S.S. and B.R.-P., unpublished work); therefore, we confirmed this interpretation with restriction enzyme analysis and sequencing. This analysis revealed that the patient is indeed homozygous for allele B and an additional alteration was found, changing a C → A (Fig. 2), creating a stop codon identical to the mutation found in patient *9318*. This mutation was present in blood DNA and the tumor was heterozygous for this alteration. Thus, this tumor has two different alterations in the *WT1* gene, one germ-line nonsense mutation in exon 7 and one tumor-specific nonsense mutation in exon 8, fulfilling Knudson's classical two-hit hypothesis. To our knowledge, this is the second example where two different mutations in the *WT1* gene could be identified (36). In addition, several examples of a mutation in the remaining allele in tumors from WAGR patients with a germ-line deletion have been reported.

A summary of all alterations found in the *WT1* gene in our collection of patients is given in Fig. 3. The details of the alterations with the position within the gene, LOH data and patient descriptions are summarized in Table 2.

DISCUSSION

We have analyzed 64 WT patients for *WT1* mutations. The first analysis was done on consecutive cases and later patients with associated anomalies or bilateral WTs were selected. None of the patients studied in this report had Denys–Drash syndrome. Alterations were found in 19 cases; most of these occurred in tumors with stromal–predominant or mixed triphasic histology and only one was found in a blastemal–predominant tumor. Of the 21 stromal–predominant tumors analyzed, 62% had *WT1* gene mutations, including 4 deletions of the entire gene in three WAGR and one WT/GU patient. In cases where no alterations were found with SSCP, we have sequenced several exons (exons 1a, 1b, 3, 6, 7, 8, 9, and 10) and no further mutations have been identified so far. When the frequencies of mutations were determined according to histopathology, the numbers were 13 of 21 (62%) in stromal–predominant, 3 of 16 (19%) in triphasic, and 1 of 8 (12.5%) in epithelial or blastemal–predominant tumors.

Most of the mutations are nonsense mutations leading to protein truncation at different positions of the protein. If a protein were synthesized from the mutant genes the shortest would be 89

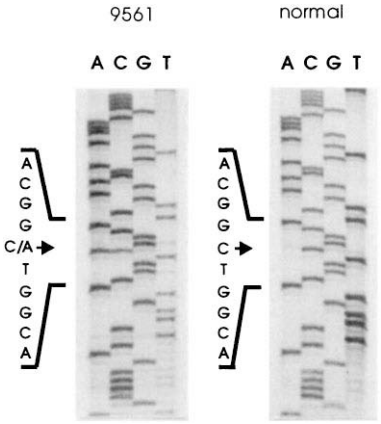

FIG. 2. *Bsi*EI digests of exon 7 PCR products from blood (B) and tumor (T) DNA from patient *9318* with a bilateral WT and a normal control (NEK). Loss of the recognition sequence by the mutation results in an undigested fragment of 235 bp; the digested products are 80 and 155 bp. M, 1-kb size marker. Schematic shows the mutation with loss of *Bsi*EI site and the stop codon.

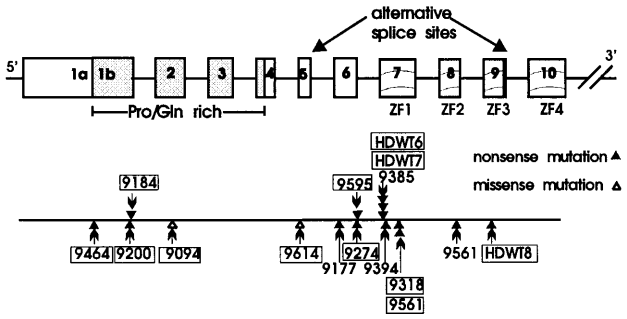

FIG. 3. Summary of all *WT1* gene mutations described here. Exons are boxed, the Pro/Gln-rich region is shaded, and the ZF exons contain wavy lines. The positions of the alternative splice sites are indicated. Patients with constitutional alterations are boxed. The clustering of mutations in exon 7 is evident. In addition, patients *9318* and *9561* have identical germ-line mutations and patients *HDWT6*, *HDWT7* (twins), and *9385* also have identical mutations. The two missense mutations are shown as open triangles and the truncations as closed triangles.

aa in length and the longest would lack part of ZF 3 and all of ZF 4; none of these would be able to bind to their normal target sequence. Mutations in most exons were detected, even in the alternatively spliced exon 5. Two missense mutations were found, the first is identical to a previously described mutation in exon 2 (37), changing Pro → Ser and was a germ-line alteration in a patient who developed a blastemal–predominant tumor. The tumor has retained the wild-type allele as previously described (37), indicating that this mutation may act by a dominant-negative or gain-of-function mechanism. The altered amino acid lies at the beginning of the domain that has recently been shown to be responsible for protein oligomerization (38). The second missense mutation changes a Gly → Ala next to the Ser-rich region in exon 5 corresponding to the alternative splice I. A mutation in this exon has to our knowledge never before been described. To our knowledge, this germ-line alteration demonstrates for the first time the importance of this splice variant for the normal function of the WT1 protein.

All other mutations led to protein truncations. No correlation was found between the site of the mutation in the gene and the development of either GU, unilateral, or bilateral tumor. GU was present in patients with protein truncations in exons 3, 7, and 9. Bilateral tumors were found in patients with stops in exons 1b, 2, and 7. Unilateral or bilateral tumors were observed in patients with identical mutations: *9318* and *9561* in exon 7 and *HDWT6*, *HDWT7*, and *9385* with another mutation in exon 7. This shows a clear difference in expressivity of identical germ-line mutations. Two of the 4 male patients without GU had germ-line truncation mutations and the other two tumor-specific mutations. Lack of the entire gene as in WAGR patients or in patient *HDWT2* almost always led to GU malformations. Truncation in exon 1b in the male patient *9464* should probably lead to a complete loss of the protein as in WAGR patients, however, he has no GU. Therefore, it seems possible that truncated proteins are produced, having a partial function and that there may be subtle differences between the lack of the entire gene and the presence of a truncated protein. A similar observation has been made in familial adenomatous polyposis (FAP) patients with mutations leading to truncation of the APC protein at the amino terminus, resulting in an attenuated form of FAP with fewer polyps and later age of onset (39). It has been postulated that in these cases the short protein may have a protective effect.

The three alterations in exon 1 and one in exon 7 are deletions. It has been suggested that exon 1 could be a hot spot for deletion/insertion mutations due to the presence of multiple tri- and tetranucleotide repeat sequences (40). The CCTG (CAGG) sequence was found within 6 nt of deletion breakpoints in 25% of all human gene deletion mutations (41). Four previously described mutations (40) and the three exon 1 mutations described here are all deletion/insertion mutations occurring within 6–9 nt of such sequences, further supporting the role of these repeat sequences in the creation of the deletions.

The mutations at nucleotide positions 1279, 1316, and 1546 involve a CpG dinucleotide, suggesting that deamination of a methylated cytosine generated the mutation. We found the 1279 mutation three times, the 1316 mutation two times, and the 1546 mutation once. The 1546 mutation was previously found in three other WTs (36, 42, 43). It was observed that the triplet CGN coding for Arg is a frequent site of spontaneous mutations in the p53 gene (44). Here we show that mutations in triplets with a CpG (CGN or TCG) also occur frequently in the *WT1* gene.

Table 2. Clinical and molecular features of patients with WT1 alterations

Patient	sex	Clinical features	Tumor age at diagnosis	Pathology	Tumor LOH	Location of mutations ex/in	nt	Codon	Mutation	Effect on protein (codon)	c/t
9464	m	Normal	bl/3 mo	stage V, tri	LOH	ex1a	408–436	10–20	del 29 bp	Stop ex1b (89)	c
9200	f	Normal	bl/15 mo	st V; l: s, r, tri	LOH	ex1b	746–771	123–131	del 26 bp	Stop ex2 (178)	c
9184	m	ul testicular aplasia	ul/12 mo	stage I, s	LOH	ex1b	787–793	137–139	del 7 bp	Stop ex3 (217)	c
9094	f	Normal	ul/30 mo	stage III, b	HT	ex2	919	181	C → T	Pro → Ser	c
9614	f	Normal	ul/29 mo	stage III, s	HT	ex5	1136	253	G → C	GLY → Ala	c
9177	m	Normal	ul/30 mo	stage I, s	HT	ex6	1192	272	ins TACG	Stop ex6 (276)	t
9274	m	amb ext	ul/9 mo	stage I, s	LOH	in6	+1	—	G → C	Stop ex7 (306	c
9595	m	Hypospadias	ul/7 mo	stage I, s	ND*	in6	+2	—	T → G	?	c
HDWT6	f	Normal	ul/19 mo	stage III, stand	ND*	ex7	1279	301	C → T	Arg → Stop	c
HDWT7	f	Normal	bl/18 mo	stage V, tri	ND*	ex7	1279	301	C → T	Arg → Stop	c
9385	m	Normal	ul/12 mo	stage II, s	LOH	ex7	1279	301	C → T	Arg → Stop	t
9394	f	Normal	ul/8 mo	stage III, s	LOH	ex7	1297–1312	307–312	del 16 bp	Stop ex9 (380)	t
9318	f	Normal	bl/7 mo	stage V, s	LOH	ex7	1316	313	C → A	Ser → Stop	c
9561	m	Normal	ul/34 mo	stage II, s	HT	ex7	1316	313	C → A	Ser → Stop	c
						ex8	1494	372	ins GA	Stop ex9 (380)	t
HDWT8	m	mald testis, prot	u/24 mo	stage I, s	ND*	ex9	1546	390	C → T	Arg → Stop	c
HDWT2	m	Hypospadias, crypt	ul/87 mo	stage II, s	ND*	del WT1			del 1300 kb		c
ANS1	m	WAGR	ul/23 mo		ND*	del WT1			del 1800 kb		c
ANS2	m	WAGR	ul/21 mo	stage II, tri	HEM	del WT1			del 1700 kb		c
ANS3	m	WAGR	ul/20 mo	stage I, s	HEM	del WT1			del 11p13		c

Patient descriptions and details on all alteratons. c, constitutional and t, tumor specific alteration. HT, heterozygous; HEM, hemizygous; amb ext, ambigious external genitalia; mald, maldescended; prot, proteinuria; crypt, cryptorchidism; del, deletion; ins, insertion. All other abbreviations are described in legend of Table 1.

*ND, no tumor was available for LOH analysis.

The *WT1* gene is expressed during the normal induction phase of the nephrogenic mesenchyme. For epithelial differentiation a high level of *WT1* expression is needed in the cells giving rise to specific structures of the nephron. After completion of the differentiation process, *WT1* gene expression is restricted to podocytes. It has been observed that the expression level of *WT1* correlates with specific histologic subtypes of WTs, with stromal–predominant tumors showing the lowest level (15, 18–20). This correlates with low expression of *WT1* in normal stromal components of the kidney (15, 19, 20).

Patients with germ-line mutations have only one intact copy of the *WT1* gene, resulting in a reduced level of the normal protein in the induced mesenchymal cells. Higher levels required for differentiation along the epithelial pathway may not be reached. The development of stromal–predominant WTs could be explained by two mechanisms. In the first, induced cells may be blocked early in their differentiation process due to the low level of *WT1*, leading to an increase in proliferation of these cells. If in one of these cells the second allele is also lost and no normal WT1 protein is present, this cell eventually develops into a tumor with stromal–predominant phenotype. It has been suggested that the term "stromal component" in a WT may be a misnomer and that the stromal cells have characteristics of nonaggregated-induced nephrogenic mesenchyme. If this is the case, the stromal cells may therefore correspond to the induction phase of normal renal development (22). The second possibility is that the lack of *WT1* expression drives cells into a stromal differentiation pathway by default, because epithelial differentiation cannot proceed. In this case, stromal cells in a tumor would correspond to more differentiated cells, e.g., skeletal muscle. The two possibilities can now be distinguished in further studies. In the light of our data that *WT1* mutations are found mostly in tumors of stromal–predominant histology, it could be postulated that mutational *WT1* inactivation is the cause of stromal–predominant histology. Our data strongly suggest that WTs of stromal–predominant histology and inactivating *WT1* mutations constitute a new specific molecular subset, which arises by complete loss of a functional WT1 protein, following a classical two-hit inactivation mechanism of a tumor suppressor gene as proposed by Knudson and Strong (45). In contrast, epithelial/blastemal–predominant tumors, which show a low frequency of *WT1* mutations, probably occur by a different mechanism and/or involve different genes. A large proportion of the mutations that we have identified are present in the germ line, increasing the risk in developing a second tumor. Many of the patients with a tumor of this histologic subgroup have either bilateral WT or GU anomalies, suggesting that a germ-line mutation in *WT1* predisposes to this subtype of WT. These patients may also transmit this mutation to their offspring. Therefore, it seems warranted that patients with stromal–predominant WT should be analyzed for *WT1* mutations.

We are currently analyzing tumors with *WT1* mutations for the presence of truncated WT1 proteins. Oligomerization of the WT1 protein has been mapped to the first 180 amino acids at the amino terminus and interestingly truncated proteins, lacking the zinc fingers, have a higher activity in oligomerization (38). This could indicate that these truncated proteins may act in a dominant-negative fashion.

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