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RESEARCH ARTICLE

Infrequent Mutation of the *WT1* Gene in 77 Wilms' Tumors

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Homozygous deletions in Wilms' tumor DNA have been a key step in the identification and isolation of the *WT1* gene. Several additional loci are also postulated to contribute to Wilms' tumor formation. To assess the frequency of *WT1* alterations we have analyzed the *WT1* locus in a panel of 77 Wilms' tumors. Eight tumors showed evidence for large deletions of several hundred or thousand kilobasepairs of DNA, some of which were also cytogenetically detected. Additional intragenic mutations were detected using more sensitive SSCP analyses to scan all 10 *WT1* exons. Most of these result in premature stop codons or missense mutations that inactivate the remaining *WT1* allele. The overall frequency of *WT1* alterations detected with these methods is less than 15%. While some mutations may not be detectable with the methods employed, our results suggest that direct alterations of the *WT1* gene are present in only a small fraction of Wilms' tumors. Thus, mutations at other Wilms' tumor loci or disturbance of interactions between these genes likely play an important role in Wilms' tumor development.

KEY WORDS: **Wilms' tumor, *WT1*, Zinc finger gene, Tumor suppressor gene, Nephroblastoma, Deletion analysis, SSCP analysis, Mutation screening**

INTRODUCTION

Wilms tumor, an embryonic renal tumor, is thought to arise through the loss or functional inactivation of one or more tumor suppressor genes, first postulated from epidemiological data (Knudson and Strong, 1972). This concept is supported by deletions including chromosome 11p13 found in sporadic tumors (Douglas et al., 1985) and the high incidence (40–60%) of Wilms' tumors in children with constitutional hemizygous deletions of chromosome band 11p13—a condition known as the WAGR syndrome (*W*ilms' tumor, *a*niridia, genitourinary anomalies, and mental *r*etardation) (Francke et al., 1979). Studies on loss of heterozygosity (LOH) in tumor DNA also showed a frequent involvement of chromosome 11p. Later it could be shown, however, that this loss of alleles is often limited to chromosome 11p15 where a second locus involved in Wilms' tumor formation, *WT2*, has been postulated (Mannens et al., 1988; Koufos et al., 1989; Reeve et al., 1989; Wadey et al., 1990). More recently, a third locus on chromosome 16q was shown to be lost in about 20% of Wilms' tumors (Maw et al., 1992). In addition, none of these loci seems to confer susceptibility to the familial form of Wilms' tumor in several well-studied kindreds, suggesting the existence of yet

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another contributing gene (Grundy et al., 1988; Huff et al., 1988, 1992).

Until now only the 11p13 locus, *WT1*, has been isolated by a positional cloning strategy using homozygous deletions found in tumor DNA (Call et al., 1990; Gessler et al., 1990). The gene encodes a zinc finger protein that binds DNA in a sequence specific manner and can regulate transcription of target genes (Madden et al., 1991). The role of *WT1* as a tumor suppressor gene has been supported by the detection of intragenic homozygous deletions in tumor DNA and more recently by point mutations within the zinc finger domain (Haber et al., 1990; Huff et al., 1991; Ton et al., 1991; Pelletier et al., 1991b; Baird et al., 1992; Brown et al., 1992; Little et al., 1992; Coppes et al., 1993; Gessler et al., 1993). These latter studies, however, were mainly focused on alterations within the zinc finger domain encoded by exons 7 through 10, and provided less information regarding the entire 5′ region of the gene.

Despite these clear examples of inactivation of the *WT1* gene in Wilms' tumors, the overall frequency of *WT1* alterations is still unclear. Here we have characterized the type of mutations affecting the *WT1* gene and evaluated their frequency in a panel of 77 Wilms' tumor DNAs using both, gene dosage studies, and single-exon SSCP analysis, supplemented by other approaches in some of the cases. Results on three of the tumors from this panel, WT8A, PER, and S86-1334 (Gessler et al., 1990, 1993), have already been reported previously, and are included here to provide a more complete description of the type of *WT1* mutations we have found.

MATERIALS AND METHODS

DNA Samples

Tumor tissue was kept at −70°C and DNA was extracted according to standard protocols (Sambrook et al., 1989). In some cases either normal adjacent kidney tissue or blood samples from the respective patient were available for comparison. DNA from normal lymphoblastoid cell lines were used as controls.

All hybridization probes used have been described in Gessler et al. (1989, 1990).

Gene Dosage Analysis

The preparation of Southern blot filters, hybridization conditions, and evaluation of autoradiograms have been described previously (Gessler et al., 1989). In brief, tumor DNAs (4 μg) were digested with *Eco*RI and gel loading was adjusted for all samples according to DNA concentrations determined by flourometric measurements. Equal loading was verified by hybridization with non chromosome 11 probes. Filters were hybridized with up to three probes simultaneously and they were reused for subsequent probings to facilitate comparison of signal strength. Control DNAs and matching DNA samples from normal tissue, if available, were used for comparison. Evaluation of gene dosage was done by visual inspection and laser scanning densitometry as described (Gessler et al., 1989). A reduction in signal strength to about 50% of control values was interpreted as a hemizygous deletion.

Analysis of Allele Loss

For studies on loss of heterozygosity pairs of normal and tumor DNA from the same patient were analyzed with polymorphic markers as described in Koufos et al. (1989). Allele status for HRAS and TH was determined by PCR amplification of microsatellite markers (Polymeropoulos et al., 1991; Tanci et al., 1992).

RT-PCR Analysis

Preparation of first strand cDNA from Wilms' tumor RNA, PCR amplification of the *WT1* coding region, and sequencing of the subcloned cDNA have been described in Gessler et al. (1993). A segment corresponding to nucleotides 779–1605 of the *WT1* cDNA sequence (HSWT1) was analyzed from three independent plasmid clones.

Oligonucleotide Primers

All oligonucleotide primers were designed using the PRIMER program developed by Lander et al. (Whitehead Institute), based on the genomic sequence of *WT1* (EMBL: X61631–X61640; Gessler et al., 1992). Generally, the primers flank one exon with intronic splice consensus sequences to give PCR products of 174 to 253 nucleotides. For exons 1 and 10 only the coding region was analyzed. The sequences of the respective primers are listed in Table 1. Crude oligonucleotides (MWG-Biotech, Ebersberg) were ethanol-precipitated twice and stored in water at −20°C. All sequence manipulations and comparisons were done using the GCG/HUSAR sequence analysis package at DKFZ (Heidelberg).

SSCP Analysis

DNA fragments were amplified from genomic DNA and labeled by [^{32}P]dCTP incorporation

TABLE 1. Primers for the Amplification of *WT1* Exons

Exon	Sense primer	Antisense primer	Annealing temperature (°C)	Product length (bp)
1	cgaggagcagtgcctgagcg	gcggagagtccctggcgc	69	249
2	cgagagcaccgctgacactg	gagaaggactccacttggttccg	66	199
3	ccaggctcaggatctcgtgt	aaggacccagacgcagagc	63	237
4	tgcttttgaagaaacagttgtg	ggaaaggcaatggaatagaga	58	178
5	gggcttttcactggattctg	ccatttgctttgccatctcc	60	174
6	gtgagccacactgagccttt	ggccggtaagtaggaagagg	60	200
7[a]	ggcttaaagcctcccttcct	tgagagcctggaaaaggagc	60	253
8	gagatccccttttccagtatca	acagctgccagcaatgagaa	60	176
9	cattgttagggccgaggcta	cttttccaatccctctcatca	60	218
10	tgtgcctgtctctttgttgc	gttcacacactgtgctgcct	60	224

[a]1 mM spermidine must be added with this primer pair to suppress artifactual bands.

(modified from Orita et al., 1989). In general, a master mix for up to 40 reactions was prepared containing 1 × reaction buffer (50 mM KCL, 10 mM Tris-HCl pH8, 1.5 mM $MgCl_2$), 20 μM each dGTP, dATP, dTTP, 2 μM dCTP, 20 μCi [α-^{32}P]dCTP, primers (1 μg each), 50 μg/ml BSA, and 20 units *Taq* polymerase in a total volume of 380 μl. Reactions were assembled on ice by mixing 9.5 μl master mix with 0.5 μl DNA (20–200 ng) and overlayed with mineral oil. Amplification was started with denaturation at 94°C for 5 min, followed by 30 cycles of 94°C (30 sec), 60–66°C (30 sec), and 72°C (30 sec) and a final extension at 72°C for 5 min. One microliter of the PCR products was diluted in 20 μl loading buffer (95% formamide, 10 mM NaOH, 0.1% bromphenol blue, 0.1% xylene cyanol) and denatured for 5 min at 90°C. After quick cooling on ice 2 μl of each sample was loaded on 6% polyacrylamide gels and run in 0.5 × TBE with 30W either at 4°C or with the addition of 10% glycerol to the gel at room temperature. Gels were dried onto filter paper and exposed to X-ray film for 12–48 hr. DNAs from normal lymphoblastoid cell lines served as additional controls. Nondenatured PCR products were included to identify weak signals from renatured doublestranded DNA.

Direct Sequence Analysis of PCR Products

For homozygous alterations 100–500 ng of genomic DNA were amplified under standard conditions in a 100 μl reaction volume. Heterozygous DNA samples were analyzed by cutting the normal and aberrant fragments from dried SSCP gels followed by standard PCR amplification with part of the gel piece present. In both cases PCR products were purified by chloroform extraction and binding to glassmilk (BIO101). The size and amount of DNA products were checked by gel electrophoresis of a small aliquot. Generally 1/4 of a 100 μl reaction (20–200 ng) was used for sequence analysis according to the instructions provided with the fmol kit (Promega). In brief, one of the amplification primers (20 ng) was end-labeled with [γ-^{32}P]ATP (1 μCi) and the four termination reactions (5 μl) were assembled on ice and covered with paraffin oil. Samples were transferred to a hot thermoblock (95°C) and subjected to 30 cycles of linear amplification with 30 sec each at 94, 60, and 72°C. Sequencing reactions were stopped by adding 4 μl of formamide/dye mix and separated on denaturing polyacrylamide gels (8%) followed by drying of the gel and exposure to X-ray film for 6–24 hr.

RESULTS

Deletion Analysis

A panel of 77 Wilms' tumors was assembled during this study. The clinical, pathological, and cytogenetic features of cases with *WT1* alterations are detailed in Table 2. In some cases DNA from blood or normal kidney of the same patient was available for comparison. Initially, the tumors were examined for deletions and gross rearrangements around the *WT1* locus by Southern blot analysis with the *WT1* cDNA clone and a panel of hybridization probes that cover a 15 Mbp region from 11p13-p14 (Gessler et al., 1989, 1990; Gessler and Bruns, 1989). In all cases gene dosage was measured to detect hemizygous deletions. About 20% of tumors showed varying degrees of DNA degradation and could not be evaluated unambiguously.

Surprisingly, only the two tumors described previously (WT 8A and PER, Gessler et al., 1990) showed evidence of homozygous deletion of the *WT1* gene. In one case, PER, both chromosomes

TABLE 2. Clinical and Molecular Features of Wilms' Tumors

Tumor sample	Clinical features	Sex/ karyotype	Age at diagnosis	Pathology	Loss of heterozygosity	Status of 11p/WT1	
						Allele 1	Allele 2
WT 8A	Clinically normal	Male	18 m	Stage III, favorable histology	n.d.	Large deletion	Large deletion
PER	N/A	N/A	N/A	N/A	n.d.	Deletion, 170 kbp	Deletion, 170 kbp
S86-1334	Aniridia, hypospadias, cryptorchidism (WAG)	46 XY, del 11p14.1 -p13	7 yr	Blastemal tumor, focal tubular differentiation, no nephroblastomatosis	Heterozygous for HRAS, TH	Large deletion	1 bp deletion in exon 7, stop codon
S87-52	Clinically normal	Male	13 m	Stage I, favorable histology, multicentric tumor, multifocal intralobar nephroblastomatosis	Heterozygous for HRAS, INS, HBB, CAT	Large deletion	c to t, His to Tyr in exon 8
S87-877	Clinically normal	Female	11.5 m	Favorable histology, interstitial nephritis and moderate glomerulosclerosis, no nephroblastomatosis	Heterozygous for HRAS, INS, CALCA	Large deletion	5 bp deletion in exon 1, stop codon
P.N.	Clinically normal	Male	1 yr, 10m	Stage II, favorable histology	n.d.	Large deletion	No mutation found
WT 2A	Aniridia, perineal hypospadias, failure of scrotal fusion and persistent cloaca (WAG)	46 XY	4 yr, 5 m	Stage III, favorable histology	Heterozygous for HRAS, TH	Large deletion very likely	c to t in exon 9, stop codon
WT 12A	Low set ears, cardiomegaly, developmental delay, no aniridia!	46 XY, del 11p13	7 m	Stage I, favorable histology, possible intralobar nephrogenic rests, recurrence after chemotherapy	Heterozygous for HRAS, TH, HBB, CALCA, FSHB	del 11p13 in tumor karyotype	5 bp deletion in exon 2, stop codon
BT1	Minor foot anomaly	46 XY	3 yr, 11 m	Stage IV, no nephroblastomatosis	n.d.	ccc to tcc; P to S in exon 2	No mutation found
S86-169	Clinically normal	46 XX	2 yr	Bilateral, favorable histology, nephroblastomatosis, metastatic recurrence of tumor	Not informative for HRAS, TH	g to t at −20, upstream exon 3	g to t at −20, upstream exon 3
MR1	Clinically normal	Male	3 yr	Stage II, stroma-rich tumor	n.d.	Insertion a at −58, exon 3	No mutation found

11 showed the identical deletion of 170 kbp, as probes from the breakpoint region detected only one type of junction fragment with normal copy number. This implies that homozygosity for the deletion was achieved through mitotic recombination or chromosome loss with duplication of the deletion chromosome. The second homozygous deletion of *WT1*, seen in Wilms' tumor 8A, is flanked on both sides by large regions that show reduced copy number for multiple probes suggesting a hemizygous deletion. The homozygous deletion thus appears to result from either two independent and partially overlapping deletions or a combination of a smaller deletion region that is contained within a large deletion on the other chromosome (see Fig. 1).

In addition to these homozygous deletions four other tumors (P.N., S87-877, S86-1334, and S87-52) showed evidence of a hemizygous deletion of *WT1*. The common feature of all these deletions is their size in the range of millions of base pairs. No smaller deletions that would only encompass the *WT1* gene or single intragenic restriction fragments were detected.

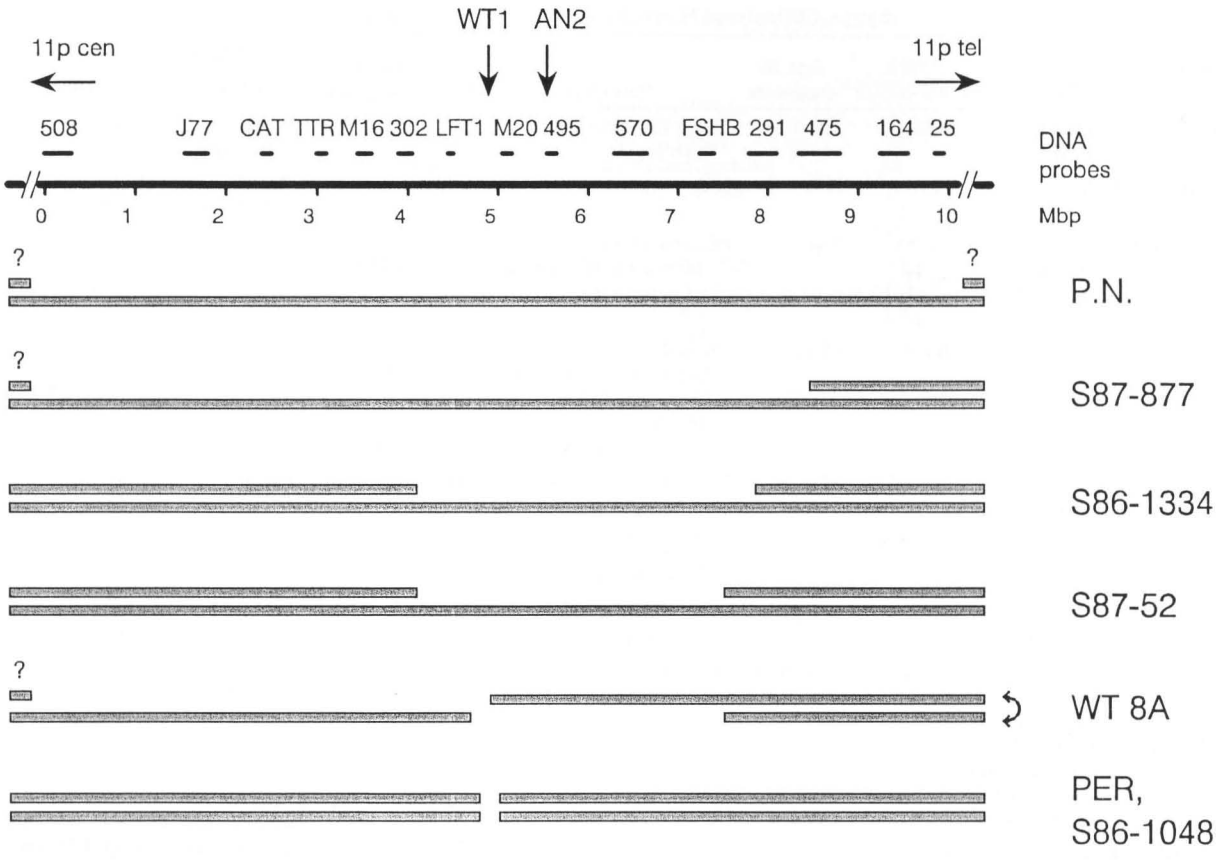

FIGURE 1. **Deletions of 11p13 in Wilms' tumors spanning the *WT1* locus. Gene dosage analysis was used to establish the extent of deletions on chromosome 11p. DNA probes used in this study are shown above the scale bar. In most cases several additional DNA probes, some from other chromosomes, were used to confirm and extend the results depicted in this diagram. Horizontal boxes represent the parts of chromosome 11p material present in each tumor. For tumor WT 8A it is unclear whether the areas of hemizygous deletion affect only one chromosome 11 or both. Question marks above short boxes indicate uncertainty about the extent of the deletion outside the mapped area.**

RNA Analysis

Northern blot analysis could be performed on RNA from 16 tumors, revealing apparently normal transcript sizes in all cases. The amount of *WT1* mRNA, however, varied greatly between different tumor samples—a phenomenon that may reflect the different developmental stage of the cells from which the tumor originated (Beckwith et al., 1990).

The deletion of one allele of the *WT1* gene in DNA from four Wilms' tumors led to the assumption that the second copy likely suffered a more subtle alteration. In two cases (S86-1334 and P.N.) available RNA showed a normal-sized transcript. The *WT1* coding region was then analyzed by RT-PCR and sequencing of the amplified and subcloned fragment. For tumor S86-1334 a deletion of a single nucleotide in exon 7 leading to a frameshift mutation was detected (Gessler et al., 1993). For patient P.N. no alteration could be found in the cDNA fragment analyzed (nucleotides 779–1606). This, however, does not rule out a mutation especially in the 5′-region of the cDNA. This region could not be amplified from the cDNA preparation, perhaps due to the high G + C content in that sequence. For the other two tumors showing hemizygous deletions no tissue was available for RNA analysis.

SSCP Analysis

In order to screen the entire panel of tumors for subtle alterations in the *WT1* gene, SSCP analysis was employed. Based on the genomic sequence of each of the 10 *WT1* exons (Gessler et al., 1992, EMBL X61631-X61640) PCR assays were developed to analyze single exons with a small amount of flanking intron sequence containing splice donor and acceptor consensus sequences. Only the coding region was used and exon 1 was divided into two parts to obtain short PCR products (<300

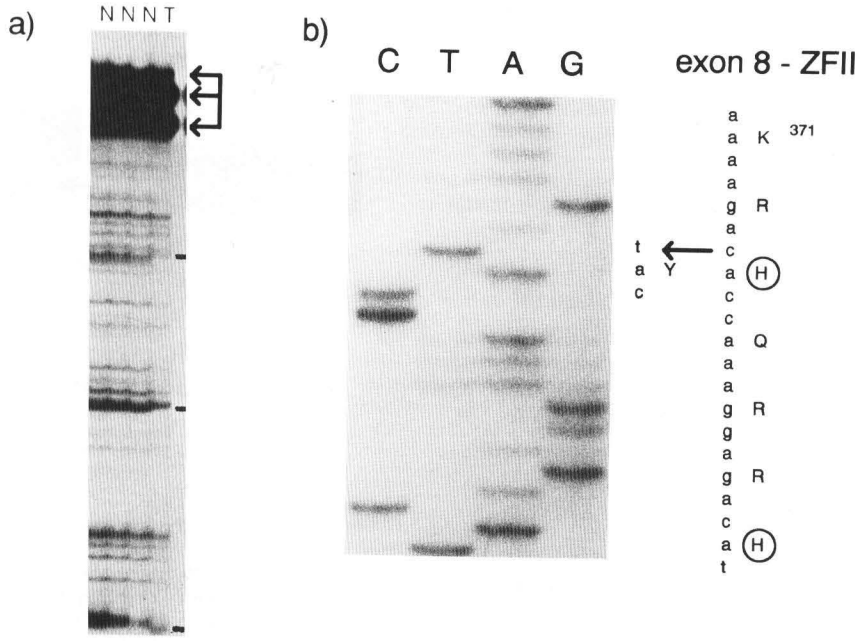

FIGURE 2. **Wilms' tumor S87-52 shows a point mutation in exon 8. (a) SSCP analysis showed indistinguishable major bands (arrows) in tumor DNA (T) and normal samples (N). Only additional weak bands displayed alterations in tumor DNA. (b) Direct sequence analysis of amplified exon 8 DNA revealed a clear c→t substitution shown on the right side with the corresponding amino acid translation.**

bp) that give a high sensitivity of mutation detection. Due to the high G+C content, the first part of exon 1 could only be amplified reproducibly from genomic DNA using thermostable Vent polymerase (NEB) at high temperature. Even then, many tumor and control DNAs did not give a PCR product in repeated experiments and this part of the coding region was therefore not analyzed any further.

All other primer pairs produced characteristic patterns of two to four bands upon electrophoresis in nondenaturing gels. For exon 7 two different sets of bands could be distinguished with individuals being either heterozygous or homozygous for one of the variants (data not shown). This polymorphism was also seen in control DNAs and has been recently described as a silent A to G mutation in exon 7 by Groves et al. (1992).

Several tumor DNAs exhibited additional variant bands that could be due to mutations within the amplified DNA segment. These DNA fragments were then amplified from genomic DNA or cut out from dried gels and sequenced from both ends using the amplification primers.

Tumor S87-52, that was shown to be hemizygously deleted for *WT1*, revealed altered SSCP bands with primers for exon 8 (Fig. 2). These alterations were only seen as additional weak bands that were detected upon prolonged exposure of the gel. The main amplification product appeared unaltered under different gel running conditions. Direct sequencing of the PCR product revealed a point mutation C→T in the remaining *WT1* allele, thereby replacing the first histidine residue of zinc finger 2 by tyrosine. This amino acid residue is a key element of zinc binding and the mutation would be expected to produce a nonfunctional finger structure with concomitant loss of sequence-specific DNA binding. A second tumor, S87-877, that was hemizygously deleted for *WT1* showed very weak and altered bands for exon 1 (Fig. 3). In this case a deletion of 5 base pairs was found, leading to a reading frame shift that results in a stop codon immediately after the deletion with a very short residual open reading frame. Interestingly, the deletion involves one copy of a direct repeat of 5 nucleotides, suggesting a polymerase slippage error during replication.

Evidence for *WT1* inactivating mutations was apparent in two additional cases. Wilms' tumor 12A displayed only altered SSCP bands for exon 2 which resulted from a 5 base pair deletion in exon 2, again leading to a premature stop codon within this exon (Fig. 4). In this case gene dosage analysis suggested a normal copy number for the *WT1* locus, but these results remain tentative due to par-

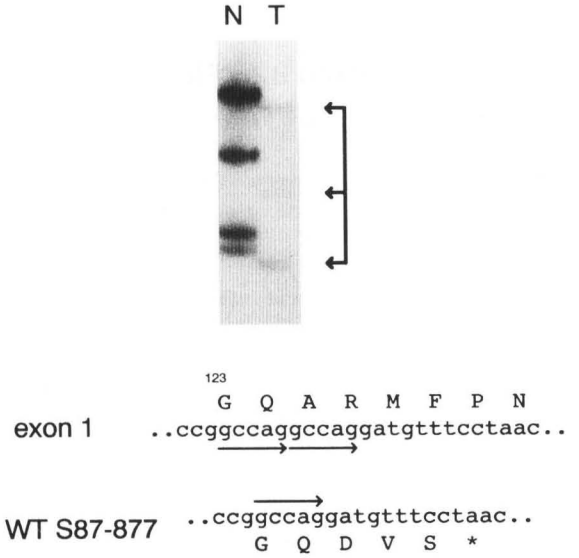

FIGURE 3. Deletion of a five nucleotide direct repeat from exon 1 in Wilms' tumor S87-877. SSCP analysis displayed weak and altered bands in tumor DNA (T). The pentanucleotide stretch deleted in this tumor is depicted in the diagram. A stop codon occurs in the new reading frame after three amino acids.

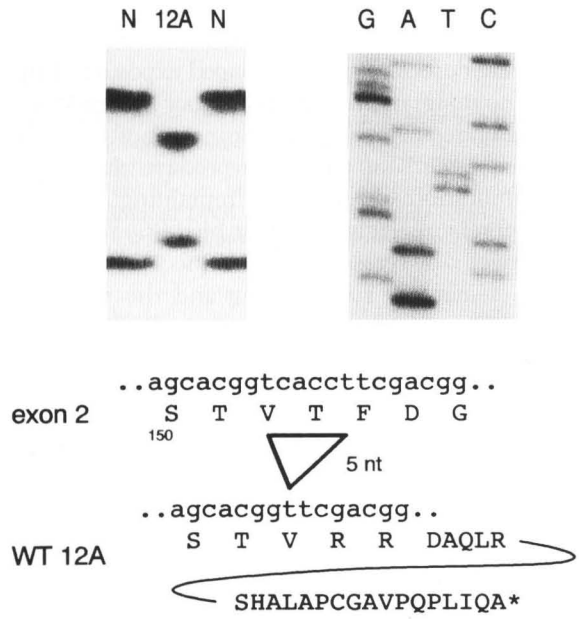

FIGURE 4. SSCP analysis (left) and direct sequence analysis (right) of exon 2 reveals a five base pair deletion in Wilms' tumor WT 12A. The reading frame shift leads to a premature stop codon within exon 2 (shown below).

tial degradation of the tumor DNA sample. Cytogenetic analysis of blood cells as well as direct analysis of short-term cultures from a lung metastasis, on the other hand, revealed a 46 XX, del 11p13 karyotype. Thus, the microdeletion de-

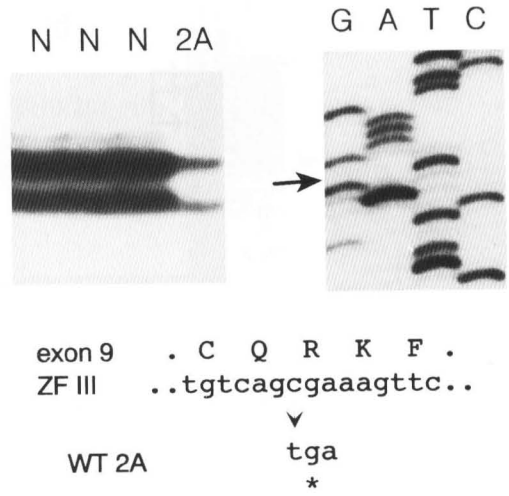

FIGURE 5. A stop codon is created by a c→t substitution in exon 9 of Wilms tumor WT 2A. SSCP analysis (left) showed minimal alterations in the mobility of the amplified exon strands from the tumor (2A). Sequence analysis unambiguously identified the introduction of a stop codon in exon 9 of the tumor DNA.

tected in exon 2 likely represents the second mutation inactivating the remaining *WT1* allele in a precursor cell. DNA from tumor 2A showed minor alterations upon SSCP analysis of exon 9 (Fig. 5). Direct sequencing of the PCR product revealed a C to T mutation in codon 390 of exon 9 resulting in a translation stop codon. Again, gene dosage analysis in tumor DNA did not yield unambiguous results due to partial DNA degradation. The clinical description of the patient with aniridia and genitourinary anomalies, however, suggests the presence of a constitutional WAGR deletion including both the *WT1* and *AN2* genes. The point mutation in exon 9 would be expected to be the second alteration that abrogates *WT1* function in tumor cells.

Another mutation in exon 2 was detected in DNA from Wilms' tumor BT1 (Fig. 6). In this case a C → T mutation on one of the *WT1* alleles leads to a change from Pro to Ser in the amino-terminal half of the protein. This amino acid replacement may represent a diagnostic missense mutation of a functionally important amino acid residue of the *WT1* polypeptide. As there are no distinct properties assigned to specific regions of the amino-terminal part of the *WT1* protein it is difficult to test whether some *WT1* functions might be affected by such a mutation based solely on sequence information.

There were additional cases where single base-pair changes in introns were detected by SSCP

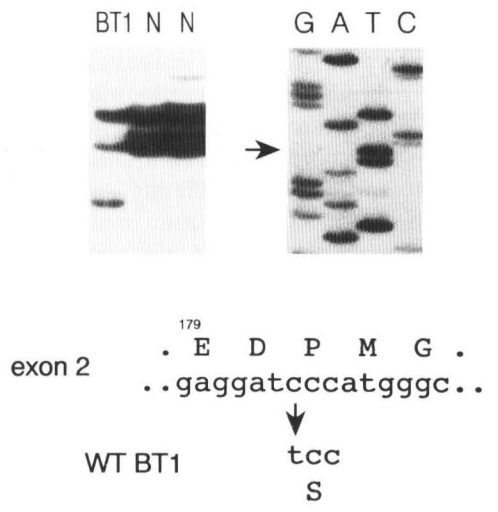

FIGURE 6. **Hemizygous point mutation in exon 2 of Wilms' tumor BT1. One additional band of faster mobility can be observed upon SSCP analysis in BT1 DNA. Sequence analysis of the anomalous migrating band isolated from the SSCP gel revealed a c→t mutation, resulting in a proline to serine change.**

analysis of tumor DNA: Two of these affect the region upstream of exon 3 (Fig. 7). Tumor S86-169 shows a G→T mutation at nucleotide −20 (relative to the splice site). In tumor MR1 there is a single base insertion (+A at −58, data not shown). In both cases the significance of the mutations is unclear. The mutations have not been found in other tumor DNAs or control samples, but they may well represent rare polymorphic variants. Sequence requirements for efficient splicing are still poorly defined on the other hand and it cannot be assessed whether these mutations may affect correct assembly of the *WT1* mRNA as there is no RNA available from these tumors.

Studies on Loss of Heterozygosity

It is still unclear whether mutations in *WT1* can cooperate with alterations at the other proposed Wilms' tumor genes to override growth control. For several of the tumors that were shown to contain *WT1* alterations LOH studies with polymorphic markers for chromosome 11p15 were performed (Table 2). In all informative cases there was no loss of alleles at chromosome 11p15. Identical *WT1* mutations on both chromosomes 11 in tumors PER and S86-169, however, suggest that a loss of the normal allele has occurred in these cases.

DISCUSSION

The detection of homozygous deletions, with some being nonoverlapping and affecting only the 5′ or 3′ part of the gene or internal exons has firmly established *WT1* as the 11p13 Wilms' tumor gene (Call et al., 1990; Gessler et al., 1990; Huff et al., 1991; Ton et al., 1991). In parallel studies, however, increasing evidence has been found suggesting the presence of several additional genes that may be involved in Wilms tumor development. A significant fraction of Wilms' tumors show loss of heterozygosity for chromosome 11p, but limited to the telomeric band p15 where the *WT2* locus has been genetically mapped (Mannens et al., 1988; Koufos et al., 1988; Reeve et al., 1989) and for which functional tumor suppression has been demonstrated (Dowdy et al., 1991). Another locus that may be involved in the genesis of these tumors has been mapped by loss of heterozygosity to chromosome 16q in as many as 20% of tumors (Maw et al., 1992). Finally, the location for the predisposing gene in rare familial Wilms' tumors has been excluded by genetic linkage from any of these regions (Grundy et al., 1989; Huff et al., 1989, 1992).

Only the 11p13 Wilms' tumor gene, *WT1*, has been cloned so far and is amenable to mutation screening in tumor tissue. Here a panel of 77 Wilms' tumors was analyzed by gene dosage and SSCP analyses to detect most of the mutations that may have occurred at this locus. While gene dosage studies can be very difficult in tumors showing DNA degradation, SSCP analysis appears to be the method of choice to analyze large numbers of samples with high sensitivity and the potential to detect even single basepair changes. SSCP analysis is postulated to detect around 70–95% of all possible single base mutations (Sheffield, 1993). Nevertheless, it must be kept in mind that using only this technique one will inevitably overlook some of the alterations present.

Examples of cases that may well go undetected during SSCP screening are represented by the mutations in exon 9 of tumor WT2A and especially in exon 8 of tumor S87-52. By scoring the main amplification products—as done usually—this exon would have been classified as unaltered. Only faint additional bands that could be derived from rare internal priming events or represent shorter and incomplete products whose conformation is more sensitive to alterations identified the mutation in this case.

The results from this study suggest an unexpectedly low frequency of mutations at *WT1* in Wilms' tumors. In 85% of tumors no evidence for alterations of *WT1* was found with any of the methods employed. Taking in account that a small region of

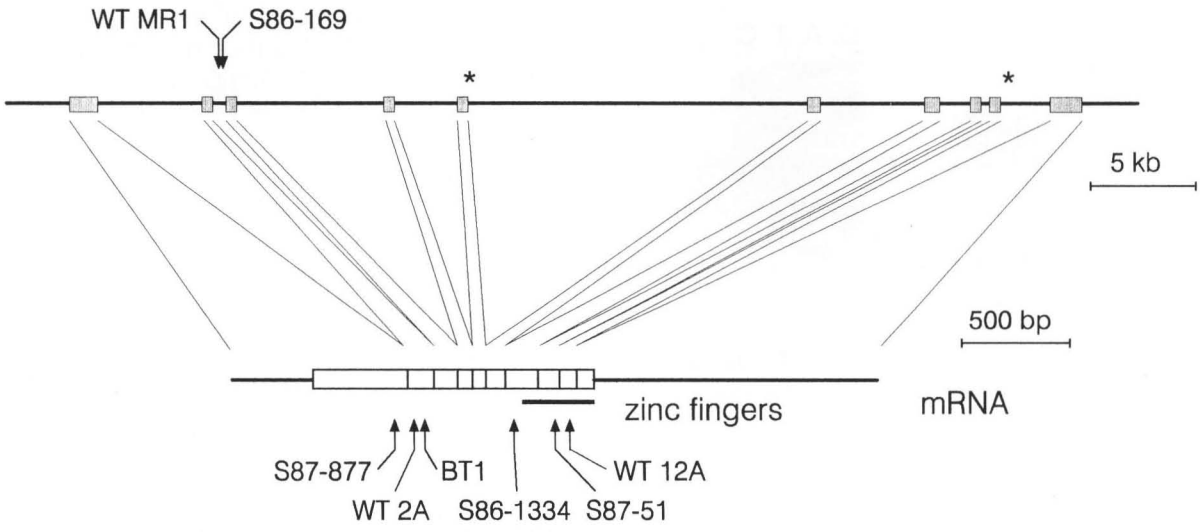

FIGURE 7. **Localization of mutations within the *WT1* gene. The exon–intron organization of the *WT1* gene is shown (adapted from Gessler et al., 1992). Mutations within the coding sequence are shown below the cDNA. The thin line represents noncoding sequence of the cDNA. The two mutations upstream of exon 3 are indicated above the genomic sequence.**

the 5′ end of the *WT1* gene including the promotor and the 1.3 kbp 3′ untranslated region have not been screened for subtle mutations it seems likely that the overall mutation frequency at *WT1* is below 20%.

Large deletions of 11p13 and adjacent chromosomal regions, that should also be cytogenetically visible, are present in about 10% of tumors. These deletions are reminiscent of the typical WAGR deletions and three of the patients either have these deletions in constitutional DNA or present clinical features that make an 11p13 deletion very likely. These deletions, however, affect only one of the chromosomes 11 or if present on both, the region of overlap seems to be limited to a much smaller area of a few hundred kbp, as seen in WT8A and PER. The low frequency of these deletions is in agreement with the paucity of chromosome 11p13 deletions found in tumor karyograms (Douglas et al., 1985; Slater et al., 1992). Also in agreement with this is the low overall incidence of smaller rearrangements that can be detected in tumor DNA upon Southern blot analysis with *WT1* probes (Royer-Pokora et al., 1991; Cowell et al., 1991).

The second type of mutations in Wilms' tumor DNA—besides large deletions that likely remove several genes—are subtle alterations that directly inactivate the *WT1* gene. Of the total of six cases with larger, hemizygous deletions the second *WT1* allele suffered point mutations or small deletions in five of them. These mutations introduce premature stop codons, either directly or through reading frame shifts, or change a key amino acid in the protein product. These alterations would be expected to lead to either complete *WT1* loss or to the translation of a truncated and nonfunctional protein.

Unlike the situation with p53 in different tumor types where a number of diagnostic missense mutations can be found, there are few alterations of this type in Wilms' tumors. Most of the *WT1* mutations appear to affect the zinc finger domain as in tumor S87-52, where the replacement of the key amino acid histidine by tyrosine should destroy the important DNA binding domain. Similar mutations altering key amino acids in Wilms' tumors and the Denys–Drash syndrome have been described by others (Little et al., 1992; Pelletier et al., 1991a). There is only one mutation outside the finger region in exon 2 of tumor BT1 where the hemizygous replacement of proline by serine may affect the proline-rich putative transacting domain of the *WT1* protein. To date functional domains of *WT1* outside the zinc finger region have not been characterized enough to evaluate the significance of this mutation. It will be interesting, however, to test whether the BT1 mutation represents a dominant "gain of function" mutation that interferes with the transcriptional repression properties of *WT1* (Madden et al., 1991).

The detection of several additional loci that may be involved in Wilms' tumor formation raises the question whether these genes may cooperate in

some way. Henry et al. (1989) described a WAGR patient with a constitutional deletion of 11p13 where studies on loss of heterozygosity revealed allele loss on chromosome 11, but limited to 11p15. Although the status of the remaining WT1 allele has not been reported, this finding suggested that there may be some interaction between the 11p13 and 11p15 loci. Surprisingly, none of the six tumors in our series with *WT1* alterations that were also informative for 11p15 markers showed allele loss. This does not, however, rule out other alterations of the presumed *WT2* gene that may result in a cooperative effect on cell growth.

One of the Wilms' tumor DNA samples in the present study was derived from a familial Wilms' tumor (patient IV-14 in Grundy et al., 1988). Both gene dosage and SSCP analyses did not reveal any WT1 alterations (data not shown). This is in agreement with the report of Schwartz et al. (1991) who found no cosegregation between familial predisposition to Wilms' tumors and the WT1 gene.

The assays for *WT1* integrity we have used here will certainly not reveal all possible cases of *WT1* inactivation. Studies of protein expression and functional tests of the DNA binding capacity of the *WT1* protein in tumor extracts may complement the present approaches. Our analysis, however, revealed an unexpectedly low frequency of homozygous inactivation of the *WT1* gene. Thus, the high frequency of allele loss reported for chromosome 11p may reflect alterations of the *WT2* gene in 11p15. The test of this notion awaits the cloning and analysis of these other WT loci.

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