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Homozygous somatic *WT1* point mutations in sporadic unilateral Wilms tumor

(mutation analysis/renal tumor/childhood cancer)

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ABSTRACT **Wilms tumor may be caused by loss of function of genes at different loci. A Wilms tumor suppressor gene, *WT1*, at chromosome 11 band p13, has recently been cloned and characterized. *WT1* has been implicated in the development of Wilms tumor by virtue of mutations in patients with genitourinary anomalies and susceptibility to Wilms tumor. Homozygous intragenic mutations have been reported in Wilms tumors, but usually not in sporadic unilateral Wilms tumors, which constitute the majority of Wilms tumor cases. Using the single-strand conformational polymorphism assay, we have identified three sporadic unilateral Wilms tumors with homozygous point mutations: one with a *de novo* germ-line nonsense point mutation within *WT1* exon 8, and two carrying a somatic mutation within *WT1* exon 10. In all three cases loss of the wild-type allele was demonstrated by tumor loss of heterozygosity. This report provides an example of two somatic mutations in the same tumor expected to inactivate *WT1* function.**

Wilms tumor, or nephroblastoma, is an embryonal malignancy of the kidney that affects approximately 1 in 10,000 children younger than 16 years of age. Cytogenetic deletions in patients with Wilms tumor, aniridia, genitourinary malformations, and mental retardation (the WAGR syndrome) suggested the inactivation of a gene at 11p13, at least in a subset of Wilms tumors. This was supported by genomic analyses of sporadic Wilms tumors showing loss of heterozygosity (LOH) for markers at 11p13 (1–4). Molecular analysis of DNA mapping to a homozygously deleted region at 11p13 in two cases of sporadic Wilms tumor with apparent normal karyotype (5, 6) led to the identification and isolation of *WT1* independently by three groups (6–8). The predicted WT1 protein contains four zinc fingers and a proline/glutamine-rich region, suggesting a transcriptional regulatory function. The WT1 zinc finger region has been shown to bind the promoter consensus sequence of early growth response genes (9) and it seems likely that the WT1 protein has a regulatory function in the expression of genes in early embryonic differentiation.

The role of *WT1* as a Wilms tumor susceptibility gene has been supported by reports demonstrating homozygous deletions of the region encompassing all or part of *WT1* (5, 6, 10–13), constitutional intragenic *WT1* deletions followed by 11p13 tumor LOH (14–20), and by a case with early chromosomal reduplication followed by nondisjunction that preceded a somatic 25-bp *WT1* deletion spanning an exon–intron junction (21). Homozygous somatic point mutations in sporadic unilateral Wilms tumor have thus far not been reported. We report on two such cases as well as on one with a *de novo* germ-line point mutation as first event. In all three, loss of the wild-type allele is expected to result in inactivation of *WT1*.

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MATERIALS AND METHODS

Fifty Wilms tumors from as many patients were analyzed in this study. Five of these patients had bilateral disease, one hemihypertrophy, one aniridia, one the Prader–Willi syndrome, and one Bloom syndrome. None of the remaining 41 patients showed aniridia, genitourinary malformations, mental retardation, hemihypertrophy, or any other dysmorphic feature. The family history in all cases was negative for Wilms tumor. Patient Wit-24 is a male who presented with a sporadic unilateral Wilms tumor at 4 years of age, patient Wit-26 is a female who presented with a unilateral sporadic Wilms tumor at the age of 2 years, and patient Wit-29 is a female who presented with a unilateral sporadic Wilms tumor at the age of 11 months.

Isolation of constitutional and tumor DNA and determination of LOH by Southern blot hybridization of restriction enzyme digests of unamplified genomic DNA, as well as by direct visualization of genomic DNA restriction fragments amplified by the polymerase chain reaction (PCR), were performed as described (4).

Single-Strand Conformational Polymorphism (SSCP) Analysis. Exonic sequences from genomic DNA were amplified by PCR and the PCR products were analyzed for SSCP (22) in order to detect possible deletions, insertions, or point mutations. In the analysis of *WT1* exon 7, the primers used were Luc7 (sense), 5′-ACCTACGTGAATGTTCACATGT-GCTTA-3′, and LucAS7 (antisense), 5′-TCTTGAACCAT-GTTTGCCCAAGACTGGA-3′. For *WT1* exon 8, (A-2)8 and (S-2)8, and for *WT1* exon 9, (A-2)9 and (S-2)9, which have been described (16), were used. For *WT1* exon 10, Luc10 (sense), 5′-GTTGCAAGTGTCTCTGACTGGCAATTGT-3′, and LucAS10 (antisense), 5′-TGAAAGCAGTTCACA-CACTGTGCTGCCT-3′, were used. PCR was carried out in a GeneAmp PCR system 9600 (Perkin–Elmer) with a final volume of 100 μl, containing 250 μM each dATP, dGTP, and dTTP; 20 μM dCTP; 1 mCi (37 MBq) of [α-^{32}P]dCTP, 200–800 ng of DNA, 50 pmol of each PCR primer, 2.5 units of *Taq* polymerase, and 10 μl of *Taq* polymerase reaction buffer. Following PCR, a 5-μl aliquot of the amplified product was diluted with 40 μl of 0.1% SDS/10 mM EDTA; 2 μl of this mixture was added to 2 μl of Sequenase stop mix [95% formamide/20 mM EDTA/0.05% bromophenol blue/0.05% xylene cyanol (United States Biochemical)]. The DNA samples were denatured at 95°C for 3 min and immediately placed on ice before being run on 6% polyacrylamide nondenaturing gels containing 10% glycerol. Electrophoresis was performed in TBE (0.09M Tris base/0.09M boric acid/2.5 mM EDTA) running buffer at 30 W at room temperature for 4–6 hr, at 30 W at 4°C for 4–6 hr, or at 5 W at room temperature for 16 hr. The gels were dried and exposed to XAR-5 film (Kodak) at room temperature for 16–96 hr without an intensifying

Abbreviations: LOH, loss of heterozygosity; SSCP, single-strand conformational polymorphism.

screen. We targeted our analysis to exons 7–10 because the zinc fingers are considered to be critical for the putative transcriptional regulatory function of the WT1 protein.

Sequencing. The mechanisms used for direct sequencing of biotinylated PCR products have recently been described (23). Briefly, when the PCR product was to be used for sequencing, PCR amplification was performed with one biotinylated primer at the 5′ end and one nonbiotinylated primer. The final volume was 100 μl, [^{32}P]dCTP was omitted, and the amount of primer was decreased to 5–15 pmol. Adsorption of a 50-μl aliquot of the biotinylated PCR product on streptavidin-coated magnetic Dynabeads (DYNAL, Merseyside, U.K.) was performed utilizing a magnetic particle concentrator (MPC) (DYNAL) as recommended by the manufacturer. The supernatant was removed and the beads were suspended in 100 μl of 0.15 M NaOH to denature the double-stranded DNA. Biotinylated and nonbiotinylated strands were separated on the MPC. The nonbiotinylated strands were precipitated in 100% ethanol and the DNA was recovered by centrifugation. The biotinylated strands were washed with 100 μl of 10 mM Tris·HCl, pH 8.0/1 mM EDTA/0.1 M NaCl and subsequently with 100 μl of water. Both single-strand DNA samples (biotinylated and nonbiotinylated) were resuspended in 5 μl of water. Dideoxy sequencing was performed on each strand with a Sequenase kit (United States Biochemical) using the complementary DNA primer.

RESULTS

The 50 Wilms tumors were examined for deletions, insertions, or base-pair mutations within *WT1* exons 7–10 (encoding the four zinc fingers) by PCR/SSCP analysis. LOH for the 11p13–pter region, which includes *WT1*, in the tumor DNA of Wit-24 has previously been reported (4); analysis of a representative sample using the *D11S9* probe, which lies proximal to the Wilms tumor locus at 11p13, is shown in Fig. 1*A*. Tumor DNA of Wit-26 did not demonstrate LOH for either chromosome 11p or 16q markers (4); sequence analysis, however (see below), demonstrated LOH at *WT1* exon 10. Tumor DNA of Wit-29 showed LOH for a previously reported CA repeat within *WT1* (21) (Fig. 1*B*). *WT1* mRNA levels were low in all three cases (ref. 24; B.R.G.W. and H.Y., unpublished data). Southern blot analysis of genomic DNA with 31E1, a human *WT1* cDNA (25), as a probe showed no difference between the tumor DNAs of patients Wit-24, Wit-26, and Wit-29 and the DNA of normal controls, demonstrating that there were no gross rearrangements of the *WT1* gene (data not shown).

PCR/SSCP analysis of exon 10 (zinc finger 4) demonstrated a mobility shift for the Wit-24 and Wit-26 tumor DNAs when compared with DNA from unaffected individuals, whereas exon 8 (zinc finger 2) showed a shift for Wit-29 tumor DNA. The constitutional DNA of Wit-29 was shown to be heterozygous (Fig. 2). Subsequent sequence analysis revealed a C^{1084} → T transition, which would convert the Arg^{362} codon to a premature stop codon in tumor DNA of Wit-29 (Fig. 3*A*). In the sequence of tumor DNA of Wit-24, a C^{1297} insertion would convert Arg^{433} to Pro (Fig. 3*B*). In the tumor DNA of Wit-26, a C^{1298}-G^{1299} deletion was demonstrated, which would convert Arg^{433} to Pro (Fig. 3*B*). The constitutional DNAs of Wit-24 and Wit-26 were normal (data not shown). However, the constitutional DNA of Wit-29 showed both the wild-type allele and mutant allele sequence (Fig. 3*A*). The constitutional DNAs of both parents were normal (Fig. 3*A*).

The mutations described here were confirmed by sequence analysis of at least five additional samples from any given specimen. In addition, SSCP analysis of the coding region of exons 1–6 was performed for tumors Wit-24, Wit-26, and Wit-29. No additional SSCP shift was noted for these tumors (data not shown).

DISCUSSION

We have analyzed 50 Wilms tumors, 41 of which were sporadic unilateral cases, for *WT1* zinc finger mutations and have demonstrated the occurrence of two genetic events which are predicted to inactivate *WT1* in three cases (7.3%). In Wit-29, a *de novo* germ-line point mutation was followed by loss of the wild-type allele in the tumor, as demonstrated by tumor LOH for the CA repeat within *WT1*. The C^{1084} → T point mutation in Wit-29 causes early termination of translation in the second zinc finger and is predicted to result in a truncated WT1 protein which contains one single intact zinc finger (zinc finger 1). Interestingly, this germ-line nonsense point mutation does not result in ambiguous genitalia, nephropathy, and Wilms tumor (Denys–Drash syndrome). The germ-line mutations described in patients with the Denys–Drash syndrome are missense mutations resulting in a modified WT1 protein in which at least three out of the four zinc fingers remain intact. Recently, another germ-line nonsense mutation (in exon 9) has been described in a patient

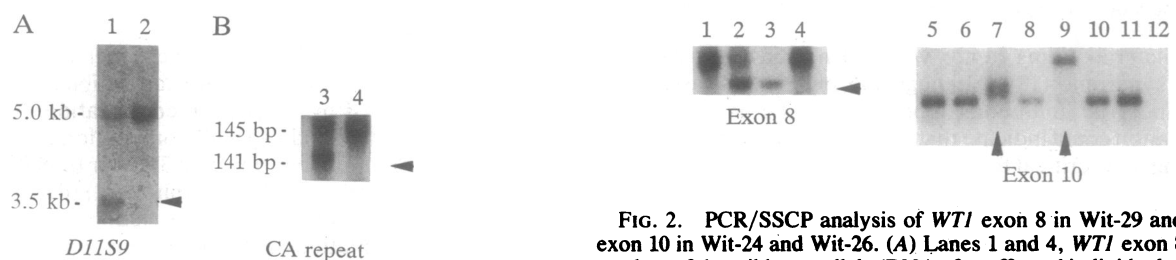

FIG. 1. Tumor LOH in patients Wit-24 and Wit-29. (*A*) Representative Southern hybridization, with probe *D11S9* [pES1-2(*Taq* I)], of constitutional DNA (lane 1) and tumor DNA (lane 2). LOH is indicated by the arrowhead. Densitometric scanning of Wit-24 hybridization signals demonstrated reduplication of the chromosome segment that showed LOH (data not shown). (*B*) Reduction to homozygosity of Wit-29 tumor DNA at a CA repeat within 31E1. A 50-bp CA repeat is present in the 3′ untranslated sequence of 31E1. Primers flanking this repeat that have previously been published (21) were used to amplify genomic DNA. PCR products were electrophoresed in a denaturing 7% polyacrylamide gel. Difference in size of the bands represents variations in the length of the CA repeat. PCR product from tumor DNA (lane 4) shows loss of the wild-type allele present in the constitutional DNA (lane 3).

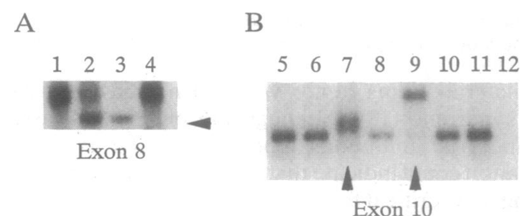

FIG. 2. PCR/SSCP analysis of *WT1* exon 8 in Wit-29 and *WT1* exon 10 in Wit-24 and Wit-26. (*A*) Lanes 1 and 4, *WT1* exon 8 PCR product of the wild-type allele (DNA of unaffected individual used as control); lane 2, Wit-29 constitutional DNA demonstrating both the wild-type PCR product and a second PCR product with a different mobility shift (arrowhead); lane 3, Wit-29 tumor DNA showing loss of the wild-type PCR product and retention of the shifted PCR product (arrowhead). (*B*) Lanes 5, 10, and 11, *WT1* exon 10 PCR product of the wild-type allele (DNAs of three unaffected individuals used as controls); lane 6, Wit-24 constitutional DNA showing the wild-type pattern; lane 7, Wit-24 tumor DNA showing a SSCP mobility shift (arrowhead); lane 8, Wit-26 constitutional DNA showing the wild-type pattern; lane 9, Wit-24 tumor DNA showing a SSCP mobility shift different from the one demonstrated in lane 7 (arrowhead); lane 12, nondenatured sample used to delineate the position of migration of the double-stranded DNA product (not visible on this photographic mount).

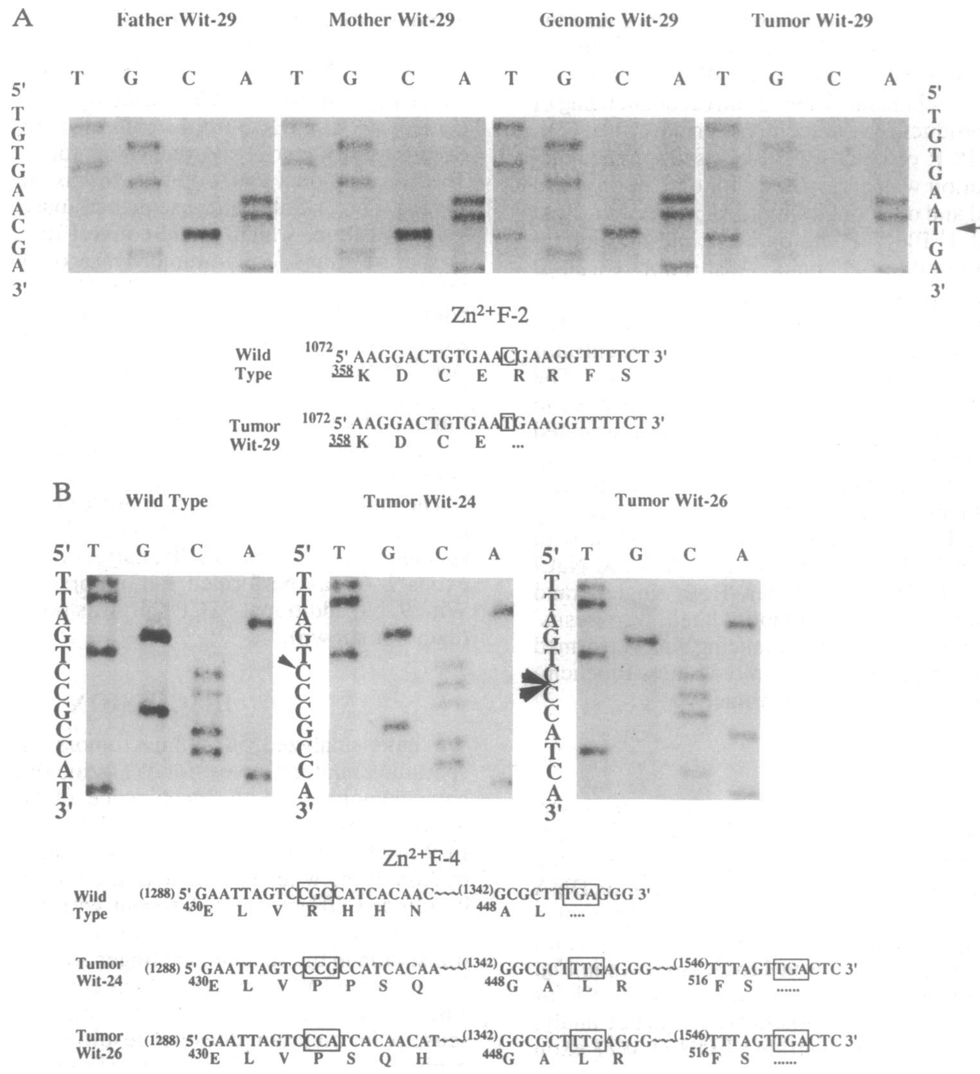

FIG. 3. Sequence analysis of *WT1* exons 8 and 10 in Wit-24, Wit-26, and Wit-29. The nucleotide numbering system is based on the coding sequence only and considers the A of the initiator ATG as +1. The numbering system for the amino acids considers the initiator methionine as +1. (*A*) *WT1* exon 8 [zinc finger 2 (Zn^{2+} F-2)] sequence analysis of constitutional and tumor DNA of patient Wit-29 showing a $C^{1084} \rightarrow T$ point mutation (heterozygous in the genomic DNA and homozygous in the tumor DNA) which converts the Arg^{362} codon to a premature stop codon. Constitutional DNA from the father and mother of patient Wit-29 are shown in the first two lanes and represent the normal sequence of this region. (*B*) *WT1* exon 10 [zinc finger 4 (Zn^{2+} F-4)] sequence analysis of Wit-24 shows a C^{1297} insertion and analysis of Wit-26 shows a homozygous C^{1298}-G^{1299} deletion. Both mutations convert Arg^{433} to Pro. Note the disruption of the normal reading frame, eliminating the wild-type stop codon at 1348–1350 and introducing a novel stop codon at 1549–1551.

with bilateral Wilms tumor who did not have the Denys–Drash syndrome (18). Although the number of cases with germ-line *WT1* mutations reported thus far is small, this observation could indicate that the number of functional WT1 zinc fingers is critical for a given germ-line mutation to result in a gain-of-function or dominant-negative lesion, a mechanism postulated in Denys–Drash syndrome (16).

The C^{1297} insertion in Wit-24 converts the sixth residue of the α-helix, Arg^{433}, to Pro, which results in the disruption of the last of four residues involved in base contacts used by the zinc finger motif (26). The C^{1298}-G^{1299} deletion in the tumor DNA of Wit-26 also results in a conversion of Arg^{433} to Pro. In addition, both mutations in Wit-24 and Wit-26 eliminate the third zinc ligand of the fourth zinc finger (His^{434}) and the wild-type stop codon of WT1 (TGA at 1348–1350). This results in the translation of 68 additional codons with the new TGA stop codon at 1549–1551. The loss of the wild-type allele in Wit-24 is demonstrated by extensive LOH in the tumor. In Wit-26, LOH is indicated by sequence analysis, which shows the deletions to be homozygous. The previously reported CA repeat (21) used to demonstrate LOH in Wit-29 was not informative when used to determine tumor LOH for Wit-26. The data presented for Wit-26 are compatible with a somatic mutation followed by a double recombination event. However, we cannot exclude a small constitutional deletion (not detected by Southern blotting with 31E1 or by SSCP analysis of three polymorphic markers within *WT1*) (data not shown) followed by a somatic C^{1298}-G^{1299} deletion.

Each of the mutations described in this report occurs at an arginine residue. In Denys–Drash syndrome, ≈75% of the reported constitutional *WT1* mutations also affected an arginine residue (17–20). These observations seem of particular interest, as transitions at CpG dinucleotides contribute heavily to the p53 mutation frequency in many cancers, although the fraction of tumor mutations that are transitions at CpG sites varies greatly from one cancer type to another (27). Moreover, over one-third of all point mutations giving rise to human genetic disease are due to mutation from CpG to TpG, despite the rarity of CpG and the existence of a dedicated repair system (28). The unusual mutability of CpG dinucleotides is attributed to the presence of 5-methylcytosine residues found at these dinucleotide sites.

The findings presented in this report show that subtle somatic lesions in the ≈40-kb *WT1* genomic sequence can serve as the first "hit" proposed in the two-hit model for development of Wilms tumor. The two-hit hypothesis, based on the age-specific incidence of familial and sporadic forms of Wilms tumor, proposes hereditary tumors in which the first mutation is provided in the germ-line, and somatic cases in which both mutations occur within the same somatic cell (29). According to this concept both mutations in most unilateral sporadic cases are somatic. However, unlike retinoblastoma, in which both sporadic and hereditary forms can be attributed to the loss of function of one single locus, Wilms tumor may be caused by loss of function at multiple loci. LOH at 11p15 in association with a constitutional *WT1* deletion in two cases (30), with a somatic *WT1* deletion in one case (21), and with low *WT1* mRNA levels in two other cases (4) has led to the suggestion of a possible interaction between *WT1* and *WT2*, the putative Wilms tumor gene at 11p15, in the development of some Wilms tumors. It is conceivable that one of these two loci predisposes to tumorigenesis whereas the other is required for tumor initiation or progression.

A role for *WT1* in Wilms tumorigenesis has been established by the demonstration of constitutional intragenic *WT1* mutations followed by 11p13 tumor LOH (13–20). However, all but one (13) of the cases reported in these studies did not have unilateral sporadic Wilms tumor [bilateral involvement (14, 16), bilateral Wilms tumor and genitourinary malformations (15), genitourinary malformations and a positive family history for Wilms tumor (15), and several individuals with the Denys–Drash syndrome (17–20)]. These data suggest that inactivation of *WT1* might be sufficient for the development of some Wilms tumors, although it remains unclear whether complete loss of function at this locus is required for the development of Wilms tumor (18). It has been suggested that mutation of only one *WT1* allele could result in suppression of the normal function of the wild-type protein, a phenomenon called "trans-dominant suppression" (31), which has previously been proposed in the case of p53 (32). The observation of two somatic events described in this report provides molecular evidence for the two-hit mutational model for a subset of sporadic unilateral Wilms tumor cases.

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