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Genetics

Zinc finger point mutations within the *WT1* gene in Wilms tumor patients

(zinc finger genes/tumor suppressor genes/protein–DNA binding/chemical mismatch cleavage)

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ABSTRACT **A proposed Wilms tumor gene, *WT1*, which encodes a zinc finger protein, has previously been isolated from human chromosome 11p13. Chemical mismatch cleavage analysis was used to identify point mutations in the zinc finger region of this gene in a series of 32 Wilms tumors. Two exonic single base changes were detected. In zinc finger 3 of a bilateral Wilms tumor patient, a constitutional *de novo* C → T base change was found changing an arginine to a stop codon. One tumor from this patient showed allele loss leading to 11p hemizygosity of the abnormal allele. In zinc finger 2 of a sporadic Wilms tumor patient, a C → T base change resulted in an arginine to cysteine amino acid change. To our knowledge, a *WT1* gene missense mutation has not been detected previously in a Wilms tumor. By comparison with a recent NMR and x-ray crystallographic analysis of an analogous zinc finger gene, early growth response gene 1 (*EGR1*), this amino acid change in *WT1* occurs at a residue predicted to be critical for DNA binding capacity and site specificity. The detection of one nonsense point mutation and one missense *WT1* gene point mutation adds to the accumulating evidence implicating this gene in a proportion of Wilms tumor patients.**

One of the genes implicated in the pediatric kidney neoplasm Wilms tumor (*WT*) is located at chromosome 11p13. This chromosome region was first implicated by the observation of constitutional 11p13 deletions in patients with WAGR syndrome (WT with aniridia, genitourinary dysplasia, and mental retardation) (1). Cytogenetically visible 11p13 deletions are also found in 1% of WTs themselves (2), and 30–40% of tumors show loss of constitutional heterozygosity (LOCH) for markers along the short arm of chromosome 11 (3). It was therefore hypothesized that at least some WTs arise due to the loss of function of both copies of a recessive tumor suppressor gene situated at 11p13 (4). Comprehensive mapping of the deletions present in WAGR patients confined the putative gene to a particular 325-kilobase (kb) *Not* I fragment (5–7) and indicated that this gene may also be involved with the genital anomalies seen in WAGR patients (8). Two groups (9, 10) recently cloned a candidate *WT* gene from the 11p13 smallest region of overlap. This gene, *WT1*, encodes a DNA-binding zinc finger (ZF) protein with four consecutive ZF motifs at the 3′ end of the transcript, each encoded by a separate exon (11). WT1 is in a family of ZF proteins that includes the transcription regulators named early growth response gene 1 (*EGR1*), *EGR2*, *Sp1*, *Krox-20*, *GLI*, and Krüppel (*Kr*), all of which show a Cys_2-His_2 ($F/YXCX_{2-4}CX_3FX_5LX_2HX_{3-4}HX_5$) motif and bind to guanidine-rich DNA sequences (12, 13). *WT1* is specifically expressed in the developing kidney and gonadal ridges (14), thereby suggesting a role for *WT1* in normal kidney development and strengthening its case as the 11p13 *WT* gene. However, many WTs express high levels of apparently normal length *WT1* transcript. If *WT1* is implicated in the development of these tumors, there may be point mutations or small deletions undetectable by Northern blotting. The ZF region of the *WT1* gene is believed to be responsible for DNA binding in a manner critically dependent on the amino acid sequence and it is therefore an obvious region in which to begin to search for point mutations. To this end, four pairs of oligonucleotide primers flanking the ZF-encoding regions of the four ZF exons were designed for polymerase chain reaction (PCR) amplification of tumor DNA from a series of 32 tumors arising in 27 WT patients. Using the chemical mismatch cleavage technique (15), these tumors were investigated for genomic single base changes within the ZF region of *WT1*. Two exonic point mutations and two intronic possible polymorphisms were detected.

MATERIALS AND METHODS

DNA was prepared from 32 tumors of 27 WT patients. Two of these were bilateral patients, contributing tumor NP27 (left kidney recurrence) from one patient and tumors NP57 (right kidney) and NP58 (left kidney) from the other patient. DNAs from the primary WT (NP38) and two separate lymph node metastases (NP39, NP68) of one unilateral sporadic patient and the primary WT (NP52) and two separate lung metastases (NP53, NP54) of another unilateral sporadic patient were examined. The remaining tumors were single neoplasms from unilateral sporadic patients. None of the patients displayed the WAGR syndrome; however, one tumor (NP59) had a cytogenetically visible deletion encompassing the *WT1* gene. The wild-type DNA used was from an unaffected father of a sporadic WT patient, in whom the genomic *WT1* sequence concurred with the published sequence of the gene (9, 10). Constitutional DNA was also prepared from lymphoblastoid cell lines established from the patients, from 34 unrelated Caucasian adults with no history of renal cancer and, when available, from the parents of patients found to have genomic mutations.

Oligonucleotides and PCR. The oligonucleotide primers were designed to amplify DNA covering each of the four ZF coding sequences. In the case of ZF2 and ZF3, the primers are in the preceding and following intronic sequences. For ZF1 and ZF4, one primer was intronic and the other was within an exon but either preceding, for ZF1, or following, for ZF4, the ZF motif itself (Fig. 1 *a* and *b*). Primers 439 (5′ CTT GTA CGG TCG GCA TCT 3′) and 945 (5′ ACA ACA CCT GGA TCA AGA CCT 3′) were used for PCR amplification of a 255-base-pair (bp) fragment covering ZF1, primers 953 (5′ GCC TTA ATG AGA TCC CTT TCC 3′) and 796 [5′ GGG GAA ATG TGG GGT GTT TCC 3′ (11)] were used for a 339-bp fragment covering ZF2, primers 798 [5′ TGC AGA

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Abbreviations: WT, Wilms tumor; ZF, zinc finger; WAGR, WT with aniridia, genitourinary dysplasia, and mental retardation; LOCH, loss of constitutional heterozygosity.

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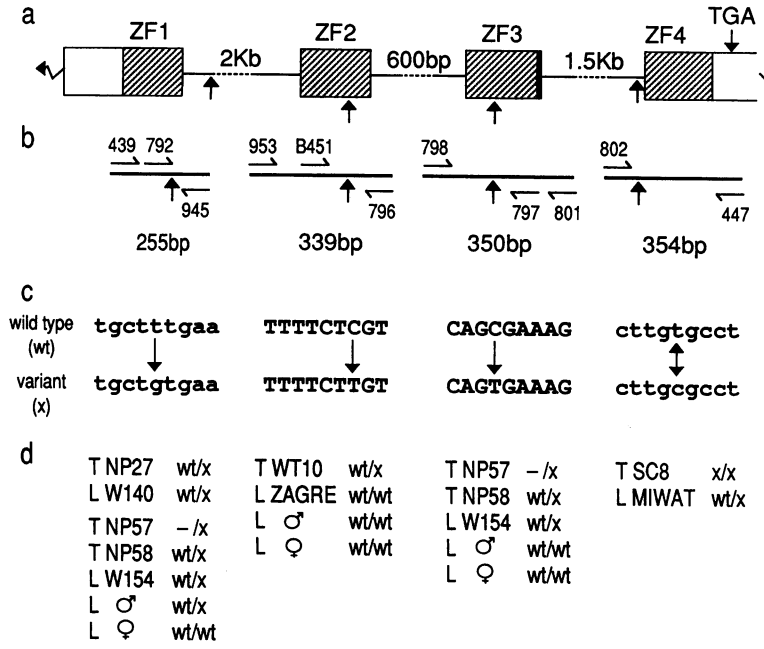

FIG. 1. Diagram of the ZF region of the *WT1* gene and the point mutations detected in this region. (*a*) Exon (boxes) and intron (lines) structure of the *WT1* gene within the ZF region. Shaded boxes represent ZF-encoding portions of the exons. The 9-bp alternative splice is shown as a blackened box. (*b*) PCR fragment sizes and primers used. In *a* and *b*, arrows mark positions of the point mutations. (*c*) Sequence of the point mutations in each ZF. Uppercase letters indicate exonic sequences; lowercase letters indicate intronic sequences. (*d*) Tumor (T), somatic (L), and parental (OO) genotypes for the patients carrying base changes. wt, Wild-type allele; x, variant allele.

CAT TGC AGG CAT GGC AGG 3′ (11)] and 801 [5′ GCA CTA TTC CTT CTC TCA ACT GAG 3′ (11)] were used for a 350-bp fragment covering ZF3, and primers 802 [5′ ACA ATA TTT CGA TCC TTA AAG CCC 3′ (11)] and 447 (5′ TCC CGG GGACAC TGA ACG 3′) were used for a 354-bp fragment covering ZF4. The same primers were used for direct sequencing of the resultant PCR fragments. In ZF1, ZF2, and ZF3, sequencing was also performed using the internal primers 792 [5′ GCT GTC CCA CTT ACA GAT GC 3′ (11)] for ZF1, B451 (5′ GTG TGA CTT CAA GGA CTG TG 3′) for ZF2, and 797 [5′ GGG TCT TCA GGT GGT CGG ACC GGG 3′ (11)] for ZF3. PCR reaction mixtures were 50 μl total volume containing 200 ng of genomic DNA, 200 μM nucleotides, 1 μM of each primer, 1× Promega PCR buffer, and 1 unit of Promega Taq polymerase. Reactions were denatured at 92°C for 45 s, annealed at the appropriate temperature for 1 min, and elongated at 72°C for 1–1.5 min for 30 cycles (Hybaid Thermal Cycler; tube control). All PCR fragments were purified from low-melting-temperature agarose (SeaPlaque) using Mermaid (Bio 101, La Jolla, CA).

Chemical Mismatch Cleavage. Chemical mismatch cleavage analysis was performed as described (15–17). When a mismatch was detected, homoduplex mismatch analysis was used to determine whether the mutation was present in one or both copies of the gene (18). Direct sequencing of independently amplified fragments was used to characterize specific base changes and confirm homo- or heterozygosity for these base changes (19).

Base-Change-Specific Oligonucleotides. Mutant-specific oligonucleotides were designed to investigate the prevalence of two of the detected single base changes. Primer B496 [5′ GAC TGT GAA CGA AGG TTT T (C/T) T 3′] was designed to detect the ZF2 C → T point mutation found in WT10; B502 (5′ TGG ATC AGG ACC TTT CAC 3′) was designed to detect the intronic T → G base change found in both of the bilateral patients. The bases in bold type refer to those detected as base changes and not seen in the wild-type gene.

EXPERIMENTAL RESULTS

Exonic Base Changes. Four distinct single base sequence variants were detected and characterized. Two of these sequence variants were exonic, whereas the other two were intronic single base mutations (Fig. 1*c*).

Patient NP57/NP58 presented with simultaneous bilateral WT at the age of 11 months. There were no associated congenital abnormalities nor was there a family history of renal tumor. The histology of the tumors from both kidneys was classic triphasic with no evidence of anaplasia, metastasis, or nephroblastomatosis in the kidney tissue adjacent to the tumor. Previous restriction fragment length polymorphism analysis of this patient had revealed extensive 11p allele loss in the right kidney tumor NP57 extending from *HRAS* (11p15.5) to *CAT* (11p13) but not across the centromere (data not shown). Allele intensity suggested this was due to hemizygosity for the region. However, there was no allele loss in the left kidney tumor NP58. A point mutation at a CpG dinucleotide in ZF3 (Fig. 1*c*), changing a C to a T and resulting in an arginine becoming a stop codon, was detected constitutionally and in both tumors of this patient (Fig. 2). The point mutation was present heterozygously in NP58 and the somatic cells, whereas, due to hemizygosity, NP57 carried only the mutant allele (Fig. 2). Neither parent carried the mutation, so it must have arisen *de novo* either during gametogenesis or very early embryogenesis.

In tumor WT10, a C → T point mutation, again at a CpG dinucleotide, was detected, resulting in an arginine to cysteine amino acid change in ZF2. The patient was a 3 year old with unilateral sporadic WT and no family history of renal neoplasia and no associated anomalies or cytogenetic abnormalities. The histology of the tumor was triphasic with a large amount of epithelial glomerular differentiation. The mutation was present only in one allele of the tumor and was not present constitutionally or in either of the parents (Fig. 1*d*). To determine whether this base change was present in the general population, an oligonucleotide primer, B496, was designed with a 3′ base compatible only with the mutant allele. None of 34 normal unrelated Caucasian individuals carried the variant.

Intronic Base Changes. Three tumors from two patients (NP27, NP57, and NP58) displayed a T → G base change in the intron between the ZF1 and ZF2 exons (Fig. 1*c*). Direct sequencing of tumors NP27 and NP58 revealed heterozygosity for this mutation, whereas NP57, due to hemizygosity, carried only the variant allele. The variant was constitutionally heterozygous in both patients. In the case of NP57/58, the mutation had been inherited from the father, who also carried the variant allele (Fig. 2). Therefore, the allele lost in NP57 was from the mother, as expected (20), and the ZF3

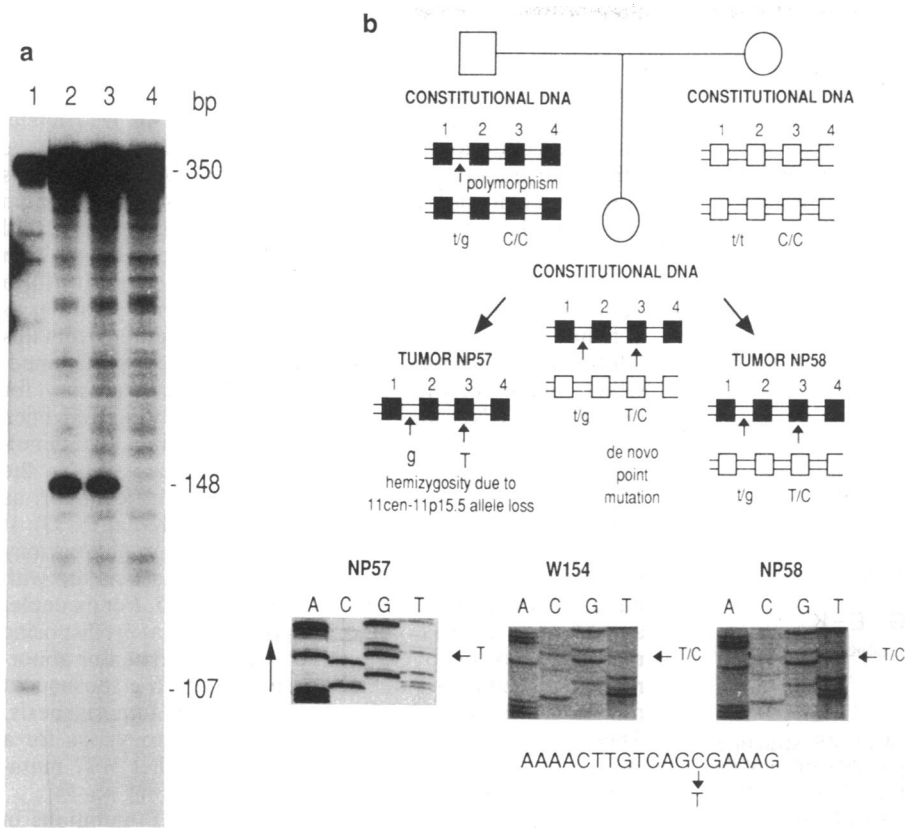

FIG. 2. Identification and characterization of exonic ZF point mutations. (*a*) Autoradiograph from a hydroxylamine chemical mismatch cleavage analysis of heteroduplexes between the ZF2 PCR fragment from control and WT10 DNA (lane 1) and the ZF3 PCR fragment from control and NP57 (lane 2), NP58 (lane 3), and NP59 (lane 4) DNA. The length of the cleavage products indicates the position of the base change. (*b*) Diagrammatic representation of the base changes found in the parents, patient, and tumors of a bilateral WT patient, together with sequencing of the ZF3 PCR fragment in the somatic DNA from the patient (W154) and DNA from both tumors (NP57 and NP58).

exonic point mutation must have occurred in the remaining paternal allele. Curiously, in seven of eight WT patients with *de novo* constitutional deletions, these have occurred in the paternal allele (21). The T → G base change in the intron between ZF1 and ZF2 was therefore present in three individuals—two patients and the unaffected father of one of the patients—and probably represents a previously unreported polymorphism. However, although several hundred base pairs from the ZF1 exon, splicing mechanisms are still too poorly understood to say that this apparently inert single base change does not interfere with correct splicing. A sequence variant-specific oligonucleotide primer, B502, was used to examine the 34 normal Caucasian individuals together with positive and negative controls. Although two Caucasian patients carried this single base change constitutionally, it was not detected in any of the normal individuals. Therefore, if it is a polymorphism, it is rare in the Caucasian population. It is curious that both patients with this base change displayed bilateral WT.

Tumor SC8 showed a single base sequence variant in the intron between ZF3 and ZF4 involving a T → C base change (Fig. 1*c*). The tumor was homozygous for the change, whereas the somatic DNA was heterozygous (Fig. 1*d*). Previous restriction fragment length polymorphism analysis of SC8 tumor DNA had shown allele loss for the *HRAS*, *CALC*, and *PTH* loci at 11p15 (data not shown). Homozygosity for this T → C base change in a *WT1* intron shows the allele loss extends as far as the *WT1* gene. This is not a tumorigenic point mutation, as it has already been reported as a polymorphism, occurring in 17.4% of the normal population (11).

DISCUSSION

WT1 and EGR1 are very similar ZF proteins, with the three ZFs of EGR1 showing high homology with fingers 2, 3, and 4 of WT1 (Fig. 3*a*). Recent NMR and x-ray crystallographic studies (22) of the mouse immediate early protein Zif268, also known as EGR1 (we will refer to it here as EGR1), have shown that each ZF motif is composed of an antiparallel β-sheet, containing the two consensus cysteines, and an α-helix, containing the two consensus histidines (Fig. 3*a*). The three fingers of EGR1 lie within the major groove of B-DNA allowing binding to the critical target site. Specific amino acids in the NH_2-terminal half of each α-helix directly contact the bases of the DNA.

In each finger, the amino acid side chains directly contacting the DNA primary strand bases are those immediately preceding the α-helix (R_{18}, R_{46}, and R_{74} in EGR1; Fig. 3*a*) and either the third or the sixth residue of the α-helix itself (R_{24}, H_{48}, and R_{80} in EGR1). In the *WT1* ZF2 mutation, it is an arginine immediately prior to the α-helix that is mutated to a cysteine (C) in tumor WT10. We would predict that an uncharged cysteine residue at this point would fail to interact with its target guanidine and might destroy binding of the WT1 protein to its target DNA sequence. Although an arginine immediately prior to the α-helix in each ZF is quite common in the family of ZF proteins with guanidine-rich DNA target sequences (12, 13), it is not in other ZF families where a number of other amino acids may be present. In the *Drosophila* hunchback (*hb*) gene (23), which has a very adenine-rich consensus binding sequence (G/C A/C ATAAAAAA) (24), a cysteine is found in this position in the fourth finger, as is the case in the *WT10* point mutation. It is therefore conceivable that this mutation may not only decrease the binding capacity of WT1 for its native target sequence but may create a protein with binding capacity for a different target sequence altogether. Such a mutation could possibly act in a dominant way. In fact, since our study was completed, it has been shown that Drash syndrome patients, who are characterized by congenital nephropathy and genital dysplasia, carry a single constitutional point mutation in the *WT1* gene always involving an amino acid involved in target binding (25). In 8 of 10 cases, this altered the arginine preceding the α-helix, with 7 being arginine to tryptophan mutations in ZF3 and 1 being an arginine to histidine mutation

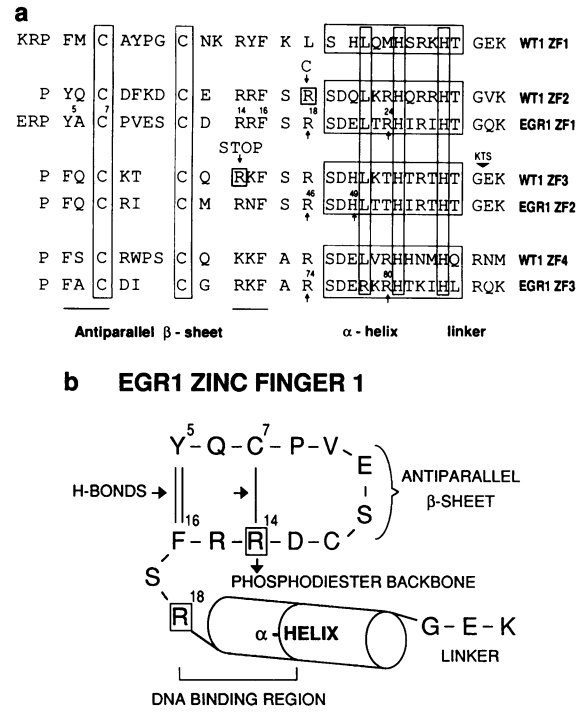

FIG. 3. Comparison between EGR1 and WT1 ZF structure, demonstrating the folding of the finger and the possible effect of the point mutations detected. (*a*) Amino acid sequence of the four WT1 and three EGR1 fingers aligned to indicate the antiparallel β-sheet region (underlined), α-helical region (horizontal enclosed area), and linker regions. The two cysteines (C) and histidines (H) highly conserved between ZF proteins are indicated. Arrows are positioned underneath each of the amino acids in EGR1 known to interact with the bases of the DNA. The amino acids altered by WT1 point mutations are boxed with the mutant amino acid shown above them. (*b*) Diagram of the folding of a ZF (EGR1 ZF1), showing the H bonds stabilizing the β-sheet, the site of interaction with the DNA backbone, and the portion of the α-helix binding to the bases. The amino acids analogous to those mutated in WT1 are boxed.

in ZF2. It is known that a null mutation or a deletion of one copy of the *WT1* gene does not lead to such a severe developmental anomaly. Therefore, in these cases it is clear that a specific point mutation of a critical amino acid within the ZF region has conferred a dominant activity on the mutated protein resulting in severe developmental anomalies in the urinary and genital systems. This is the same arginine that is mutated in *WT10*, supporting our hypothesis that the product of this mutation may also act in a dominant or even a dominant-negative or antimorph (26) fashion to disrupt or compete with the activity of the remaining normal allele. This may be studied by cloning the mutated *WT1* transcript from this tumor and investigating its binding capacity and target sequence specificity compared to a normal *WT1* ZF region.

When LOCH for 11p markers was first observed in WT, it was thought to reinforce the Knudson and Strong two-hit hypothesis (4) with 11p allele loss representing the second hit removing the remaining normal allele of an already heterozygously mutated tumor-suppressor gene at 11p13. This model appears to hold true for retinoblastoma, where allele loss for the *RB1* gene at 13q14 is seen in 80% of tumors (27) and mutations occur in the other allele in most of cases (28). A far lower percentage of WTs shows 11p13 allele loss (3), and the involvement of genes elsewhere on chromosome 11p and elsewhere in the genome in WT has been clearly demonstrated, initially by 11p15-specific allele loss (29) and subsequently by the lack of linkage to 11p in familial WT (30, 31). Certainly, in some WT patients, there may be no involvement of the 11p13 region. In this study, only two WT1 ZF mutations were detected in 3 tumors from 27 WT patients. Of these tumors, 11p13 allele loss was seen in only one tumor (NP57). In this case, a point mutation followed by generation of hemizygosity for a large portion of the normal chromosome 11p did serve to remove the remaining normal *WT1* allele. However, allele loss was not a tumorigenic mechanism in the other two tumors with mutations. In *WT10*, the patient is constitutionally normal, while the mutation is only present in only one allele of the tumor. A WT heterozygous for a small deletion of a ZF exon/intron boundary has previously been reported (11). In these cases showing heterozygosity for a mutation or deletion it is possible that there may be other as yet undetected *WT1* changes, particularly in tumors from this study where only the ZF region of the gene was examined. Other regions of the gene are likely to be necessary for determining DNA target sequence specificity, as has been seen in a single motif ZF gene (32). The (proline, glutamine)-rich region 5′ of the ZFs is probably necessary for the functioning of this protein as a transcriptional regulator, as has been seen for the alanine-rich region of Krüppel (33). It is also possible that certain single allele *WT1* mutations may be sufficient for WT development or act in association with mutations of other WT genes, perhaps at 11p15. For example, a single hit in *WT1* could simply act as a predisposing mutation, allowing overgrowth of undifferentiated or abnormally differentiated kidney blastema in which a subsequent mutation at another locus actually leads to tumorigenesis. Therefore, in these tumors apparently heterozygous for a *WT1* mutation/deletion, there may be no other *WT1* mutations present.

This study suggests that there are *WT1* ZF mutations in 7.3% of WT patients. The prevalence of such mutations in other parts of the gene is yet to be established. The clinical data currently available suggest that 1–2% of WT patients have cytogenetically visible 11p13 deletions, all encompassing *WT1*, and 20% show LOCH extending to 11p13. Southern blot analysis does suggest that few WT patients show gross rearrangements of *WT1* (34). However, our results, together with the identification of intragenic deletions in *WT1* by several groups, strongly implicate *WT1* as the 11p13 gene involved in this subgroup of 11p13-related WT patients. There have now been four cases of small intragenic *WT1* deletions in WT patients (11, 35, 36). Three of these cases were present constitutionally (35, 36) and resulted in frameshifts creating premature stop codons 5′ of the ZF region. The fourth was only found in the tumor of a sporadic patient and in the presence of one apparently normal allele (11). In the three constitutional nonsense mutations (35, 36), there was homozygosity for the mutation in the tumors. In our one patient with a nonsense mutation within the ZF region, this had only reached homozygosity in one tumor. *WT10* is the only reported missense mutation in the tumor of a WT patient.

In summary, one nonsense point mutation and one missense exonic ZF point mutation in *WT1* have been detected in the tumors of WT patients. By analogy with *EGR1*, we hypothesize that the missense mutation detected may result in decreased binding of the WT1 protein to its target sequence by removing binding to one of the target guanidines. It may even facilitate binding to an incorrect target sequence. In addition, the presence of a *de novo* constitutional point mutation in one individual who subsequently developed bilateral WT implies that this gene may act as a predisposition gene. Two intronic base changes were found, one previously reported (11) and one newly identified and undetected in 34 members of the general population but presenting in two bilateral WT patients. Taken together with other reports of small intragenic deletions in *WT1*, these findings further implicate the *WT1* gene in at least a proportion of WT cases. The identification of a naturally occurring ZF missense point

mutation will also facilitate investigations into the effect of removing a residue critical for DNA binding on ZF structure, function, and target specificity.

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