A Point Mutation within Exon 5 of the WT1 Gene of a Sporadic Unilateral Wilms' Tumor Alters Gene Function

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ABSTRACT

The Wilms' tumor suppressor gene, wt1, encodes a zinc-finger transcription factor, WT1, that represses transcription of a number of growth-promoting genes and inhibits cell growth. The transcripts of wt1 undergo two alternative splicing events, giving rise to four isoforms of mRNA in constant ratios. The first alternative splice introduces an extra exon 5, which encodes 17 amino acid residues inserted between the transcription regulatory domain and the DNA binding domain of WT1. Previously, we demonstrated that the 17-amino acid domain functioned as a transcription repressor when it was fused with the DNA binding domain of WT1. We have now identified a point mutation within exon 5 of wt1 in a sporadic unilateral Wilms' tumor patient. The mutation changes the last of the 17 amino acids from asparagine to serine. The protein isoform of WT1 carrying this mutation exhibited a 2-3-fold lower transcription-repressing activity than wild-type WT1 in transient cotransfection assays. The mutant amino acid change also decreased growth-inhibiting activity of WT1 in two osteosarcoma cell lines, U2OS and Saos-2. By diminishing transcription-repressing and growth-inhibiting activities of WT1, this naturally occurring mutation may disturb the normal function of the protein and lead to the uncontrolled cell growth characteristic of Wilms' tumor.

INTRODUCTION

WT, or nephroblastoma, is the most common interabdominal solid tumor of childhood. It arises in 1 per 10,000 children worldwide (1). WT is associated with certain congenital defects, including sporadic aniridia; hemihypertrophy; Beckwith-Wiedemann syndrome (a congenital overgrowth syndrome); Denys-Drash syndrome (which consists of the triad WT, intersex disorders, and nephropathy); WAGR syndrome; and various abnormalities of the genitourinary tract (1).

Recently, a WT susceptibility gene, wt1, mapping to chromosome locus 11p13 was isolated (2-4). The wt1 transcript is ~3.5 kb long and encodes a zinc-finger protein, WT1, with a predicted molecular weight, M, 47,000 to 49,000, depending on the presence or absence of two alternatively spliced exons (5). The first alternative splicing introduces exon 5, encoding 17 amino acids (+17 aa), just proximal to the first of four zinc fingers. The second results in an insertion of three amino acids (+KTS) disrupts the distance between zinc fingers 3 and 4 and thus alters its DNA-binding specificity (6, 7). The NH2 terminus of WT1 contains a proline- and glutamine-rich domain that exhibited transcription-regulating activity in transient cotransfection assays (7, 8).

Consistent with the view that WT1 functions as a tumor suppressor, experimental evidence has demonstrated that WT1 represses transcription of a number of growth-related genes. These include, PDGF A-chain, insulin-like growth factor II, epidermal growth factor receptors, and the wt1 gene itself (9-12). Furthermore, recent experiments have shown that introducing wt1 cDNA into a Wilms' tumor cell line, RM1 (13), and two osteosarcoma cell lines, Saos-2 and U2OS (11), suppresses growth of the cells. The classification of WT1 as a tumor suppressor is based on the detection in tumor specimens of mutations within the gene that inactivate the normal function of the protein. To date, a small set of sporadic WTs have been found to have homozygous deletions that extend from adjoining DNA to the 5' exons of the gene (14, 15) or the 3' exons of the gene (16), as well as small mutations that are entirely contained within the wt1 locus (17, 18). However, only ~15% of sporadic WT have thus far been detected with intragenic wt1 mutations, although ~99% of WTs arise as sporadic unilateral lesions (19, 20). Thus, the molecular mechanisms of the involvement of the wt1 gene in the genesis of sporadic WTs are not clear.

A recent experiment has revealed a third functional domain of WT1 that is encoded by alternatively spliced exon 5 (7). This domain functions as a transcription repressor when it was tethered directly to promoter DNA through fusion with the zinc-finger DNA-binding domain of WT1 (7). Isoforms of WT1 with this extra domain have a different effect on cell growth than WT1 isoforms lacking this domain (21). Thus, the alternatively spliced exon 5 encodes an important functional domain, and mutations in this domain would disturb the normal function of WT1 and contribute to the genesis of sporadic WT.

We decided to search mutations within this domain in patients with sporadic unilateral Wilms' tumor. We have analyzed seven samples of sporadic unilateral Wilms' tumor using RT-PCR and sequencing of PCR product. In one patient, we found a novel G to A point mutation within exon 5. The mutation, an asparagine to serine substitution at the last of the 17 aa encoded by exon 5, altered the functional properties of WT1, decreasing both transcription-repressing and growth-inhibiting activities.

MATERIALS AND METHODS

RT-PCR and Mutational Analysis. Samples of sporadic unilateral WT were obtained from the XinHua Hospital, the RenJin Hospital, and the Children's Hospital at Shanghai Medical University in Shanghai, Puerto Rico, China. The samples were snap frozen in liquid nitrogen at the time of surgical resection and kept at −70°C. Total cellular RNA was extracted from frozen tumor tissues and neighboring normal tissues as described previously (22), and the first-strand synthesis was prepared as published by Noonan and Roninson (23). Conditions of PCR amplification were: an initial 3 min at 94°C, 35 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C, followed by a 5-min final extension at 72°C. Primer sequences are the same as those designed by Brown et al. (24). PCR fragments were cloned into the Smal site of Puc118, and at
least five clones from each PCR reaction were sequenced with a sequenase version 2.0 kit (United States Biochemicals).

Cell Culture, DNA Transfections, and Colony Formation Assays. Osteosarcoma cell lines (Saos-2 and U2OS) and human embryonic kidney cells (293) were grown in DMEM with 10% FCS. Cells were transfected by the calcium phosphate DNA precipitation method (25). Drug-resistant colonies were selected by growth in G418 (0.5 mg/ml) for 3 weeks. The G418-resistant colonies were then stained with crystal violet (4% in 20% methanol) and counted.

Construction of Expression Plasmids. For the transient cotransfection assays and colony formation assays, full-length cDNAs of WT1 isoforms were cloned into the pCB6+ expression vector as described previously (7). The mutation in exon 5 of wt1 cDNA was introduced into wild-type wt1 cDNA using the Quick Change Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) according to the method recommended by the manufacturer. The plasmid expressing wild-type 17 aa/zinc finger fusion protein used was described previously (7). The plasmid expressing the mutated 17 aa/zinc finger fusion protein was constructed as follows. Oligonucleotides encoding 17 aa (with the last one changed from Asp to Ser) with a kozak translational start site and appropriate restriction enzyme sites at both ends were synthesized and fused in-frame to CMV-WT1 294–429, which contains the zinc-finger domain of the ΔKTS form of WT1 (7).

CAT Assays. CAT reporter plasmid p-153 PDGF-CAT was constructed as described previously (9). Human embryonic kidney cells (293) were transfected by the calcium-phosphate DNA precipitation method (25) using a reporter plasmid (5 μg) and a β-galactosidase expression plasmid (pCMVβ; Clontech; 1 μg) to establish transfection efficiency. The total amount of expression vector added in each transfection was adjusted to 20 μg by addition of pCB6 + vector alone. Forty-eight h after transfection, cell extracts were prepared, aliquots were normalized for transfection efficiency by assay of β-galactosidase activity, and CAT activity was determined. After autoradiographic exposure, the TLC plates were assayed by scintillation counting, and the percent conversion values were calculated.

RESULTS

Mutational Analysis. Previously, we have demonstrated that the 17-aa residues encoded by alternatively spliced exon 5 of WT1 functioned as a transcription repressor when fused with the zinc-finger domain of WT1 or the DNA binding domain of transcription factor Gal4 (7). Thus, exon 5 encodes a functional domain, and any mutation that disrupts the normal activity of this domain may disturb the functional balance of WT1 that is required for normal growth of cells in the developing kidney.

We analyzed tumor specimens from seven patients with sporadic unilateral WT (none of them has signs of Beckwith-Wiedemann, WAGR, or Denys-Drash syndrome). Total cellular RNA was extracted from frozen WT and neighboring normal tissue, and wt1 transcripts were amplified by PCR with primers flanking exon 5, cloned into plasmids, and sequenced. WT1 mRNA isolated from the tumor sample of one patient (case 222) contained a missense mutation. Five PCR-derived clones were sequenced, and all five contained an A→G mutation in exon 5. This mutation results in an asparagine → serine change at residue 242 (R242S), the last residue of the 17 aa encoded by alternatively spliced exon 5. The wild-type asparagine at this position is highly preserved, being present in both the human and mouse wt1 transcripts. However, because karyotype analysis was not available, it was not clear whether this mutation is caused by a homozygous point mutation or a "second hit" after loss of heterozygosity by chromosome deletion. The patient’s germ line exhibited no wt1 abnormality, and wt1 mRNA amplified from neighboring normal kidney did not contain the mutation seen in the tumor specimen (five of five clones sequenced, Fig. 1), indicating that it had arisen somatically.

Functional Analysis. The location of the R242S mutation within the regulatory domain encoded by exon 5 raised the possibility that it might alter the transcription-repressing activity of WT1. To test this possibility, we reconstructed the point mutation in wt1 cDNA encoding the isoform with 17 aa but lacking the KTS insertion (WT1 + m17aa). This mutated wt1 cDNA was then cloned into a CMV-driven expression vector (Fig. 2A). The trans-regulatory property of this mutant WT1 and the wild-type WT1 were compared by transient cotransfection of expression constructs along with the PDGF A-chain promoter-driven CAT reporter gene into human embryonic kidney cells (293 cells; Fig. 2B). The promoter region of the PDGF A-chain gene contains several perfect binding sites for WT1 isoforms and the regulatory domain encoded by exon 5 raised the possibility that it functioned as a transcription repressor when fused with the zinc-finger domain of WT1 or the DNA binding domain of transcription factor Gal4 (7). Thus, exon 5 encodes a functional domain, and any mutation that disrupts the normal activity of this domain may disturb the functional balance of WT1 that is required for normal growth of cells in the developing kidney.

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MUTATION IN EXON 5 OF WTI

A. Expression Plasmids:

CMV • • PolyA

WTI+17aa

VAAGSSSVKWTBGQSN

WTI+ml7aa

VAAGSSSVKWTBGQSN

B. Reporter Plasmids:

pPDGFA-CAT

Vector p17aa+ZF

pm17aa+ZF

C.

Fig. 2. The point mutation relieves the transcriptional-repressing activity of WT1. A. expression plasmids. The CMV-IE promoter was used to drive expression of human cDNAs of wild-type WTI and mutated WTI. In B, the reporter plasmid p-153 PDGF A-CAT contains the DNA sequences -153 to 388 of the PDGF A-chain promoter. Three WT1 binding sites are also shown. In C, the reporter p-153 PDGF A-CAT (5 µg) was cotransfected with 1 µg of internal control plasmid pCMVß and different expression plasmids at the amounts indicated at the bottom of each lane. A corresponding amount of pCB6 + was added to make 20 µg of total transfected plasmid. CAT assays were carried out as described in "Materials and Methods." Relative activities are shown with activity of the empty vector transfectant as 1.0. Transfection was repeated four times. Bars, SD.

The mutation in exon 5 of WTI reduces suppression of colony formation in U2OS and Saos-2 osteosarcoma cells U2OS and Saos-2 cells were transfected by calcium phosphate DNA precipitation with CMV-driven constructs encoding wild-type WTI isoforms and linked to the neomycin resistance gene. G418-resistant colonies were stained and counted after 3 weeks. The numbers of colonies/dish shown were derived from representative experiments (±SD).

<table>
<thead>
<tr>
<th>Construct</th>
<th>G418-resistant colonies/dish</th>
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</thead>
<tbody>
<tr>
<td>U2OS</td>
<td>Saos-2</td>
</tr>
<tr>
<td>Mock</td>
<td>0</td>
</tr>
<tr>
<td>Vector</td>
<td>486 ± 28</td>
</tr>
<tr>
<td>WTI + 17 aa</td>
<td>108 ± 2</td>
</tr>
<tr>
<td>WTI + m17 aa</td>
<td>298 ± 27</td>
</tr>
<tr>
<td>WTI + 17 aa(-KTS)</td>
<td>120 ± 8</td>
</tr>
<tr>
<td>WTI + m17 aa(-KTS)</td>
<td>295 ± 16</td>
</tr>
</tbody>
</table>

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To test the effect of this point mutation on the function of the 17-aa domain directly, we synthesized a DNA oligonucleotide encoding the 17 aa with the last residue changed from asparagine to serine, directly fused it in-frame with the zinc-finger DNA binding domain of WT1, and tested the effects of the fusion protein on the promoter activity of PDGF A/CAT reporter in a cotransfection assay. The fusion protein of the wild-type 17 aa with the zinc-finger domain of WT1 (p17aa + ZF; Fig. 3A) repressed the promoter activity of the reporter plasmid about 4--5-fold (Fig. 3B), as described previously (7). The point mutation, however, relieved the transcription-repressing activity of fusion protein (Fig. 3B), indicating that the R242S mutation directly diminishes transcription-repressing activity encoded in the 17-aa domain.

To determine the effects of the R242S mutation on the growth-inhibiting activity of WT1, we chose two osteosarcoma cell lines, U2OS and Saos-2, in which growth has been shown to be suppressed by WT1 (11). Cells were transfected with expression vectors containing isoforms of wild-type and mutated wt1 cDNA driven by the CMV IE promoter and linked to the neomycin-resistant (neoR) gene and selected with G418 for 3 weeks.

In U2OS cells, both isoforms of wild-type WT1 carrying the 17 aa significantly reduced the number of drug-resistant colonies. However, the mutant WT1 consistently gave rise to more colonies than did the wild-type WT1 (Fig. 4). Table 1 summarizes the results of several
MUTATION IN EXON 5 OF WT1

Fig. 4. The mutation in exon 5 of wt1 relieves suppression of colony formation mediated by WT1 in U2OS osteosarcoma cells. U2OS cells were transfected by calcium phosphate DNA precipitation with CMV-driven constructs encoding wild-type and mutated WT1 isoforms with exon 5 (20 μg) linked to the neomycin-resistant gene. G418-resistant colonies were stained after 3 weeks. A representative experiment is shown.

independent experiments. In both osteosarcoma cell lines, the two isoforms of WT1 carrying this mutation increased the number of colonies about 2.5-fold compared with wild-type WT1. These data suggest that the R242S mutation not only relieved the transcription-repressing activity but also diminished the growth-inhibiting activity of the WT1 isoform with this 17-aa domain.

DISCUSSION

In this report, we described a naturally occurring mutation within exon 5 of the wt1 gene in a sporadic unilateral WT. This point mutation, an asparagine to serine substitution at codon 242, reduced the transcription-repressing activity of WT1 and also relieved the growth-inhibiting activity of WT1. To our knowledge, this is the first missense mutation reported in exon 5 of the wt1 gene. Thus far, most of the wt1 point mutations reported in WTs have resulted in either a frameshift and premature termination of translation, producing a non-functional WT1, or an amino acid substitution within the zinc-finger domain, presumably abolishing the DNA-binding activity of WT1 (19).

Recently, a point mutation of wt1 has been identified in WAGR-associated WT, resulting in a substitution of one aa in the regulatory domain and converting WT1 from a transcription repressor to an activator of its target promoter (26). Our finding, however, demonstrated that a point mutation in the 17-aa domain decreased the transcription-repressing activity of WT1 but did not totally abolish or convert it. We further demonstrated that this asparagine to serine substitution mutation directly relieved the repression activity encoded by the 17 aa domain. However, the molecular mechanisms by which the mutation does so are unclear. We have also fused the mutated 17 aa with a Gal4 DNA binding domain, and the fusion protein exhibited identical results as the fusion protein of 17 aa and zinc-finger binding domain of WT1 (data not shown). Therefore, the possibility that the point mutation might reduce the DNA-binding affinity of mutated WT1 is excluded. Previously, we have reported that deletion of four consecutive serine residues in the 17-aa domain totally abolished transcription repression mediated by the 17-aa domain (7), indicating that these serines play a critical role in transcriptional repression, presumably through interaction with components of the transcription machinery. The asparagine at the last residue of the 17-aa domain may also be involved in the interaction of the 17-aa domain with transcription machinery. The mutation of this residue may weaken the interaction and thus decrease the repression activity.

The 17-aa domain contains a second transcription-repressing domain that functions independently of the more NH2-terminal repression and activation domains of WT1 (7). The functional balance between these two repression domains and between activation and repression domains within a WT1 molecule must be critical for the overall effect of WT1 on transcription activity and for normal regulation of target genes. Thus, the reduction of repression activity of the 17-aa domain by mutation may result in a functional imbalance of WT1, leading to misregulation of target genes and then the chaotic, uncontrolled proliferation characteristic of neoplasia.

Furthermore, the relative ratios of the four species of wt1 transcripts are constant in both human and mouse tissues expressing this gene (5). Thus, the different isoforms of WT1 must be carefully balanced to perform their normal function of regulating target genes. However, it has recently been reported that splicing of exon 5 in the wt1 gene is disrupted in some sporadic WTs, resulting in an isoform imbalance with an increase in isoforms in which the 17-aa domain is missing (27). These data suggest that disrupted alternative splicing of exon 5 may play a role in the etiology of sporadic WT. We also found alterations in the ratios of the alternatively spliced isoforms of WT1 in four of seven cases of sporadic unilateral WT. Interestingly, the patient reported in this paper was found to express a lower level of WT1 isoforms carrying the 17-aa domain compared with normal fetal kidney. Thus, the combined effects of the point mutation and the reduced amounts of the +17-aa isoform of WT1 greatly reduced the functional activity of WT1 isoforms with this extra domain. Further-

4 Z. Y. Wang, unpublished data.
more, it has been demonstrated that the WT1 isoform containing the 17-aa insertion is a much stronger repressor than the isoforms that lack this insertion (28). Thus, the reduction of transcription-repressing activity by the mutated 17-aa domain would significantly disturb the functional balance of WT1 isoforms and lead to the misexpression of target genes.

It has been shown that the WT1 protein can both repress and activate transcription. WT1 also has been shown to inhibit cell growth. However, it was unclear which transcription function is critical for inhibition of cell growth. This mutation has no effect on the transcription-activating activity of WT1 (data not shown), and the reduction of repression activity correlated well with the loss of ability to inhibit cell growth, strongly suggesting that transcription-repressing activity is important for WT1 to function as a growth inhibitor.

These assays are limited in that only individual isoforms of WT1 were assayed for their effects on cell growth; we did not analyze the effect of combinations of WT1 isoforms, as are found in vivo, on cell growth. Additional experiments must be performed to determine the effects of the mutation on cell growth when multiple WT1 splicing variants are present.

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