

# A Novel *WT1* Gene Mutation Associated with Wilms' Tumor and Congenital Male Genitourinary Malformation

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## ABSTRACT

*WT1* is located on the short arm of human chromosome 11 and consists of 10 coding exons. Mutations of this gene have been reported to be the cause of Wilms' tumor, congenital male genitourinary malformations, and/or renal disorders. We describe here a novel *WT1* gene mutation, *i.e.* a point mutation at intron 7 (+2) in both the tumor and the germline cells of a patient with Wilms' tumor and congenital male genitourinary malformation, but without renal disorder. The position of the mutation is at a splice donor site of intron 7, which causes the splicing out of exon 7 and generates a truncated protein. This type of mutation in the *WT1* zinc finger domain has not been reported before. The mutation is of paternal origin and is heterozygous in the germline cells. In the tumor cells, however, the maternal allele is largely lost, from 11p12 to 11p15, which results in maternal loss of

heterozygosity. These results, together with the data from previous reports, suggest that *WT1* may function in gonadogenesis, nephrogenesis, and Wilms' tumor tumorigenesis. (*Pediatr Res* 50: 337–344, 2001)

### Abbreviations:

**WT**, Wilms' tumor  
**GU**, genitourinary  
**WT+GU**, Wilms' tumor and congenital male genitourinary malformation in the absence of renal disorder  
**LOH**, loss of heterozygosity  
**DDS**, Denys-Drash syndrome  
**SSCP**, single-strand conformation polymorphism  
**RFLP**, restriction fragment length polymorphism

WT is an embryonal renal solid tumor that affects about 1 in 10,000 children (1) and accounts for about 6.5% of all childhood malignancies (2). The median age at onset is 38 mo, and nearly 80% of the patients develop the tumor before 5 y of age (3). In patients with congenital malformations (*e.g.* aniridia), the age at diagnosis may be even earlier—before 30 mo (3).

*WT1* has been localized to human chromosome 11p13 by positional cloning in studies of patients with WAGR (WT, aniridia, GU malformation, and mental retardation) syndrome (4, 5). The gene spans approximately 50 kb and contains 10 coding exons: exons 1–6 encode an amino-terminus Gln/Pro-rich domain, which may function in transcriptional suppression or activation, and exons 7–10 encode carboxyl-terminus four consecutive (Cys)<sub>2</sub>-(His)<sub>2</sub> zinc finger domains, which share a high degree of homology with those of the early growth response gene 1 (*EGRI*) and have DNA binding activity that recognizes the same GC-rich target as *EGRI* (6, 7) or a second

TC-rich target (8). This gene product has four splicing isoforms, reflecting the presence or absence of two alternatively spliced exons [17 amino acids of exon 5 and the 3 amino acids, Lys-Thr-Ser (KTS), in the last part of exon 9]. The relative distribution among variants and respective binding ability to the target have been investigated in detail (9).

Although *WT1* was initially thought to be the putative WT suppressor, the frequency of mutations in this gene in children with sporadic WT is <10% (10). Therefore, recent attention has focused more on the role of *WT1* in the development of the GU system and kidney rather than in tumorigenesis. A knock-out study of murine *wt1* revealed failure of kidney and gonad development, and the result was lethal when the mutation was homozygous (11). Nevertheless, this gene is still considered to be related to WT tumorigenesis in cases with certain congenital anomalies (3, 12, 13). WT and congenital male GU malformations, in particular, are postulated to be closely related, and the incidence of such malformations in WT patients is estimated to be much higher than in the general population (3, 14, 15).

In the present study, we report a novel *WT1* gene mutation in a case displaying unilateral WT and congenital male GU malformation in the absence of renal disorder (referred to

Received January 30, 2001; accepted May 24, 2001.

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“WT+GU” in this study), and we discuss the possible involvement of the WT1 protein in four *WT1*-related syndromes [Denys-Drash syndrome (DDS), WAGR syndrome, WT+GU, and Frasier syndrome (FS)] by comparing the data in the literature.

## MATERIALS AND METHODS

### Patient

The patient was a 1-y-old boy in whom ambiguous genitalia, hypospadias, and cryptorchism were noted at birth. No aniridia or any other anomalies were observed, and no signs of proteinuria or any other renal dysfunction have been detected to date (he is now 5 y old). The patient's karyotype is 46XY, and he has no family history of WT. He developed left unilateral WT at 1 y of age, and was admitted to the University of Tokyo Hospital. The tumor was successfully removed and diagnosed as stage I (C1, N0, V0, U0, M0).

### DNA and RNA Preparation

After obtaining informed consent, we collected tumor tissue samples and peripheral blood from the patient and his parents. As a control, normal kidney samples were collected from an aborted fetus with parental permission. All these procedures were approved by the Research Ethical Board of Keio University School of Medicine. Genomic DNA was isolated from tissue samples and peripheral blood lymphocytes by the phenol-chloroform method. Total RNA was extracted from the same specimens with ISOGEN (Wako Pure Chemicals, Tokyo, Japan) and purified by the thiocyanate method and DNase I treatment. cDNA libraries were synthesized with a First-Strand cDNA Synthesis Kit (Pharmacia, Uppsala, Sweden).

### Analysis of WT1 Gene

Genomic DNA from each sample was digested by *Eco*RI and submitted to Southern blotting with the WT33 probe (16). PCR-SSCP analysis was performed for all 10 coding exons with appropriate PCR primers, as described previously (17, 18), with some modifications. The PCR products were denatured and resolved on a high-density nondenaturing gel (20% total polyacrylamide, 30% ethylene glycol) using the Phast-System (Pharmacia). Direct sequencing analysis without intermediate cloning steps was performed with the same samples using an A.L.F DNA Sequencer 2 (Pharmacia).

We used the reverse transcriptase (RT)-PCR method to identify mutant transcripts with a sense primer designed against exon 6 (5'-AACCACACAACGCCATCCT-3') of the *WT1* and antisense primers against exon 8 (5'-CTCCTTTGGT-GTCTTTTGGAGC-3') and exon 10 (5'-GCCACCGACAGCT-GAAGGGC-3'). PCR was also performed with total RNAs without reverse transcriptase for negative controls. The RT-PCR products were sequenced after subcloning to confirm the splicing errors.

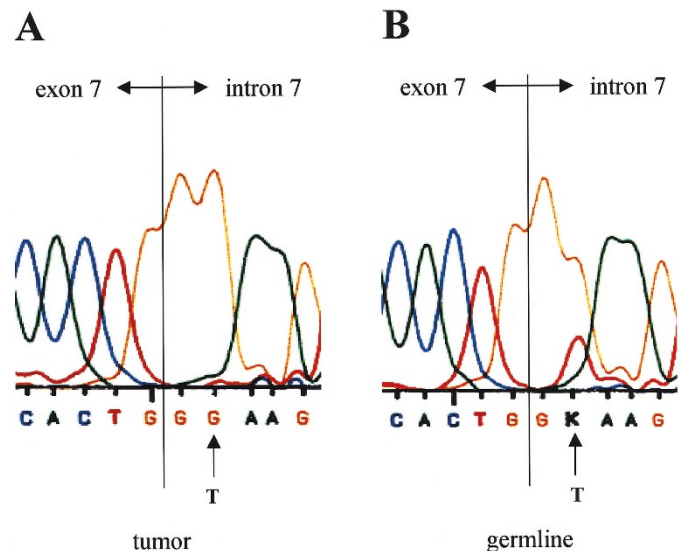
### Immunohistochemistry

After 3- $\mu$ m thick serial paraffin sections of the fixed tumor specimens for immunohistochemistry were prepared, they were treated with 0.4% pepsin in 0.2 N HCl for 30 min at 37°C and reacted with a 1:200 dilution of anti-human WT1 antibody, 6F-H2 (DAKO, Carpinteria, CA, U.S.A.), which recognizes the peptide corresponding to the N-terminus 84 amino acid residues of WT1 for 18 h at 4°C. After reaction with secondary antibody with peroxidase-labeled polymer, color was developed with Dotite DAB (Wako Pure Chemicals). Control sections were immunostained with nonimmune immunoglobulins, and immature glomeruli adjacent to the tumor were evaluated as an internal positive control. Immunohistochemistry of desmin and actin was then performed. Sections were also immunostained with anti-human D33 antibody or anti-human HHF35 antibody, respectively, as described above.

### Study of the LOH on Chromosome 11

We investigated polymorphism to detect LOH on chromosome 11 and to determine which allele (paternal or maternal) had been lost. We identified 11 markers on chromosome 11 between 11q13 and 11p15, all of which could be examined by the PCR amplification method and were suitable for polymorphic analysis. We used the PhastSystem and high-density nondenaturing gels for denaturing PAGE. Some of the RFLP markers were resolved on 2% agarose gels.

The markers used were *PYGM* (11q13) (19), D11S991 (11p11) (20), D11S554 (11p12) (21), *WT1* (11p13) (17, 22), *PAX6* (11p13) (23), *BDNF* (11p13) (24), D11S865 (11p14) (25), *PTH* (11p15) (26), *TH* (11p15) (27), *IGF2* (11p15) (28), and *H19* (11p15) (29).



**Figure 1.** Sequence of *WT1* intron 7 splice donor site in the tumor and the germline cells of the present case. (A) Mutation in the tumor cells. T→G mutation is recognized at intron 7 (+2). (B) Mutation in the germline cells. The “K” in the sequencing line means that the sequencer could not distinguish between T or G at this base. Hence, although the T→G mutation is recognized at the same position in the tumor cells, the sequencing data suggest the possibility of the heterozygosity at this base in the germline cells.

## RESULTS

## WT1 Mutation Analysis

No deletions or mutations were detected by Southern blotting or PCR-SSCP analysis in *WT1* coding exons from any of the samples (data not shown). However, direct sequencing revealed a point mutation at intron 7 (+2, T→G) in the patient's tumor cells (Fig. 1A) and the patient's peripheral blood lymphocytes (the germline cells) (Fig. 1B). In the germline cells, the sequencer showed the "K" at this position, which meant that the sequencer could not tell T or G at this base (Fig. 1B). This result strongly suggested that the point mutation was heterozygous in the germline cells.

Because intron 7 (+2) is a splice donor site, a mutation at this position should induce splicing out of exon 7. Accordingly, RT-PCR was used to detect this splice error. Analysis with the primer-pairs for exon 6 and 8 should yield a 305-bp band if splicing is normal. However, if exon 7 is spliced out, the base pairs would be shortened to 154 bp (Fig. 2A). The normal fetus kidney showed only a normal 305-bp band, whereas the tumor cells showed an aberrant 154-bp band (Fig. 2B). By contrast, both 305-bp and 154-bp bands were detected in the germline cells (Fig. 2B). These results also indicate that the mutation is heterozygous in the germline cells. Both bands were sequenced after subcloning passage, and the results confirmed that the products were consistent with the splicing theory described above (data not shown). When RT-PCR analysis was extended to exon 10, a stop codon was detected at the beginning of exon 9 in the mutant mRNA due to a frame shift (Fig. 2C). The tumor showed two extra miss-splicing bands at positions between 305-bp and 154-bp, but these products were faint in intensity and not investigated further.

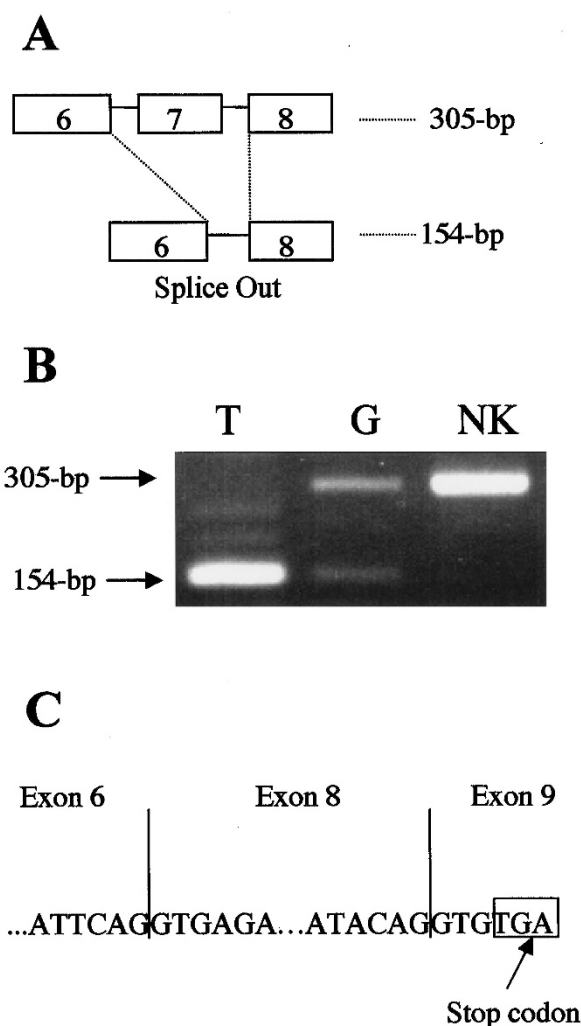
## Pathologic Findings and Immunohistochemical Study

The size of the tumor was 11 × 10 × 9 cm, and it weighed 511 g. On gross section, the tumor was found to be centrally located in the left kidney. Microscopic examination of the tumor revealed a nephroblastoma with triphasic histology (Fig. 3A). Blastemal cells showed a nodular pattern, and were surrounded by rich stromal components, including ectopic myogenic cells (Fig. 3A). This myogenic differentiation was confirmed by desmin and actin immunohistochemistry (data not shown). The abortive regions, which suggested intralobar nephrogenic rests, were found at the margin of the tumor. No glomerular sclerotic changes were found in the normal kidney tissue removed with the tumor (data not shown).

We performed an immunohistochemical study to demonstrate the loss of DNA binding function of the aberrant WT1. The tumor and the nephrogenic rests did not stain with anti-WT1 antibody (Fig. 3B), although the immature glomeruli adjacent to the tumor stained with the antibody (Fig. 3C).

## Polymorphic Analysis of Chromosome 11

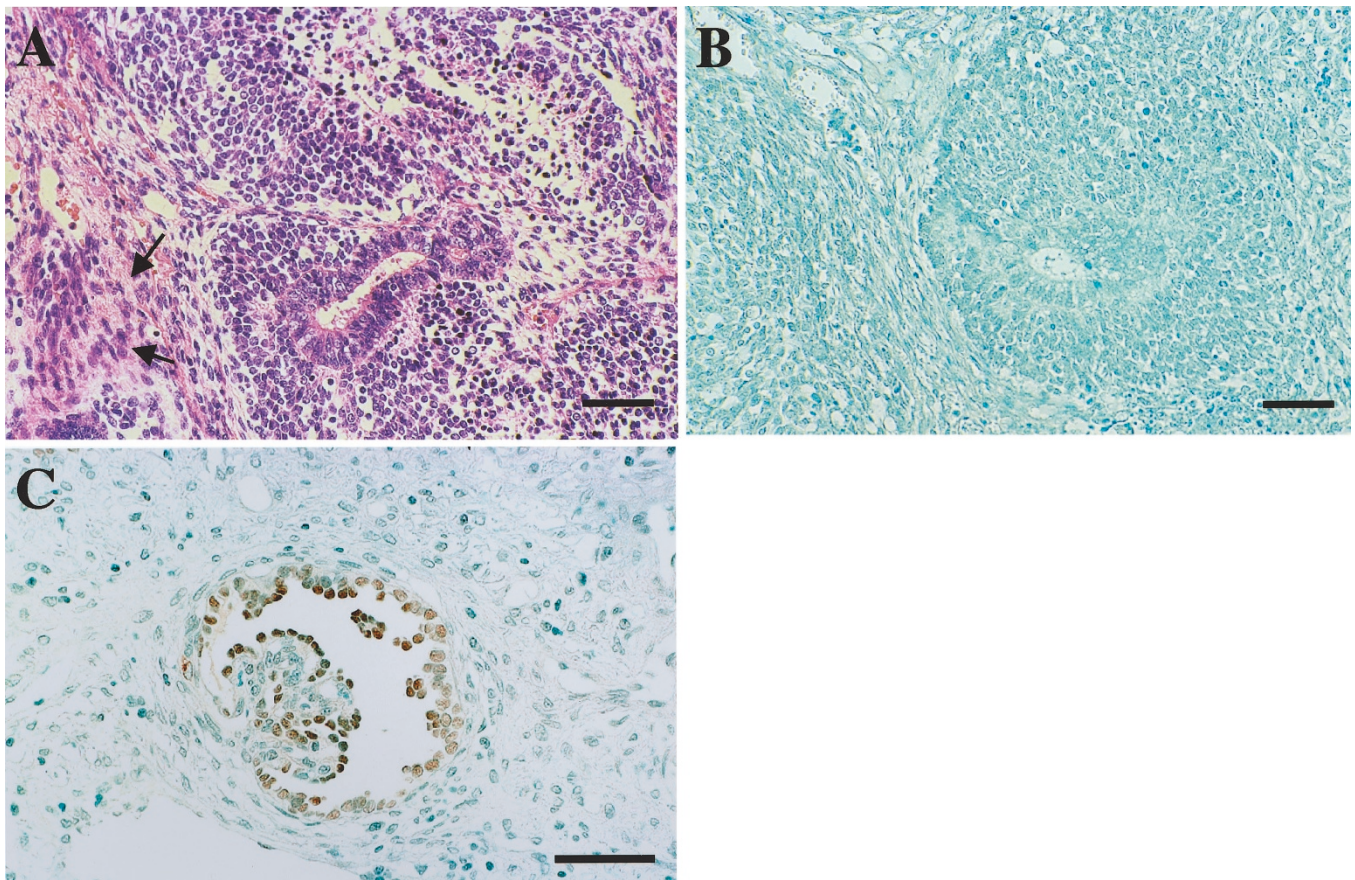
We then investigated whether the mutation detected in *WT1* in the tumor cells was homozygous or hemizygous as a result of LOH. However, polymorphic analysis of *WT1* region in all the DNA samples in this family was not informative (data not



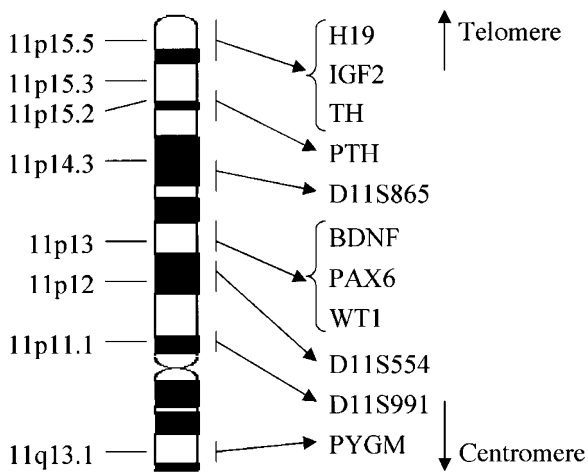
**Figure 2.** Mutant transcripts from the *WT1* intron 7 (+2) mutation. (A) Illustration of the mutant transcripts. The rectangles represent exons, and the numbers in them represent those of each exon. Normal mRNA including exon 7 generates a 305-bp band, whereas aberrant mRNA in which exon 7 is spliced out generates a shorter 154-bp band. (B) RT-PCR at exon 6–8. The tumor cells show the aberrant 154-bp band instead of the intact 305-bp band, whereas the germline cells have both the 154-bp and 305-bp bands. The normal fetus kidney cells show only the 305-bp band. T, tumor; G, germline; NK, normal fetus kidney sample. (C) Sequence of the aberrant mRNA in the tumor and the germline cells. The sequencer shows that exon 8 is directly connected to exon 6 and detects a stop codon (TGA) at the beginning of exon 9 due to a frame shift.

shown). We therefore selected 10 polymorphic markers on chromosome 11 encompassing *WT1* between 11q13 and 11p15 (Fig. 4).

*PYGM* (11q13) showed a different genetic background between the father (A/A') and the mother (B/B'). The patient's germline cells had received one of the alleles from each parent (A/B), and the tumor cells contained the same two alleles (A/B). We therefore concluded that heterozygosity was retained in the tumor cells at this region (Fig. 5A). On the other hand, although D11S554 (11p12) showed a similar tendency in the germline cells, one of the alleles inherited from his mother was lost in the tumor cells, indicating maternal LOH (mLOH) in this region (Fig. 5A). *TH* and *IGF2* (both 11p15), which was electrophoresed on an agarose gel, also showed mLOH in this



**Figure 3.** Histology of the tumor and immunohistochemistry in the present case. (A) Triphasic histology of the tumor. Ectopic myogenic cells are found in stromal components surrounding the blastemal cells (arrows). (B) The tumor tissue is unstained by the anti-human WT1 antibody. (C) Immature glomerulus. The Bowman's capsule epithelium and podocytes are clearly stained. (A) Hematoxylin and eosin stain; (B, C) Immunohistochemistry with the anti-human WT1 antibody. Scale bars: 100  $\mu\text{m}$ .



**Figure 4.** Site map of the loci analyzed in chromosome 11. The loci are arranged in order, top to bottom, from the telomere to the centromere of 11p. PYGM at the bottom is a region in 11q (11q13).

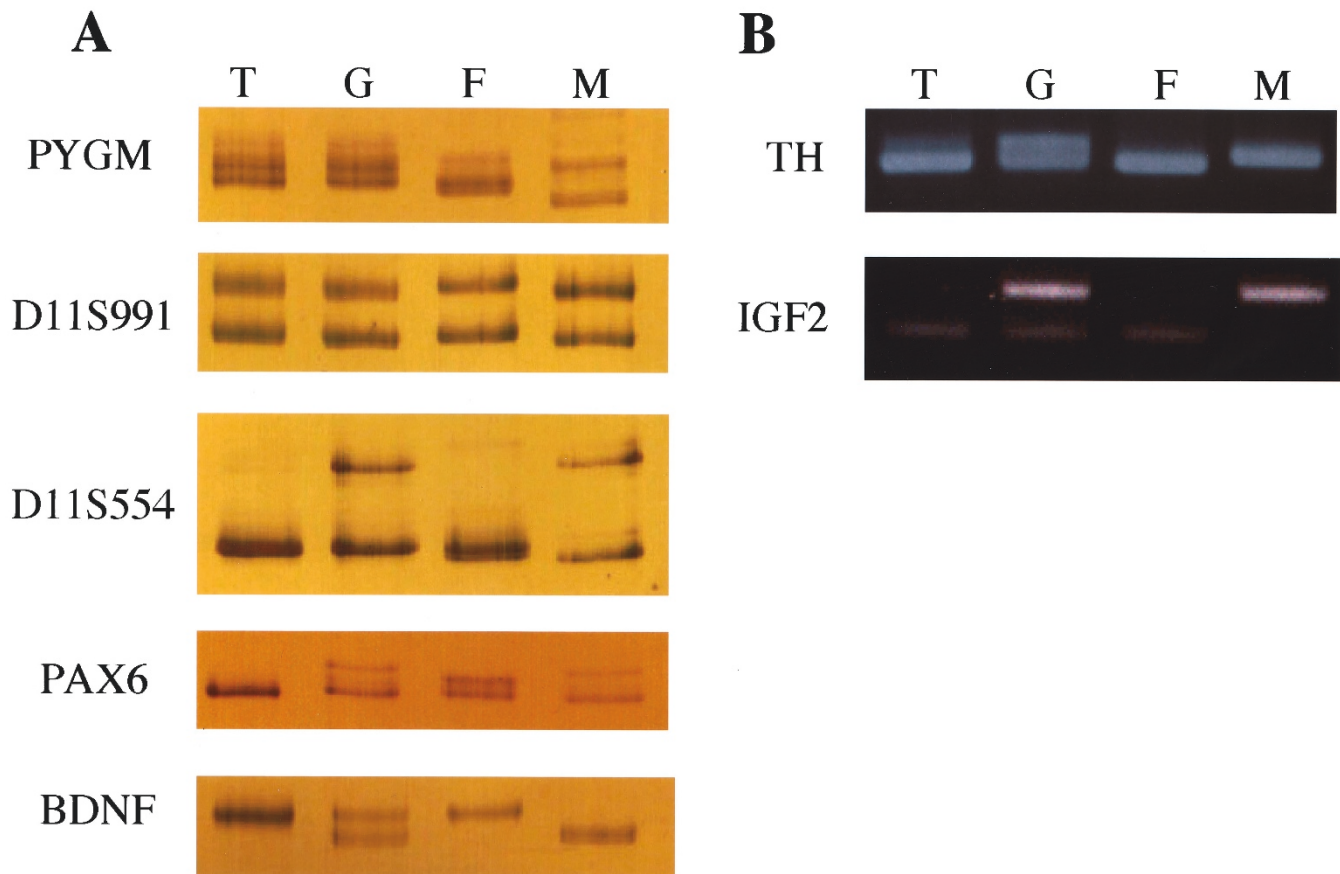
region in the tumor cells (Fig. 5B). Together with the results for *PAX6* and *BDNF* (both 11p13) (Fig. 5A), we concluded that both alleles were retained in the germline cells, and that the mutation, which was of paternal origin, was heterozygous. On the other hand, there was a broad loss of the maternal allele from 11p12 through 11p15 in the tumor cells that resulted in a

hemizygous mutation (see Table 1). It is unclear whether this mLOH in the tumor cells was the result of a deletion or a mitotic recombination. The parental alleles were all apparently normal (data not shown).

## DISCUSSION

In the present study, we demonstrated a novel *WT1* gene mutation in a patient with WT+GU. Polymorphic analysis indicated that the mutation was of paternal origin and heterozygous in the germline cells, whereas the maternal allele was largely lost in the tumor cells. This mutation was concluded to be *de novo* because the parental alleles were intact.

Concerning *WT1* mutation in patients with WT+GU, only nine cases have been reported so far (12, 30–35). The genetic characteristics of these cases, including the present one, are summarized in Table 2. Different from the previous reports, the present case is the first with a splicing error in the zinc finger domain of *WT1*. In addition, we reviewed the literature and have summarized the characteristics of the four *WT1*-related syndromes in 46XY patients in Table 3, although the features described are limited to those that are most commonly observed and typical, e.g. Breslow *et al.* (36) reported that patients with WT+GU have a moderate risk of long-term renal failure and that those with WAGR have a high risk of long-



**Figure 5.** Polymorphism of the present case in the short arm of chromosome 11. The germline cells show retention of heterozygosity (ROH), but the tumor cells show extensive mLOH from 11p12 to 11p15. *T*, tumor; *G*, germline; *F*, father; *M*, mother. (A) Polymorphic markers electrophoresed by the PhastSystem. PYGM and D11S991 show ROH in the tumor cells, but D11S554, PAX6, and BDNF show mLOH in the tumor cells. (B) Polymorphic markers electrophoresed on a 2% agarose gel. TH and IGF2 show mLOH in the tumor cells.

**Table 1.** Extent of maternal LOH on 11p in the present case

Region	Locus	Method of detection	Tumor	Germline	Father	Mother	Allele status of the tumor cells
11p13	<i>PYGM</i>	Compound(CA)(GA) repeat	A/B	A/B	A/A'	B/B'	ROH
11p11	D11S991	Dinucleotide repeat	A/A'	A/A'	A/A'	A/A'	ROH
11p12	D11S554	Short tandem repeat	A/-	A/B	A/A'	B/B'	mLOH
11p13	<i>WTA</i>	RFLP and dinucleotide repeat	A/A?	A/A?	A/A'	A/A	NI
11p13	<i>PAX6</i>	Dinucleotide repeat	A/-	A/B	A/A'	A/B	mLOH
11p13	<i>BDNF</i>	Dinucleotide repeat	A/-	A/B	A/A	B/B	mLOH
11p14	D11S865	Dinucleotide repeat	A/A?	A/A?	A/A	A/B	NI
11p15.2	<i>PTH</i>	Tetranucleotide repeat	A/A?	A/A?	A/A	A/A	NI
11p15.5	<i>TH</i>	Tetranucleotide repeat	A/-	A/B	A/A	B/B'	mLOH
11p15.5	<i>IGF2</i>	RFLP	A/-	A/B	A/A	B/B	mLOH
11p15.5	<i>H19</i>	RFLP	A/A?	A/A?	A/A	A/A	NI

\*A, A', B, and B' represent the respective alleles, - = allele loss; ? = uncertain; ROH = retention of heterozygosity; mLOH = maternal LOH; NI = not informative.

term renal failure. However, because theirs is the only report to have described such findings, we have added a question mark, indicating that there is no clear evidence, in Table 3.

LOH on chromosome 11 was investigated in some of the previous cases, as shown in Table 2. In the present case, the lost allele was maternal, and LOH extended from 11p12 to 11p15. In the other cases, it was uncertain whether the lost allele was maternal or paternal, or how extensive the LOH was. LOH in 11p15 region has been suggested to be critical for WT tumorigenesis in certain cases (37), and a series of reports

demonstrated the nonrandom maternal allele loss of 11p in the tumor cells of WT children (38–40). Because there are several imprinted genes in 11p15, some of which may be related to WT tumorigenesis, LOH, especially the maternal loss of 11p15, may be critical for WT tumorigenesis in association with a *WT1* mutation effect. For example, the constitutional genetic event in the WAGR patients is an 11p LOH, which is followed by a second hit *WT1* mutation when the somatic cells transform to WT, whereas in the WT+GU patients, a constitutional *WT1* mutation may be followed by a second hit 11p

**Table 2.** Nine cases with WT1 mutation associated with GU malformation

Patient no.	Karyotype	GU malformation	Age at diagnosis	WT	Mutation	Effect on protein	Result	Tumor LOH		Ref.
								11p13	11p15	
1	46XY	Ambiguous genitalia, hypospadias, bl. cryptorchism	12 mo	ul.	Point mutation at intron 7 (+2) T→G	Splicing out of exon 7	Truncated in exon 4	mLOH	mLOH	The present study
2	46XY	Hypospadias, left cryptorchism	nd	bl.	17 bp deletion within exon 4	Frameshift	Truncated in exon 4	nd	nd	12
3	46XY	Hypospadias, bl. cryptorchism	36 mo	ul.	1 bp deletion within exon 6	Frameshift	Truncated in exon 7	nd	nd	12
4	46XY	Hypospadias, bl. cryptorchism	9.5 mo	ul.	Point mutation at intron 6 (+1) G→C	Splicing out of exon 6 or exon 5 and 6	Truncated in exon 7	NI	LOH	30
5	46XY	Genitourinary malformation	nd	ul.	5 bp insertion within exon 1	Frameshift	Truncated in exon 1	LOH	LOH	31
6	46XY	Bl. cryptorchism	12 mo/33 mo	bl.	Point mutation at exon 6 C→G	Tyr→stop	Truncated in exon 6	LOH	LOH	32
7	46XY	Ul. testicular aplasia	12 mo	ul.	7 bp deletion within exon 1	Frameshift	Truncated in exon 3	LOH	nd	33
8	46XY	Hypospadias, bl. cryptorchism	15 mo	bl.	Point mutation at exon 8 C→T	Arg→stop	Truncated in exon 8	nd	nd	34
9	46XY	Testicular ectopia, hypospadias	6 mo	ul.	Point mutation at exon 4 G→A	Trp→stop	Truncated in exon 4	nd	nd	35

nd = no data; ul. = unilateral; bl. = bilateral; ref. = reference numbers.

**Table 3.** Four WT1-related syndromes in 46XY patients

WT1-related syndromes	External genitalia	Gonads and internal reproductive organs	Renal disorder	WT1 mutations in germline cells/allele	WT1 mutations in WT cells/allele	Result of WT1 mutation	Wild-type WT1 in the germline cells	Wild-type WT1 in the tumor cells	WT incidence rate	Mean onset of WT (mean ± SD)
DDS	Female phenotype or ambiguous genitalia	Dysgenetic testes and persistent Müllerian duct	Diffuse mesangial sclerosis with early onset	Missense point mutations in ZF domain/hetero	Missense point mutation in ZF domain/homo	Gain of dysfunctional protein	Decrease	Null	74%*	19.3 ± 14.4 m**
WAGR	Abnormal male or ambiguous genitalia	Streak gonads or cryptorchism	Long-term renal failure?	No mutation/hemi (11p deletion)	Nonsense mutations at various points/hemi (LOH)	Gain of nonfunctional protein	Decrease	Null	62%†	25.9 ± 16.0 m†
WT+GU	Abnormal male or ambiguous genitalia	Streak gonads or cryptorchism	Long-term renal failure?	Nonsense mutations at various points/hetero	Nonsense mutations at various points/hemi (LOH)	Gain of nonfunctional protein	Decrease	Null	<4.4%‡	18.5 ± 11.1 m§
FS	Female phenotype	Streak gonads and persistent Müllerian duct	Focal segmental glomerular sclerosis, delayed renal failure	Intro 9 splicing donor site point mutation/hetero	Loss of +KTS isoform	+KTS-KTS imbalance	-	-	-	-

The features described here are cited from references (ref.) 3, 10, 14, 35, 41–43, 46, and 47. \* ref. 47; \*\* ref. 42; † ref. 41; ‡ ref. 14; § the present study. In regard of Fraiser syndrome (FS), we refer to the report of Barbaux et al., Nat Genet 17: 467–470, 1997. “-?” means without such a feature, and “?” means without a clear evidence. WAGR = WT, an iridia, GU malformation, and mental retardation.

LOH (Table 3). Because meiotic recombinations are thought to be more frequent than mitotic recombinations, the higher risk of WT in the WAGR patients (62%) (41) than in the WT+GU patients (<4.4%) (14) could be explained by the probability of LOH. However, to demonstrate the relation between 11p LOH and WT tumorigenesis in WT+GU, more cases need to be accumulated, and it is recommended that polymorphic studies be carried out in WT+GU patients.

The patients described in Table 2 had an earlier onset of WT [ $18.5 \pm 11.1$  mo (mean  $\pm$  SD)] than in most WT cases ( $43.3 \pm 31.6$  mo) reported by Breslow *et al.* (3), suggesting that the presence of a predisposing *WT1* mutation affects the onset, as shown in DDS patients (13, 42). In addition, all the *WT1* mutations shown in Table 2 resulted in a truncated protein. Huff *et al.* (43) compared four cases that had been reported earlier (patients 2, 3, 4, and 5) with DDS patients in 1996. They hypothesized that the truncated protein is nonfunctional and causes GU malformation by a dose effect of decreasing normal WT1 protein, whereas the aberrant protein produced by the mutation in DDS is dysfunctional and induces additional, more severe symptoms, such as renal failure, as a result of a dominant-negative effect on an unknown target. However, both DDS and truncated WT1 mutants lose DNA binding activity for selected targets (44), and these mutants are known to associate with wild-type WT1 and act in a dominant-negative fashion if they retain the first 160 amino acids (45). These results suggest that both mutants act dominant-negatively. Additionally, the possibility of long-term renal failure in patients with WAGR or WT+GU has been suggested by a statistical study (36). Based on the results of these recent studies, we speculate that a decreasing dose of wild-type WT1 due to either mutant affects male GU malformation and causes long-term renal failure and that the dysfunctional DDS mutants may cause more severe GU anomalies, acute renal disorder (46), and an even higher incidence of WT (47), although their function remains to be elucidated.

The loss of *WT1* expression within the tumor in the present case was demonstrated by the results of our immunohistochemical study. The regions suggesting nephrogenic rests also did not stain, and this further implies that the maternal allele was already lost in the nephrogenic rest cells, confirming the previous report of genetic events in the nephrogenic rests (48). Also, we found rich stromal components and ectopic myogenesis in the tumor. These findings are consistent with the previous report of a *WT1* null effect (33, 49). With respect to tumorigenesis, we must not overlook *WT1* null effect. The complete loss of transcriptional regulation and/or apoptosis induced by the WT1 protein (50) may affect WT tumorigenesis in some cases, because the incidence of WT in patients with the *WT1*-related syndromes is definitely higher than in sporadic cases (Table 3). Baudry *et al.* (51) recently reported that they had found quantitative and/or qualitative alterations of *WT1* mRNA expression, including *WT1* splicing alterations, in 90% of sporadic WT. Their results may affect the present view of the relation between *WT1* and sporadic WT. In addition, although we did not demonstrate *WT1* splicing variants expression, imbalance among them may also function in cases with WT+GU.

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