

# A point mutation found in the *WT1* gene in a sporadic Wilms' tumor without genitourinary abnormalities is identical with the most frequent point mutation in Denys–Drash syndrome

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We have analyzed exon 9 of the *WT1* gene of 18 non-familial/sporadic unilateral Wilms' tumors (WTs) from Japanese patients, by the polymerase chain reaction single-strand conformation polymorphism (PCR-SSCP) method. After screening these WTs, a nucleotide alternation, which was present on both alleles, was found in only one case. Furthermore, PCR-SSCP analysis of the constitutional DNA revealed that this patient carried the mutation on only one allele in the germline. Sequence analysis showed that the tumor carried a point mutation (C-1180 to T-1180) in *WT1* exon 9 of both alleles, resulting in an Arg-394 to Trp-394 amino acid substitution within the third zinc finger domain of the *WT1* product. Interestingly, this mutation is identical with the most frequent point mutation associated with the Denys–Drash syndrome. However, the classical triad of Denys–Drash syndrome does not apply to this patient. This is in the first report of the point mutation in the zinc finger domain of both *WT1* alleles in a sporadic unilateral WT without genitourinary abnormalities, and the mutation suggests that some sporadic WTs carry the Denys–Drash *WT1* mutations.

Wilms' tumor; *WT1* gene; Denys–Drash syndrome; Point mutation

## 1. INTRODUCTION

Wilms' tumor (WT) is an embryonal malignancy of the kidney arising from a metanephric blastoma [1]. The occurrence of both sporadic and hereditary forms of WT and the early age of bilateral WT onset suggest that WTs develop when a predisposing germline mutation is accompanied by a second mutation [2]. A tumor suppressor gene for WT, *WT1*, has recently been isolated from the distal 11p13 region [3,4]. The *WT1* gene contains 10 coding exons that produce four distinct *WT1* mRNAs [5], and encodes a DNA-binding protein with a serine- and proline-rich NH<sub>2</sub> terminus and 4 zinc fingers [4]. These findings indicate that *WT1* has several features characteristic of transcription factors and that *WT1* zinc finger domains can bind to a specific DNA sequence [4,6].

There are two disease syndromes in which a genetic predisposition to WT is observed, with similar genital system malformations. The first is WAGR syndrome (Wilms' tumor (W), aniridia (A), genitourinary abnormalities (G), and mental retardation (R) [7]; the second

is the Denys–Drash syndrome [8,9]. The latter is a syndrome characterized by the association of nephropathy, dysgenetic male pseudohermaphroditism and WT. The nephropathy is characterized by its very early onset (before the age of 2 years), and in most patients it progresses to chronic or end-stage renal failure within a few months to 2 years from the onset [10]. From a morphological point of view, the nephropathy is often characterized by a lesion described as diffuse mesangial sclerosis [10]. Recently, Pelletier et al. made a detailed analysis of the *WT1* gene in 10 Denys–Drash patients [11]. They found point mutations in the *WT1* gene in all 10. Seven out of the 10 patients have a specific point mutation (C-1180 to T) in exon 9 (zinc finger III). These findings indicate that *WT1* plays a direct role in the pathogenesis of the Denys–Drash syndrome [11].

In this report, we present one case of a point mutation in *WT1* exon 9 in a unilateral sporadic WT from a Japanese girl. Furthermore, the patient carries the mutation on one allele in the germline. Sequence analysis of *WT1* exon 9 revealed that the tumor has the same point mutation as that of WTs from the majority of Denys–Drash individuals. However, she has neither renal failure nor malformation of the external genitalia, and is alive and well at 7 years of age without renal failure. In these respects, the triad of the Denys–Drash syndrome does not apply to this patient. This is the first report of a point mutation of the zinc finger domain of

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both *WT1* alleles in a sporadic unilateral WT without genitourinary abnormalities [11,12], and the mutation suggests that some sporadic WTs carry the Denys-Drash *WT1* mutations.

## 2. MATERIALS AND METHODS

### 2.1. Genomic DNA isolation

Genomic DNAs were prepared from tumorous and normal kidneys of WT patients by the SDS-proteinase K method [13] with slight modification. Ground tissues were suspended in 100 mM ethylenediamine tetraacetic acid (EDTA), 0.5% sodium dodecyl sulfate (SDS), and 0.5 mg of RNase A per ml for 3 h at 37°C, then incubated for 3 h at 50°C with an additional 0.1 mg of proteinase K per ml. The DNA was extracted with phenol and chloroform, then precipitated with 2-isopropyl alcohol.

### 2.2. Polymerase chain reaction (PCR) amplification

The PCR was performed by using a DNA thermocycler (Perkin-Elmer-Cetus) with 0.1 µg of genomic DNA in a total volume of 10 µl. Thirty cycles of amplification were carried out (7 min at 94°C once, followed by 1 min at 94°C, 1 min at 55°C and 1 min at 72°C for 30 cycles, then 10 min at 72°C) with two pairs of primers (9A and 9S, 9a and 9s). Prior to the PCR, the primers were radiolabeled with T4 polynucleotide kinase (Takara Biochemicals) and [ $\gamma$ -<sup>32</sup>P]ATP.

### 2.3. Single-strand conformation polymorphism (SSCP) gel analysis

After the PCR reaction, the PCR products were diluted 10-fold in a solution containing 95% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol. The products were heated at 90°C for 5 min and cooled on ice. The samples were loaded onto 5% nondenaturing polyacrylamide gels (49:1 polyacrylamide/methylene bis-acrylamide) containing 1 × TBE (90 mM Tris-borate pH 8.3, 2 mM EDTA) and 5% glycerol. Electrophoresis was carried out at 40-W constant power at room temperature in an electrophoretic apparatus (Bio-Rad, Model 3000Xi). The gel was dried on filter paper and exposed to X-ray film at -70°C for 12 h.

### 2.4. DNA sequencing

The PCR products were sequenced by the linear PCR method [14]. Briefly, one pmol of the primer was 5'-end-labeled in 20 µl of a reaction mixture containing 60 mM Tris-HCl (pH 7.5), 9 mM MgCl<sub>2</sub>, 10 mM DTT, 2 U of T<sub>4</sub>polynucleotide kinase (Takara Biochemicals), and 5 µCi of [ $\gamma$ -<sup>32</sup>P]ATP for 20 min at 37°C. One µl of this reaction mixture was added to a mixture of 16.6 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 67 mM Tris-HCl (pH 8.8), 6.7 mM MgCl<sub>2</sub>, 10 mM DTT, 2 mg of BSA per ml, 2.5 µM each of dATP, dCTP, dGTP, dTTP, 0.6 unit of Taq DNA polymerase (Perkin-Elmer-Cetus), and either 1 mM ddT, 50 µM ddG, 500 µM ddA or 500 µM ddC (final volume 20 µl) and the solution was overlaid with 50 µl of mineral oil. Thirty cycles of PCR were carried out at 95°C (1 min), 45°C (1 min) and 72°C (2 min). Two µl of each mixture was loaded on a 6% polyacrylamide-urea sequencing gel.

## 3. RESULTS

### 3.1. Detection of mutations on *WT1* exon 9 in sporadic WTs

The PCR-SSCP method [15] was used to screen for mutations in *WT1* exon 9 (zinc finger domain III) of DNAs extracted from 18 sporadic unilateral WTs. Table I summarizes the clinical and pathological data of the 18 patients. None of these patients presented with genital malformations, and 16 of the 18 tumors were histologically diagnosed as triphasic nephroblastoma [16]. By Southern blot analysis, we did not detect any

gross deletion or DNA rearrangement within the *WT1* gene in any of the 18 WTs. Therefore, we examined the WTs for small nucleotide changes in *WT1* exon 9 by the PCR-SSCP method using specific primers (9A and 9S) designed by Pelletier et al. (Table II) [11]. Primer 9a, which contains a mutated base against primer 9A, is used with 9S to show that a point mutation can be detected under our PCR-SSCP conditions. The SSCP pattern in Fig. 1A clearly indicates that the point-mutated *WT1* gene can be distinguished from the normal *WT1* gene by mobility shift. None of the PCR products from 17 WTs and the control human placenta showed a mobility shift, indicating no structural alteration. The only exception was a fragment resulting from case 12 (Fig. 1B).

### 3.2. SSCP analysis of *WT1* in a germline of a patient

We analyzed the *WT1* gene in the constitutional DNA to determine whether germline mutation could occur in this patients (case 12). Fig. 1B shows the PCR-SSCP analysis of the *WT1* exon 9 in the normal kidney of the patient. The SSCP pattern in Fig. 1B indicates that the WT of this patient carries the mutation in exon 9 of both *WT1* alleles, and that the patient carries the same mutation of one *WT1* allele in the germline.

### 3.3. Sequence analysis of the *WT1* gene in WT from the same patient

To elucidate the structural change in *WT1* exon 9 from WT of this patient, we subjected the PCR product to direct sequencing. The results shown in Fig. 2 revealed a cytosine-1180 (C) to thymine-1180 (T) transition in exon 9 of both *WT1* alleles. This mutation results

Table I  
Clinical information and histology of 18 unilateral WTs

Case	Age(yr/mo)	Sex	Histology	Abnormality*
1	0/2	F	NBW, triphasic	
2	0/6	M	NBW, triphasic	
3	1/4	M	NBW, triphasic	
4	1/4	M	NBW, triphasic	
5	1/4	M	NBW, triphasic	
6	1/4	F	NBW, triphasic	
7	1/5	F	NBW, triphasic	
8	1/6	F	NBW, triphasic	
9	2/0	M	NBW, triphasic	
10	2/1	F	NBW, triphasic	
11	2/1	M	NBW, triphasic	
12	2/6	F	NBW, triphasic	a point mutation
13	3/0	F	NBW, triphasic	
14	3/2	F	NBW, triphasic	
15	3/8	F	NBW, CPDN	
16	4/0	F	NBW, Mesenchymal	
17	4/2	M	NBW, triphasic	
18	4/2	M	NBW, triphasic	

NBW, nephroblastic Wilms' tumor; CPDN, cystic partially differentiated nephroblastoma.

\*Abnormality detected by PCR-SSCP analysis

in the conversion of arginine-394 (Arg) to tryptophane-394 (Trp) within the third zinc finger domain of the *WT1* product. Interestingly, this mutation is identical with the most frequent point mutation, as described by Pelletier et al. [11], associated with the Denys-Drash syndrome.

### 3.4. Patient and pathological observation

The patient (case 12) was a girl who was 2 1/2 years old at the time of the operation. The tumor arose in the upper portion of her right kidney. Thorough computerized tomography, X-ray and echo examinations revealed no evidence of a tumor in her left kidney. There was no family history of a renal tumor. She did not present with nephropathy (proteinuria/elevated serum creatinine). Furthermore, she had normal female external genitalia, and had no other abnormalities such as aniridia and mental retardation. After nephrectomy of the tumorous kidney, she is alive and well at the age of 7 years without renal failure. On histopathological examination, the WT of this patient showed an admixture of three components (epithelial, stromal and blastemal), indicating that the tumor was of the nephroblastic, triphasic type [16]. Furthermore, histological examination of the renal tissue adjacent to the tumor showed no apparent features of diffuse mesangial sclerosis which are considered to be the specific histology of the Denys-Drash syndrome (Fig. 3) [10].

## 4. DISCUSSION

The incidence of gross genomic alteration of *WT1* in WTs is very low [4,17–20]. Our previous study also revealed only three partial deletions, which include one case of an intragenic deletion, in 25 unilateral sporadic WTs [21]. Therefore, investigations detecting more subtle DNA abnormalities such as small deletions or point mutations is required to understand the role of the *WT1* gene in the development of WT.

Since subtle DNA abnormalities in the zinc finger domains of the *WT1* gene have been shown to affect DNA binding [6], we reasoned that such DNA abnormalities in the *WT1* zinc finger may occur and contribute to the tumorigenesis in sporadic unilateral WTs. Therefore, we chose to search unilateral WTs for subtle DNA abnormalities in the *WT1* zinc finger by the PCR-SSCP method. In this paper, we have described one case of a point mutation in *WT1* exon 9 in a unilateral

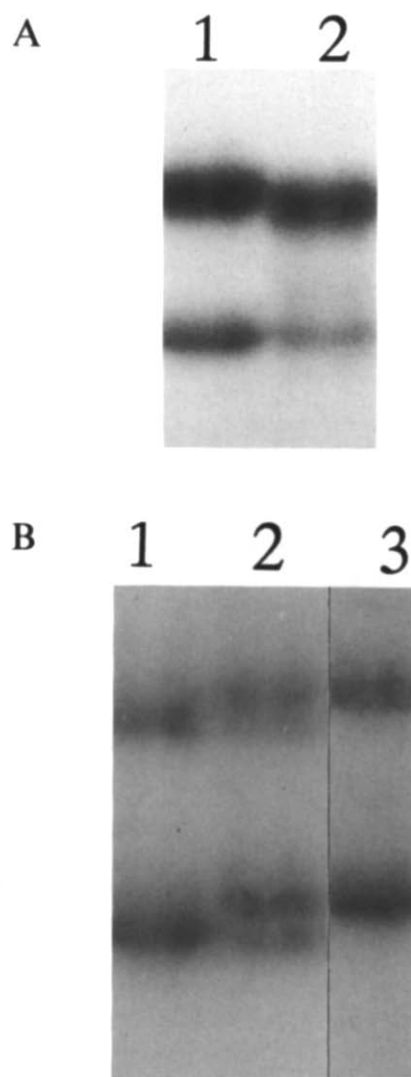


Fig. 1. (A) PCR-SSCP patterns obtained from the control unaffected human placental DNA. Genomic DNAs were subjected to PCR-SSCP analysis with two pairs of primers (lane 1, 9A and 9S; lane 2, 9a and 9S) flanking exon 9 of the *WT1* gene. Primer 9a contains the mutated base (replacement of C [underlined] by T [underlined] in the primer 9A), as indicated in Table II. (B) PCR-SSCP analysis of *WT1* exon 9. Genomic DNAs were isolated from WT of case 12 (lane 1), normal kidney of case 12 (lane 2) and unaffected normal human placenta (lane 3), and subjected to PCR-SSCP analysis with a pair of primers (9A and 9S). The separated strands of fragment (lane 1) from WT moved to downward positions from the control human placenta (lane 3). Moreover, we could not detect any fragment in WT with the same mobility as that of the fragments from the control. In lane 2, the amplified fragments from normal kidney of case 12 were separated into four single-stranded DNAs. In four strands, two out of them had the same mobility as that from the unaffected normal human placenta, and the other two had the same mobility as that of the fragment from WT of this patient (lane 1). Electrophoresis was carried out in 5% polyacrylamide gel with 5% glycerol at room temperature.

Table II

Primers for PCR-SSCP of *WT1* exon 9

Name	Sequence
9A	5'-CCCCGAATTCAAGATAGCCACGCACTATTCC-3'
9a	5'-CCCCGAATTCAAGATAGCCATGCACTATTCC-3'
9S	5'-CCCCAAGCTTCAGGAAATGCTGGGCTCC-3'

sporadic WT, and this is the first report of the point mutation in the zinc finger domain of both *WT1* alleles in a sporadic WT without genitourinary abnormalities [11,12].

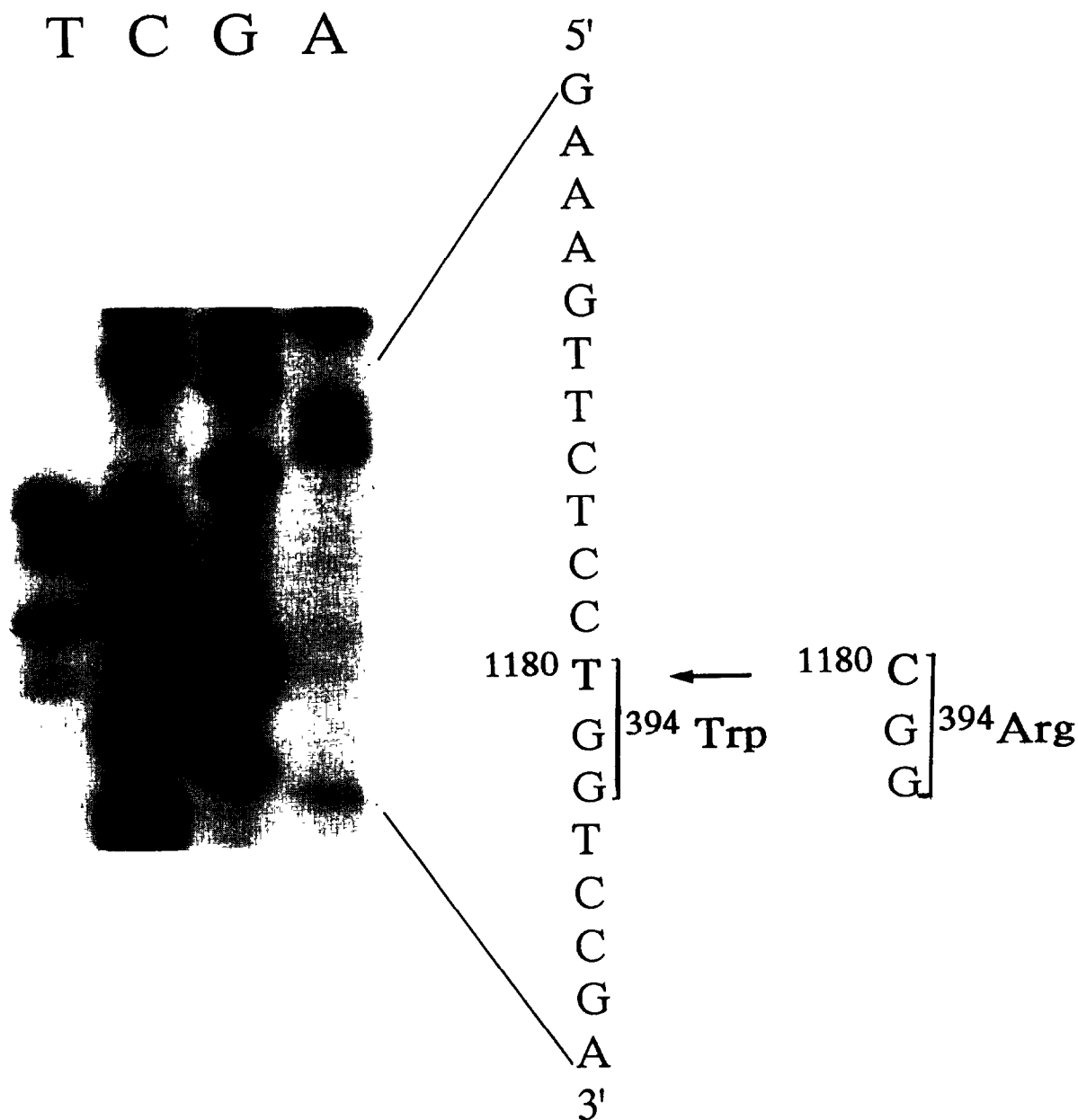


Fig. 2. Nucleotide sequence analysis of PCR products obtained from WT of case 12 were sequenced as described in section 2. The arrowhead represents the site of the point mutation (replacement of C by T).

Sequence analysis revealed that the point mutation in this patient is identical with the most frequent point mutation in the Denys-Drash syndrome [11]. However, clinically, the Denys-Drash syndrome could not apply to this patient in terms of nephropathy [10]. In the study of 10 Denys-Drash patients, seven cases of nephropathy with WT were found [11]. Interestingly, six of the seven cases had the common point mutation in exon 9 of one *WT1* allele in the germline. Those authors postulated that the germline hemizygous inactivation of *WT1* contributes to the development of apparently non-WT-

associated genitourinary abnormalities [11,22,23]. Our patient carries the same point mutation as that of the above cases in the germline. However, their model does not apply to our case since the nephropathy associated with this syndrome has not occurred in this patient. Why is the effect of such a hemizygous mutation abrogated in this patient? One possible explanation is that the renal failure may be caused by a truncated form of the *WT1* gene product [24]. Another possible explanation is the influence of other genetic or environmental factors. Although there have been no reported cases

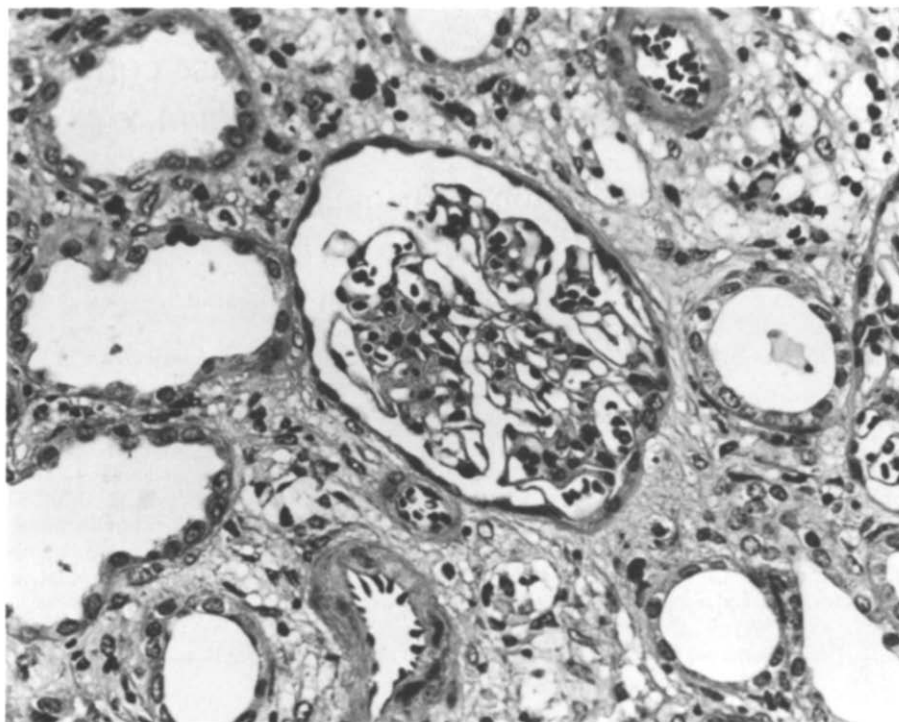


Fig. 3. Microscopic observation of the normal kidney of case 12. The histology of the renal tissue adjacent to WT did not show the apparent features of diffuse mesangial sclerosis which is considered to be a specific histology for the Denys-Drash syndrome.

which are parallel to this one, this discrepancy will be clarified by detailed analysis after accumulation of cases like this one.

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