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Mutations in *WT1* in boys with sporadic isolated steroid-resistant nephrotic syndrome

Y. Yang[1,2,3]*, F. Zhao[1,2,3]*, X. Tu[4] and Z. Yu[1,2,3]

[1]Department of Pediatrics, Fuzhou Dongfang Hospital, Fuzhou, Fujian, China
[2]Department of Pediatrics, Fuzhou Clinical Medical College, Fujian Medical University, Fuzhou, Fujian, China
[3]Department of Pediatrics, Affiliated Dongfang Hospital, Xiamen University, Fuzhou Fujian, China
[4]Research Center for Molecular Diagnosis of Genetic Diseases, Fuzhou Dongfang Hospital, Fuzhou, Fujian, China

*These authors contributed equally to this study.
Corresponding author: Z. Yu
E-mail: zihuayu@vip.sina.com

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ABSTRACT. Mutations in the Wilms' tumor gene, *WT1,* can lead to syndromic steroid-resistant nephrotic syndrome and isolated steroid-resistant nephrotic syndrome. *WT1* mutations have been identified in the majority of children with Denys-Drash or Frasier syndrome. *WT1* mutations have not previously been identified in boys with sporadic isolated steroid-resistant nephrotic syndrome, but, recently, four boys with isolated nephrotic syndrome were identified to have *WT1* mutations. However, whether boys with sporadic isolated steroid-resistant nephrotic syndrome should be routinely subjected to mutation analysis of *WT1* has not been established. We examined 35 boys with sporadic isolated steroid-resistant nephrotic syndrome for mutations in *WT1*. Mutation analysis of all 10 exons of *WT1* was performed by polymerase chain reaction and direct sequencing. Karyotype analysis or Y chromosome identification was performed for all patients. A Y chromosome or a 46, XY karyotype was demonstrated for

all 35 patients. No causative *WT1* mutation was identified in any of the patients. The *WT1* mutation, IVS4+14T>C, which is not predicted to affect splicing, was identified in one patient who achieved complete remission after 8 weeks of oral prednisone treatment, indicating that IVS4+14T>C is not a causative mutation. Five *WT1* polymorphisms were also identified in some patients and controls. Our results suggest that mutation analysis of *WT1* should not be routinely performed for genetically defined boys with sporadic isolated steroid-resistant nephrotic syndrome.

Key words: Male; Mutation; Polymerase chain reaction; *WT1;* Steroid-resistant nephrotic syndrome

INTRODUCTION

Idiopathic nephrotic syndrome (INS) is characterized by proteinuria, hypoalbuminemia, hyperlipidemia, and edema and is the most common childhood glomerular disease (Yu et al., 2005). INS is divided into steroid-sensitive nephrotic syndrome and steroid-resistant nephrotic syndrome (SRNS) based on the patient's response to steroid treatment. Most children with sporadic INS respond to steroids and have a favorable long-term prognosis; however, 10-20% of patients do not respond to steroids and may progress to end-stage renal disease (ESRD) (Weber et al., 2004; Ruf et al., 2004a). Mutations in genes encoding podocyte proteins such as *NPHS1* (OMIM 256300), *NPHS2* (OMIM 604766), *CD2AP* (OMIM 607607832), and the Wilms' tumor gene, *WT1* (OMIM 607102), are responsible for SRNS in children (Weber et al., 2004; Mucha et al., 2006; Löwik et al., 2007; Philippe et al., 2008).

The *WT1* gene, located on chromosome 11p13 and consisting of 10 exons, plays a crucial role in kidney and genital system development (Call et al., 1990; Haber et al., 1991; Bruening et al., 1992). Mutations in *WT1* can lead to syndromic forms of SRNS, such as Denys-Drash syndrome (OMIM 194080) and Frasier syndrome (OMIM 136680), and can cause isolated SRNS (Pelletier et al., 1991; Barbaux et al., 1997; Mucha et al., 2006). Denys-Drash syndrome is characterized by the triad of infantile SRNS, ambiguous genitalia, and Wilms' tumor (Chernin et al., 2010). Frasier syndrome is characterized by the association of SRNS with male pseudohermaphroditism (Chernin et al., 2010). Isolated SRNS discussed here refers to SRNS without accompanying genital abnormalities, Wilms' tumor, ocular abnormalities, audiological abnormalities, or mental retardation.

WT1 mutations have been identified in the majority of children with Denys-Drash syndrome or Frasier syndrome (Niaudet and Gubler, 2006). A *WT1* mutation is also a frequent cause of sporadic isolated SRNS in girls, occurring in 10.8% of girls in a worldwide cohort (Muchaet al., 2006). No *WT1* mutations have been detected in boys with sporadic isolated SRNS (Ruf et al., 2004b; Aucella et al., 2006; Mucha et al., 2006; Cho et al., 2008; Li et al., 2010). Our previous study identified no mutations in exons 8 and 9 of *WT1* in 38 boys with sporadic isolated SRNS (Yang et al., 2013b). However, *WT1* mutations in sporadic isolated SRNS children may occur in exons other than 8 and 9 (Takata et al., 2000), and recently four boys with isolated nephrotic syndrome were identified to have *WT1* mutations (Takata et al. 2000; Tajima et al. 2003; Chernin et al. 2010; Yang et al. 2013a). There is currently no guideline regarding whether boys with sporadic isolated SRNS

should be routinely subjected to *WT1* mutation analysis. This study aims to examine mutations in all 10 exons of *WT1* in 35 boys with sporadic isolated SRNS using polymerase chain reaction (PCR) and direct sequencing.

MATERIAL AND METHODS

Subjects

We enrolled 35 boys based on the following inclusion criteria: 1) no family history of renal diseases and not born from consanguineous parents; 2) older than 3 months and younger than 18 years at disease onset; 3) diagnosed with SRNS; 4) absence of Wilms' tumor based on renal ultrasound; 5) audiometry showed a lack of symmetrical deficits for high-frequency sounds; 6) ophthalmologic assessment confirmed a lack of ocular lesions; 7) clinical and laboratory examinations demonstrated a lack of post-infectious glomerulonephritis and systemic diseases; 8) not mentally retarded; and 9) had no *NPHS2* mutations. Nephrotic syndrome was diagnosed based on 24-h urinary protein excretion greater than 0.05 g/kg, with serum albumin less than 25 g/L. Steroid resistance was defined as the absence of remission after an initial four weeks of steroid therapy at a dose of 2 $mg \cdot kg^{-1} \cdot day^{-1}$. As control subjects, we also studied 100 unrelated adult volunteers with normal urinalyses. This study was approved by the Ethics Committee of Fuzhou Dongfang Hospital (China) and was performed in accordance with the Declaration of Helsinki. Informed consent was obtained from patients' parents and control subjects.

Karyotype analysis or Y chromosome identification

To confirm the gender of patients, karyotype analysis was performed on five patients. For the other 30 patients, the sex-determining region Y gene, *SRY* (a specific marker for the Y chromosome), was identified by PCR amplification (Harley et al., 2003). The *SRY* primers used were described previously (Tu et al., 2008).

WT1 mutation analysis

For genetic analysis of *WT1*, genomic DNA was isolated from peripheral blood. All 10 exons of *WT1* were amplified from genomic DNA by PCR. Genomic DNA (50 ng) was subjected to PCR amplification in a 25-μL volume consisting of 1 μL 5 μM sense primer, 1 μL 5 μM antisense primer, 1.5-3.5 μL 25 mM $MgCl_2$, 1 μL 2.5 mM deoxyribonucleotide triphosphates, and 0.125 μL 5 U/μL Taq polymerase (Promega Corporation, Madison, WI, USA). The PCR conditions were as follows: 94°C for 7 min, followed by 36 cycles of 94°C for 30 s, 56°-64°C for 30 s, and 72°C for 1 min, followed by 72°C for 7 min. Because of the high GC content of exon 1, 2X GC buffer I and LA polymerase (Takara, Shiga, Japan) was added to the reaction mixture. The PCR products were visualized using 1.5% agarose (w/v) gel electrophoresis. PCR amplicons were directly sequenced using an ABI 3730XL DNA Analyzer (Shanghai Invitrogen Biotechnology, Shanghai, China). Mutations were confirmed by sequencing in both directions and by repeated amplification and sequencing.

Splice site predictions

We used programs hosted by the NetGene2 Server (http://www.cbs.dtu.dk/services/NetGene2/) and the Berkley *Drosophila* Genome Project (http://www.fruitfly.org/seq_tools/splice.html) to predict splice sites in *WT1*.

RESULTS

Clinical data

The 35 boys with sporadic isolated SRNS did not have genital abnormalities, Wilms' tumor, ocular abnormalities, hearing abnormalities, or mental retardation (Table 1). Their age at onset was 4.3 ± 3.8 years (range: 0.4-12.7 years). Renal biopsy was performed on 12 patients and revealed focal segmental glomerulosclerosis in five patients, minimal change nephrotic syndrome in four patients, and mesangial proliferative glomerulonephritis in three patients. The other 23 patients did not consent to undergo renal biopsy. All 35 patients received prolonged steroid or immunosuppressive agent treatment. Eight patients did not respond to either steroids or immunosuppressive agents. One of these eight patients died from pneumonia with heart failure within seven weeks after disease onset. Twenty seven patients responded to either prolonged steroid therapy or immunosuppressive agents. During the follow-up period, two patients progressed to ESRD. One patient, who responded to cyclosporin A, progressed to ESRD within 71 months after disease onset; another patient, who showed no response to immunosuppressive agents, progressed to ESRD within 74 months after disease onset.

Patient 31 (Table 1) initially presented with eyelid edema at 4.2 years of age. Physical examination revealed eyelid edema and normal male external genitalia. Blood pressure was normal and a urine dipstick revealed 3+ albumin. His 24-h proteinuria was 4.8 g and serum albumin was 10.6 g/L. Serum cholesterol was 8.85mM. He screened negative for Wilms' tumor by kidney ultrasound. He failed to respond to prednisone treatment (2 $mg^{-1} \cdot kg^{-1} \cdot 24\ h^{-1}$) over a 4-week course. However, after eight weeks of oral prednisone treatment, complete remission was achieved. A renal biopsy was refused by his parents, and he was diagnosed with isolated SRNS. Neither of his parents had proteinuria.

Karyotype analysis or Y chromosome identification

Karyotype analysis showed a 46, XY karyotype in five patients. *SRY* PCR analysis indicated the presence of a Y chromosome in the remaining patients (Table 1).

WT1 mutational analysis

The *WT1* variant, IVS4+14T>C, was identified in patient 31 (Figure 1) and was not observed in controls, indicating that it is a mutation of *WT1*. However, no *WT1* mutations were found in the other 34 patients. We also identified one variant (5'-UTR-7G>T) in the 5'-untranslated region of *WT1* in two patients and four controls, one silent mutation (126C>T) in exon 1 in 27 patients and 46 controls, one variant (IVS3+16G>A) in intron 3 in one patient and two controls, one silent mutation (903A>G) in exon 7 in 30 patients and 48 controls, and one variant (IVS7-32C>A) in intron 7 in three patients and two controls, indicating that these variants and silent mutations are *WT1* polymorphisms (Table 2).

Table 1. Clinical data for 35 boys with sporadic isolated steroid-resistant nephrotic syndrome.

Patient	Age of onset (years)	Creatinine (μM)	BUN (mM)	Renal biopsy	Karyotype/Y chromosome status	Therapy and response	ESRD after disease onset (months)
1	12.2	74	10.9	ND	Y†	GC, CTX, MMF (NR)	N
2	0.4	25	3.7	FSGS	Y†	GC (NR)	N
3	3.5	33	6.7	FSGS	Y†	GC, CsA (R)	N
4	7.6	37	4.1	FSGS	Y†	GC, CsA (NR)	74
5	3.7	30	3.3	MCNS	Y†	GC (R)	N
6	0.7	74	1.4	ND	Y†	GC (NR)	N
7	4.6	41	4.2	FSGS	Y†	GC, CsA (R)	N
8	0.7	30	1.3	ND	46,XY	GC, CTX (NR)	N
9	12.3	114	7.4	MsPGN	Y†	GC, CsA (R)	71
10	5.2	42	4.6	ND	Y†	GC (R)	N
11	1.5	15.8	2.5	ND	Y†	GC, CsA (R)	N
12	1.3	56.3	6. 9	ND	Y†	GC, CsA (R)	N
13	6.5	40	5.8	ND	Y†	GC (R)	N
14	2.6	29.7	1.6	ND	Y†	GC (R)	N
15	2.0	25	2.7	ND	Y†	GC (R)	N
16	0.8	26	4.8	ND	Y†	GC, CsA (NR)	N
17	8.0	80	14.5	MCNS	Y†	GC, CsA (NR)	N
18	5.4	14	3.0	ND	Y†	GC (R)	N
19	2.4	23	5.6	ND	Y†	GC, CsA (R)	N
20	3.5	23	1.7	ND	Y†	GC, TG (NR)	N
21	1.8	30.6	4.9	ND	Y†	GC, CsA (R)	N
22	12.7	40	2.6	MCNS	Y†	GC (R)	N
23	2.0	30.2	4.1	ND	Y†	GC, CsA (R)	N
24	5.8	47	7.6	FSGS	Y†	GC, TG (R)	N
25	5.8	79	7.3	MCNS	Y†	GC, CsA (R)	N
26	4.8	22	7.0	MsPGN	Y†	GC (R)	N
27	11.0	38	6.7	MsPGN	Y†	GC, CsA (R)	N
28	2.6	42.3	2.7	ND	Y†	GC (R)	N
29	2.1	29.9	4.3	ND	Y†	GC (R)	N
30	1.7	20.2	4.7	ND	Y†	GC, CsA (R)	N
31	4.2	59	14.8	ND	Y†	GC (R)	N
32	5.1	81.3	21.4	ND	46,XY	GC, CsA (R)	N
33	1.2	10	4.0	ND	46,XY	GC, CsA (R)	N
34	1.6	23	4.9	ND	46,XY	GC, CsA (R)	N
35	1.8	27	3.7	ND	46,XY	GC, CsA (R)	N

BUN = serum urea nitrogen; ESRD = end-stage renal disease; ND = not determined; GC = glucocorticosteroid; CTX = cyclophosphamide; MMF = mycophenolate mofetil; NR = not in remission; N = not progressed to ESRD; FSGS = focal segmental glomerulosclerosis; CsA = cyclosporine; R = complete remission; MCNS = minimal change nephrotic syndrome; MsPGN = mesangial proliferative glomerulonephritis; TG = tripterygium glycosides. †*SRY* PCR indicated presence of the Y chromosome.

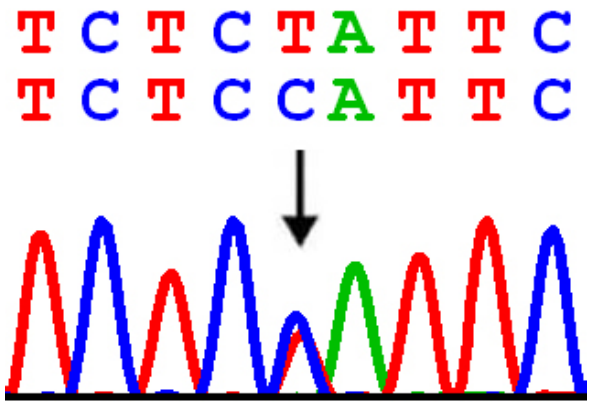

Figure 1. IVS4+14T>C mutation in intron 4 of *WT1* was identified by sequencing. Arrow indicates mutant nucleotide.

Table 2. *WT1* mutation and polymorphisms detected in 35 patients.

Patient	5′-UTR-7G>T	126C>T (P42P)	IVS3+16G>A	IVS4+14T>C[†]	903A>G (R300R)	IVS7-32C>A
1		Hom			Hom	
2		Het			Hom	
3		Het			Het	
4		Hom			Hom	
5	Het	Het	Het		Het	Het
6		Het			Hom	
7		Hom			Hom	
8		Hom			Hom	
9		Het			Hom	
10		Het			Het	
11		Hom			Hom	
12		Het			Het	
13		Hom			Hom	
14		Het			Het	
15						
16		Hom			Hom	
17						
18						
19		Hom			Hom	
20					Het	
21		Hom			Hom	
22		Het			Het	
23					Het	
24		Hom			Hom	
25		Hom			Hom	
26						
27		Hom			Hom	
28		Het			Het	
29						Het
30		Hom			Hom	
31	Het	Het		Het	Het	Het
32		Hom			Hom	
33					Het	
34		Het			Het	
35		Het			Het	

Hom = homozygous variant; Het = heterozygous variant. [†]Novel mutation.

Splice site prediction

IVS4+14T>C was not predicted to affect splicing using prediction algorithms hosted by the NetGene2 Server and the Berkley *Drosophila* Genome Project.

DISCUSSION

In this study, using direct sequencing, we detected no causative mutations in any of the 10 exons of *WT1* in 35 boys with sporadic isolated SRNS. Our results suggest that routine mutation analysis of *WT1* should not be recommended for boys with sporadic isolated SRNS.

We identified the *WT1* variant, IVS4+14T>C, in one patient (patient 31). This variant was absent in the controls, indicating that it is a *WT1* mutation; however, it was not predicted to affect splicing. This patient presented with SRNS at 4.2 years. He had a normal male phenotype and a Y chromosome. No tumor was identified using renal ultrasound and he was, therefore, diagnosed with isolated SRNS. However, after 8 weeks of oral prednisone treatment, complete remission was achieved. It is possible that a subset of SRNS with a response to immunosuppressive agents has an underlying immune defect (Benoit et al., 2010). Therefore, we do not consider IVS4+14T>C to be a causative mutation of SRNS.

We also detected five other *WT1* variants, 5'-UTR-7G>T, 126C>T, IVS3+16G>A, 903A>G, and IVS7-32C>A in some patients and controls and believe them to be *WT1* polymorphisms. All of these *WT1* polymorphisms were identified in our previous study, which showed that there was no association between the five *WT1* polymorphisms and SRNS in Chinese children (Wang et al., 2009).

No mutations in the *WT1* gene have been identified in boys with sporadic isolated SRNS (Table 3) (Ruf et al., 2004b; Aucella et al., 2006; Mucha et al., 2006; Cho et al., 2008; Li et al., 2010). Mucha et al. (2006) screened a worldwide cohort (Central European, Turkish, African-American, Hispanic, or Asian backgrounds) of 84 boys with sporadic isolated SRNS for mutations in all 10 exons of *WT1*. No *WT1* mutations were identified in these patients. They used multiplex capillary heteroduplex analysis, the sensitivity of which was 91%, and direct sequencing. Li et al. (2010) screened 43 boys with sporadic isolated SRNS for mutations in exons 8 and 9 of *WT1*, but no mutations were found. Ruf et al. (2004b) examined 57 boys with sporadic isolated SRNS originating from Central Europe, Turkey, or India for mutations in exons 6-9 of *WT1*. They were unable to identify *WT1* mutations in any of these patients. Aucella et al. (2006) performed mutation analysis of exons 8 and 9 of *WT1* in 32 Italian boys with sporadic isolated SRNS, and no *WT1* mutations were found in these patients. Cho et al. (2008) examined exons 8 and 9 of *WT1* in 30 Korean boys with sporadic isolated SRNS and identified no mutations.

Table 3. Summary of detection rates of *WT1* mutations in boys with sporadic isolated SRNS.

Ethnic background	Number of patients	Detection rate of *WT1* mutations	Exons	Mutation detection method	References
Worldwide cohort	57	0% (0/57)	Exons 6-9	DHPLC and direct sequencing	Ruf et al. (2004b)
Worldwide cohort	84	0% (0/84)	All 10 exons	MCHA and direct sequencing	Mucha et al. (2006)
Italian	32	0% (0/32)	Exons 8 and 9	DHPLC and direct sequencing	Aucella et al. (2006)
Korean	30	0% (0/30)	Exons 8 and 9	Direct sequencing	Cho et al. (2008)
Chinese	43	0% (0/43)	Exons 8 and 9	Direct sequencing	Li et al. (2010)
Chinese	35	0% (0/35)	All 10 exons	Direct sequencing	This study

SRNS = steroid-resistant nephrotic syndrome; DHPLC = denaturing high-performance liquid chromatography; MCHA = multiplex capillary heteroduplex analysis.

Four boys with isolated nephrotic syndrome have been reported to carry *WT1* mutations. Takata et al. (2000) reported a boy with nephropathy without genital abnormalities or Wilms' tumor caused by the *WT1* mutation R312Q in exon 7. His age of onset was 2 years, and he rapidly progressed to ESRD. His karyotype was 46, XY. Tajima et al. (2003) reported a boy with focal segmental glomerulosclerosis without genital abnormalities or Wilms' tumor caused by the *WT1* mutation IVS9+5G<A. His age of onset was 5 years, and at 8 years of age, his serum creatinine level was 0.8 mg/dL. Chernin et al. (2010) reported a boy with isolated nephrotic syndrome caused by the *WT1* mutation R394W in exon 9. His age of onset was 1.5 years and he progressed rapidly to ESRD by the age of 1.7 years. His karyotype was 46, XY. Recently, we reported a 6.3-year-old boy with isolated nephrotic syndrome caused by the *WT1* mutation K351E in exon 8. He rapidly progressed to ESRD and his karyotype was 46, XY (Yang et al., 2013a).

In conclusion, no causative mutations were identified in any of the 10 exons of *WT1* in 35 boys with sporadic isolated SRNS. Our results suggest that mutation analysis of *WT1* should not be routinely recommended for boys with sporadic isolated SRNS.

Conflicts of interest

The authors declare no conflicts of interest.

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