



## Mutational analysis of podocyte genes in children with sporadic steroid-resistant nephrotic syndrome

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**ABSTRACT.** Recent studies have demonstrated that mutations in 4 podocyte genes, *NPHS1*, *NPHS2*, *CD2AP*, and *WT1*, are associated with the pathogenesis of steroid-resistant nephrotic syndrome (SRNS). Systematic investigation of all 4 genes for sporadic SRNS in China has not been performed. We examined 10 Chinese children with sporadic SRNS who showed no response to immunosuppressive agents and 20 SRNS controls who exhibited a response to prolonged steroid or immunosuppressive treatment and achieved complete remission. We analyzed mutations in the 4 podocyte genes, *NPHS1*, *NPHS2*, *CD2AP*, and *WT1*. Mutational analysis was performed using polymerase chain reaction and direct sequencing. Of the 10 SRNS children who showed no response to immunosuppressive agents, the compound heterozygous *NPHS1* mutations 2677A>G (T893A)

and \*142T>C were identified in 1 patient, while a heterozygous mutation in *WT1*, 1180C>T (R394W), was found in another patient. Of the 20 SRNS children showing complete remission who responded to prolonged steroid therapy or immunosuppressive agents, 4 heterozygous *NPHS1* mutations, 928G>A, IVS8+30C>T, IVS21+14G>A, and IVS25-23C>T, were identified in 4 patients and a heterozygous *CD2AP* mutation, IVS7-135G>A, was identified in 1 patient. Our results indicate the necessity of genetic examination for mutations in podocyte genes in Chinese SRNS children who show no response to immunosuppressive agents.

**Key words:** Immunosuppressive agents; Mutation; Podocyte genes; Steroid-resistant nephrotic syndrome; Steroid treatment

## INTRODUCTION

Idiopathic nephrotic syndrome (NS) is a very common childhood glomerular disease. It is characterized by the association of gross proteinuria with hypoalbuminemia, edema, and hyperlipidemia, a condition that typically requires a prolonged course of steroids or immunosuppressive treatment. Approximately 10% of patients do not respond to corticosteroids and are at risk for additional renal complications of NS and the development of end-stage renal disease (ESRD) (Benoit et al., 2010). Recent studies have demonstrated that mutations in a number of podocyte genes, including *NPHS1*, *NPHS2*, and *CD2AP*, are associated with the pathogenesis of steroid-resistant nephrotic syndrome (SRNS) (Boute et al., 2000; Ruf et al., 2004b; Löwik et al., 2007; Philippe et al., 2008). *NPHS1*, *NPHS2*, and *CD2AP* code for structural elements of the slit diaphragm in the glomerular filtration barrier and have been identified as the causative genes in autosomal-recessive (AR) SRNS. When these genes are mutated, patients with AR SRNS can show resistance to steroids and immunosuppressive agents (Boute et al., 2000; Ruf et al., 2004a; Ehrich et al., 2007; Löwik et al., 2007; Philippe et al., 2008; Büscher et al., 2010). Mutations in *NPHS1*, which is located on chromosome 19q13.1 and contains 29 exons that encode nephrin, cause not only congenital NS of the Finnish type but also childhood-onset SRNS (Kestilä et al., 1998; Philippe et al., 2008). Mutations in *NPHS2*, which is located on chromosome 1q25-31 and contains 8 exons that encode podocin, are associated with AR SRNS (Boute et al., 2000). Mutations in *CD2AP*, which contains 18 exons and encodes CD2AP, have been identified in sporadic NS and focal segmental glomerulosclerosis (FSGS) (Löwik et al., 2007; Gigante et al., 2009). The *WT1* gene is located on chromosome 11p13, contains 10 exons, and encodes a zinc finger transcription factor involved in kidney and gonadal development (Call et al., 1990). Mutations in *WT1* have been shown to cause Denys-Drash syndrome (DDS), Frasier syndrome (FS), and isolated SRNS (Machuca et al., 2009; Benoit et al., 2010). DDS is characterized by the association of early onset NS and diffuse mesangial sclerosis, which rapidly progresses to ESRD, XY pseudohermaphroditism, and Wilms' tumor (Ruf et al., 2004b; Benoit et al., 2010). Incomplete DDS is characterized as NS associated with Wilms' tumor or XY pseudohermaphro-

dism (Schumacher et al., 1998). FS is also characterized by male pseudohermaphroditism as well as nephropathy (Ruf et al., 2004b; Benoit et al., 2010). Most SRNS patients with mutations in the podocyte genes *NPHS1*, *NPHS2*, *CD2AP*, and *WT1* are also resistant to immunosuppressive agents (Boute et al., 2000; Ruf et al., 2004a,b; Löwik et al., 2007; Philippe et al., 2008; Benoit et al., 2010; Büscher et al., 2010; Li et al., 2010; Megremis et al., 2011; Stefanidis and Querfeld, 2011).

Classification of SRNS can be modified by the results of gene analysis. Therefore, understanding causative mutations in podocyte genes in Chinese children with SRNS is important; however, a systematic investigation has not been performed. To clarify the role of these genetic mutations, all exons and exon-intron boundaries for all 4 genes were analyzed in 10 Chinese SRNS children who showed no response to immunosuppressive agents.

## MATERIAL AND METHODS

### Subjects

This study included children (N = 10) who were suffering from sporadic SRNS. Inclusion criteria included: 1) children of non-consanguineous marriages with onset between 3 months and 18 years of age; 2) no family history of renal diseases; 3) no response to immunosuppressive agents; 4) renal histology showed changes of focal segmental glomerulosclerosis, minimal-change NS, or mesangial proliferative glomerulonephritis; and 5) other systemic diseases were excluded following clinical and laboratory examinations. The control group included SRNS children (N = 20) who responded to either prolonged steroid therapy or immunosuppressive agents. We also studied unrelated adult volunteers (N = 50) with normal urinalyses as controls.

NS was diagnosed based on urinary protein excretion  $>50 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$  with hypoalbuminemia  $<25 \text{ g/L}$ . Complete remission was defined as normal urinalysis or trace protein on dipstick analysis for 3 consecutive days and normalization of serum albumin levels ( $>35 \text{ g/L}$ ). Partial remission was defined as the resolution of edema, proteinuria from  $4\text{--}40 \text{ mg}\cdot\text{m}^{-2}\cdot\text{h}^{-1}$ , and normalization of serum albumin. Steroid resistance was defined as a failure of induction of complete remission after 4 weeks of standard therapy with prednisone ( $2 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ , given in 3 divided doses, maximum 60 mg/day). Patients with late steroid resistance were excluded from the study. ESRD was defined as a glomerular filtration rate below  $10 \text{ mL/min per } 1.73 \text{ m}^2$ , or the necessity of any renal replacement therapy.

Treatment with cyclophosphamide, cyclosporine, mycophenolate mophetil, or tacrolimus of most patients with no response to steroids was determined by disease activity. This study was approved by the Ethics Committee of Fuzhou Dongfang Hospital (China). Informed consent was obtained from patients or their parents.

### Mutational analysis

Mutational analysis was carried out using blood samples. Genomic DNA was extracted from peripheral blood leukocytes using an EZNASE Blood DNA Kit (OMEGA

Bio-Tek, Inc., Norcross, GA, USA) according to the manufacturer protocol. Mutational analysis was performed by direct sequencing of all 29 exons of *NPHS1*, all 8 exons of *NPHS2*, all 18 exons of *CD2AP*, and all 10 exons of *WT1*. Exons were amplified by polymerase chain reaction (PCR) using intron-flanking primers as described previously (Lenkkeri et al., 1999; Yu et al., 2005; Li et al., 2010; Meng et al., 2011), except for 3 sets of primers that amplified exons 6, 9, and 16 of *CD2AP*. Primers were designed using the Primer 3 software based on the human genomic sequence (NT\_007592.15 and NM\_012120.2). PCR primers for amplifying exons 6, 9, and 16 were as follows: exon 6, TGTC AAGCGTTTTGTGATTCT and CTGTGCTAACTAAAAACCCTAAAG; exon 9, TGGGAGACCAAATCAAGAGG and CAGTCGAGAAAGAAACAAAGGAA; exon 16, CTGTTACATTCTTGCCCTGTTTCA and TACCACACCCAGCCACAGATTAGAT. PCR products were visualized by 1.5% (w/v) agarose gel electrophoresis and sequenced using an ABI 3730XL DNA Analyzer (Applied Biosystems, Foster City, CA, USA). Mutations were confirmed by sequencing in both directions and by repeated amplification and sequencing. Genomic DNA from 50 healthy controls was used to confirm mutations and to exclude DNA polymorphisms.

## RESULTS

### Clinical data for patients with SRNS

The clinical findings of the 10 SRNS children who showed no response to immunosuppressive agents and 20 SRNS controls are summarized in Table 1. Of the 10 patients (6 males, 4 females) who experienced no sustained reduction in proteinuria, the average age of disease onset was 53.7 months (range: 8-147 months); renal biopsy, which was available for 3 patients, revealed FSGS, mesangial proliferative glomerulonephritis (MsPGN), and minimal change nephrotic syndrome (MCNS). Of the 20 SRNS controls (15 males, 5 females) who exhibited a response to prolonged steroid or immunosuppressive treatment and achieved complete remission, the average age of onset was 59 months (range: 15-149 months); renal biopsy, which was available for 9 patients, revealed MsPGN in 5 patients, FSGS in 2 patients, MCNS in 1 patient, and membranous nephropathy in 1 patient. One child, who did not show a sustained reduction in proteinuria following prolonged steroid treatment, progressed to ESRD within 6 months after disease onset. None of the 20 SRNS controls with complete response reached ESRD during the follow-up time of 8.2-82.7 months.

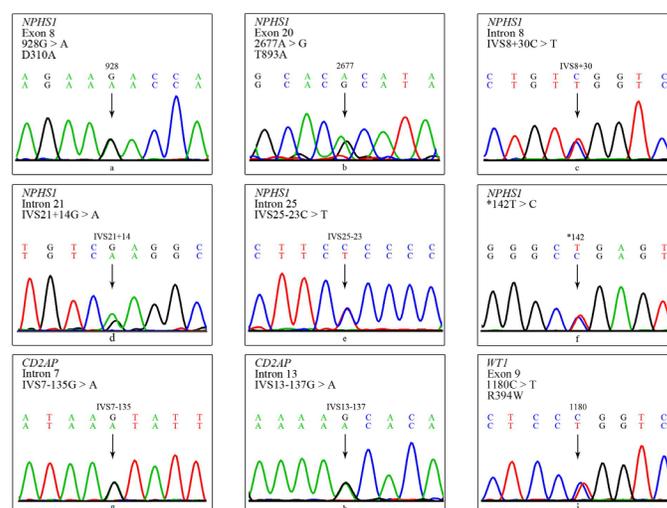
### DNA sequence analysis

Six *NPHS1* variants, 928G>A, 2677A>G, IVS8+30C>T, IVS21+14G>A, IVS25-23C>T, and \*142T>C, were detected in 5 patients (Figure 1 and Table 2). Two heterozygous *CD2AP* variants, IVS7-135G>A and IVS13-137G>A, were detected in 2 patients (Figure 1 and Table 2). One heterozygous *WT1* mutation, 1180C>T, was identified in 1 patient (Figure 1 and Table 2). None of the above variants were found in the 50 healthy controls. No mutation in the *NPHS2* gene was found in any patient.

**Table 1.** Clinical data of 30 patients with sporadic steroid-resistant nephrotic syndrome.

Patient	Gender	Karyotype	Age at disease onset (months)	Renal biopsy	Follow-up period (months)	Therapy and response	ESRD after disease onset (months)
1	F	46,XY	14	ND	6.0	GC (NR)	6
2	M	ND	96	MCNS	44.7	GC, MMF (NR)	N
3	M	ND	147	ND	8.4	GC, CTX, MMF (NR)	N
4	M	46,XY	19	ND	33.3	GC, CsA, MMF (NR)	N
5	M	46,XY	8	ND	7.9	GC, CTX (NR)	N
6	M	ND	90	MsPGN	11.0	GC, CsA (NR)	N
7	F	ND	58	FSGS	18.7	GC, CsA (NR)	N
8	F	ND	34	ND	12.2	GC, CsA, TAC (NR)	N
9	F	ND	28	ND	14.0	GC, CsA, TAC (NR)	N
10	M	ND	43	ND	10.4	GC, CsA (NR)	N
11	M	ND	63	ND	26.7	GC (R)	N
12	M	ND	31	ND	9.6	GC (R)	N
13	M	ND	24	ND	26.0	GC (R)	N
14	M	ND	44	MsPGN	10.3	GC (R)	N
15	M	ND	32	ND	74.7	GC (R)	N
16	M	ND	66	ND	10.1	GC (R)	N
17	F	ND	15	ND	27.0	GC (R)	N
18	F	ND	80	ND	8.2	GC (R)	N
19	M	ND	118	MsPGN	24.8	GC (R)	N
20	M	ND	42	FSGS	31.3	GC, CsA (R)	N
21	F	46,XX	99	MN	36.9	GC, CsA (R)	N
22	F	ND	138	MsPGN	38.6	GC, CsA (R)	N
23	M	ND	18	ND	26.1	GC, CsA (R)	N
24	M	ND	16	ND	28.6	GC, CsA (R)	N
25	M	ND	56	FSGS	55.2	GC, CsA (R)	N
26	M	ND	108	MCNS	42.5	GC, CsA (R)	N
27	M	ND	149	MsPGN	36.4	GC, CsA (R)	N
28	F	ND	35	MsPGN	82.7	GC (R)	N
29	M	ND	22	ND	22.3	GC, CsA (R)	N
30	M	ND	24	ND	14.4	GC, CsA (R)	N

ESRD = end-stage renal disease; ND = not determined; MCNS = minimal-change nephrotic syndrome; MsPGN = mesangial proliferative glomerulonephritis; FSGS = focal segmental glomerulosclerosis; MN = membranous nephropathy; GC = glucocorticosteroid; NR = not remission; MMF = mycophenolate mofetil; CTX = cyclophosphamide; CsA = cyclosporine; TAC = tacrolimus; R = complete remission; N = not progressed to ESRD.



**Figure 1.** Mutations in podocyte genes *NPHS1*, *CD2AP*, and *WT1* were identified by sequencing in 8 patients. (a.-f.) Chromatograms show 6 *NPHS1* mutations: 928G>A, 2677A>G, IVS8+30C>T, IVS21+14G>A, IVS25-23C>T, and \*142T>C, respectively. (g, h.) Chromatograms revealed 2 *CD2AP* mutations: IVS7-135G>A and IVS13-137G>A. (i.) Chromatogram revealed 1 *WT1* mutation: 1180C>T. Arrows indicate mutant positions.

**Table 2.** Mutations detected in 8 patients with sporadic steroid-resistant nephrotic syndrome.

Patient	<i>NPHSI</i>			<i>CD2AP</i>			<i>WT1</i>		
	Nucleotide substitution	Effect on protein	Mutation status	Nucleotide substitution	Effect on protein	Mutation status	Nucleotide substitution	Effect on protein	Mutation status
1							1180C>T	R394W	het
2				IVS13-137G>A		het			
7	2677A>G *142T>C	T893A	het het						
12	IVS21+14G>A		het						
13	928G>A	D310N	het						
16	IVS8+30C>T		het						
21	IVS25-23C>T		het						
25				IVS7-135G>A		het			

\*The first nucleotide of the ATG translation codon is considered nt +1. Patient numbers correspond to the numbers in Table 1. het = heterozygous mutation.

## DISCUSSION

Mutational analysis of 4 podocyte genes (*NPHSI*, *NPHS2*, *CD2AP*, and *WT1*) was performed on 10 Chinese SRNS children who showed no response to immunosuppressive agents, using PCR and direct sequencing. Pathogenic mutations were identified in 2 of the 10 patients (20%) who experienced no sustained reduction in proteinuria following prolonged steroid or immunosuppressive agent treatment. No mutations were detected in the 20 SRNS controls who achieved complete remission following prolonged steroid or immunosuppressive agent treatment. The data suggest that mutational analysis of podocyte genes should be performed on SRNS patients who show no response to either prolonged steroid or immunosuppressive agent treatment.

As shown in Table 2, compound heterozygous variants of *NPHSI*, 2677A>G and \*142T>C, were identified in patient 7, while the heterozygous variant of *WT1*, 1180C>T, was identified in patient 1. Variants 2677A>G and \*142T>C were not found in the 50 controls, indicating that these are *NPHSI* mutations. Several lines of evidence suggest that the *NPHSI* mutation 2677A>G is pathogenic. First, the *NPHSI* mutation of 2677A>G leads to a threonine to alanine substitution at codon 893 of nephrin (T893A). Second, sequence conservation of the 2677 nucleotide in human *NPHSI* mRNA, counted from the beginning of the start codon, has been established by alignment analysis of *Homo sapiens* (GenBank NM\_004646.3), *Pan troglodytes* (GenBank XM\_524228.4), *Macaca mulatta* (GenBank XM\_001112314.1), *Bos taurus* (GenBank NM\_001192512.1), *Canis lupus familiaris* (GenBank XM\_541685.3), *Rattus norvegicus* (GenBank NM\_022628.1), *Mus musculus* (GenBank NM\_019459.2), and *Xenopus laevis* (GenBank NM\_001095706.1) *NPHSI* mRNA. Third, alignment analysis of nephrin in humans (GenBank NP\_004637.1), *P. troglodytes* (GenBank XP\_524228.3), *M. mulatta* (GenBank XP\_001112314.1), *B. taurus* (GenBank NP\_001179441.1), *C. lupus familiaris* (GenBank XP\_541685.2), and *X. laevis* (GenBank NP\_001089175.1) has also shown that the 893rd amino acid of human nephrin is threonine, while the corresponding amino acids in rats (GenBank NP\_072150.1) and mice (GenBank NP\_062332.2) are isoleucine and lysine, respectively. All 3 amino acids with polar side chains are hydrophilic. Finally, nephrin is a major component of the zipper-like isoporous filter structure for assembly into the slit diaphragm and is crucial for the selective filtration function of the kidneys (Ruotsalainen et al., 1999). Amino acid residues located in Ig-like modules of nephrin are critical for the function of the

modules (Lenkkeri et al., 1999). The missense mutant T893A affects the conservative residues of the 8 nephrin Ig domains. Whether the other heterozygous *NPHS1* mutation, \*142T>C, in the 3'-untranslated region actually causes disease remains unknown. Patient 7 was clinically diagnosed with NS at the age of 58 months; she failed to respond to either steroid or cyclosporin A treatment, and her renal pathology showed changes of FSGS. These clinical features were similar to those of patients with homozygous mutations or compound heterozygous mutations, or a heterozygous pathogenic mutation, in their *NPHS1* genes, as reported previously (Heeringa et al., 2008; Philippe et al., 2008; Caridi et al., 2009; Santín et al., 2009). It is likely that the compound heterozygous state of the *NPHS1* mutations caused development of SRNS in patient 7.

The *WT1* variant, 1180C>T, in patient 1 was absent in the 50 normal controls, indicating that it is a *WT1* mutation. Having the 1180C>T variant results in the substitution of arginine with tryptophan at codon 394 of WT1 (R394W). Karyotype analysis of patient 1 showed 46 chromosomes; she was an XY phenotypic girl, and ultrasound examination showed no renal tumors. Patient 1 was diagnosed at the age of 14 months with DDS and failed to respond to steroids. She reached ESRD at the age of 20 months. Previous studies have reported that the *WT1* mutation R394W can cause DDS, FS, or isolated SRNS (Call et al., 1990; Gigante et al., 2009); therefore, we considered this mutation to be responsible for the phenotype observed in patient 1.

A heterozygous mutation of *CD2AP* of IVS13-137G>A with no identified pathogenicity was detected in patient 2, who carried no mutations in *NPHS1*, *NPHS2*, or *WT1*. Patient 2 presented NS at 96 months of age and failed to respond to steroids. His renal biopsy showed MCNS. He received treatment with mycophenolate mophetil, but did not reach remission of proteinuria. Whether the heterozygous mutation of *CD2AP* is a rare *CD2AP* polymorphism or is a pathogenic *CD2AP* mutation could not be determined because of the unavailability of a functional assay.

In our study, we found pathogenic mutations in the podocyte genes *NPHS1*, *NPHS2*, *CD2AP*, and *WT1* in 20% of the 10 SRNS patients who did not respond to either steroids or immunosuppressive agents. Our results are consistent with those of Löwik et al. (2008) who identified mutations in the podocyte genes *NPHS1*, *NPHS2*, *CD2AP*, and *WT1* in 4 (26.7%) of 15 SRNS patients who showed no response to prolonged steroid or immunosuppressive treatment. In addition, Santín et al. (2011) detected mutations in podocyte genes in *NPHS1*, *NPHS2*, *CD2AP*, and *WT1* in 7 (22.2%) of 36 SRNS patients who showed no response to prolonged steroid or immunosuppressive treatment.

In the 20 SRNS controls with remission following prolonged steroid or immunosuppressive treatment, each patient (Table 2; patients 12, 13, 16, and 21) carried only 1 heterozygous mutation in *NPHS1*. One patient (Table 2, patient 25) carried only 1 heterozygous mutation in *CD2AP*. However, from a genetic perspective, *NPHS1* mutations are responsible for the autosomal recessive form of SRNS and should produce an evident phenotype in either homozygosity or compound heterozygosity, and an *NPHS1* heterozygous mutation cannot explain the pathogenesis. In contrast to the SRNS patients with homozygous or compound heterozygous mutations, all 4 patients with heterozygous mutations in *NPHS1* responded to prolonged steroid or immunosuppressive treatment and went into remission. Patient 25 was found to carry 1 heterozygous mutation in the *CD2AP* gene, IVS7-135G>A, which showed no effect upon altering a splice site using Splice Site Prediction by Neural Network ([http://www.fruitfly.org/seq\\_tools/splice.html](http://www.fruitfly.org/seq_tools/splice.html)), suggesting that IVS7-135G>A is not a causative mutation.

Patient 25 was diagnosed at 56 months of age with NS and failed to respond to steroids, but went into remission after receiving cyclosporin A. This cannot be explained based on inherited structural defects of the glomerular filtration barrier.

In summary, we identified mutations in the podocyte genes in 2 of 10 patients who were resistant to both steroids and immunosuppressive agents. Our investigation supports the necessity for genetic examination of mutations in podocyte genes in Chinese SRNS children who do not respond to either steroid or immunosuppressive agent treatment.

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