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WT1 Gene Mutations in Chinese Children With Early Onset Nephrotic Syndrome

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ABSTRACT: In Chinese children with steroid-resistant nephrotic syndrome (SRNS), it was reported that *NPHS2* mutation was detected in 4.3%, which was lower than that in Caucasians (10–30%). However, there were no data on *WT1* mutation in nephrotic syndrome (NS), especially in early-onset NS of Chinese children. Thus, a study, which enrolled 36 Chinese children with early-onset (before 3 y old) NS and steroid resistance if failing steroid therapy (early-group), was conducted. As control, 35 children with SRNS and with disease onset age after 3 y old were also analyzed (control-group). *WT1* gene was examined by PCR and direct sequencing. The result showed that in the early-group 6/36 (16.7%) were detected with *WT1* mutations. Further analysis according to different onset age revealed that the mutation detection rates of *WT1* were 26.3% (5/19), 6.3% (1/16), and 0 (0/1) in children younger than 1 y, 1–2 y, and 2–3 y, respectively. In control-group, no *WT1* (0/35) mutation was detected. *WT1* mutation combined with *NPHS2* variant was detected in a girl. In conclusion, *WT1* mutations seemed more common in Chinese children with early-onset NS. **(*Pediatr Res* 68: 155–158, 2010)**

Nephrotic syndrome (NS) is often a life-threatening condition when manifesting as early-onset, especially in the first year of life. In recent years, several causative genes related to NS have been identified by applying molecular genetic approaches, including *NPHS1*, *NPHS2*, *WT1*, *LAMB2*, *PLCE1*, *ACTN4*, *TRPC6*, and *CD2AP* (1–8). These genes have been analyzed in a large cohort of patients with NS worldwide, especially *NPHS1*, *NPHS2,* and *WT1*. The results suggested that, in these genes, *NPHS1* mutations were mainly detected in children with congenital NS (CNS), and *WT1* and *NPHS2* might be more common in children with primary steroid-resistant nephrotic syndrome (SRNS) (9–11). As to *WT1* gene, previous studies by other groups revealed that the incidence of *WT1* mutations in isolated SRNS children (6.8 y in average age) was 6–7% (9,10), and the two hot spots were exons 8 and 9 in *WT1* gene. Nevertheless, there were few reports on the incidence of *WT1* mutations in children with early-onset NS, especially in the first year of life. In China, there are numerous children with early-onset NS for whom neither the incidence nor clinical characteristics of *WT1* gene mutations are clear. The identification of gene mutations associated with early-onset NS will definitely help Chinese childhood patients to obtain the correct diagnosis, to prevent excessive drug therapy, and to obtain the suitable genetic consultation. Therefore, we detected *WT1* mutations in a large cohort of children with early-onset NS and analyzed the phenotypic characterization in children with *WT1* mutations.

METHODS

Patients. A total of 36 unrelated children of Chinese ethnicity with early-onset NS (within 3 y, early-group) was enrolled in this study. There were 30/36 children in the early-group who received steroid therapy (1.5–2 mg/kg daily for 8 wk) and presented with steroid resistance. The other 6/36 cases had not received steroid therapy for the early onset age younger than 4 mo (including five cases with CNS). All children of early-onset NS were further divided into three groups according to the onset ages, younger than 1 y, 1–2 y, and 2–3 y, respectively. Some of the infectious diseases resulting in CNS were excluded through screening blood serum antibodies of toxoplasma, rubella virus, cytomegalovirus, herpes simplex virus, and syphilis. Another 35 SRNS children with onset age more than 3 y were enrolled as a control-group in this study. All the children both in the early-group and the control-group were diagnosed between 2003 and 2008. The study was approved by the Ethics committee of Peking University First Hospital, Beijing, China (2006023). All the parents provided informed consent.

WT1 mutation analysis. Genomic DNA was extracted from peripheral blood cells from probands and their parents by standard methods (12). Direct sequencing of exons 8 and 9 in *WT1* gene was performed. For those children suspected of having hereditary NS, including 5 with CNS and a boy with NS combined with cryptorchidism, other exons of *WT1* gene were also analyzed. Using Primer 3 software, the primers of *WT1* exons 1–10 were designed as done in our previous studies (Table 1) (12). The PCR for *WT1* exons 2–10 were performed in 25 μL consisting of 12.5 μL 2× *Taq* plus Master Mix (Tiangen Biotech, Beijing Co., Ltd.), 1 μL sense primer (5 μM), 1 μL anti-sense primer (5 μM), and 1 μL DNA (50 ng/L). Takara La TaqE (5 MU/L) and 2× GC Buffer II were used in the PCR reaction for the GC-rich *WT1* exon 1. The amplification was performed using Touchdown PCR with an annealing temperature from 64 to 58°C, descending 1°C every two cycles, and subsequent annealing at 58°C for 26 cycles. The PCR products were visualized by 2% agarose gel electrophoresis and sequenced using the ABI 3730XL DNA Analyzer (SinoGenoMax Company Ltd). *WT1* gene in probands' parents was also analyzed if mutations of *WT1* were detected in the proband. All mutations were confirmed by two independent PCR reactions as well as forward and reverse sequencings. As normal control, *WT1* was analyzed in 50 unrelated adult volunteers whose urinalysis was normal.

Karyotype analysis or Y chromosome identification. To confirm the sex of patients, karyotype analysis was performed on four patients with *WT1* mutations. For the two patients, for which karyotype analysis was not performed, SRY gene (specific marker on Y chromosome) was identified by amplifying *SRY* gene with PCR technique and confirmed by electrophoresis (13).

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Abbreviations: CNS, congenital nephrotic syndrome; **NS,** nephrotic syndrome; **SRNS,** steroid-resistant nephrotic syndrome

RESULTS

Clinical data. In the 36 children of the early-group, the ratio of male to female was 21:15. There were 19 children with onset age in the first year of life, of which five children with early onset age, <3 mo after birth (in accordance with the definition of CNS). There were 16 children with onset age between 1 and 2 y and one child with onset age between 2 and 3 y. In the 35 children of the control-group, the ratio of male to female was 23:12 (Table 2). There was no family history of renal diseases in 71 children of both the early and control-groups.

In 36 children of the early-group, renal biopsy, which was available for 15 childhood patients, revealed focal segmental glomerulosclerosis in 10 patients, minimal change lesions in three patients, and diffuse mesangial sclerosis in two patients. In the control-group, renal biopsy, which was available for 15 children, revealed focal segmental glomerulosclerosis in 12 patients and minimal change lesions in three patients (Table 2).

WT1 mutations in children of early-group and control-group. Of 36 children in the early-group, *WT1* mutations (Table 3) were detected in six children (16.7%). There were 19 of 36 children with onset age in the first year of life. Among the 19 children (10 girls), *WT1* mutations were detected in five children (patients 1–5), of whom four were girls. In this subage group, the mutation detection rate was 26.3% (5/19), and the mutation detection rate in phenotypic girls was 40% (4/10). There were 16 of 36 children with onset age between 1 and 2 y old, and only one child (patient 6, 1/16, 6.3%) had a *WT1* mutation detected in this subage group. Of 36 children, there was only 1 child with onset age between 2 and 3 y old, and no *WT1* mutation was detected in this subage group. In control-group, no *WT1* mutation was detected in all the 35 children. In addition, no *WT1* mutation was detected in all the parents of the children in both the groups.

A *WT1* (IVS9 + 4 C>T) mutation along with a heterozygous *NPHS2* variant 860A>G het leading to Q287R was detected in an 8-mo-old girl (patient 2). Although the mutation analysis only detected *NPHS2* variant in the girl's mother, she did not present proteinuria. In our previous study, *NPHS2* gene was detected and analyzed in all children of the early and control groups, which showed an *NPHS2* compound heterozygous mutation only in one child who had been excluding from this study group. There was no *NPHS1* mutation detected in all five children with onset age of 3 mo after birth in the early-group (data not shown).

Karyotype analysis or Y chromosome identification. Karyotype analysis or Y chromosome identification were performed on the six children with *WT1* mutations, which showed 46, XY karyotype or Y chromosome in three of five phenotypic girls. Another two of five girls were revealed 46, XX karyotype or no Y chromosome. There was only one male (patient 5) on phenotype and genotype among the 6 children with *WT1* mutations who had urinary-genital malformations. In addition, one of six children had dysembryoma (patient 6, Table 3).

Table 1. *Sequences of primers used for PCRs*

Gene	Primer sequences (5′→3′)	PCR products (bp)
Exon 1	F: CAGCGCTGAACGTCTCCA	573
	R: GGGTGTCCTAGAGCGGAGAG	
Exon 2	F: CCCGTGGCTGGTTCAGAC	339
	R: TGCCATTGGGGTAATGATTT	
Exon 3	F: GCTCAGGATCTCGTGTCTCC	328
	R: GTCTCGTGCCTCCAAGACC	
Exon 4	F: ATGTGGAGGCTTGCACTTTC	343
	R: ACCAACTAGGGGAAGGAGGA	
Exon 5	F: CAGTGGGACTGGGGACTTAG	328
	R: GAGATTCTTCCCATCCACCA	
Exon 6	F: CCATCATTCCCTCCTGATTG	329
	R: GAGCAGGTGTCCCTGATGTT	
Exon 7	F: CAGTGCTCACTCTCCCTCAA	314
	R: CCTGGGTCCTTAGCAGTGTG	
Exon 8	F: TCCAGCGAAGTGCCTTAGGC	407
	R: GGGGAAATGTGGGGTGTTTCC	
Exon 9	F: TGCAGACATTGCAGGCATGGCAGG	349
	R: GCACTATTCCTTCTCTCAACTGAG	
Exon 10	F: AATTCAGAGTGGGTGCCTTG	309
	R: TGAGGAGGAGTGGAGAGTCAG	

F, forward; R, reverse.

DISCUSSION

Early-onset NS represents a clinically refractory condition, in whom most are resistant to steroid and immunosuppressive therapy and tend to progress to end-stage renal disease. Several causative genes related to proteinuria have been identified and analyzed in the patients with early-onset NS or primary SRNS in many countries. The results suggested that, in these genes, *NPHS1* mutations were mainly detected in CNS; *WT1* and *NPHS2* might be more common in childhood SRNS (9–11). In European countries, the incidence of *NPHS2* mutations in primary SRNS was 10–30% (11). Nevertheless, in Asian countries such as Korea, Japan, and China, the incidence of *NPHS2* mutations in sporadic SRNS was low (14,15), which suggested that the relative frequency of these genetic defects might be influenced by patient ethnic origin.

Previous studies have confirmed that *WT1* mutation was a well-known cause of Denys-Drash syndrome and Frasier syn-

Table 2. *Distribution of age, renal pathology, and mutation in early-group and control-groups*

Groups	Age of onset (mo)	Number of patients	Sex		Renal biopsy FSGS/MCL/DMS	Number of patients with *WT1* mutations	Number of patients with ESRD
			M	F			
Early-group	<3	5	2	3	1/0/1	1	1
	4–12	14	7	7	3/1/0	4	2
	13–24	16	11	5	5/2/1	1	2
	25–36	1	1	0	1/0/0	0	0
Control-group	>36	35	23	12	12/3/0	0	8

F, female; M, male; FSGS, focal segmental glomerulosclerosis; MCL, minimal change lesion; DMS, diffuse mesangial scleroses; ESRD, end-stage renal disease.

Table 3. *Clinical data of patients with WT1 mutations*

Cases	Phenotype/ karyotype	Onset age (mo)	Diagnosis age (y)	Edema	Proteinuria (mg/kg.24 h)	Age at CRF	Renal pathology	Therapy	*WT1* mutation	Genital status
P1	F/46, XX	3	2.0	Eyelid	4 + /198.0	No (3 y)	FSGS	SR CsA/NR	IVS9 + 5 G>A	Female, normal
P2	F/46, XY	8	1.5	Eyelid	3 + /NA	Yes (8 mo)	NA	PD	IVS9 + 4 C >T	Female, streak gonads
P3	F/46, XY	12	8.0	Eyelid	3 + /221.4	No (11 y)	FSGS	SR CsA/NR	IVS9 + 5 G>A	Female, streak gonads
P4	F/46, XX	12	1.3	No	3 + /99.0	No (1.3 y)	NA	SR CTX/NR	IVS9 + 5 G>A	Female, normal
P5	M/46, XY	6	0.6	Eyelid	4 + /NA	No (1 y)	NA	SR	1186G>A het, D396N	Hypospadias cryptorchidism
P6	F/46, XY	24	4.8	No	4 + /90.5	No (4.8 y)	FSGS	SR MMF/ NR CTX/NR	IVS9 + 5 G>A	Dysembryoma

F, female; M, male; FSGS, focal segmental glomerulosclerosis; SR, steroid resistant; NA, not available; CRF, chronic renal failure; NR, no responsive; PD, peritoneal dialysis; CTX, cyclophosphamide; CsA, cyclosporine; MMF, mycophenolate mofetil; het, heterozygous.

drome, which are rare diseases. In recent years, studies indicated that *de novo* mutations in exons 8 and 9 of the *WT1* gene might be the causative gene for sporadic SRNS. Some studies reported that the incidence of *WT1* mutations in isolated SRNS children younger than 18 y was 6–7% (9,10). Nevertheless, *WT1* mutation in children with early-onset NS was only reported from two study groups. Hinkes *et al.* (16) reported that the prevalence of *WT1* mutations in NS manifesting in the first year of life was 3.8%, and Sako *et al.* (17) reported that no *WT1* mutation was detected in Japanese children with CNS. In China, the incidence and the phenotypic features of children with *WT1* mutations, especially in children with early-onset NS are unknown. In this study, the prevalence of *WT1* mutations in children of early-group was 16.7% (6 of 36 children). Furthermore, in subage groups, the mutation detection rates of *WT1* were 26.3% (5/19), 6.3% (1/16), and 0 (0/1) in children of younger than 1 y, 1–2 y, and 2–3 y, respectively. The *WT1* mutation seemed predominant in girls, onset age younger than 1-y-old (40.0% detection rate in this study). However, no *WT1* mutation was detected in children of the control-group, and the result suggested that, in Chinese children younger than 1 y, the rate of *WT1* mutations was obviously higher than that reported in European countries (3.8%) (16). In CNS, 1/5 (20.0%) children were found to have the *WT1* mutation in this study, whereas no *WT1* mutation was detected in 13 Japanese children (17). Therefore, we proposed to carry out the detection of *WT1* mutation in Chinese children with early-onset NS, especially in female phenotypic children with onset age in the first year of life.

Patients with *WT1* mutations exhibited an inconsistency between the extent of edema and the levels of proteinuria. In six children with *WT1* mutations and predominantly massive proteinuria, four of them presented with intermittent eyelid edema and two of them developed proteinuria without edema. Similar presentation features were also described in previous studies reported by others (9,10,14). On the contrary, patients with *NPHS1* or *NPHS2* mutations often presented with severe anasarca and massive proteinuria.

The *WT1* gene encodes a nuclear zinc finger domain, which is significant to normal kidney and gonadal system development, especially in male patients (18). In a number of studies, including our investigation, children of the 46, XY karyotype with *WT1* mutations developed NS with genitourinary malformation, including abnormal extra-genitourinary manifestations and pseudohermaphroditism. Children of the 46, XX karyotype with *WT1* mutations often developed NS without genitourinary anomalies. These results suggest that male phenotypic children with *WT1* mutations tend to have genitourinary malformations. In other words, for children with the normal 46, XY phenotypic male, the probability of *WT1* mutations was rare and *WT1* mutational analysis is not regularly recommended. Compared with children without the *WT1* mutation, there was no significant difference on the renal pathology.

In this study, *WT1* mutation combined with *NPHS2* variant was detected in a girl. The child presented with some characters of Frasier syndrome, such as male pseudohermaphroditism and the *WT1* IVS 9+4 C>T mutation. However, renal failure occurred at 8 mo of age, which was earlier than other children of the Frasier syndrome reported previously by other authors. The girl also had an *NPHS2* variant (860A>G het leading to Q287R) in the conserved region of podocin, which had not been detected in 50 unrelated adult volunteers of Chinese ancestry and has not been reported by other studies. Thus, we speculate that the deterioration of renal function might be accelerated by two mutations of *NPHS2* and *WT1* genes. Di-genic heterozygosity has been reported in some patients, including *NPHS2* combined with *NPHS1* or *CD2AP* mutations, and a mutation in *NPHS1* combined with a *WT1* mutation (19,20). Our study, for the first time, demonstrated the di-genic heterozygosity of *NPHS2* mutation combined with a *WT1* mutation might exist in Chinese patients with NS. Nevertheless, the clinical implication of the di-genic heterozygosity as well as the interaction of the two genes and the role of the combined genetic defects in the onset and severity of proteinuria is still unclear. Therefore, further study is needed to clarify the pathogenic role of the di-genic heterozygosity in NS.

In conclusion, our study demonstrates that *WT1* mutations seemed more common in Chinese children with early-onset NS and more *WT1* mutations were detected in Chinese female NS children with early-onset age. The child with *WT1* mutation and single heterozygous *NPHS2* variant is the first report in the literature, which showed earlier renal failure. The pathogenesis of *WT1* mutation combined with *NPHS2* mutation still needs to be investigated.

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