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ORIGINAL ARTICLE

Report of novel genetic variation in *NPHS2* gene associated with idiopathic nephrotic syndrome in South Indian children

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Abstract
Background Steroid-resistant nephrotic syndrome (SRNS) is found in 10–20 % of children with idiopathic nephrotic syndrome (INS). In SRNS patients, common histopathological subtypes are Focal segmental glomerulosclerosis (FSGS) (53 %) and minimal change disease (MCD) (27 %). Familial forms of FSGS constitute podocyte diseases with varying severity and age of onset. Podocin gene (*NPHS2*) mutations cause childhood-onset steroid-resistant FSGS and MCD to adult-onset FSGS. In view of genetic variations and susceptibility to the disease, the present investigation was undertaken to study the pattern of genetic mutation in children from South India.
Methods Mutation analysis was carried out by direct sequencing of the entire *NPHS2* gene (eight exons) using specific primers in 200 INS (100 SRNS and 100 steroid sensitive) children and 100 healthy controls. The allele and genotype frequencies of *NPHS2* gene were calculated for both cases and controls as per Hardy–Weinberg equilibrium.
Results Among the SRNS patients, 18 % revealed both heterozygous and homozygous mutations. Out of 12 mutations, 8 were homozygous and 4 were heterozygous. Interestingly, we found two novel SNPs in exon 4 of *NPHS2* gene, which are documented and submitted to dbsnp database (Ref rs12401711 and rs12401708).
Conclusion Mutational analysis of *NPHS2* would be advisable at the start of treatment. The genetic variations detected in the study would serve as the important molecular marker in treating the children's at early stage, which also enables to detect carriers, prenatal diagnosis and provide genetic counseling to couples at risk.

Keywords Steroid-resistant nephrotic syndrome · *NPHS2* mutations · Polymorphism

Introduction

Idiopathic Nephrotic syndrome (INS) is a glomerular disorder occurring mainly in children [1, 2]. The annual incidence of INS is projected around 2–7/100,000 children and a prevalence rate of 16/100,000 children below 16 years of age [3]. Based on the response to steroid therapy, INS is classified into steroid-sensitive NS (SSNS) and steroid-resistant NS (SRNS) [4]. In most patients, the severity of the disease was controlled after steroid treatment. Nevertheless, 10 % of children with INS did not respond to either steroids or to any other immunosuppressive therapy, and progressed to end stage renal disease (ESRD) [5]. In SRNS, 75 % of patients revealed renal histologic features of FSGS, while 25 % of the cases exhibited MCNS.

FSGS and MCNS diseases are due to aberrations of genes that govern podocyte structure and function [6]. One

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of the causative genes for this disease, *NPHS2*, encodes a novel protein named podocin, which is expressed only in the podocytes at the cytoplasmic part of the slit diaphragm in both fetal and mature kidney [7]. Mutations in the *NPHS2* gene are a main cause of autosomal-recessive SRNS in childhood with approximately 6.4–30 % of sporadic and 20–40 % of familial cases [8–10]. Previous studies have shown that SRNS patients with an *NPHS2* mutation had more severe clinical manifestations compared to SRNS patients without the *NPHS2* mutation [10–12].

Podocin performs a mechanotransduction function by interacting with the cytoskeleton and slit-diaphragm nephrin to stabilize the permeability unit of podocytes [8, 13]. There are different *NPHS2* mutations (missense, nonsense and frameshift mutations), which play a key role in the glomerular filtration barrier function. Huber et al. [12] demonstrated that mutations in the *NPHS2* cause disruption of nephrin targeting to lipid raft microdomains. The prevalence of *NPHS2* mutations in Indian children with SRNS is limited. The aim of the study was to evaluate the frequency of *NPHS2* gene mutations in correlation with the pathogenesis of INS in South Indian children.

Materials and methods

Subjects and blood samples

The study group consisted of 200 children with INS and 100 healthy controls without a familial history of renal disease were included in the study. The mean age of onset for INS is 5.39 and for controls 5.86. All cases fulfilled the criteria of the International Standard of Kidney Disease in Children (1981) for the diagnosis of the NS. Of the 200 children included, 100 children were steroid resistant and biopsy confirmed FSGS and rest were SSNS. All these children had massive proteinuria >40 mg/m^2/day or >50 mg/kg body weight/day or a random sample of urinary protein-to-creatinine ratio exceeding 2 mg/mg which effectively resulted in severe hypoalbuminemia of serum albumin less than 2.5 g/dL. On ethical approval, 3 ml of blood samples was collected from the study subjects with the written informed consent from the parents.

All these children had initially a course of prednisolone when found resistant had a course of either IV cyclophosphamide or oral cyclophosphamide, followed either oral cyclosporine or oral tacrolimus and then mycophenalate mofetil along with steroids. None of the children had progressed into chronic kidney disease stage 2 or more during the study period. The study was approved by the Institutional Medical Ethics Committee (Ref No: IEC-N1/10/June/17/15). Written informed consent was obtained from the parents of both cases and healthy controls before collecting 3 ml of peripheral blood from the study subjects.

Mutation and genotyping analysis

Genomic DNA from blood sample was isolated and purified using the QIAamp blood kit (QIAGEN GmbH, Germany). All eight exons of the *NPHS2* gene were amplified using exon-flanking primers [8] and the primers are listed in Table 1. The PCR reaction mixture was made up in ddH2O water containing 100 ng genomic DNA, 0.2 μM of each primer, and PCR Taq master mix (Amplicon). The cycling conditions had a range of annealing temperatures from 50 to 60 °C (50 °C-exon 6, 55 °C-exons 2, 3, 4 and 5 and 60 °C exons 1, 7 and 8) [8]. Yield and purity were determined by electrophoresis on 0.8 % agarose gel. PCR was carried out. The amplified PCR products were separated on 2 % agarose gel electrophoresis and visualized under UV transilluminator after staining with ethidium bromide. The amplified products were subjected to mutation analysis using Big Dye Terminator V.1.1. (Applied Biosystems, Foster City, California) by direct sequencing of all exons in ABI Prism^{TM} 3730 Genetic Analyzer (Applied Biosystems, Carlsbad, CA). Sequences were evaluated with Seq scape analysis software V2.5.

Statistical analysis

Mutational analysis was carried out using Seqscape analysis software. The allele frequency and distribution of genotypes for the SNPs in cases and controls were calculated, and their relationship with drug resistance was statistically evaluated using SPSS Statistical Software (Version 16.0). Hardy–Weinberg equilibrium performed for both cases and controls. The odds ratio at 95 % confidence interval (CI) facilitated the comparison of genotype frequency distribution between the controls and cases. A p value of less than 0.05 was accepted as significant. Pair wise linkage disequilibrium was calculated for the SNPS and the D′ and r′ statistic measures showed a strong association coefficient of 0.9995 for both SRNS and SSNS against control group.

Results

Patient characteristics

NPHS2 gene mutation screening was performed in 200 INS patients and 100 healthy children without a familial history of renal disease as healthy controls; among the patients 100 patients were SRNS and 100 patients were SSNS. Age of onset between 1 and 16 years with the mean age of

Table 1 Primer sequence of *NPHS2* gene

Exon (*n*)	Forward primer	Reverse primer
1	GCAGCGSCTCCACAGGGACT	TCAGTGGGTCTCGTGGGGAT
2	AGGCAGTGAATACAGTGAAG	GGCCTCAGGAAATTACCTA
3	TTCTGGGAGTGATTTGAAAG	TGAAGAAATTGGCAAGTCAG
4	AAGGTGAAACCCAAACAGC	CGGTAGGTAGACCATGGAAA
5	CATAGGAAAGGAGCCCAAGA	TTCAGGCATATTGGCCATTA
6	CTCCCACTGACATCTGA	AATTTAAAATGAAACCAGAA
7	CTAAATCAATGGCTGCACCACC	TTCCTAAAGGGCAGTCTGG
8	GGTGAAGCCTTCAGGGAATG	TTCTATGGCAGGCCCCTTTA

5.39 ± 14.8 years for INS children's and the mean age for healthy controls is 5.86 ± 15.3 years. There was a predominance of 62 % male children and 38 % females. Among 100 children with SRNS a similar gender distribution was seen. All the 100 children with SRNS were biopsy proved FSGS. Among 100 children 76 % were late steroid resistant while 24 % were initial steroid resistant.

NPHS2 gene mutation analysis

Pathogenic mutations were defined as variations which are reported to be present in less than 1 % population. The results obtained showed that there was no mutation in the *NPHS2* in SSNS patients and healthy controls. In contrast, 12 mutations were observed of which 8 were homozygous 4 were heterozygous which accounts for 18 % among SRNS group of patients. Of the 8 homozygous mutations, three at exon 4 [nt21237 (T>C) in 1 %, nt21240 (G>A) in 3 % and nt21260 (C>T) in 1 %], two at exon 5 [nt23771 (C>T) in 1 % and nt23841 (A>T) in 1 %], one at exon 8 [nt29680 (C>T) in 1 %] and two at exon 1 [nt5221 (T>C) in 1 % and nt5250 (G>A) in 1 %], respectively. The heterozygous mutations was observed one in exon 4 [nt21253 (G>A) in 2 %], one in exon 5 [nt23795 (C>A) in 2 %], one in exon 8 [nt29515 (C>T) in 3 %] and one in intronic region [nt21306 (A>G) in 1 %]. Seven mutations reported to be novel at exon 1, exon 4, exon 5, and exon 8 and in intronic region sequences of which few sequences submitted in Genbank (Ref KR349908-KR349916) are tabulated in Table 2.

Genotype and allele frequency of *NPHS2* Exon 4, polymorphism between cases and healthy controls

In exon 4 analysis, 2 novel SNPs has been observed and reported to dbsnp database (Ref rs12401711 and rs12401708). The genotype frequency of SNPs among the cases and the controls were tabulated in Table 3. Among the controls, 66 % had GG genotype, 32 % had GA genotype, 2 % had AA genotype. In cases the SRNS group showed 71 % had GG genotype, 25 % had GA genotype and 4 % had AA genotype and in SSNS group, 78 % had GG genotype, 20 % had GA genotype and 2 % had AA genotype at nucleotide position nt21143 and nt21168.

The distribution of genotype for the SNPs in both the patient groups did not show any significant differences as compared to control group ($p > 0.05$). Distribution of genotypes studied follows Hardy–Weinberg equilibrium tabulated in (Table 4). The pair wise linkage disequilibrium was calculated for the SNPS and the D′ and r′ statistic measures showed a coefficient of 0.9995 for both SRNS and SSNS against control group. Hence, both the SNPs showed strong linkage between the two groups (control and SSNS, control and SRNS).

Discussion

Podocin, an integral structural protein of the podocyte is encoded by the *NPHS2* gene. A truncated protein will result in a glomerular barrier dysfunction, which leads to nephrotic syndrome. Therefore, any change in *NPHS2* gene as well as the resulting defect in the protein podocin, are considered to be the disease-causing mechanism among patients with nephron dysfunction [6]. These defects remain persistent even after immune suppressive therapy, as indicated by the non-responsiveness of these patients to steroid therapy [14]. The present study confirms this notion as the mutations observed in the exon were confined only to SRNS.

In the present study, out of 12 mutations observed, 8 were homozygous and 4 were heterozygous leads with conservative changes in the amino acid. Whereas, there was no amino acid change in two mutations (one homozygous and one heterozygous). Of the 12 mutations identified, five were reported (rs id:530318579; rs id:12568913; rs ID: 199506378; rs ID: 199837664; rs ID: 113058664), while the others, i.e. L167P at nt21237(T>C), nt21260 (C>T) P175S (novel) and nt21253 (G>A) in exon 4, nt5250 (G>A) and nt5221 (T>C) S46P (novel) at exon1, nt29680 (C>T) S192F (novel) at exon 8 and one heterozygous (nt21306 A>G) mutation in intron are of novel findings. Among the five mutations reported, one was

Table 2 Summary of *NPHS2* gene variations in cases (INS) and controls detected in the study

Sample details	Gene location	Nucleotide change	Aminoacid change	Mutation status
Control ($N = 100$)	All exons	No change	Not applicable	Not applicable
SSNS ($N = 100$)	All exons	No change	Not applicable	Not applicable
SRNS-number of patients (P) = 100				
P44	Exon 4	nt21237 (T>C)	L167P (novel)	Homozygous
P7, P73 and P96	Exon 4	nt21240 (G>A)	R168H (reported) rs ID:530318579	Homozygous
P23	Intron	nt21306 (A>G)	H52R (novel)	Heterozygous
P24	Exon 5	nt23771 (C>T)	R196G (reported) rs ID:12568913	Homozygous
P47, P3 and P76	Exon 8	nt29515 (C>T)	A297V (reported) rs ID: 199506378	Heterozygous
P46 and P4	Exon 5	nt23795 (C>A)	L204L (reported) rs ID: 199837664	Heterozygous
P43 and P48	Exon 4	nt21253 (G>A)	No change	Heterozygous
P51	Exon 1	nt5221 (T>C)	S46P (novel)	Homozygous
P62	Exon 1	nt5250 (G>A)	No change	Homozygous
P78	Exon 5	nt23841 (A>T)	Q219L (reported) rs ID: 113058664	Homozygous
P93	Exon 8	nt29680 (C>T)	S192F (novel)	Homozygous
P98	Exon 4	nt21260 (C>T)	P175S (novel)	Homozygous

SSNS steroid sensitive nephrotic syndrome, *SRNS* steroid resistant nephrotic syndrome, *NT* nucleotide

Table 3 *NPHS2*, Exon 4 G/A polymorphism frequency distribution of patients with INS and healthy subjects for SNPs (ss#1130236897) and (ss#1130236897)

Sample	Genotypes			OR	CI	*P*
	GG	GA	AA			
Control ($N = 100$)	66 (66 %)	32 (32 %)	2 (2 %)			NS
SSNS ($N = 100$)	78 (78 %)	20 (20 %)	2 (2 %)	0.5475	0.2920–0.0265	
SRNS ($N = 100$)	71 (71 %)	25 (25 %)	4 (4 %)	0.7929	0.4359–1.4420	

CI confidence interval, *INS* idiopathic nephrotic syndrome, *NS* Non significant

Table 4 Distribution of genotype and allele frequency of *NPHS2* exon 4, polymorphism in study subjects and control group

SNPs (rs12401711 and rs12401708)	Genotypes			Allele frequency		*P*
	GG	GA	AA	G	A	
Controls ($N = 100$)						
Observed	66	32	2	0.82	0.18	NS
Expected	67.2	29.5	3.2			
SSNS ($N = 100$)						
Observed	78	20	2	0.89	0.11	NS
Expected	78.2	19.6	1.2			
SRNS ($N = 100$)						
Observed	71	25	4	0.83	0.17	NS
Expected	69.2	27.6	2.7			
Expected	69.2	27.6	2.7			

SNP single nucleotide polymorphism, *NS* non significant

synonymous and 4 were missense type. Of the four missense mutations, one R168H at nt21240 (G>A) in exon 4 and the other A297V at nt29515 (C>T) in exon 8 were already published [10, 15, 16]. Nevertheless, clinical symptoms were not different in patients showing homozygous or heterozygous mutations.

The mutation R168H nt21240 (G>A) observed in exon 4 was widely investigated in various populations in relation to kidney disorders [15, 17]. Yu et al. [18] were the one identified this homozygous *NPHS2* mutation for the first time in Chinese children with FSGS, which showed a substitution of histidine for arginine at 168th position (podocin R168H). This change has been shown to induce apoptosis, a marked loss and aggregation of actin filaments and activation of extracellular signal-regulated kinase (ERK) pathway in podocytes [19]. The abnormal retention of podocin R168H in endoplasmic reticulum (ER) significantly up-regulated ER stress markers and also led to the mis-localizations of other crucial slit diaphragm molecules like nephrin, CD2-associated protein (CD2AP) and the transient receptor potential-C channel-6 (TRPC6) [19, 20]. These studies implicate that podocin R168H induce different degrees of podocyte injury, which may disrupt glomerular filter function, ultimately leading to nephrotic syndrome.

The missense mutation A297V is located in an alanine- and glutamate-rich region of stomatin protein family. Tory et al. [21] reported that this mutation was found to be pathogenic, with a milder phenotype and a disease onset in late childhood or adulthood. Caridii et al. [10] reported the absence of podocin in the glomeruli of a patient with composite heterozygous mutation of NPHS2 (R229Q and A297V). It has been reported that more than 116 pathogenic mutations have been identified with the disease. These mutations may change the expression of the gene and presumably, the structure of the protein. Among missense variants, severity of the disease appears to be determined by the amino acid substitution on specific functional domains and on the intracellular trafficking of podocin.

The existence of *NPHS2* gene mutations only in SRNS group and their absence in SSNS and healthy controls observed in the present study indicates a strong relationship between the occurrence of SRNS and these *NPHS2* gene mutations. Among the 18 % of children, who showed *NPHS2* mutations, 12 % were male and 6 % were females. These children were treated with various immunosuppressive medications. 3 % children were lost for follow up. 4 % children were under remission for the past 3 years. 6 % children were under partial response and continued to be under small doses of prednisolone. 4 % children have shown no response and continued to be under active treatment. One child expired due to renal failure and its complications. According to the guidelines of International Study for Kidney Disease in children (1981), initial steroid resistance is defined as NS that does not respond to 1 month's treatment with oral prednisolone at a dosage of 60 mg/m^2/day or 2 mg/kg/day. Children with late resistance is defined as initial steroid responders who developed resistance later than $\geq$6 months of treatment. In the present study, 24 % children showed initial steroid resistance while 76 % children were later non-responsive.

The detection of these *NPHS2* mutations is of clinical importance as the preponderance of these mutations only in SRNS not only confirms genetic heterogeneity in SRNS but also underscores molecular defect leading to the non responsiveness of these patients to steroid therapy. Failure to respond to steroid treatment has an important ramification for the risk of developing progressive renal failure later in life leading to ESRD. The higher frequency of these mutations (18 %) observed in SRNS is of noteworthy, though not significant, confirming the previous findings that mutations in the *NPHS2 is* known to cause SRNS, occurring in both sporadic and familial cases of SRNS [10, 11, 22, 23]. Lipska et al. [24]. also reported that *NPHS2* mutations were to be found in the highest prevalence in patients with SRNS in Polish population.

In the present study, the detection of *NPHS2* mutations is of clinical importance as the identification of the underlying gene defect in SRNS has underscored the understanding of the pathogenesis of nephrotic syndrome. Earlier studies have shown that in addition to the structural injury to podocyte, identification of genetic mutations in numerous podocyte and podocyte-related proteins have been shown to play pivotal roles in the development of podocyte injury and proteinuria and are associated with SRNS and/or FSGS [2, 7, 9, 12]. The most implicated mutation involves the genes encoding the proteins such as nephrin, podocin, CD2-associated protein and alpha-actinin-4 that are important in maintaining its structure and function of podocytes. *NPHS1* gene encodes nephrin, a key component of the podocyte slit diaphragm and is responsible for the Finnish-type congenital nephrotic syndrome [9, 13]. *NPHS2* gene encodes, an integral membrane protein, podocin which interacts with nephrin and is responsible for FSGS. In case of alterations in some of the genes encoding these proteins, the podocyte foot process along with the glomerular basement membrane and slit diaphragm can lose its normal structure and alter its function leading to protein leakage or proteinuria causing NS and often the renal biopsy tissue will show a picture of FSGS [12, 28].

Further, mutational analysis in SRNS would help in preventing unnecessary exposure to immuno-suppressants and their adverse effects, besides helping in prognostication. Although 12 mutations were observed in SRNS, the significance of these mutations is not much known. Moreover, there was no subtle phenotypic difference between patients who bear or do not harbor these mutations. Further analyses are required to study the protein structure modifications and its implications to the diseases. Caridi et al. [10] have reported homozygous or compound

heterozygous mutations in *NPHS2* for 14 of 120 patients with SRNS (12 %). In contrast to those data, Maruyama et al. [25] did not detect any mutation in *NPHS2* in a study conducted in 36 Japanese children with SRNS. Vasudevan et al. [26] reported that the prevalence of *NPHS2* mutations observed in a group of Indian children with sporadic SRNS is low (4 %) and is similar to the Chinese (3 %), Korean (0 %), and the Japanese (0 %) populations [16, 25, 27]. In contrast, *NPHS2* gene mutations are more prevalent in Europe and North America affecting 10.5–28 % of the sporadic SRNS children [28, 29]. Diversity in the *NPHS2* gene mutation pattern is thought to be due to variations in ethnicity and environment [10, 11, 29]. The small number of *NPHS2* exons and the detection of a frequent mutation will allow rapid screening of these individuals for mutation of *NPHS2*. This gives the advantage of avoiding unnecessary immunosuppressive therapeutic trials and renal biopsy.

In conclusion, our results demonstrate that mutational analysis of *NPHS2* would be advisable at the start of treatment. The genetic variations detected in the study would serve as the important molecular marker in treating the children's at early stage, which also enables to detect carriers, prenatal diagnosis and provide genetic counseling to couples at risk. Still, furthermore studies required to rule out the protein modifications and its implications to the diseases to know about the functional significance of the protein.

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Compliance with ethical standards

Conflict of interest The authors report no conflicts of interest and they are responsible for the content and writing of this article.

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