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NPHS2 Gene Mutation and Polymorphisms in Indonesian Children with Steroid-Resistant Nephrotic Syndrome

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Abstract

Objective: Although several NPHS2 gene mutations and polymorphisms were described and associated with clinical manifestation of steroid-resistant nephrotic syndrome (SRNS), the occurrence of these genetic abnormalities or variants appeared to be influenced by race and ethnic group. We have investigated probable mutations and variants in NPHS2 gene involved in SRNS and their association with clinical manifestations. Methods: We examined 28 children with primary SRNS who visited the pediatric nephrology division of 10 teaching hospitals in Indonesia. Molecular genetic studies of the NPHS2 gene were conducted through screenings for the exon 1, exon 2, and exon 8. The mutational analysis of NPHS2 was performed by DNA sequencing. Fisher's Exact Test was used to determine the correlation between NPHS2 polymorphisms and clinical manifestations. Results: Seven females (25%) and 21 males (75%) participated in the study. The mean age of the subjects with 95% CI is: 7.6 (6.1 - 9.0) years while the mean age at onset of disease with 95% CI is: 5.4 (3.9 - 7.0) years. Sixteen patients (57.14%) were younger than 6 years at the onset of disease. Seventeen (60.7%) subjects had normal eGFR, while 11 (39.3%) had chronic renal insufficiency. The mean eGFR of the subjects with 95% CI is: 111.4 (87.7 - 135.1) ml/min/1.73 m^2. The mean systolic blood pressure with 95% CI is: 117.0 (108.9 - 125.1) mmHg and the mean diastolic blood pressure with 95% CI is: 77.0 (70.3 - 83.7) mmHg. We identified 6 NPHS2 polymorphisms, *i.e.* g.-52G>T, c.101A>G, g.-117C>T, c.288C>T, c.954C>T, and c.1038A>G and no mutation was found. There was no correlation between NPHS2 polymorphisms and clinical manifestations ($p > 0.05$). Conclusion: The results demonstrate no mutation of NPHS2 gene, and the 6 NPHS2 gene polymorphisms that were identified

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have no correlation with the clinical manifestation in Indonesian children with SRNS.

Keywords

Steroid-Resistant Nephrotic Syndrome, NPHS2 Gene, Polymorphism

1. Introduction

Steroid-resistant nephrotic syndrome (SRNS) is defined as a condition where nephrotic syndrome patients do not achieve remission after a full dose of single drug prednison therapy during the first four weeks [1] [2]. Other authors suggested a period of after 4 - 6 weeks [3] and 8 weeks of standard steroid treatment with no remission [4]. Steroid-resistant nephrotic syndrome accounts about 10% - 15% of nephrotic syndrome in children and tends to progress to end stage-renal disease within 10 years [5]. Molecular genetic studies have demonstrated that mutations in NPHS2 gene are responsible for structurally defective podocytes or deficient basement membrane, resulting in severe proteinuria [6]-[9].

About 50 NPHS2 gene mutations and variants and/or nonsilent polymorphisms have been reported and recognized as potentially involved in proteinuria [6]. Information about NPHS2 variants for different racial and ethnic groups are lacking in terms of the variant population frequency and their association with clinical manifestations. The available data as described in HuGe review suggest that large epidemiological studies to examine the association between NPHS2 variants and nephrotic syndrome are warranted [10]. In Indonesia, knowledge on NPHS2 mutations and variants in children with SRNS is lacking. We decided to evaluate the presence of probable mutations and variants in NPHS2 gene which are involved in SRNS and their association with clinical manifestations.

2. Materials and Methods

Subjects were patients with steroid resistant nephrotic syndrome (SRNS) who visited the pediatric nephrology division of several educational hospitals in Indonesia. The inclusion criteria were Indonesian, aged between 1 - 14 years old, with primary SRNS. This study was approved by the ethical committee of the Faculty of Medicine, Universitas Padjadjaran/Dr. Hasan Sadikin General Hospital, Bandung. The definitions for nephrotic syndrome (NS) and steroid resistant nephrotic syndrome (SRNS) used were definitions as defined by the International Study of Kidney Disease in Children (ISKDC) criteria, *i.e.* edema, severe proteinuria, and hypoalbuminemia (<2.5 g/dL) for NS [11] while SRNS was defined as NS patients who do not achieve remission after the full-dose single drug prednisone therapy during the first four weeks [1]. NS and SRNS were diagnosed by pediatric nephrologists or pediatricians supervised by pediatric nephrologists. Renal insufficiency was defined as an estimated glomerulo filtration rates (eGFR) of <90 mL/min per 1.73 m^2. Data analysis was performed using SPSS™ version 20.0. Fisher's Exact Test was used to determine the correlation between NPHS2 polymorphisms and clinical manifestations, *i.e.* eGFR, hematuria, and hypertension.

1) Mutation analysis

DNA was extracted and purified from peripheral blood which was collected after informed consent from the subjects was obtained in accordance with the standard protocol. These procedures were conducted at the Health Research Unit of the Faculty of Medicine, Universitas Padjadjaran, Bandung. The mutation analysis was then performed at the Laboratory of Pediatrics and Neurology of the Radboud University Medical Centre Nijmegen. The exons of the NPHS2 gene were PCR-amplified using specific primers. Three sets of primers were designed to cover the sequences of introns adjacent to each NPHS2 exon. The sequences of the forward and reverse primers, PCR conditions, and the sizes of PCR products are given in **Table 1**.

2) Sequence reaction protocol

We sequence a PCR product (single bright band on agarose gel), by adding to each primer: 2.5 uL diluted PCR product (10 × diluted with MilliQ), 0.5 uL RR-sequence mix (keep on ice containing 2.5 × sequence buffer), 1.75 uL 5 × sequence buffer, 0.25 uL 10M primer, and 5.0 uL MilliQ-water. We spin the content and then put the tube(s) in a PCR machine and run the sequence program with the following instructions: 0.96˚C for 60 sec; 1. 96˚C for 60 sec; 2. Ramp 1˚C/sec to 50˚C; 3. 50˚C for 5 sec; 4. Ramp 1˚C/sec to 60˚C; 5. 60˚C for 2 min

Table 1. Primers and PCR conditions used in the study, NPHS2 Genbank References: NM_014625, AJ279254, NP_055440.

Exon	Primer sequences 5' → 3'	T anneal (˚C)	Fragment (bp)
1	F: GCA GCG ACT CCA CAG GGA CT	56	420
	R: TCC ACC TTA TCT GAC GCC CC		
2	F: AGG CAG TGA ATA CAG TGA AG	58	203
	R: GGC CTC AGG AAA TTA CCT A		
8	F: GGT GAA GCC TTC AGG GAA TG	58	380
	R: TTC TAT GGC AGG CCC CTT TA		

0 sec; 6. Go to 1.27 times; 7.15˚C forever; 8. End.

We add 1 uL 125 mM EDTA and 1 uL 3 M NaAc and mix the content and add 30 uL abs. EtOH.

We leave the samples at room temperature for 15 minutes and centrifuge for 30 minutes at 3000 g −4˚C, then remove the supernatant by short-spinning the opened tubes upside down on a tissue and then wash the samples by adding 35 uL 70% EtOH and centrifuge for 15 minutes at 1650 g −40˚C. We store the pellets at −20˚C until electrophoresis. Just before electrophoresis, we dissolve the pellet in 10uL HiDi, denature 1 minute at 92˚C, cool on ice and run the samples in the automatic DNA sequencer.

3. Results

The study population consisted of 59 Indonesian pediatric patients with primary steroid resistant nephrotic syndrome. Subjects were recruited from patients who visited the pediatric nephrology division of 10 education hospitals in several cities in Indonesia, *i.e.* Bandung, Jakarta, Yogyakarta, Semarang, Surabaya, Denpasar, Medan, Palembang, Makassar, and Manado. The molecular genetic studies of the NPHS2 gene were performed for all subjects but only 28 subjects were successfully screened for exon 1, exon 2, and exon 8. The other subjects and the other exons were failed to performed. The 28 subjects consisted of 7 females (25%) and 21 males (75%). The mean age of the subjects with 95% CI: 7.6 (6.1 - 9.0) years while the mean age at onset of disease with 95% CI: 5.4 (3.9 - 7.0) years. Seventeen (60.7%) subjects had normal eGFR, while 11 (39.3%) had chronic renal insufficiency (eGFR < 90 mL/min/1.73 m^2). The mean eGFR of the subjects with 95% CI: 111.4 (87.7 - 135.1) ml/min/1.73 m^2. The mean systolic blood pressure with 95% CI: 117.0 (108.9 - 125.1) mmHg and the mean diastolic blood pressure with 95% CI: 77.0 (70.3 - 83.7) mmHg. The clinical characteristics of the patients with NPHS2 gene polymorphisms are presented in **Table 2**.

The nomenclature for describing the sequence variations of NPHS2 used here was based on the reference sequence NM_014625, AJ279254, NP_055440 (Gen Bank Database). DNA sequence analysis of exon 1, exon 2, and exon 8 of the subjects did not find any mutation, but six polymorphisms were detected. In exon 1, 3 kinds of polimorfisms were revealed, *i.e.* g.-52G>T (heterozygous) in 4 of 28 patients (14%), c.101A>G (homozygous) in 12 of 28 patients (43%), and g.-117C>T (heterozygous 8 and 1 homozygous) in 9 patients (32%). Polymorphism c.288C>T (heterozygous) in exon 2 was found in 4 of 28 patients (14%), whereas in exon 8 polymorphism c.954C>T were detected in 24 patients (85.7%), which consisted of 15 heterozygous and 9 homozygous. heterozygous c.1038A>G polymorphism was also found in exon 8 of 5 of 28 patients (17.8%). The polymorphisms are presented in **Table 3**.

The results of the univariate analysis with Fisher's Exact Test on the correlation between NPHS2 polymorphisms and clinical manifestations, *i.e.* eGFR < 90 (ml/min/1.73 m^2), hematuria, and hypertension, were not significant (**Table 4**).

4. Discussion

Almost 50 NPHS2 gene mutations and variants and/or non-silent polymorphisms have been reported as potentially involved in structurally defective podocytes or deficient basement membrane that lacks perm-selectivity, causing proteinuria [6]. Previous studies have shown that NPHS2 gene mutations and variants were associated with different clinical features, such as early childhood onset proteinuria, rapid progression to ESRD, late onset nephrotic syndrome, and renal histology of FSGS [5] [12] [13]. In sporadic SRNS, these mutations are responsible for 10% - 30% of diseases [13]-[17]. Most published studies on SRNS cases associated

Table 2. Characteristics of the subject (n = 28).

No	Characteristics	n (%)
1	Sex	
	Male	21 (75)
	Female	7 (25)
2	Hematuria	
	(+)	8 (28.6)
	(-)	20 (71.4)
3	Hypertension	
	(+)	12 (42.9)
	(-)	16 (57.1)
4.	Age (years)	
	Mean (SD): 7.6 (3.8)	
	Median: 7.0	
	Range: 2.5 - 13.9	
5.	Age onset (years)	
	Mean (SD): 5.4 (4.1)	
	Median: 4.1	
	Range: 1.0 - 13.0	
6	Creatinine (mg/dL)	
	Mean (SD): 0.91 (1.07)	
	Median: 0.6	
	Range: 0.2 - 6.0	
7	eGFR (ml/min/1.73 m^2)	
	Mean (SD): 111.4 (61.2)	
	Median: 96.5	
	Range: 12 - 295	

Table 3. NPHS2 polymorphisms in 28 patients with steroid-resistant nephrotic syndrome.

Exon	Polymorphism	Effect	Heterozygous/ Homozygous	Patients (n = 28)
1	g.-52G>T	p.Arg6Arg	Heterozygous	4 (14.2%)
1	c.101A>G	p.Arg34Arg	Homozygous	12 (42.8%)
1	g.-117C>T	p.Phe39Phe	Heterozygous	8 (28.5%)
			Homozygous	1 (3.5 %)
2	c.288C>T	p.Ser96Ser	Heterozygous	4 (14.2%)
8	c.954C>T	p.Ala318Ala	Heterozygous	15 (53.5%)
			Homozygous	9 (32.1%)
8	c.1038A>G	p.Leu346Leu	Heterozygous	5 (17.8%)

with biopsy-proven focal segmental glomerulosclerosis (FSGS) [5] [10] [15]. In addition, NPHS2 gene mutation studies in different populations and countries have shown that ethnicity plays an important role in disease genes [5] [18] [19]. NPHS2 412C→T and 419delG gene mutations are the risk factors for SRNS in Indonesian children [20].

The result of this study shows a ratio of male and female of 3:1, which is similar to those from previous SNRS studies. The ISKDC has reported a male and female ratio of 2:1 [11]. However, Caridi *et al*. observed a male to female ratio of patients with an NPHS2 gene mutation of 7:2 [14]. A study about NPHS2 412C→T and

Table 4. Correlation between NPHS2 polymorphisms and clinical manifestations in 28 patients with steroid-resistant nephrotic syndrome.

Polymorphisms	eGFR < 90 (ml/min/1.73 m^2)		p*	Hematuria		p*	Hypertension		p*
	+	-		+	-		+	-	
g.-52G>T			1.000			1.000			0.113
+	2	2		1	3		0	4	
−	9	15		7	17		12	12	
c.101A>G			0.705			0.691			0.136
+	4	8		4	8		3	9	
−	7	9		4	12		9	7	
g.-117C>T			1.000			0.091			0.434
+	4	5		5	5		3	7	
−	7	12		3	15		9	9	
c.288C>T			1.000			0.555			1.000
+	2	2		2	2		2	2	
−	9	15		6	18		10	14	
c.954C>T			0.355			0.311			1.000
+	10	12		5	17		9	13	
−	1	5		3	3		3	3	
c.1038A>G			0.353			0.123			1.000
+	3	2		3	2		2	3	
−	8	15		5	18		10	13	

*Fisher's Exact Test.

419delG gene mutations in Indonesian children with SRNS concluded that male gender was risk factor for SRNS [20]. The age at onset of disease in this study is 1 to 13 years with 57% of the patients who were younger than 6years at the disease onset. Weber *et al*., found that NPHS2 gene mutation R138Q in SRNS patients is associated with early onset (12 ± 3 months) [13]. Polymorphism of NPHS2 gene R229Q is associated with late-onset nephrotic syndrome [21] as well as increased risk of microalbuminuria in the general population [22]. This R229Q variant presents in approximately 4% of Western population, encoding a protein with lower affinity for binding to nephrin [23]. About 36% patients with SRNS exhibited progression to ESRD 5 - 6 years after onset. Aucella *et al*., studied 33 patients adult onset FSGS which showed that glomerular filtration rate (GFR) was in the normal range in 19 subjects and 14 patients had a variable degree of renal failure [24]. In our study, 17 (60.7%) of the subjects had normal eGFR, while 11 (39.3%) had chronic renal insufficiency (eGFR < 90 mL/min/1.73 m^2). Our previous study about NPHS2 412C→T and 419delG gene mutations shows that no differencein clinical manifestations is found between SRNS with mutation and SRNS without mutation, except for serum creatinin in 412C→T mutation [25].

This study reports the identification of 6 NPHS2 polymorphisms, *i.e.* g.-52G>T, c.101A>G, g.-117C>T, c.288C>T, c.954C>T, and c.1038A>G, in patients with clinical diagnosis of SRNS. Homozygous NPHS2 c.101A>G polymorphism was found in 12 subjects, leading to p.Arg34Arg. Homozygous NPHS2 g.-117C>T was found only in 1 subject and Homozygous NPHS2 c.954C>T was found in 9 subjects, leading to p.Ala318Ala. The other NPHS2 polymorphisms were heterozygous. The NPHS2 homozygous and heterozygous polymorphisms have not been implicated in clinical manifestation of SRNS, such as decrease of GFR, hypertension and

hematuria, in this study (**Table 4**).

In our study, we could not find any mutation of NPHS2 gene as causative SNRS, suggesting that NPHS2 gene mutations are not major cause of SNRS in Indonesian children. In addition, we also suggests that there is an inter-ethnic difference in the occurrence of NPHS2 gene mutations or their variants, so the 6 NPHS2 gene polymorphisms that were identified have no correlation with the clinical manifestation. The strength of our study lies in the collection of sample from multicenter of various teaching hospitals in Indonesia, while the weakness of this study is only 28 of 59 samples were successfully screened, due to limitation of time and research finance.

5. Conclusion

In conslusion, no mutation was found in this study; however, due to our limitations, more studies are needed. Other exons of podocin or other podocyte proteins in Indonesian children may play a role in the pathogenesis of SRNS.

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