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Research article

Prevalence of NPHS2 gene R229Q polymorphism in Bangladeshi children with nephrotic syndrome

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ABSTRACT

Background: Limited and contradictory pharmacogenetic studies of *NPHS2* gene R229Q polymorphism in nephrotic syndrome (NS) children of different ethnicities steered us to investigate the genotype frequency and associated risk of this polymorphism in Bangladeshi NS children.

Methods: A prospective case-control study was conducted which comprised a total of 142 children having nephrotic syndrome (NS), divided into 2 groups: case group consisted of 40 children with steroid-resistant nephrotic syndrome (SRNS), and control group involved 102 children with steroid-sensitive nephrotic syndrome (SSNS). Both were genotyped by using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method for R229Q polymorphism.

Results: The results indicate the presence of R229Q polymorphism in 27.50% of SRNS and 12.75% of SSNS children. SRNS children possess 2.94-fold greater risk ($p = 0.025$) of carrying Arg/Gln genotype compared to SSNS children. Moreover, R229Q variant in SRNS children was observed as in a compound heterozygous form with p.Ala297Val located in exon 8. Age of onset (4–6 years) presents as a significant contributing factor (adjusted OR = 1.06; 95% CI = 1.023–1.094; $p = 0.001$) for SRNS susceptibility in Bangladeshi children. Contrarily, though the incidence of SRNS was higher in male children than female (80% vs 20%), gender remains to be a neutral factor ($p = 0.257$) in relation to SRNS susceptibility.

Conclusion: Compound heterozygosity of *NPHS2* p.R229Q gene variant with p.Ala297Val may cause pathogenic SRNS in Bangladeshi children. Large scale studies are warranted to establish the genotype-phenotype correlation. It is recommended to screen for p.R229Q first and, if positive, for p.Ala297Val in Bangladeshi SRNS children.

1. Introduction

Nephrotic syndrome (NS) is a nonspecific renal disorder which is characterized by heavy proteinuria, edema and hypoalbuminemia [1, 2] accompanied by several pathomorphologic entities with glomerular lesions [3]. All these characteristic features primarily develop from the alteration of permselectivity barrier of the glomerular capillary wall and as a result the capillary wall loses its ability to restrict the loss of protein to <100 mg/m^2 body surface per day. In children, the treatment is strongly based on corticosteroid therapy [4]. Depending on the response to steroid therapy, NS is classified into steroid sensitive nephrotic syndrome (SSNS) and steroid resistant nephrotic syndrome (SRNS) [2, 3]. Most of the patients respond to steroid therapy; however, approximately 10–20% of the patients fail to respond to steroid therapy [2, 5, 6].

Podocin is a membrane protein belonging to the stomatin family of lipid-raft associated protein and exclusively expressed in the podocytes, the key component of glomerular basement membrane [7]. It is an integral protein consisting of 383 amino acids having a unique topology (a single short hairpin-like transmembrane domain and cytosolic N- and C-terminal domains) [8]. Podocin oligomerizes and these podocin clusters along with other podocyte proteins such as nephrin and CD2AP in lipid rafts ensure the proper functioning of filtration slits [9].

Mutations in *NPHS2* gene, encoding podocin result in a reduction or absence of functional protein and thus impair the formation of normal slit diaphragms [3, 10, 11, 12]. This leads to SRNS before 6 years of age and

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Table 1. Demographics and clinicopathological features of SRNS and SSNS children.

Characteristics	SRNS (n = 40)	SSNS (n = 102)	*p value*
Male:Female	32:8	72:30	-
Age of onset (year)	5.39 ± 1.42	4.79 ± 0.65	0.0007*
Weight (kg)	19.43 ± 2.57	18.25 ± 2.76	0.0209*
Height (cm)	117.5 ± 3.39	116.9 ± 4.66	0.4603
Body surface area (m^2)	1.0 ± 0.01	0.99 ± 0.01	0.0001*
Systolic blood pressure (mm Hg)	106.92 ± 2.62	105.71 ± 2.97	0.0257*
Diastolic blood pressure (mm Hg)	78.09 ± 1.50	77.40 ± 0.97	0.0015*
Serum albumin (g/dL)	1.98 ± 0.52	2.07 ± 0.37	0.2496
Serum cholesterol (mg/dL)	393.7 ± 155.37	347.2 ± 129.8	0.0718
Serum creatinine (mg/dL)	0.80 ± 0.04	0.78 ± 0.05	0.054
Serum urea nitrogen (mg/dL)	13.49 ± 2.54	12.50 ± 3.07	0.0725
24-hr urine protein (mg/L)	384.1 ± 71.3	339.6 ± 44.2	0.0001*
Histopathological types (n)	23	35	
FSGS	10	13	
MCNS	6	9	
MPGN	4	5	
Others	3	8	

FSGS, Focal segmental glomerulosclerosis; MCNS, Minimal change glomerulonephritis; MPGN, Membranoproliferative glomerulonephritis.
All data are presented as Mean ± SD.
* Result is statistically significant ($p < 0.05$).

reach end stage renal disease (ESRD) during their first decade of life [3]. Several studies have investigated many potential genetic variants of *NPHS2* gene. The variant p.R229Q (c.686G > A; rs61747728), a non-synonymous variant in exon 5, is affirmed to be one of the most important predictive factors in the pathogenesis of SRNS [13, 14]. Evidences confirmed that the R229Q allele is a disease-causing, rather than a benign polymorphism [15]. However, the susceptibility to R229Q polymorphism varies among different ethnic groups. The susceptibility of NS in Asian children is 9–16 per 100,000 [16,17], which is 2–7 per 100,000 children in USA [18] and 2–4 new cases per 100,000 children in UK [17, 19]. The rate of such incidence in Bangladesh is not reported yet. Therefore, we aimed to investigate the genotypic distribution of R229Q variant of *NPHS2* gene and thus finding its associated risk with SRNS in Bangladeshi children.

2. Materials and methods

2.1. Subject selection

The current study was conducted on 40 SRNS (cases) and 102 SSNS (controls) children. Patients who achieved complete remission with steroid therapy were classified as having SSNS. And those who failed to achieve remission following 4-week of 60 mg/m^2/day prednisolone treatment were identified as having SRNS. So, volunteers of SRNS and SSNS were selected according to expert physicians' diagnosis of the type of NS after treating with prednisolone. The study was carried out by recruiting patients from Bangladesh Sheikh Mujib Medical University (BSMMU), Dhaka and Mymensingh Medical College Hospital (MMCH), Mymensingh between the period of January 2018 and September 2019. The study protocol was approved by the ethical committees of the respective hospitals and the study was conducted in accordance with the declaration Helsinki and its subsequent revisions (WMADH, 2008). Freely-given informed consent document was obtained from the legal guardian of each patient after ensuring that they have understood the purpose of the study. The genetic analyses were conducted in the Laboratory of Pharmacogenetics and Pharmacokinetics at the Department of Clinical Pharmacy and Pharmacology, Faculty of Pharmacy, University of Dhaka, Bangladesh.

2.2. Genotyping

3 ml of venous blood was collected from all patients in sterile tubes containing EDTA-Na_2 and stored at -80 °C until genomic DNA (gDNA) extraction. gDNA was isolated from blood by previously published method [20]. The quantity and purity of the isolated DNA were assessed by using a UV Spectrophotometer (UV Prove v2.1) at 260 nm. PCR-RFLP method was employed for genotyping the selected single nucleotide polymorphism (SNP). The relevant genomic target region, containing the SNP of interest, was amplified by means of primer-directed PCR using thermostable DNA polymerase and subsequently digested with specific restriction endonuclease, *ClaI* (NEB®, USA). Visualization of the digested PCR fragments was done by 2% agarose gel electrophoresis after staining with ethidium bromide. To avoid false positive genotype, 20% of the samples were sequenced. Moreover, exons 7 and 8 of R229Q heterozygous patients were PCR amplified from gDNA using predesigned primers and directly sequenced by capillary electrophoresis according to previously published methods [21, 22] with necessary optimization. All primer sequences can be found in the supplementary table 1.

2.3. Statistical analyses

Student t-test and chi-square ($\chi 2$) test were applied to compare the demographic data between SSNS and SRNS patients. Deviation of genotype frequencies in the control group from case group under Hardy–Weinberg equilibrium (HWE) was measured by chi-square test ($\chi 2$). A multivariate logistic regression analysis was performed to assess the odd ratios (ORs) and 95% confidence interval (95% CI) adjusted to age and sex. In all of the analyses, $p < 0.05$ was considered statistically significant. All statistical analyses were done applying the SPSS software, version 23.0.

3. Results

3.1. Characteristics of study population

The study included a total of 142 NS children with mean age of 4.9 years. Detailed demographics and clinicopathological features can be

Table 2. Association of age of onset and sex with nephrotic syndrome children.

Characteristics	SRNS (n = 40) (%)	SSNS (n = 102) (%)	Odds Ratio (OR)	(95% CI)	*p value*
Age of Onset, years					
<4	4 (10.0)	3 (2.94)	0.24	0.029–2.028	0.191
≥4- ≤6	25 (62.5)	97 (95.10)	1.06	1.023–1.094	0.001*
>6	11 (27.5)	2 (1.96)	Ref	-	-
Sex					
Male	32	72	1.67	0.689–4.035	0.257
Female	8	30	Ref	-	-

* Result is statistically significant ($p < 0.05$).

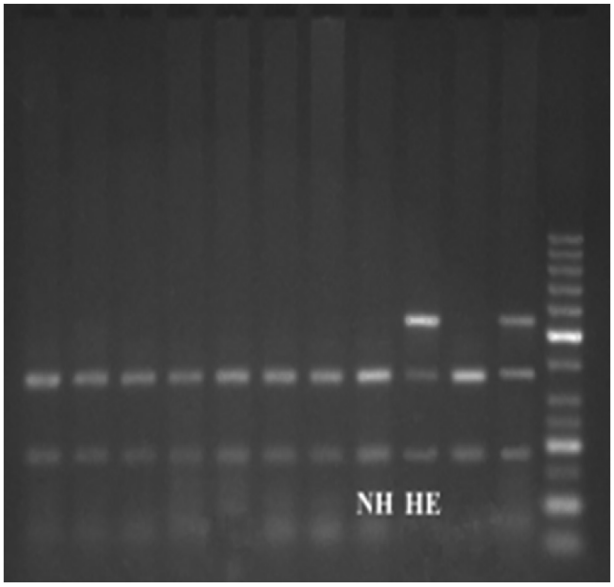

Figure 1. Restriction Endonuclease (ClaI) digestion fragment of NPHS2 gene with R229Q polymorphism (2% agarose gel). NH: Normal homozygous (wild type) variant having two bands at 184 bp and 361 bp; HE: Heterozygous variant having three bands at 184 bp, 361 bp and 545 bp.

found in Table 1. Age of onset, weight, body surface area, blood pressure and 24-h urine protein level were found to be statistical different between SRNS and SSNS children groups. Focal segmental glomerulosclerosis (FSGS) was reported as the predominant (39.66%) histopathological finding from 58 available biopsy reports. All the children exhibited scanty micturition, puffed face and generalized edema. Around 85.92% children were diagnosed with NS at the age of 4–6 years. Besides, 62.5% SRNS represented early onset between the age 4–6 years. Age was found to be a statistically significant factor for SRNS (adjusted OR = 1.06; 95% CI = 1.023–1.094; p = 0.001) when compared to SSNS patients. Of 102 SSNS patients, 72 were male and 30 were female, whereas there were 32 males and 8 females in the SRNS patients. However, high prevalence of the male patients was not found to be a risk factor for SRNS in Bangladeshi children (adjusted OR = 1.67; 95% CI = 0.689–4.035; p = 0.257) (Table 2).

3.2. Correlation of R229Q polymorphism with SRNS

Figure 1 shows the DNA fragment pattern of *ClaI* digestion of the PCR products carried out to identify the variations at codon 229. Two bands at 184bp and 361bp demonstrated Arg/Arg homozygous genotype, three bands at 184bp, 361bp and 545bp indicated Arg/Gln heterozygous genotype and one band at 545bp represented Gln/Gln mutant homozygous genotype.

The genotypic frequencies of SSNS and SRNS patients are demonstrated in Table 3. The percent distribution of Arg/Arg homozygous genotype was significantly lower in SRNS patients than in SSNS patients (72.50% vs 87.25%). Significant relationship was found between the Arg/Gln heterozygous genotype and susceptibility to SRNS, with an adjusted OR of 2.94 (95% CI = 1.147–7.557; p < 0.05) when compared to Arg/Arg homozygous genotype. The Gln/Gln mutant homozygous genotype was not found in any of the study group (SRNS and SSNS). In addition, analysis of exons 7 and 8 of R229Q heterozygous children revealed the presence of trans-associated p.Ala297Val variant in exon 8.

4. Discussion

In the present study, we investigated whether there is any risk association between *NPHS2* gene R229Q polymorphic variant and SRNS susceptibility in Bangladeshi NS children. We also investigated the relation of this polymorphism with different demographic characteristics in NS children.

R229Q polymorphism is one of the most commonly found podocin sequence variations. The arginine residue at protein position 229 is highly conserved across species, which is substituted by glutamine in case of polymorphism. This results in consequent disruption of the functional properties of podocin in vitro and possibly in vivo [23]. In vitro studies have provided an explanation of the pathogenesis by decreased binding of the p.R229Q mutant protein to nephrin [15]. This variant is abundant in patients with SRNS which indicates that it has certain role in the SRNS disease state [13]. In our findings, SRNS children possess 2.94-fold increased risk of carrying the Arg/Gln heterozygous genotype compared to SSNS children which is consistent with several

Table 3. Genotype frequencies of *NPHS2* gene R229Q polymorphism in nephrotic syndrome children.

Genotypes	SRNS n = 40 (%)	SSNS n = 102 (%)	Adjusted Odds Ratio (AORs)	95% CI	*p value*
GG (Arg/Arg)	29 (72.50)	89 (87.25)	Ref.	-	-
GA (Arg/Gln)	11 (27.50)	13 (12.75)	2.94	1.147–7.557	0.025*
AA (Gln/Gln)	0	0	0	0	0

* Result is statistically significant ($p < 0.05$).

other case-control studies conducted in South Asian population [5, 24, 25]. On the contrary, a few studies conducted in SRNS patients found no correlation between this variant and NS [26, 27]. Moreover, a study conducted by Tory et al. [28] reported that the pathogenicity of R229Q variant of *NPHS2* gene in SRNS is dependent on the trans-association of some specific mutations i.e. p.Ala284Val, p.Ala288Thr, p.Arg291Trp, p.Ala297Val, p.Glu310Lys, p.Glu310Val, p.Leu327Phe and p.Gln328Arg located in exons 7 and 8. This is due to the fact that R229Q variant protein coexpressed with any of these specific mutations shows altered dimerization resulting in the retention of R229Q variant protein within the cytoplasmic compartments. Therefore, further analysis into the exons 7 and 8 of our 11 SRNS and 13 SSNS children, we observed that all 11 SRNS children contain only p.Ala297Val mutation in exon 8 corroborating with the findings of Tory et al [28]. Having such rarity of other above mentioned mutations might reflect a true finding or inadequate sample size, differences among ethnicities which is yet to be determined. Therefore, it is cautionary to analyze exon 8 along with R229Q heterozygosity in order to confirm its pathogenicity in Bangladeshi SRNS children.

The current study also investigated the genotype-phenotype correlations between R229Q polymorphism and age of onset. We reported all 11 heterozygous genotype carriers in SRNS children aged between 4-6 years but failed to correlate R229Q polymorphism with the risk of developing early-onset SRNS. This is in disagreement with Hinkes et al. and Boute et al. who confirmed *NPHS2* p.R229Q variant causes early-onset SRNS, ultimately leading to ESRD before the age of 10 years [10, 29]. In contrast, two independently conducted studies found the correlation of late-onset SRNS with this polymorphic variant. Tsukaguchi et al. [15] reported *NPHS2* variants in 23% of late-onset familial cases and in 2% of sporadic ones and Machuca et al. [13] identified *NPHS2* substitutions in 14% of cases presenting with SRNS after 18 years of age. Furthermore, Santin et al. supported that late childhood and adult-onset SRNS had a similar *NPHS2* p.R229Q detection rate than those with early childhood-onset [30].

In this study, statistically, we could not differentiate any gender-specific risk for developing steroid resistance (p value < 0.257) in NS patients. However, several studies reported contradiction to our finding. Ali et al. established male predominance with statistical significance in case of p.R229Q [31]. Similar findings were observed in studies of different region [32, 33, 34, 35]. In our study, the total SRNS ratio of male vs female is 80% vs 20% which indicates the much higher rate of male susceptibility towards the disease. Besides, the gender discrepancy was also observed in case of p.R229Q frequency distribution (male 80% vs female 20%). The statistical dissimilarity to the male susceptibility might be due to small sample size.

In conclusion, this is the first study to identify *NPHS2* p.R229Q heterozygosity trans associated with specific mutation in exon 8 may cause pathogenic steroid resistance in Bangladeshi NS children. Future studies with a larger sample size in different ethnicities must be conducted to evaluate the statistical significance.

Declarations

Author contribution statement

S. Jyoti and F. Islam: Performed the experiments; Wrote the paper.

I. Shrabonee: Contributed reagents, materials, analysis tools or data.

T. Sultana and N. Chaity: Performed the experiments.

N. Nahid, M.R. Islam and M.S. Islam: Conceived and designed the experiments; Analyzed and interpreted the data.

M. Apu: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

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