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Assessment of the Link of *ABCB1* and *NR3C1* gene polymorphisms with the prednisolone resistance in pediatric nephrotic syndrome patients of Bangladesh: A genotype and haplotype approach

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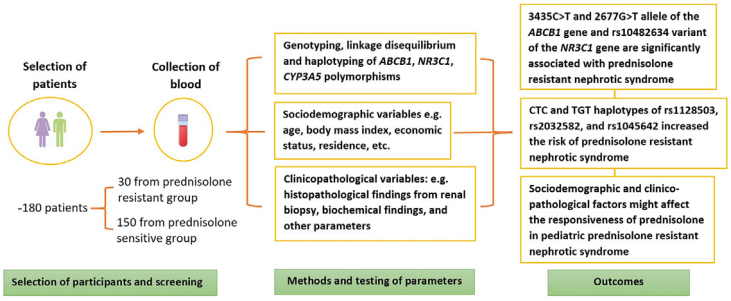
GRAPHICAL ABSTRACT



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ABSTRACT

Introduction: Nephrotic syndrome is a common pediatric kidney disease. Investigations on several genetic polymorphisms revealed an inconsistent influence on the resistance of patients to steroids.
Objectives: This study aimed to identify the association of *ABCB1* (1236C > T, 2677G > T, 3435C > T), *NR3C1* (rs10482634, rs6877893), and *CYP3A5* (CYP3A5*3) gene polymorphism as well as sociodemographic and clinicopathological parameters with the risk of developing prednisolone resistance in pediatric patients with nephrotic syndrome.
Methods: A case-control analysis was performed on 180 nephrotic syndrome patients. Among them, 30 patients were classified as prednisolone resistant group, and 150 were classified as prednisolone sensitive group. Genotyping was performed by the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method.
Results: No significant association of 1236C > T polymorphism with the risk of prednisolone resistance ($p > 0.05$) was found. The GT heterozygous of 2677G > T was found to be significantly associated with

Abbreviations: NS, Nephrotic syndrome; SSNS, Steroid-sensitive nephrotic syndrome; SRNS, steroid-resistance nephrotic syndrome; *MDR1*, multidrug resistance gene 1; GC, Glucocorticoids; P-gp, Permeability glycoprotein; PR, Prednisolone resistance; *NR3C1*, nuclear receptor subfamily 3, group C, member 1; GR, Glucocorticoid receptor; PRNS, Prednisolone resistance nephrotic syndrome; PRG, Prednisolone resistance group; PSG, Prednisolone sensitive group; PCR-RFLP, polymerase chain reaction-restriction fragment length polymorphism; OR, odds ratio; 95%CI, 95% confidence intervals; HWE, Hardy-Weinberg equilibrium; LD, Linkage disequilibrium; MesPGN, mesangioproliferative glomerulonephritis.

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the development of prednisolone resistance (OR = 3.9, p = 0.034). In the case of 3435C > T, a statistically significant association was observed in TC heterozygous and TT mutant homozygous genotypes (OR = 0.38, p = 0.047; OR = 3.06, p = 0.038, respectively) with prednisolone resistance. For rs10482634 polymorphism, the AG heterozygous and AG+GG genotypes were significantly linked with prednisolone resistance (OR = 2.40, p = 0.033; OR = 2.36, p = 0.034, respectively). We found no association with the risk of prednisolone resistance with rs6877893 and CYP3A5*3 polymorphism (p > 0.05). CTC and TGT haplotypes of *ABCB1* and GA haplotype of *NR3C1* were also associated with the increased risk of pediatric prednisolone resistance (OR = 4.47, p = 0.0003; OR = 2.71, p = 0.03; and OR = 4.22, p = 0.022, consecutively). We also observed the correlation of different sociodemographic and clinicopathological factors with prednisolone resistance in pediatric nephrotic syndrome.

Conclusion: Our findings showed a significant association of *ABCB1* and *NR3C1* gene polymorphisms with prednisolone resistant pediatric nephrotic syndrome.

Introduction

Nephrotic syndrome (NS) or nephrosis is one of the most frequent types of end-stage renal disease among children in developing and developed countries. It is described as a structural and functional deficiency in the glomerular filtration barrier [1,2], which causes excessive loss of protein from blood to the urine through the glomeruli [3]. NS leads to heavy proteinuria, hypoalbuminemia, hyperlipidemia, hypercholesterolemia, lowered oncotic plasma pressure, weight gain, and edema following the buildup of body fluid [4]. Pediatric NS is more common in boys than in girls (2:1) and can affect children at any age, from infancy to adolescence [5].

According to genetic, racial, geographical, environmental differences, as well as nephrological practices, the pattern of childhood kidney diseases varies [6]. In 2–8% of cases, family NS appears to be inherited in a polygenic manner, and in most cases, monozygotic twins are involved [7]. It is considered an important cause of morbidity and mortality, which imposes a considerable burden on health services in developing countries [8]. The prevalence of NS is almost 16:100,000 children throughout the world, with an incidence of 2–7:100,000 children [9]. Among Asian countries, the incidence of NS is higher in the Indian subcontinent, constituting 9–10:100,000 population [10]. A study reported that 50–60% of in-patients at the pediatric nephrology unit of Bangabandhu Sheikh Mujib Medical University of Bangladesh are NS patients [11].

Steroid-sensitive nephrotic syndrome (SSNS) is presently thought to entail reversible conformational changes of podocytes, whereas irreversible conformational changes are associated with steroid-resistance and disease progression [12]. Among the NS patients, more than 85% are steroid-responsive [13], with a favorable long-term prognosis. The underlying genetic defect is usually considered as a primary steroid-resistance nephrotic syndrome (SRNS), causing damage to the filtration barrier of podocytes [14]. Furthermore, polymorphisms related to gene encoding for the podocyte proteins, including podocin, nephrin, CD2-associated protein, and alpha-actinin-4, contribute to structural changes in podocytes [15]. The discovery of more than 50 monogenic reasons of SRNS has reported dysfunction in these podocyte proteins in proteinuria progression. Plenty of genes are presently known to be associated with inherited SRNS, such as congenital nephrotic syndrome type 1 (*NPHS1*), congenital nephrotic syndrome type 2 (*NPHS2*), aarF domain-containing-kinase 4 (*ADCK4*), coenzyme Q6 (*COQ6*), crumbs homolog 2 (*CRB2*), WD repeat-containing protein 73 (*WDR73*), epithelial membrane protein 2 (*EMP2*), CD2-associated protein (*CD2AP*), phospholipase C epsilon 1 (*PLCE1*), alpha-actinin-4 (*ACTN4*), transient receptor potential channel C6 (*TRPC6*), inverted formin 2 (*INF2*), etc. The continuous discovery of genetic abnormality in podocyte-related proteins has led to understand the genetic basis of NS with respect to onset, pathophysiology, diagnosis, prognostic assesments, and therapeutic treatments [16]. In addition, alternating factors that modify disease responsiveness to drug therapies is also another risk factor for SRNS, like the expression of P-glycoprotein (P-gp), a multidrug-resistance gene 1 (MDR1) product [15]. Steroid resistance, however, may occur in NS patients independently of the expression P-gp [15,17]. Several studies have shown that polymorphisms of membrane carrier gene or metabolizing enzymes also affect patients' responses to therapeutic regimens [15].

Glucocorticoids (GC), especially prednisone or prednisolone, have been the hallmark for more than 60 years to treat pediatric NS, as over 80–90% of the patients have achieved a complete remission. Unfortunately, 80% of these patients will experience one or more relapses, requiring additional GC therapy courses. Besides, about 10–20% of children with NS are steroid-resistant and therefore do not respond due to interindividual and intraindividual variability. Given that optimal GC dosing schemes are still under discussion and large-scale clinical trials are insufficient, existing medical practice among physicians is variable, specifically for the treatment of consequential relapses and the choice of second-line immunosuppressive agents [5].

Permeability glycoprotein, abbreviated as P-gp, is a well-characterized ABC-transporter of the MDR/TAP subfamily in humans that is encoded by the *ABCB1* or *MDR1* gene [18]. GC are known substrates for P-gp and may also induce its expression [19]. Previous studies showed greater *ABCB1* expression and increased P-gp activity in children with SRNS [20]. However, the understanding of the effect of genetic polymorphisms on P-gp expression is vague and may vary based on the type of tissue, pathological status, and ethnicity [21]. Multiple studies have investigated the association of P-gp polymorphisms with the responsiveness of GCs that are contradictory [15,22-28]. An increased risk of steroid-resistance was observed for *ABCB1* 1236C > T (Gly412Gly, rs1128503) polymorphism in children with NS [24,29]. A study showed that NS patients carrying homozygous mutations in 2677G > T are more prone to developing steroid-resistance [30]. The presence of 2677G > T (Ala893Ser/Thr, rs2032582) and 3435C > T (Ile1145Ile, rs1045642) polymorphisms of the *ABCB1* gene in different combinations may enhance the possibility of developing SRNS [17]. Numerous genetic polymorphisms exist within the *ABCB1* gene, and some are in strong linkage disequilibrium [31]. So, we tried to assess the distribution of the three frequent exonic polymorphisms, including 1236C > T, 2677G > T, and 3435C > T SNPs of the *ABCB1* gene, for their role in developing prednisolone resistance (PR).

The *NR3C1* (nuclear receptor subfamily 3, group C, member 1) or glucocorticoid receptor (GR) is expressed in about every cell in

the body and regulates gene transcription, controls the development, metabolism, and immune response [32]. Sensitivity to GCs depends on both the functionality and expression of the GR [33]. Wasilewska et al. [34] found a temporary decrease of GR number in lymphocytes and monocytes during GC treatment in children with SSNS, which resolved spontaneously. Different studies suggest that the genetic variations of *NR3C1* are associated with GC sensitivity. Huizenga et al. [35] showed that the common *NR3C1* polymorphism is associated with an increased sensitivity to GCs that was also proven by a specific GR haplotype [36]. Furthermore, it was found that mutations in the *NR3C1* gene are associated with both familial [37] and acquired steroid-resistance in some diseases, such as Cushing's disease [37], leukemia [38], lupus nephritis [39], and female pseudohermaphroditism [40]. Therefore, we hypothesized that *NR3C1* variants (rs10482634 and rs6877893) might be associated with SRNS in pediatric patients.

The human *CYP3A5* (cytochrome P450 3A5) gene encodes a member of the cytochrome P450 3A subfamily of enzymes, which plays an important role in drug metabolism, including prednisolone [24]. CYP3A5*3 is an A to G transition (6986A > G) within intron 3 of polymorphic expression of *CYP3A5* that may account for interindividual variations in the clearance of *CYP3A* substrates [24]. The G allele of CYP3A5*3 produces a truncated protein with a loss of enzyme activity [41]. Several studies [42] have demonstrated the predictive role of the *CYP3A5* genotype of Tacrolimus doses for lung and kidney transplant recipients. Indeed, genetic polymorphisms of *CYP3A5* and *MDR1* could have a role in the pharmacokinetics of prednisolone [43]. Different studies found that CYP3A5*3 polymorphism showed a trend of association to GC resistance in pediatric NS patients, but these associations did not reach statistical significance. Therefore, we have tried to find out the role of CYP3A5*3 polymorphism in prednisolone resistance nephrotic syndrome (PRNS).

In Bangladesh, no previous pharmacogenetic studies have been carried out on pediatric PRNS patients. Because of the poor prognosis in the past, the majority of the children have received intensive treatment regimens, and many of them have been over treated. Therefore, to develop genotype and haplotype-based prediction for the safety and efficacy of the treatment, we have studied the association of *ABCB1* 1236C > T (rs1128503), 2677G > T (rs2032582), and 3435C > T (rs1045642); *NR3C1* rs10482634 and rs6877893; and CYP3A5*3 (rs776746) polymorphisms with the risk of developing childhood PRNS.

Materials and methods

Selection of subjects

This case-control study was carried out on 180 pediatric NS patients in Bangladesh. Among them, 30 patients were in the prednisolone resistance group (PRG), and 150 patients were in the prednisolone sensitive group (PSG) recruited from different hospitals, namely, Bangladesh Institute of Child Health, Dhaka Medical College Hospital, and Bangabandhu Sheikh Mujib Medical University from the mid of October 2015 to the end of February 2018. The study protocol and consent form were approved by the Ethical Committee of the Bangladesh Institute of Child Health (BICH-ERC-10/2/2015). Before joining the study, each patient's parent/-guardian signed an informed consent document and was free to withdraw from the research at any stage.

Genotyping was conducted in the "Laboratory of Pharmacokinetics and Pharmacogenetics" of the Departments of Clinical Pharmacy and Pharmacology, University of Dhaka, Bangladesh. The blood samples were obtained from the patient's routine blood tests and followed up at the blood collection area of the different hospitals in the hematology department. This research was conducted following the International Conference of Harmonization guidelines for Good Clinical Practice and in compliance with the Declaration of Helsinki and its further amendments [44]. Different sociodemographic information of participants such as age, body mass index (BMI), residence, the socioeconomic condition was collected. The clinical data, including histological findings of renal biopsy, biochemical findings, and other related parameters, were retrospectively obtained from the relevant patient's medical records.

All patients met the criteria for the diagnosis of NS of the International Study of Kidney Disease in Children [45]. They were diagnosed as NS patients if they had severe proteinuria of $\geq$40 mg/m^2/h with hypoalbuminemia of $\leq$2.5 g/dl without knowing the causes [46]. Steroid-sensitive patients met the following conditions: the disappearance of proteinuria (negative to trace in urine for three consecutive days, or a urinary protein/creatinine level of <0.2 mg/mg) within the first 4 weeks of full dose prednisolone therapy (2 mg/kg/day or 60 mg/m^2/day). While steroid-resistant NS patients had nephrotic range proteinuria >40 mg/m^2/h, serum albumin level <2.5 g/dl, age >one year, the secondary causes of NS are absent; the follow-up duration was $\geq$6 months from the period of diagnosis and resistant to the steroid treatment of 60 mg/m^2/day for 4 months, followed by three pulse methylprednisolone dose of 1 g/1.73 m^2/48 h [47].

Genotyping of SNPs

Venous blood was collected in a sterile eppendorf tube containing ethylenediaminetetraacetic acid from each patient and was stored at −80 °C until the extraction of DNA. We isolated DNA by using a chemical method routinely used in our laboratory [48]. The purity and concentrations of the DNA were assessed by using a UV spectrophotometer (UV Prove v2.1) at 260 nm and evaluated 5 μl (50–70 ng/μl) of the sample at 2% (w/v) agarose gel. Genotyping was carried out using the technique of a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). *ABCB1* rs1128503 and rs2032582 SNPs were amplified according to the method published by Cizmarikova et al. [28] whereas rs1045642 and rs776746 were genotyped according to the methods of Balram et al. [49] and Islam et al. [50], respectively. The primers for *NR3C1* rs10482634 and rs6877893 SNPs were designed by primer blast, and their PCR-RFLP methods were developed in our laboratory. PCR products were observed on a 2% (w/v) agarose gel and visualized by UV light and photographed using a Gel Documentation and Analysis System. The sequences of the primers used in the study are listed in Table 1, and the required PCR conditions with fragmentation patterns are presented in Table 2.

Statistical analysis

Distributions of sociodemographic and clinicopathological variables were compared between PRG and PSG using χ^2-test and a two-sided unpaired *t*-test. The correlations between different sociodemographic and clinicopathological variables were established by using Pearson's correlation test. Genotype and allele frequencies were reported as a percentage. χ^2-test was also used to estimate the odds ratios (ORs) and their 95% confidence intervals (CIs) with the statistical software package SPSS (SPSS, v 25.0). The deviation of genotype frequencies was measured under the Hardy-Weinberg equilibrium (HWE) by using χ^2-test. Linkage disequilibrium (LD) and haplotype frequency calculations were performed SHEsis web-based program (http://analysis.bio-x.cn/) (53). The $p < 0.05$ (two-tailed) was considered statistically significant in all the analyses.

Table 1
Sequences of the designed primer with their size and melting temperature.

Genes & SNPs	Primer	Primer sequence (5′-3′)	T_m (°C)	Size (bp)
ABCB1	FP	TTACCCATCTCGAAAAGAAGTTAAGGT	65.5	27
1236C > T (rs1128503)	RP	TGCCCACTCTGCACCTTCATGTTC	73.3	24
ABCB1	FP	TTACCCAGAATATAGCAAATCTTGG	62.9	25
2677G > T (rs2032582)	RP	CATATTTAGTTTGACTCACCTTCTCAG	62.0	27
ABCB1	FP	TGCTGGTCCTGAAGTTGATCTGTGAAC	60.5	27
3435C > T (rs1045642)	RP	ACATTAGGCAGTGACTCGATGAAGGCA	61.6	27
NR3C1	FP	CACAGATACTTGACTTGGCTATGG	55.3	24
rs10482634	RP	AACACCTACTTATTTGAGCAGCTT	53.2	24
NR3C1	FP	AGAACTGGAGATTGCCAAGG	54.5	20
rs6877893	RP	AAAGCTGCATTTTAGCAGCA	52.2	20
CYP3A5	FP	CCTGCCTTCAATTTTTCACT	51.2	20
CYP3A5*3 (rs776746)	RP	GGTCCAAACAGGGAAGAGGT	56.9	20

*FP = Forward Primer; RP = Reverse Primer; T_m = Melting Temperature

Table 2
The restriction enzymes, digestion condition, PCR conditions and length of the expected fragments on digestion to diagnose genes.

Genes and SNPs	Restriction enzymes	Digestion condition	PCR conditions (35 cycles)	PCR product size (bp)	Expected fragments (bp)
ABCB1	*HaeIII*	Incubation at	95 °C for 30 sec	234	NH; TT: 234
1236C > T (rs1128503)		37 °C overnight	56 °C for 30 sec		HE; TC: 27,207,234
			72 °C for 30 sec		MH; CC: 27,207
ABCB1	*Hpy1881*	Incubation at	95 °C for 30 sec	198	NH; GG: 198
2677G > T (rs2032582)		37 °C overnight	56 °C for 30 sec		HE; GT: 25,173,198
			72 °C for 30 sec		MH; TT: 25,173
ABCB1	*MboI*	Incubation at	94 °C for 1 min	248	NH; TT: 248
3435C > T (rs1045642)		37 °C overnight	55 °C for 1 min		HE; TC: 56,192,248
			72 °C for 2 min		MH; CC: 56,192
NR3C1 rs10482634	*BstU1*	Incubation at 60 °C overnight	95 °C for 30 sec	300	NH; TT: 300
			56 °C for 30 sec		HE; TC: 118,182,300
			72 °C for 30 sec		MH; CC: 118,182
NR3C1	*HaeIII*	Incubation at 37 °C overnight	95 °C for 30 sec	225	NH; AA: 225
rs6877893			56 °C for 30 sec		HE; AG: 60,165,225
			72 °C for 30 sec		MH; GG: 60,165
CYP3A5	*RsaI*	Incubation at 37 °C overnight	95 °C for 30 sec	196	NH; AA: 94,102
CYP3A5*3 (rs776746)			56 °C for 30 sec		HE; AG: 20,74,94,102
			72 °C for 30 sec		MH; GG: 20,74,102

*NH = Normal Homozygote; HE = Heterozygote; MH = Mutant Homozygote

Results

Distributions of sociodemographic and clinicopathological characteristics

The distributions of sociodemographic and clinicopathological characteristics of all the recruited patients are summarized in Table 3. Among the enrolled patients, the mean age of onset was higher for PRG (9.07 ± 3.27 years) than for PSG (7.69 ± 3.40 years). A statistically significant difference was found in the body mass index (BMI) ($p = 0.044$) between the two groups, but no significant differences were found between the two groups for socioeconomic status and residential status.

A renal biopsy was performed among the 180 pediatric patients in 40 patients (30 from PRG and 10 from PSG). The results of the renal biopsy of 30 PRG patients were: mesangioproliferative glomerulonephritis (MesPGN) 13/30 (43.33%), membranoproliferative glomerulonephritis 6/30 (20%), focal segmental glomerulosclerosis 4/30 (13.33%), minimal change nephrotic syndrome 5/30 (16.67%), Immunoglobulin A nephropathy 1/30 (3.33%), and inadequate tissue 1/30 (3.33%). Biopsy results of the other ten patients were as follows: membranoproliferative glomerulonephritis 4/10 (40%) and minimal change nephrotic syndrome 6/10 (60%).

From the biochemical findings, the mean serum albumin level (PRG: 10.23 ± 0.65, PSG: 9.79 ± 0.79, $p = 0.003$) and mean serum cholesterol level (PRG: 435.32 ± 66.15, PSG: 404.65 ± 88.21, $p = 0.037$) were significantly greater in PRG than in PSG. However, the mean value of the urine protein/creatinine ratio of PSG (3.47 ± 0.80) was significantly higher than that of the PRG (3.14 ± 0.75 , $p = 0.011$).

We also observed some atypical and classical manifestations in the study population. Edema was the most common symptom, present in 100% of PRG and 97.33% of PSG. Massive proteinuria was presented in 100% of both PRG and PSG during their NS disease. A total of 60% of PRG was found to have microhematuria, whereas it was 20% in the case of PSG. Among others, persistent hypertension was found in 36.67% of PRG and 8.67% of PSG.

Several types of complications were seen during the NS, including 6.67% and 4.67% of PRG and PSG, respectively, had urinary tract infection, while respiratory tract infection was found in 3.33% of PRG and 2% of PSG. Cellulitis was 10% and 8% in the PRG and PSG, consecutively, whereas peritonitis was 16.67% in PRG and 14% in PSG. There were no obese patients among the PRG whereas, 2.67% of obese were found in the PSG. Malnutrition was also noted in 6.67% PRG and 4% PSG. In our study population, 6.67% of PRG had growth and development retardation.

Correlation between demographic data and clinicopathological characteristics

To established inter-variable correlations among different demographic data and clinicopathological characteristics between the PRG and PSG, we used Pearson's correlation test for the investigated data listed in Table 4. It indicated that there was a significant positive correlation between BMI and age ($r = 0.985$, $p < 0.005$), BMI and albumin ($r = 0.315$, $p = 0.045$), BMI and cholesterol ($r = 0.361$, $p = 0.025$), and age and cholesterol ($r = 0.361$, $p = 0.025$). On the other hand, age and albumin was positively

Table 3
Distribution of sociodemographic data and clinicopathological characteristics of prednisolone resistance group (PRG) and prednisolone sensitive group (PSG).

Characteristics		PRG (n = 30)	PSG (n = 150)	p-value
Age		9.07 ± 3.27	7.69 ± 3.40	0.061
Body mass index (kg/m^2)		18.62 ± 2.49	17.74 ± 2.58	**0.044**
Gender				
	Male	18 (60%)	95 (63.33%)	
	Female	12 (40%)	55 (36.67%)	0.311
Socioeconomic status				
	Upper class	2 (6.67%)	9 (6%)	
	Middle class	10 (33.33%)	54 (36%)	0.958
	Lower class	18 (60%)	87 (58%)	
Residence				
	Urban	10 (33.33%)	44 (29.33%)	0.663
	Rural	20 (66.67%)	106 (70.67%)	
Histological findings of the renal biopsy				
	Mesangioproliferative glomerulonephritis	13 (43.33%)	–	–
	Membranoproliferative glomerulonephritis	6 (20%)	4 (40%)	–
	Focal segmental glomerulosclerosis	4 (13.33%)	–	–
	Minimal change nephrotic syndrome	5 (16.67%)	6 (60%)	–
	Immunoglobulin A nephropathy	1 (3.33%)	–	–
	Inadequate tissue	1 (3.33%)	–	–
	Not done	–	140 (93.33%)	–
Biochemical findings				
	Serum albumin (gm/l)	10.23 ± 0.65	9.79 ± 0.79	**0.003**
	Serum cholesterol (mg/dl)	435.32 ± 66.15	404.65 ± 88.21	**0.037**
	Urine protein/creatinine ratio	3.14 ± 0.75	3.47 ± 0.80	**0.011**
Other parameters				
	Edema at the presentation moment	30 (100%)	146 (97.33%)	–
	Massive proteinuria	30 (100%)	150 (100%)	–
	Microhematuria	18 (60%)	30 (20%)	–
	Persistent hypertension	11 (36.67%)	13 (8.67%)	–
	Urinary tract infection	2 (6.67%)	7 (4.67%)	–
	Respiratory tract infection	1 (3.33%)	3 (2%)	–
	Cellulitis	3 (10%)	12 (8%)	–
	Peritonitis	5 (16.67%)	21 (14%)	–
	Obesity	0 (0.00%)	4 (2.67%)	–
	Malnutrition	2 (6.67%)	6 (4%)	–
	Growth and development retardation	2 (6.67%)	0 (0.00%)	–

Table 4
Correlation among different demographic data and clinicopathological characteristics of PRG and PSG of nephrotic syndrome patients.

Correlation parameters	r-value	p-value
BMI vs. Age	0.985	**<0.005**
BMI vs. Albumin	0.315	**0.045**
BMI vs. Cholesterol	0.361	**0.025**
BMI vs. Urine protein/creatinine ratio	−0.175	0.178
Albumin vs. Cholesterol	−0.337	**0.034**
Albumin vs. Urine protein/creatinine ratio	−0.309	**0.048**
Cholesterol vs. Urine protein/creatinine ratio	−0.609	0.358
Age vs. Albumin	0.284	0.064
Age vs. Cholesterol	0.361	**0.025**
Age vs. Urine protein/creatinine ratio	−0.170	0.185

*r = Correlation co-efficient; Negative values specify opposite correlation

correlated but not statistically significant (r = 0.284, p = 0.064). Same analysis revealed that albumin and cholesterol (r = -0.337, p = 0.034), albumin and urine protein/creatinine ratio (r = -0.309, p = 0.048) showed statistically significant (p < 0.05) negative correlation. The BMI and urine protein/creatinine ratio (r = -0.175, p = 0.178), cholesterol and urine protein/creatinine ratio (r = -0.609, p = 0.358), age and urine protein/creatinine ratio (r = -0.170, p = 0.185) were negatively correlated but not found statistically significant (p > 0.05).

Correlation of genetic variants with PRNS

The frequency distribution and association of 1236C > T, 2677G > T, 3435C > T, rs10482634, rs6877893, and rs776746 polymorphisms with PRNS are shown in Table 5.

In the case of *ABCB1* 1236C > T polymorphism, TC and TT genotypes were associated with 2.53 and 0.87 times lower risk of PR (OR = 2.53, 95% CI = 0.58–11.12; OR = 0.87, 95% CI = 0.37–2.04), and the results were not statistically significant (p > 0.05). Whereas, TC+TT was not significantly correlated with PR though it possesses 1.03 times higher risk (OR = 1.03, 95% CI = 0.47–2.27). The frequencies of PRG were 61.67% and 38.33% for C and T allele, respectively, while for the PSG, the distributions were 59.33% for C allele and 40.67% for T allele. The GT genotype in 2677G > T polymorphism revealed 3.9 times significantly increased association with PRNS (OR = 3.9, 95% CI = 1.11–13.70). The TT and GT+TT genotypes have shown 1.18 and 1.46 times higher influence to develop PR, respectively, but these results were not significant (OR = 1.18, 95% CI = 0.47–2.95 and OR = 1.46, 95% CI = 0.61–3.50). T allele frequency was slightly higher in PRG (63.33%) compared with PSG (62.01%) and showed a non-significant association (p = 0.846). For 3435C > T polymorphism, the C and T allele frequencies were 56.67% and 43.33%, respectively for PRG and 63.67% and 36.33%, respectively for PSG. However, T allele did not show any significant correlation with PR (p > 0.05). The TC genotype was significantly correlated with lower PR risk (OR = 0.38; 95% CI = 0.15–0.99). Again, the TT genotype was significantly associated with PR (OR = 3.06; 95% CI = 1.06–8.79), and it possesses 3.06 times more risk. TC+TT combined genotype was not significantly associated with PR (p > 0.05).

For rs10482634 polymorphism of the *NR3C1* gene, the AG genotype showed 2.40 times greater association with PR, and it was statistically significant (OR = 2.40, 95% CI = 1.07–5.40). The GG genotype was not significantly associated with PR though it possesses 1.80 times more risk (p = 0.609). On the other hand, the AG+GG genotype depicted 2.36 times significant association

Table 5
The frequency distribution and association of 1236C > T, 2677G > T, 3435C > T, rs10482634, rs6877893, and rs776746 polymorphisms with PRNS.

SNP	Genotype (Total = 180)	PRG (%) (n = 30)	PSG (%) (n = 150)	OR (95% CI)	p-value
ABCB1	CC (103)	17 (56.67%)	86 (57.33%)	Reference	
1236C > T (rs1128503)	TC (9)	3 (10%)	6 (4%)	2.53 (0.58–11.12)	0.219
	TT (68)	10 (33.33%)	58 (38.67%)	0.87 (0.37–2.04)	0.753
	TC+TT (77)	13 (43.33%)	64 (42.67%)	1.03 (0.47–2.27)	0.946
	C-allele	37 (61.67%)	178 (59.33%)	Reference	
	T-allele	23 (38.33%)	122 (40.67%)	0.91 (0.51–1.60)	0.737
ABCB1	GG (60)	8 (26.67%)	52 (34.67%)	Reference	
2677G > T (rs2032582)	GT (16)	6 (20%)	10 (6.67%)	3.9 (1.11–13.70)	**0.034**
	TT (104)	16 (53.33%)	88 (58.67%)	1.18 (0.47–2.95)	0.721
	GT+TT (120)	22 (73.33%)	98 (65.33%)	1.46 (0.61–3.50)	0.398
	G-allele	22 (36.67%)	114 (37.99%)	Reference	
	T-allele	38 (63.33%)	186 (62.01%)	1.06 (0.60–1.88)	0.846
ABCB1	CC (66)	13 (43.33%)	53 (35.33%)	Reference	
3435C > T (rs1045642)	TC (93)	8 (26.67%)	85 (56.67%)	0.38 (0.15–0.99)	**0.047**
	TT (21)	9 (30%)	12 (8%)	3.06 (1.06–8.79)	**0.038**
	TC+TT (114)	17 (56.67%)	97 (64.67%)	0.71 (0.32–1.58)	0.408
	C-allele	34 (56.67%)	191 (63.67%)	Reference	
	T-allele	26 (43.33%)	109 (36.33%)	1.34 (0.76–2.35)	0.308
NR3C1	AA (115)	14 (46.67%)	101 (67.33%)	Reference	
rs10482634	AG (60)	15 (50%)	45 (30%)	2.40 (1.07–5.40)	**0.033**
	GG(5)	1(3.33%)	4 (2.67%)	1.80 (0.19–17.31)	0.609
	AG+GG (65)	16 (53.33%)	49 (32.67%)	2.36 (1.06–5.21)	**0.034**
	A-allele	43 (71.67%)	247 (82.33%)	Reference	
	G-allele	17 (28.33%)	53 (17.67%)	1.84 (0.98–3.48)	0.059
NR3C1	AA (117)	18 (60%)	99 (66%)	Reference	
rs6877893	AG (5)	1 (3.33%)	4 (2.67%)	1.38 (0.15–13.02)	0.781
	GG (58)	11 (36.67%)	47 (31.33%)	1.29 (0.56–2.94)	0.549
	AG+GG (63)	12 (40%)	51(34%)	1.29 (0.58–2.89)	0.530
	A-allele	37 (61.66%)	202 (67.33%)	Reference	
	G-allele	23 (38.34%)	98 (32.67%)	1.28 (0.72–2.27)	0.397
CYP3A5	AA (29)	6 (20%)	23 (15.33%)	Reference	
CYP3A5*3 (rs776746)	AG (70)	9 (30%)	61 (40.67%)	0.57 (0.18–1.77)	0.327
	GG (81)	15 (50%)	66 (44%)	0.87 (0.30–2.51)	0.799
	AG+GG (151)	24 (80%)	127 (84.67%)	0.72 (0.27–1.97)	0.527
	A-allele	21 (35%)	107 (35.67%)	Reference	
	G-allele	39 (65%)	193 (64.33%)	1.03 (0.58–1.84)	0.922

(OR = 2.36, 95% Cl = 1.06–5.21). The minor G allele frequencies were 28.33% and 17.67% in PRG and PSG, respectively, and it was not associated with PR (p = 0.059). Compared with the AA genotype of rs6877893 variant, AG, GG, and AG+GG genotypes showed no significant association (p > 0.05) with PR (OR = 1.38, 95% Cl = 0.15–13.02; OR = 1.29, 95% Cl = 0.56–2.94; and OR = 1.29, 95% Cl = 0.58–2.89, respectively). The G allele percentages were 38.34% and 32.67% in PRG and PSG, consecutively and it showed no significant association (p = 0.397) with PR.

CYP3A5*3 polymorphism showed that AG, GG, and combined AG+GG genotypes were linked with lower risk of PRNS by 0.57, 0.87, and 0.72 times, respectively compared with AA genotype (OR = 0.57, 95% Cl = 0.18–1.77; OR = 0.87, 95% Cl = 0.30–2.51; and OR = 0.72, 95% Cl = 0.27–1.97, respectively) that were not statistically significant (p > 0.05). The percentage of G allele in PRG (65%) was higher than in PSG (64.33%) but was non-significantly associated with increased PR risk (p = 0.922).

Linkage disequilibrium and haplotype analysis for the ABCB1 and NR3C1

Linkage disequilibrium (LD) blocks among the haplotypes of rs1128503 (1236C > T), rs2032582 (2677G > T), and rs1045642 (3435C > T) in *ABCB1* gene (rs1128503 and rs2032582, D′ = 0.593, r^2 = 0.316; rs1128503 and rs1045642, D′ = 0.467, r2 = 0.194; rs2032582 and rs1045642, D′ = 0.360, r2 = 0.128) as well as rs10482634 and rs6877893 SNPs in *NR3C1* gene (D′ = 0.802, r^2 = 0.307) were constracted in case-control combined group (Figs. 1 and 2). Haplotype analysis results depicted in Table 6 shows that the frequencies of CTC and TGT haplotypes are significantly higher in the PRG than the PSG, and the CTC and TGT haplotypes were correlated with increased risk of PR (CTC: 16.7% vs. 4.3%, OR = 4.47, p = 0.0003; TGT: 12.3% vs. 4.9%, OR = 2.71, p = 0.03, respectively, for PRG and PSG). Similarly, the LD among the haplotypes of rs10482634 and rs6877893 of the *NR3C1* gene was detected, and the frequency of GA haplotype was relatively higher in the PRG than the PSG. It was associated with a higher risk of PR (GA: 6.8% vs. 1.7%, OR = 4.22, p = 0.022, respectively, for PRG and PSG).

Discussion

Nephrotic syndrome in children is one of the most prevalent forms of degenerative kidney disease. Glucocorticoids remain the hallmark of childhood NS treatment and response to initial oral prednisolone dictates disease prognosis. SRNS patients pose the most complex and difficult therapeutic challenge for clinicians and researchers. Despite the wide therapeutic range and efficacy of GCs in the induction of recovery, early markers that would allow optimization of their dose and duration of the therapy may improve the management of nephrotic patients [15,20]. No previous studies concerning the association of *ABCB1*, *NR3C1*, and *CYP3A5* polymorphisms with the risk of NS and patient's response to GCs have been reported in the Bangladeshi population.

No significant variations in the age distribution (p = 0.061) between PRG and PSG (PRG: 9.07 ± 3.27 years, PSG: 7.69 ± 3.40 y ears) were observed from the distribution of sociodemographic data and clinicopathological characteristics among the study

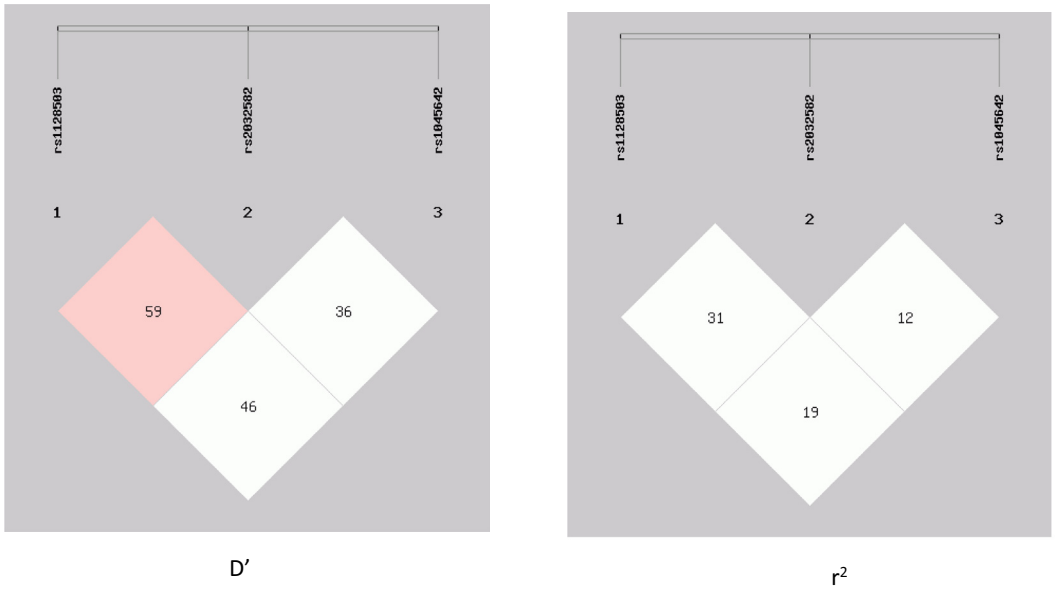

Fig. 1. D′ and r^2 values generated from the haplotyping of rs1128503, rs2032582, and rs1045642 SNPs in case-control combined group.

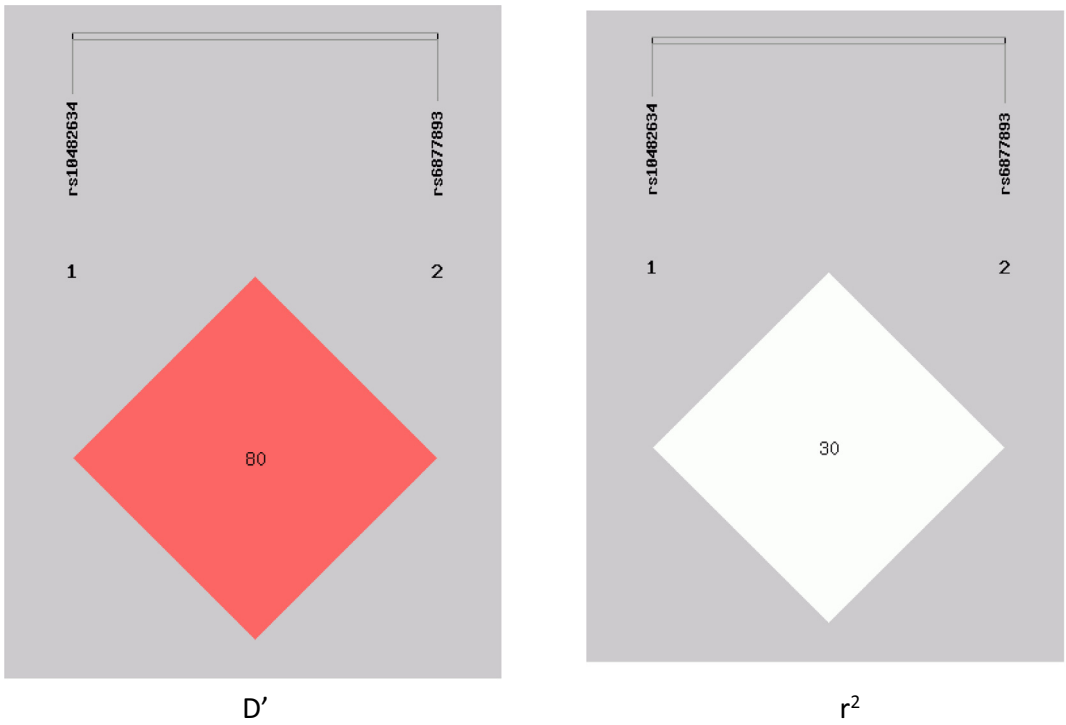

Fig. 2. Fig. 1: D′ and r2 values generated from the haplotyping rs10482634 and rs6877893 SNPs in the case-control combined group.

samples, which is comparable to the findings of Roy et al. [51]. Besides, the male and female ratio for PRG (1.5:1) and PSG (1.73:1) shows that male children were more sufferer from nephrosis than female children, which is consistent with the findings of Alam et al. and Siddique et al. [52,53]. Children from the lower socioeconomic class were more prone (60%) to develop PRNS than the middle and upper classes. This may be attributed to unhealthier lifestyles and poor food habits, and these results were close to that in other research [51,54]. In addition, a higher incidence of NS was found in rural children (66.67% in PRG and 70.67% in PRS) than that in urban children. In our country, urban peoples prefer to admit to the private hospital but not the public hospital, and we recruited all the patients from different public hospitals. So, this picture might not represent the actual situation but can be comparable with the findings of Sarker et al. [54].

We found that MesPGN was the underlying histopathology of PRNS, and its percentage was 43.33% in 30 patients of PRG. Our results were consistent with the report by Roy et al. (MesPGN = 40.63% in 32 SR patients) [51]. The occurrence of MesPGN differs from study to study as a cause of SRNS as well as SSNS with an inconvenient management protocol [55]. So, the MesPGN histopathology carrying children has the most significant risk of developing PRNS. Our study also found positive correlations among different demographic data and clinicopathological

Table 6
Haplotype analysis of *ABCB1* (1236C > T, 2677G > T, 3435C > T) and *NR3C1* (rs10482634, rs6877893) genes between PRG and PSG.

Haplotype	*ABCB1* C1236T, G2677T, and C3435T					
	PRG	PSG	χ^2	OR	95% CI	p-value
CGC	0.183	0.192	0.026	0.94	0.46–1.93	0.871
CGT	0.033	0.107	3.196	0.29	0.067–1.23	0.074
CTC	0.167	0.043	12.886	4.47	1.9–10.76	**0.0003**
CTT	0	0.064	3.147	0.001	0–0.012	0.076
TGC	0.027	0.031	0.026	0.87	0.16–4.74	0.872
TGT	0.123	0.049	4.704	2.71	1.07–6.86	**0.030**
TTC	0.056	0.097	1.014	0.56	0.17–1.77	0.314
TTT	0.410	0.416	0.007	0.98	0.56–1.72	0.935
Haplotype	***NR3C1* rs10482634 and rs6877893**					
	PRG	PSG	χ^2	OR	95% CI	p-value
AA	0.549	0.656	2.516	0.64	0.36–1.12	0.113
AG	0.168	0.167	0	1.01	0.48–2.12	0.983
GA	0.068	0.017	5.251	4.22	1.12–15.99	**0.022**
GG	0.215	0.16	1.099	1.44	0.73–2.87	0.295

characteristics between the PRG and PSG, such as BMI vs. age or albumin or cholesterol, and age vs. cholesterol. However, age and albumin were positively correlated but not statistically significant ($p > 0.05$). Albumin vs. cholesterol and albumin vs. urine protein/creatinine ratio also found to be negatively correlated.

Around 50 SNPs have been identified in the gene *ABCB1* to date [15], and 1236C > T is one of the most common variants. Multiple case-control studies have shown no association between the risk of PRNS and the 1236C > T SNP polymorphism in different ethnic groups, including North Indian [17], Slovakian [28], Indian [27], Egyptian [15,56], and Polish population [23]. We also found that 1236C > T SNP did not appear to have an association with SRNS patients. However, a study on Finnish patients found a significant association for both genotype and allele frequencies [22]. A *meta*-analysis found an association with increased SR risk in Asian and Caucasian cohorts [29]. Another study showed an increased SR risk of the 1236C > T allele when combined with 2677 T > A [17]. A significant difference in the distribution of 1236C > T genotypes suggested a linkage with SRNS ($p = 0.012$) [25]. Moreover, rs1128503 exhibits strong linkage disequilibrium with other functional polymorphisms such as rs2032582 [57].

Some of the earlier studies have reported the association between the 2677G > T polymorphism and PRNS. A study in the Egyptian population found significantly higher SR risks among the GT, GA, and TT+AA genotypes carrier patients ($p = 0.001$, $p = 0.011$, and $p = 0.001$, respectively) when compared with healthy children [15]. In our study, only GT heterozygous genotype is significantly associated with PR ($p = 0.034$). Moussa et al. [43] investigated with Tunisian children and confirmed the association with NS susceptibility (OR = 3.50, $p < 0.001$) and steroid-resistance (OR = 3.07, $p = 0.048$). Another study [17] found that the frequency of mutant homozygous genotype was significantly higher in SRNS patients than SSNS patients, and it was associated with 3.39 times more risk to develop PR ($p < 0.05$). In the present study, TT mutant homozygous genotype has 1.18 times increased, but non-significant risk association with PR ($p = 0.721$). However, GT heterozygous genotype is significantly associated compared to GG wild-type ($p = 0.034$), whereas a study on the Indian population confirmed a significant association GT genotype with steroid-resistance [27]. Some other studies also found a significant association of 2677G > T polymorphism with steroid resistance [22,29,56]. Our findings are in harmony with these studies. However, some studies disagreed with our findings, including studies on Chinese [24], South Indian [58], Slovakian [28], Korean [26], and Polish population [23].

Multiple studies have revealed that certain SNPs of the *ABCB1* gene leads to change in P-gp expression and function between different ethnicities and subjects, with the 3435C > T SNP being the target of most research [26]. Studies found an association of 3435C > T polymorphism with SRNS in South Indian children [58], Finnish patients [22]. A strong correlation with NS has been found in another study by Wasilewska et al. [23]. These findings are consistent with our study, while different studies on different ethnic groups, including studies on North Indian [17], Slovakian [28], Egyptian [15], Chinese [24], and Indian population [27] were found to be inconsistent. On the other hand, the distribution of genotypes between SS and SR patients was found substantially different [25], whereas Choi et al. found no significant difference in the distribution of genotypes and allele frequencies between the NS group and the control group.

We found an association of CTC and TGT haplotypes after performing the haplotype analysis among the rs1128503, rs2032582, and rs1045642. Gasic et al. reported similar findings for the treatment of leukemia with glucocorticoid [59]. Some other studies also reported the association of *ABCB1* haplotypes with steroid resistance [17,22,60].

It is well known that the response to GC treatment is variable in patients with idiopathic NS in childhood and has been analyzed in several studies [36,37,61]. As far as we know, no previous genetic studies or any other citation of rs10482634 polymorphism based on the NS was found. It is the world's first-reported genetic analysis based on this SNP related to SR. So, we have found no studies like this to compare our findings. However, we had tried to compare with other polymorphisms of the *NR3C1* gene in different ethnic groups. An in vitro study conducted by Sher et al. [62] has demonstrated that *NR3C1* gene polymorphisms can alter the response to GC treatment. Another study [63] has found that two polymorphisms of the *NR3C1* gene (ER22/23EK and N363S) directly affected glucocorticoid-regulated gene expression. In addition, the GR-9β polymorphism increases the expression of the mature GR-β protein leading to steroid-resistance [64]. Liu et al. [65] reported an increased expression of GR-β in peripheral blood mononuclear cells of SR patients, while Szilagyi et al. [66] found that GR-α was correlated with a positive steroid-response. Another investigation in patients with pemphigus vulgaris showed that rs11745958C > T and rs17209237A > G increase GC resistance [67]. Our study revealed that individuals carrying AG heterozygous and combined AG+GG genotype were significantly associated with PR. So, polymorphism of *NR3C1* might be involved in the development of prednisolone resistance in our study population.

In the current study, we assessed another SNP rs6877893 polymorphism of the *NR3C1* gene. We have found only two studies regarding rs6877893 SNP on the Chinese population. A study reported that the haplotype TG of two SNPs (rs6877893 and

rs4912905) was associated with a decreased risk of infantile spasms (p = 0.038), and rs6877893 SNP was associated with the responsiveness of adrenocorticotropic hormone [68]. We also found the association of GA haplotype of rs10482634 and rs6877893 of *NR3C1* gene with prednisolone resistance. The association of *NR3C1* gene haplotypes with steroid resistance was also reported by some previous studies [59,60]. However, another study [59] in Han Chinese patients with high-altitude pulmonary edema showed the A and G allele frequency of both patient and control group was 81.6%, 18.4% vs. 71.4%, 28.6%, respectively whereas, in our study, the A and G allele frequency of both cases and controls was 61.66%, 38.34% vs. 67.33%, 32.67%, respectively. Furthermore, several studies analyzed no association of different polymorphisms of the *NR3C1* gene and steroid-resistance, including Finnish [28], Dutch [70], Chinese [37] and Korean population [71]. Similarly, we found no significant association between rs6877893 polymorphism and PRNS.

Previous studies in renal transplant recipients with the G allele of CYP3A5*3 displayed higher blood tacrolimus concentrations and required a lower dosage than those with the A allele of CYP3A5*1 [72,73]. Thus, it may be expected that the frequency of the G allele would be lower in PRG than in PSG. A study showed that the frequency of the G allele of 6986A > G SNP was higher in steroid-resistant subjects (84%) than in steroid-sensitive ones (67%), showing an association but did not reach statistical significance (p = 0.059) [24]. Our study revealed that the frequency of G allele was slightly higher in PRG than PSG (65% and 64.33%, respectively) that depicts the association to PR, but it did not reach statistically significant (p = 0.922). A study on Brazilian kidney transplant patients [74] reported that the CYP3A5*3 allele was positively associated with higher tacrolimus bioavailability, confirming previous findings. A recent study on the Chinese population found no significant correlation between the CYP3A5*3 genotype and the efficacy of tacrolimus in the treatment of NS patients [75]. From a study on lung cancer patients, a non-significant increased risk was found with CYP3A5*3 polymorphism [50], and a similar result was also found in Caucasians [76], and these findings are consistent with our study population. Another study revealed a significant association of CYP3A5*3 gene polymorphism with prostate cancer risk in the Bangladeshi subjects [77]. Our results showed no significant association of CYP3A5*3 with steroid-resistance (p = 0.922) in the studied population.

In two-thirds of early-onset cases, mutations in the LAMB2, NPHS1, NPHS2 and WT1 genes are responsible for causing nephrotic syndrome. According to gnomAD and 1000 genome database, the frequencies of MAF alleles of LAMB2, NPHS1, NPHS2 and WT1 genes are very low or rare in the south Asian (SAS) population or in the Bengali in Bangladesh (BEB) population. This indicates that there may be a low chance of the involvement of the mutations of these genes in the development of SRNS in Bangladeshi children.

The drawback of this present study is the relatively small number of patients as more patients of this type was not found. So, more extensive multicentric studies and across other ethnic groups are needed to elucidate the contradictory implications of SNPs of the *ABCB1*, *NR3C1*, and *CYP3A5* genes. Besides, we did not assess the status of the podocyte monogenic genes, which should be evaluated in future studies. Large-scale cohort studies in different areas also need to be conducted to better understand the association between gene polymorphisms, allele frequency, and disease conditions. Despite these limitations, this is the first-time study on the Bangladeshi pediatric NS, and our results are also promising. In addition, we recruited 180 NS pediatric patients that are comparatively higher or quite similar in some cases to the sample size of published articles.

Conclusion

We observed that sociodemographic and clinicopathological factors might affect the responsiveness of prednisolone in pediatric PRNS. Besides, 3435C > T and 2677G > T allele of the *ABCB1* gene and rs10482634 variant of the *NR3C1* gene are significantly associated with PRNS. However, 1236C > T of the *ABCB1* gene, rs6877893 of the *NR3C1* gene and CYP3A5*3 of the *CYP3A5* gene were not significantly associated with prednisolone resistance in the Bangladeshi population. So, before starting the treatment of NS, it is essential to find out the types of disease by the genetic study with these investigated genes and other podocyte genes. After the investigation, we can give the patients a safer and effective individualized treatment plan to save many precious lives as well as costs. This would improve drug effectiveness and prevent adverse effects, particularly for drugs such as multi-toxic steroids.

Compliance with ethics requirements

The Ethical Committee of the Bangladesh Institute of Child Health approved the study protocol and consent form (BICH-ERC-10/2/2015).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author contributions

MSI (3), MH, MSI (1) collectively conceived, designed, checked, and supervised the research work. MNP, SNIR, and MMAAM undertook the collection and processing of the samples, extraction of DNA, completion of PCR and digestion of PCR products. MNP, MAA, MMAAM, SNIR, MH involved in the curation of data and data analysis. MNP, MAA, MSI (2), MSI (3), and interpreted the results and prepared the draft of the manuscript. All authors read and revised and remarked on different modified forms of the original drafted copy. MSI (3) revised and compiled the final manuscript.

Data Availability

All the data in the present study are available from the corresponding authors upon a reasonable request.

Consent for Publication

Have consent for publication.

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