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ABCB1 polymorphisms and steroid treatment in children with idiopathic nephrotic syndrome

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ABSTRACT

Background: The most common cause of nephrotic syndrome (NS) is idiopathic nephrotic syndrome (INS), also called nephrosis. Although most patients respond to steroid therapy, there is unequal response to treatment suggesting the involvement of genetic factors. The current study was conducted to evaluate the influence of two single nucleotide polymorphisms (SNPs) in *ABCB1* (C3435T and C1236T) on the steroid treatment response in INS children.

Materials and methods: Genotyping of *ABCB1* C3435T and C1236T polymorphisms by real time PCR were conducted on 120 INS children, 80 steroid sensitive (SS) and 40 steroid resistant (SR).

Results: A significant difference in the distribution of *ABCB1* C3435T and C1236T genotypes was observed between SS and SR patients. C1236T polymorphism was associated with steroid resistance in INS children (odds ratio: 2.27, 95 % confidence interval: 1.2–4.4; $P = 0.012$). The frequency of the T allele was significantly higher in SR than in SS patients (81.2 vs. 65.6%, respectively). The odds ratio for the C3435T polymorphism in response to steroid treatment was smaller than that of the polymorphism C1236T, and did not reach statistical significance (odds ratio: 1.1, 95 % confidence interval: 0.6–1.9; $P = 0.77$).

Conclusion: Our results suggested that C1236T polymorphism in *ABCB1* gene was associated with steroid resistance. A higher proportion of SR children had C1236T TT genotype and T allele, these patients may require other therapeutic strategies.

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Introduction

Nephrotic syndrome (NS) is one of the common chronic diseases observed in the childhood. In children, the most frequent type of NS is idiopathic nephrotic syndrome (INS), it constitutes about 90% of NS in the childhood and its incidence is 2–7 per 100.000.[1,2] In USA and Europe, the annual incidence in children is about 1–3 per 100,000 children below the age of 16. There is a male preponderance in children, with a male: female ratio of 2:1 but both sexes are similarly affected in adolescence.[3] INS is diagnosed by the presence of oedema, proteinuria, hypoalbuminaemia and hyperlipidaemia with non-specific glomerular abnormalities of the kidney.[4,5] The patient with INS shows varied response to standard steroid therapy, about 70–80% of patients achieve complete remission,[6] of which about 40–50% of these patients show frequent relapses.[7]. This variable response to steroid therapy is observed when glucocorticoids are recommended as the first-line therapy which may be attributed to the immune-inflammatory characteristics of the disease and/or to genetic factors.[8] Patients with a poor steroid response have an unfavourable prognosis and may develop end-stage renal failure.[9] Therefore, it is essential to identify risk factors that contribute to failure of immunosuppressive therapy in order to optimise the treatment of children with INS.

Single nucleotide polymorphisms (SNPs) have been identified in the adenosine triphosphate-binding cassette B1 *(ABCB1)* gene, also named multidrug resistance-1 (MDR1) gene which consists of 28 exons and is located on chromosomal region 7q21. A multidrug transporter P-glycoprotein is encoded by this gene and acts as an active transmembrane efflux pump and is therefore important in the absorption, tissue targeting and elimination of a broad range of drugs, therefore, changes in the levels and/or function of P-glycoprotein may be one of the possible mechanisms of drug resistance.[10,11] Previous studies have shown that gene expression and the activity of P- glycoprotein may be affected by *ABCB1* polymorphisms and the *ABCB1* haplotype derived from C1236T, G2677T/A and C3435T polymorphisms is related to the pharmacokinetics of digoxin.[12]

The primary aim of this study was to compare the genotype and allele frequency distributions of C3435T and C1236T polymorphisms among steroid sensitive (SS)

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and steroid resistant (SR) INS children and to study the influence of these two SNPs on the steroid treatment response in these children, which may be useful for predicting the treatment response of children with INS.

Materials and Methods

Patients

This study was carried out at the Medical Biochemistry and Pediatric departments, Faculty of Medicine, Menoufia University, Egypt. The study was approved by ethical committee of Faculty of Medicine, Menoufia University, and included 120 children with INS. They were diagnosed according to the criteria of the International Study of Kidney Disease in children, including hypoalbuminaemia (serum albumin <25 g/l), hyperlipidaemia (serum cholesterol >5.18 mmol/l), proteinuria (>40 mg/h/m^2) and oedema [13]. They were classified into two groups. Group I included 80 children with steroid-sensitive (SS) INS (disappearance of proteinuria within one month of prednisolone therapy, 2 mg/kg/day). They were classified into three subgroups, subgroup Ia: included 37 children with infrequent relapse (IR) (less than 2 relapses within 6 months of initial response or less than 4 relapses within a period of 1 year), subgroup Ib: included 23 children with frequent relapse (FR) (2 or more relapses within 6 months of initial response or 4 or more relapses within a period of 1 year) and subgroup Ic: included 20 children with steroid dependence (SD) (2 consecutive relapses during corticosteroid therapy or within 14 days after cessation of therapy). Group II included 40 children with steroid-resistant (SR) INS (persistence of proteinuria after one month of prednisolone therapy). A written informed consent was obtained from the legal guardians of the children included in the study.

All subjects were submitted to the following: full history taking, general and local clinical examination, urine analysis, measurement of serum levels of total cholesterol, albumin, total bilirubin, urea and creatinine, radiological investigations including abdominopelvic ultrasonography, and CT. Specific investigations included renal biopsy and histopathological examination, and analysis of the genetic polymorphisms of *ABCB1* (C1236T and C3435T) using real-time PCR.

Laboratory

After 10 h of overnight fasting, 6 ml of venous blood were withdrawn and divided into two tubes; 3 ml blood were collected into plain tube, allowed to clot for 30 min and centrifuged for 15 min at 1006 relative centrifugal force (rcf) and the serum obtained was kept frozen at –20°C for assay of serum levels of total cholesterol, albumin, total bilirubin, urea and creatinine using autoanalyzer SYNCHRON CX9ALX (Beckman Coulter Inc., CA, USA). The remaining 3 ml blood was collected into ethylene diamine tetra acetic acid (EDTA, 5.4 mg) containing tube and genomic DNA was extracted from the peripheral whole blood with the Qiagen extraction kit (Hilden, Germany), according to the manufacturer's protocol. DNA eluted in buffer AE was stored at –20 ° C for further PCR procedure.

Genotyping of the C1236T and C3435T polymorphisms of *ABCB1* was carried out by real-time PCR (RT-PCR) by allele discrimination using TaqMan® SNP Genotyping (Applied Biosystem, USA). Two primers and two probes were used for each SNP, the primers were used for the identification and amplification of the DNA sequence that contains the SNP. The two probes were designed with different fluorescent dyes, normal probe targeting the wild-type sequence and polymorphic probe targeting the mutant sequence with the SNP. For the C1236T (rs 1128503) SNP, the two probes used are probe 1: VIC labeled: 5′-CAGGTTCAGGCCCTTC-3′ and probe 2: FAM labeled: 5′-CAGGTTCAGACCCTTC-3′ and the primers are; forward: 5′-TTCTCACTCGTCCTGGTAGATCTT-3′ and reverse: 5′-CTGCCCACTCTGCACCTT-3′. For the C3435T (rs1045642) SNP, the two probes used are probe 1: VIC labeled: 5′-CCCTCACGATCTCTT-3′ and probe 2: FAM labeled: 5′-CCCTCACAATCTCTT-3′ and the primers are; forward: 5′-GCCGGGTGGTGTCACA-3′ and reverse: 5′-ATGTATGTTGGCCTCCTTTGCT-3′ (Qiagen, Applied Biosystems, USA).

Real-time PCR was performed in 25-µl reaction mixtures containing 12.5 µl of 2 × TaqMan Universal Master Mix (Applied Biosystems, U.S.A), 1.25 µl of 20 × SNP Genotyping Assay (primers and probes), 10.25 µl of RNase- and DNase-free water and 1 µl of DNA template. DNase-free water used as negative control was included in each assay run. The cycling conditions include a 10 min of pre-denaturation at 95 °C (AmpliTaq Gold® DNA polymerase activation), followed by 50 cycles with a fast denaturation at 95 °C for 15 s, annealing of the TaqMan probes to its complementary sequence and extension of the primers by AmpliTaq Gold® DNA polymerase for 1 min at 60 °C. After assay completion, the 96-well PCR plates were read on an Applied Biosystems 7500 Real-Time PCR System with endpoint analysis mode of the SDS v1.3.1, which uses the fluorescence measurements made during the plate read to plot fluorescence (Rn) values based on the signals from each well. Three genotypes for each of the two polymorphisms were detected; normal homozygous, mutant homozygous and mutant heterozygous. Allelic discrimination was performed by inspecting the fluorescence from the probe.

Statistical analysis was performed using the SPSS 20 software package. Chi-square test is used to study association between two qualitative variables. The difference between 2 groups was performed by student's *t*-test and Mann–Whitney U test for parametric and nonparametric variables, respectively. ANOVA test

Table 1. Demographic and clinical characteristics of the studied groups.

	Steroid sensitive group	Steroid resistant group	Test (*P*-value)
n (%)	80 (66.7)	40 (33.3)	–
Age of onset (year)	5.7 ± 1.1	12.2 ± 2.8	*$P < 0.001$
Gender: *n* (%)			
Male	46 (57.5)	28 (70.0)	#$P = 0.2$
Female	34 (42.5)	12 (30.0)	
Hypertension: *n* (%)	19 (23.8)	23 (57.5)	#$P < 0.001$
Haematuria: *n* (%)	3 (3.8)	27 (67.5)	#$P < 0.001$
Proteinuria: (mg/h/m^2)	2.9 ± 0.4	3.6 ± 0.3	*$P < 0.001$
Renal biopsy:			
No biopsy	57 (71.3)	0 (0.0)	#$P < 0.001$
MCD	23 (28.7)	11 (27.5)	
FSGS	0 (0.0)	18 (45.0)	
MPG	0 (0.0)	11 (27.5)	

Note: MCD: Minimal change disease, FSGS: Focal segmental glomerulosclerosis, MPG: Membranous glomerulonephritis. *t: t test. #χ^2: Chi-square test.

Table 2. Statistical comparison between the studied groups regarding laboratory parameters.

	SS group (*n* = 80)				
	IFR (*n* = 37)	FR (*n* = 23)	SD (*n* = 20)	SR group (*n* = 40)	Test (*P* value)
Proteinuria (mg/h/m^2)	2.0 ± 0.3	2.1 ± 0.2	2.8 ± 0.5	3.4±0.7	¶$P < 0.001$
		Total of SS group: 2.2 ± 0.5			*$P < 0.001$
Serum albumin (g/l)	26 ± 3.0	23 ± 3.0	22 ± 3.0	21±5.0	¶$P < 0.001$
		Total of SS group: 24 ± 3.0			*$P = 0.002$
TC (mmol/l)	8.9 ± 1.5	10.0 ± 1.1	11.6 ± 1.7	11.4±1.6	¶$P < 0.001$
		Total of SS group: 9.9 ± 1.8			*$P = 0.004$
Total bilirubin (µmol/l)	85.5 ± 15.4	78.7 ± 13.7	63.3 ± 10.3	58.1±17.1	¶$P < 0.001$
		Total of SS group: 78.7 ± 17.1			*$P < 0.001$
Serum creatinine (µmol/l)	44.2 ± 17.7	61.9 ± 26.5	61.9 ± 17.7	70.7±17.7	$P = 0.03$
		Total of SS group: 53.0 ± 17.7			§$P = 0.013$
Serum urea (mmol/l)	6.9 ± 2.1	7.5 ± 2.1	6.3 ± 1.2	10.8±3.6	¶$P < 0.001$
		Total of SS group 6.9 ± 1.9			*$P < 0.001$

Note: IFR: infrequent relapse, FR: frequent relapse, SD: steroid dependent. ¶F: ANOVA test. *t: *t* test. $K: Kruskal-Wallis. §U: Mann–Whitney, numbers are expressed as mean ± SD.

(parametric test) and Kruskal–Wallis test (nonparametric test) are used for comparison between three or more groups. Odds ratio, describe the probability that people who are exposed to a certain factor will have a disease compared to people who are not exposed to this factor. A *P*-value of <0.05 was considered statistically significant.

Results

There was a statistically significant difference among the studied groups regarding age of onset, hypertension and haematuria. Twenty-three SS patients had frequent relapse and showed minimal change disease (MCD) on renal biopsy. Focal segmental glomerulosclerosis (FSGS) was the most common histopathological subtype. MCD and membranous glomerulonephritis (MPG) were the second common histological patterns (Table 1).

SR children had statistically significant increased proteinuria compared with SS children. Serum levels of total cholesterol, creatinine and urea were significantly increased while serum levels of albumin and total bilirubin were significantly decreased in SR children compared with SS children (Table 2).

The genotypes of C1236T and C3435T polymorphisms in *ABCB1* were determined in 120 pediatric patients with INS and the correlation between the two SNPs and treatment response was analysed. Regarding the *ABCB1* C3435T genotypes of INS patients and response to prednisolone therapy, there was a significant difference between SS and SR patients with respect to genotype frequency distribution, but no significant difference between both groups regarding the allele frequency distribution (Table 3). There was no significant difference existed between SS subgroups and SR patients with respect to genotype frequency distribution (Table 4). Regarding the *ABCB1* C1236T genotypes of INS patients and response to prednisolone therapy: there was a significant difference between SS and SR patients with respect to genotypes distribution and allele frequency distributions (Table 5), whilst no significant difference existed between SS subgroups and SR patients with respect to genotype frequency (Table 6).

Discussion

Steroid response is one of the most important prognostic factors of INS. Approximately 10–20% of children with nephrotic syndrome who do not completely respond to corticosteroids are qualified as SR,[14] that is associated with poor outcomes, including progression to

Table 3. Genotype and allele frequencies of C3435T polymorphism among SS and SR patient groups.

	Genotype (*n*,%)			Allele (*n*,%)	
Steroid sensitive group (*n* = 80)	CC	CT	TT	C	T
Steroid resistant group (*n* = 40)	32 (40) 20 (50)	41 (51.25) 11 (27.5) $^{\#}P$ = **0.019**	7 (8.75) 9 (22.5)	105 (65.6) 51 (63.75) $^{\#}P$ = 0.77 OR = 1.10 95% CI: 0.6–1.9	55 (34.4) 29 (36.25)

Note: OR: Odd's ratio, CI: confidence interval. $^{\#}\chi^2$: Chi-square test. Bold value significant (P<0.05).

Table 4. Genotype frequency of C3435T polymorphism among IFR, FR, SD and SR patients.

	Steroid sensitive (*n* = 80)				
	IFR (*n* = 37) (*n*, %)	FR (*n* = 23) (*n*, %)	SD (*n* = 20) (*n*, %)	Steroid resistant (*n* = 40) (*n*, %)	Test *P*-value)
CC	12 (32.4)	12 (52.2)	8 (40)	20 (50)	$^{\#}P$ = 0.104
CT	22 (59.5)	9 (39.1)	10 (50)	11 (27.5)	
TT	3 (8.1)	2 (8.7)	2 (10)	9 (22.5)	

Note: IFR: infrequent relapse, FR: frequent relapse, SD: steroid dependent. $^{\#}\chi^2$: Chi-square test.

Table 5. Genotype and allele frequencies of C1236T polymorphism among SS and SR patient groups.

	Genotype (*n*,%)			Allele (*n*,%)	
Steroid sensitive group (*n* = 80)	CC	CT	TT	C	T
Steroid resistant group (*n* = 40)	8 (10) 2 (5)	39 (48.8) 11 (27.5) $^{\#}P$ = **0.025**	33 (41.2) 27 (67.5)	55 (34.4) 15 (18.8) $^{\#}P$ = **0.012** OR= 2.27 95% CI: 1.2–4.4	105 (65.6) 65 (81.2)

Note: OR: Odd's ratio, CI: confidence interval. $^{\#}\chi^2$: Chi-square test. Bold value significant (P<0.05).

Table 6. Genotype frequency of C1236T polymorphism among IFR, FR, SD and SR patients.

	Steroid sensitive (*n* = 80)				
	IFR (*n* = 36) (*n*,%)	FR (*n* = 24) (*n*,%)	SD (*n* = 20) (*n*,%)	Steroid resistant (*n* = 40) (*n*,%)	Test (*P*-value)
CC	2 (5.6)	4 (16.6)	2 (10)	2 (5)	$^{\#}P$ = 0.091
CT	17 (44.4)	12 (54.2)	10 (50)	11 (27.5)	
TT	18 (50)	7 (29.2)	8 (40)	27 (67.5)	

Note: IFR: infrequent relapse, FR: frequent relapse, SD: steroid dependent. $^{\#}\chi^2$: Chi-square test.

end-stage renal disease.[15] In this study, the SS children represent 66.7% and the SR children represent 33.3% of the enrolled children, these results are consistent with that reported by previous studies.[6,16] Also, in our study, there was a male predominance (1.6:1) and the mean age of onset was 5.7 years in SS and 12.2 years in SR INS patients, these results were in agreement with Hacıhamdioğlu et al. [1]

The present study showed that the three major causes of steroid resistance in INS patients were FSGS (45%), MCD (27.5%) and MPG (27.5%). In a previous study by Ibrahim et al. [14], the three major causes of steroid resistance were FSGS (30.2%), MCG (24.5%) and IgA nephropathy (13.2%). The predominance of FSGS in many studies might be explained by that FSGS could be a primary disease, or secondary complicating other glomerular lesion. [17] The reasons for disparities in the prevalence of MCD and FSGS reported in different previous studies might be due to demographic and environmental factors,[18–20] as well as the difficulty of histologic distinction between both histological pattern.

There was a statistically significant increase in proteinuria and serum levels of total cholesterol, creatinine and urea in SR compared with SS INS children, while there was a statistically significant decrease in serum albumin and total bilirubin levels in SR compared with SS INS children. These results are in agreement with that of Mortazavi and Khiavi[9]

The present study showed that *ABCB1* genotype distributions of the two tested SNPs were significantly different between the SS and SR patient groups. However, there was no significantly difference between SS subgroups and SR patients. As regards *ABCB1* C3435T polymorphism, there was no statistically significant difference between SS and SR patient groups regarding the allele frequency distribution, meanwhile, *ABCB1* C1236T allele frequency distributions were significantly different between both patient groups, the frequency of the T allele statistically significantly increased in SR patients compared with SS patients (81.2 vs. 65.6%). These results indicate that C1236T in the *ABCB1* gene was associated with an increased incidence of steroid resistance.

These results are in agreement with that of Chiou et al. [16] and Eddy and Symons [2], whilst Choi et al. [17] found significantly higher frequencies of the *ABCB1* C1236T CC genotype and C allele in the initial steroid responder than in nonresponder. Youssef et al. reported that steroid non-responders had significantly higher frequencies of *ABCB1* C3435T TT genotypes than responsive INS patients.[18] However, a previous study on SS nephrotic syndrome patients, reported that both SNPs were associated with response time to prednisone, and the frequencies of T allele of the two SNPs were higher in late responders (time to remission >7 days) than in early responders (time to remission <7 days).[21] In contrast to our study, a previous Indian study found that there was no association between C1236T polymorphism with steroid response in INS children,[22] also a previous study on Slovak children revealed no significant differences in the distribution of *ABCB1* haplotypes between initial steroid responder and non-responder.[8] This difference may be due to racial and/or ethic factors.

Changes in the P-glycoprotein (the product of *ABCB1*) are one of the possible mechanisms of drug resistance as it acts as an active transmembrane efflux pump for a variety of toxins and many drugs (including prednisone) which are also transported by P-glycoprotein and may induce its expression.[11,19] P-glycoprotein may also actively participate in the chronic inflammation in autoimmune diseases and probably participates in releasing certain inflammatory mediators.[20] Gene expression and the activity of P-glycoprotein may be affected by *ABCB1* polymorphisms, C1236T (rs1128503, silent SNP) and C3435T (rs1045642, silent SNP). The C3435T polymorphism may have some effect on DNA structure, RNA stability and P-glycoprotein function. In most studies, the T allele is associated with decreased P-glycoprotein function.[23–25] The C1236T polymorphism may affect translation regulation, RNA stability, and it was shown that TT genotype minimises P-glycoprotein activity.[26]

Inconsistent results were obtained from previous studies on different ethnic populations when they evaluated the distribution of the *ABCB1* polymorphisms in children with INS,[16,27,28] also the allele frequencies of C1236T were quite different between different ethnic populations which may affect the association results with steroid therapy. These inconsistent results may be attributed to different ethnicities, food and drug interactions,[29,30] also, the unknown pathophysiology of the diseases and the several histological patterns included in its diagnosis might make the homogeneity of the tested groups unclear, finally, the P-glycoprotein has functions other than those directly related to transport activity and there are also other gene mutations associated with steroid resistance.[31]

This work represents an advance in biomedical science because it shows that the C1236T polymorphism in *ABCB1* was associated with steroid resistance. A higher proportion of SR children had C1236T TT genotype, and the frequency of T allele was higher in SR children than SS children. These findings suggest that patients with the *ABCB1* C1236T TT genotype tend to be resistant to initial steroid treatment and may be in need of other therapeutic strategies.

Summary table

What is known about this subject
- Changes in the P-glycoprotein (the product of *ABCB1*) are one of the possible mechanisms of drug resistance.
- *ABCB1* genotype distributions of the two tested SNPs are significantly different between the SS and SR INS children.

What this paper adds:
- The genotype distributions of the two tested SNPs were not significantly different between SS subgroups (IFR, FR and SD) and SR children.
- The C1236T polymorphism in *ABCB1* is associated with steroid resistance, and patients with the ABCB1 C1236T TT genotype may be in need for other therapeutic strategies.

Disclosure statement

No potential conflict of interest was reported by the authors.

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