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Novel Variants in the *MDR1* Gene with the Potential to Discriminate Steroid Responsiveness in Iraqi Children with Idiopathic Nephrotic Syndrome

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Abstract

Steroid-resistant idiopathic nephrotic syndrome (SRNS) is a leading cause of childhood end-stage renal disease, with diverse histological features and a challenging diagnosis. SRNS has been suggested to develop due to variations in the multidrug resistance 1 (*MDR1*) gene, with variable results from different studies. This study explored the presence of novel variants in the *MDR1* gene and evaluated their association with steroid responsiveness in Iraqi children with idiopathic nephrotic syndrome. This case-control study included children with SRNS (n=30) and steroid-sensitive nephrotic syndrome (SSNS; n=30) from the pediatric nephrology clinic in Babylon Hospital for Maternity and Pediatrics. Data were collected over a three-month period (March–June/2022). Genomic DNA (gDNA) was extracted for each participant from a venous blood sample using the chemical salting-out method. The soluble gDNA was separated later on using a silica column under high salt conditions. Specifically designed primers were utilized in a polymerase chain reaction to produce the *MDR1* gene amplicons. The participants were genotyped by utilizing Sanger sequencing. This study detected three novel variants: g.87531179A>T (NV1), g.87531184T>A (NV2), and g.186784C>T (NV3). A higher risk of SRNS development was significantly associated with the NV1 A/T genotype [odds ratio (OR) and 95% confidence interval (95% CI): 3.29 (1.09–9.95), p=0.035] and the NV2 A/A genotype [OR (95% CI): 14.73 (1.64–132.64), p=0.016] compared to the wild genotype. In contrast, the NV3 C/T genotype was significantly associated with a lower SRNS risk [OR (95% CI): 0.064 (0.016–0.26), p < 0.001] compared to the wild genotype. Moreover, the haplotype analysis showed that the likelihood of developing SRNS was significantly lower among the carriers of the ATT haplotype [OR (95% CI): 0.24 (0.1–0.56), p < 0.001], which represents the wild alleles of the NV1 and NV2 variants and the mutant allele of the NV3 variant. The findings concluded that children with the NV3 wild genotype alone or in combination with the NV1 mutant heterozygous and NV2 mutant homozygous genotypes were more likely to develop SRNS, and an alternative immunosuppressive plan might be necessary. Additional research is needed to decipher the steroid pharmacogenetics, which would reveal further insight that is vital to individualizing medication treatment in nephrotic children.

Keywords: Iraq, Multidrug resistance 1 gene, Nephrotic children, Novel variant, Steroid resistance.

Introduction

The defining features of nephrotic syndrome (NS) have essentially been the episodic and concurrent development of edema, profuse proteinuria, and hypoalbuminemia. During childhood, NS is a common glomerulopathic disorder with substantial ramifications due to the associated comorbidities, fatalities, and financial burdens. Despite the diversity of the underlying etiology and histopathology of NS, a preponderance of pediatric patients during childhood have been presenting with idiopathic NS (INS) [(1,2)].

Due to the therapeutic and prognostic significance of the patients' outcomes with the steroid regimens, the current recommendations suggest treating all INS children with an initial steroid trial. Children who respond poorly to this steroid trial have been defined as having steroid-resistant NS (SRNS), which is more progressive and associated with a greater risk of multiple complications compared to steroid-sensitive NS (SSNS) [(3)]. Unfortunately, childhood SRNS has been noted to progressively grow in number around the world as well as in Iraq, which is a distressing

trend due to the fact that SRNS is one of the most frequent conditions implicated in the development of chronic renal failure and end-stage kidney disorder among children. Thus, the delineation of underlying factors involved in developing resistance to steroid therapy would be extremely insightful to optimize the management of children with INS [2,4–8].

Several biomarkers have been extensively investigated to analyze their possible link to cause SRNS [9–12]. Exploring single nucleotide polymorphisms (SNPs) has been shown to be a promising research field for a variety of health-related conditions by enabling the earlier differentiation of treatment-sensitive and treatment-resistant patients and promoting a better individualization of medication therapy [13–16]. The trans-membrane permeability glycoprotein (P-gp), encoded by the multidrug resistance 1 (*MDR1*) gene, acts as an inherent protective barrier that partakes in the biological "housekeeping" pumping of multiple xenobiotics (including prednisolone) out of the cells. Changes in P-gp expression and functional capacity were proposed as contributing factors that drive the development of resistance among patients to medication therapy [17,18].

It has also been reported that the role of P-gp is not restricted to a mere pharmacokinetic transporter but is also actively engaged in eliciting the underlying inflammation and sustaining its persistence in auto-immune diseases [19]. The extent of P-gp expression and/or its function can be altered by *MDR1* genetic polymorphisms, which prompts subsequent modifications in the medication's pharmacokinetics and pharmacodynamics [20,21]. Some studies reported that steroid resistance development among nephrotic children was linked to the C2677T SNP in the *MDR1* gene and the ensuing alterations in the P-gp efflux capability [22,23]. However, inconsistent findings were disclosed in several studies among children with various ethnic origins [24–26]. Moreover, the presence of *MDR1* genetic variations that are still undiscovered might have a significant contribution to explaining such variability, which warrants further exploration.

The discovery of novel variants would provide new insight and a better understanding of the pharmacogenetics that govern the patients' responsiveness to steroid regimens in INS children [27]. To our knowledge, no study was previously conducted to examine the presence of novel variants in the *MDR1* gene among nephrotic children of a Middle Eastern population. Additionally, no prior investigation was undertaken to assess the clinical relevance of such variants to steroid resistance development. Therefore, the aim of this work was to explore the presence of novel variants in the *MDR1* gene and evaluated their association with steroid responsiveness in Iraqi children with INS.

Materials and Methods

Study design

The case-control design was adopted to conduct this study.

Study setting

This study was undertaken in Babylon Hospital for Maternity and Pediatrics over a three-month period (March–June/2022).

Study participants

The study enrolled nephrotic children from Arabic ethnicity, with an age of 1–13 years old and a documented diagnosis of SSNS (n=30) and SRNS (n=30). The identification of SSNS was based on the lack of proteinuria (<1+) on urinalysis (early morning) with an initial prednisolone trial (2 mg per kg per day up to 60 mg per day) for four weeks. The diagnosis of SRNS was established when proteinuria was still detectable (≥1+) on urinalysis (early morning) after a prednisolone regimen of 2 mg per kg per day (up to 60 mg per day) for 4, 6, or 8 weeks. Different tapering protocols proceeded the initial prednisolone trials. SSNS children received an alternate-day regimen of prednisolone (1.5 mg per kg per day or up to 50 mg per day) over four or six weeks. The tapering of SRNS patients included the alternate-day prednisolone regimen for six months or longer.

Patients who were aged <1 or >16 years or had acute kidney injury, gross hematuria, or a family history of NS were not included because NS in such patients may likely be of genetic-based etiology, post-infectious nephritis, or autoimmune etiology rather than of idiopathic etiology (INS). Moreover, children with a diabetes history or a finding suggesting a secondary NS due to systemic lupus erythematosus (serum C3 deficiency or auto-antibody positivity) or viral infections (antibody positivity to human immunodeficiency virus, hepatitis B virus, or hepatitis C virus) were excluded. Patients who satisfied the inclusion and exclusion criteria and consented to participate were enrolled in the study. The enrolment was conducted by approaching the patients visiting the pediatric nephrology clinic for routine follow-up. The patients were recruited consecutively using simple convenience sampling.

Data collection

A data sheet was developed to collect the participants' demographical, biochemical, and clinical characteristics. The demographics included the patient's age, sex, weight, and height. The biochemical data consisted of serum albumin,

creatinine, urea, total cholesterol, serum fasting blood glucose, and urinalysis (early morning). Clinical data such as age at diagnosis, blood pressure, immunosuppressive regimens, concomitant drug intake, and prednisolone-responsiveness were also recorded. The data of height and serum creatinine was used to calculate the estimated glomerular filtration rate (eGFR) for each patient based on the revised Schwartz equation [28].

DNA extraction and genotyping

Genomic DNA (gDNA) was extracted for each participant from a venous blood sample that was stored in a sterile EDTA tube at -80 °C until the extraction assay. The gDNA extraction proceeded by isolating the insoluble polypeptides and membrane components of the cells using the chemical salting-out method and then separating the soluble gDNA in a following step, which involved the use of a silica column under high salt conditions as a purifying matrix. The product of extraction was subjected to agarose gel electrophoresis (Bio-Rad Horizontal System, US) for additional evaluation [29,30].

Specifically designed primers were utilized in a polymerase chain reaction (PCR; Biometra, Germany) to produce the *MDR1* gene amplicons. The 5' to 3' sequence of the forward primer was AGTCCAAGAACTGGCTTTGCT. The 5' to 3' sequence of the reverse primer was AGTCTCATGAAGGTGAGTTTTCAGA. The design of the primer pair was conducted using the NCBI Primer BLAST platform following the sequence retrieval of the *MDR1* gene from the NCBI website. The reaction tube consisted of 10 µl of master mixture (Syntol, Russia), 1 µl of magnesium chloride (Syntol, Russia), 2 µl of extracted gDNA, 2 µl of primer pair (Macrogen, South Korea), and nuclease-free water to make a reaction solution volume of 25 µl. The initial step of activation proceeded at 95 °C for 5 minutes. The denaturation step was executed at 95 °C for 30 seconds with 34 cycles. The annealing step proceeded at 63 °C for 30 seconds with 34 cycles. The extension step included 34 cycles at 72 °C for 30 seconds and one final cycle at 72 °C for 5 minutes. Subsequently, the PCR amplicons were evaluated in another step of agarose gel electrophoresis and were later dispatched to a sequencing company (Macrogen, South Korea). The genotypes of patients for the detected variants were identified based on Sanger sequencing. The sequencing report was emailed with the findings that were analyzed using sequence alignment editor (BioEdit 7.1).

The detected SNPs were not deposited in the dbSNP database under any given name. Thus, the identified SNPs were novel variants. The variations were determined using the GenBank accession number (NC_000007.14) and were named g.87531179A>T, g.87531184T>A, and g.186784C >T (Supplementary Figure S1).

The first novel variant (NV1; g.87531179A >T) was located at the 73th position of the 480 bp-PCR amplicon. When a single A peak was present, the genotype was identified as a homozygous A/A genotype for NV1. The detection of simultaneous A and T peaks was indicative of a heterozygous genotype (A-T) for NV1 (Supplementary Figure S2A). The second novel variant (NV2; g.87531184T>A) was located at the 78th position of the 480 bp-PCR amplicon. The finding of a T peak alone suggested that the patient has a homozygous (T/T) genotype, whereas the presence of both T and A peaks indicated the identification of a heterozygous (T/A) genotype for NV2. The homozygous (A/A) genotype for NV2 was determined based on the presence of an A peak only (Supplementary Figure S2B). Lastly, the third novel variant (NV3; g.186784C>T) was located at the 359th position of the 480 bp-PCR amplicon. The homozygous genotype (C/C) for NV3 was distinguished based on the detection of C peak only. However, patients were genotyped as heterozygous (C/T) when the C and T peaks were evident together (Supplementary Figure S2C).

Statistical analysis

The IBM SPSS Statistics software (Ver.22) was used to execute the descriptive and analytical statistics. The results of nominal variables such as demographics and clinical data were described in frequencies and percentages and analyzed using the chi-square (for variables such as sex and concomitant diuretic use) or Fisher's exact test [for variables such as blood pressure, concomitant statin use, and concomitant angiotensin-converting enzyme inhibitor (ACEI) use]. Variables with a scale level of measurement were subjected to distribution normality testing via the Shapiro-Wilk test. The mean and standard deviation were reported for normal data, whereas data that is not normally distributed were represented using the median and interquartile range (IQR). The analytical statistics included Mann-Whitney analysis for non-normal data (such as age, age at onset, weight, creatinine level, urea level, and total cholesterol level) and the unpaired t-test for variables with normal distribution (such as height, albumin level, and eGFR measurements).

With respect to the genetic variants, the association of the alleles and genotypes with steroid responsiveness was assessed using the chi-square test. Furthermore, a binary logistic regression was conducted to analyze the odds of SRNS development. The outcome variable for the binary logistic regression was steroid responsiveness, whereas the independent variable was the genotype or allele model of the study participants. The SHEsis

website provided an online tool to check for the haplotype analysis and linkage disequilibrium (LD) among the studied variants [31,32]. This online tool employs the PLCSEM (partition-ligation combination-subdivision expectation maximization) algorithm, which is specifically designed for haplotype inferences from biallelic or multiallelic loci. A result was judged statistically significant when a two-tailed p-value is less than 0.05.

Results

Analysis of participant characteristics

There were no statistically significant differences between the SRNS and SSNS groups with respect to the participants' demographics, such as age, sex, weight, and height, as well as the clinical data of blood pressure and age at diagnosis (p-value>0.05, Table 1) The majority of concomitant drug intake and biochemical characteristics were however significantly different between the two groups (p-value<0.05). The concomitant intake of diuretics and ACEIs was more frequent in the SRNS group (12 and 8, respectively) when compared to the SSNS group (5 and 1, respectively). Serum albumin was significantly lower in SRNS children (mean±SD: 32.3±10.98 gm/L) compared to SSNS patients (37.61±7.19 gm/L). Similarly, children with SRNS had a lower eGFR (64.08±14.26) than those with SSNS (73.32±15.11). Moreover, serum urea and creatinine levels were significantly higher (p-value<0.05) in the SRNS group [median (Q1,Q3): 4.2 (2.9,6.03)] and 66.5 (55.75,87.25), respectively] than in the SSNS group [2.8 (2.5,3.85) and 53 (46.5,67.75), respectively].

Table 1. The characteristics of the study participants

Type of variables	Variables	SSNS (n=30)	SRNS (n=30)	p
Demographical	Participant's age [in years; median (IQR)]	6 (5,8.25)	8.5 (5.38,11.25)	0.08§
	Participant's sex [male; frequency (%)]	19 (63.3)	21 (70)	0.584
	Age at diagnosis [in years; median (IQR)]	4 (2.88,4.63)	3 (1.88,7)	0.447§
	Weight [Kg; median (IQR)]	21.5 (19,25.75)	26 (19.63,43.5)	0.072§
	Height (cm; mean±SD)	110.53±16.92	121.33±24.93	0.055ǂ
Biochemical and functional	Albumin level (gm/L; mean±SD)	37.61±7.19	32.3±10.98	**0.031**ǂ
	Creatinine level [µmol/L; median (IQR)]	53 (46.5,67.75)	66.5 (55.75, 87.25)	**0.002**§
	Urea level [mmol/L; median (IQR)]	2.8 (2.5,3.85)	4.2 (2.9,6.03)	**0.004**§
	Total cholesterol level [mmol/L; median (IQR)]	4.05 (3.45,5.63)	4.75 (3.7,8.03)	0.117§
	eGFR (mL/min/1.73 m^2; mean±SD)	73.32±15.11	64.08±14.26	**0.018**ǂ
Findings of elevated BP (frequency, %)	Elevated systolic BP (>95 percentile)	4 (13.3)	5 (16.7)	0.718*
	Elevated diastolic BP (>95 percentile)	3 (10)	6 (20)	0.472*
Biopsy histological findings (frequency, %)	Focal-segmental glomerular sclerosis	-	1 (3.3)	NA
	Membrano-proliferative glomerulo-nephritis	-	1 (3.3)	NA
	Minimal-change disease	-	3 (10)	NA
	No biopsy	30 (100)	25 (83.3)	NA
Immunosuppressant therapy (frequency, %)	Prednisolone	30 (100)	2 (6.7)	NA
	Cyclosporine plus prednisolone	0	18 (60)	NA
	Tacrolimus plus prednisolone	0	3 (10)	NA
	Mycophenolate mofetil plus prednisolone	0	6 (20)	NA
	Chlorambucil plus prednisolone	0	1 (3.3)	NA
Concomitant therapies (frequency, %)	Angiotensin-converting enzyme inhibitor	1 (3.3)	8 (26.7)	**0.026***
	Statin use	2 (6.7)	6 (20)	0.254*
	Diuretic use	5 (16.7)	12 (40)	**0.045**

* p-value by the Fisher's exact test. § p-value by the Mann-Whitney U test. ǂ p-value by the independent samples t-test. Statistically significant p-values are in bold. SSNS: steroid-sensitive nephrotic syndrome; SRNS: steroid-resistant nephrotic syndrome; eGFR: estimated glomerular filtration rate; BP: blood pressure; SD: standard deviation; IQR: interquartile range (Q1,Q3).

The distribution of the MDR1 variants in INS children

Regarding the detected MDR1 variants, each of the NV1 wild genotype (A/A) and the NV3 heterozygous genotype (C/T) was present in about two-thirds of the studied Iraqi children with INS (n=38, 63.3%). The minor allele frequency (MAF) of the NV2 variant was greater [NV2 MAF (A) = 34.2%, see Table 2] than that of the NV3 and NV1 variants [NV3 MAF (T) = 31.7% and NV1 MAF (T) = 18.3%].

Table 2. The identified novel variants in the *MDR1* gene among children with INS

The variant presentation	Frequency (%) of NV1 in INS cases	Frequency (%) of NV2 in INS cases	Frequency (%) of NV3 in INS cases
Homozygous wild genotype	A/A: 38 (63.3)	T/T: 29 (48.3)	C/C: 22 (36.7)
Heterozygous genotype	A/T: 22 (36.7)	T/A: 21 (35)	C/T: 38 (63.3)
Homozygous mutant genotype	T/T: 0 (0)	A/A: 10 (16.7)	T/T: 0 (0)
Wild allele	A: 98 (81.7)	T: 79 (65.8)	C: 82 (68.3)
Mutant (minor) allele	T: 22 (18.3)	A: 41 (34.2)	T: 38 (31.7)

INS: idiopathic nephrotic syndrome; NV1: first novel variant named g.87531179A>T; NV2: second novel variant named g.87531184T>A; NV3: third novel variant named g.186784C>T. The variations were named using the GenBank accession number (NC_000007.14).

The analysis of the variants' genotypes and alleles with respect to the patients' steroid responsiveness

A significant association was detected between the variants' genotypes and the steroid responsiveness of Iraqi children with INS (Table 3). The NV1 A/T and NV2 A/A genotypes were associated with a higher risk of developing SRNS [odds ratio (OR) and 95% confidence interval (95%CI): 3.29 (1.09~9.95) and 14.73 (1.64~132.64), respectively; p<0.05]. Conversely, children with the NV3 C/T genotype were less likely to develop SRNS [OR (95%CI): 0.064 (0.016~0.26), p<0.001] compared to those with the C/C genotype.

With respect to the alleles' distribution, the NV3 T (mutant) allele was significantly (p=0.002) more common in the SSNS group [n=27 (71.1%)] than in the SRNS group [n=11 (28.9%)]. A higher frequency of the NV2 A (mutant) allele was found (p=0.005) among children with SRNS [n=28 (68.3%)] compared to those with SSNS [n=13 (31.7%); Table 3].

Table 3. The distribution analysis of the *MDR1* variants' genotypes and alleles with respect to steroid responsiveness

The identified *MDR1* variants	The genotypes and alleles	SSNS	SRNS	OR (95% CI)	p
NV1: g.87531179A>T	A/A genotype (n=38)	23 (60.5)	15 (39.5)	Reference	
	A/T genotype (n=22)	7 (31.8)	15 (68.2)	3.29 (1.09~9.95)	**0.035**
	A wild allele (n=98)	53 (54.1)	45 (45.9)	Reference	
	T mutant allele (n=22)	7 (31.8)	15 (68.2)	2.52 (0.95~6.73)	0.064
NV2: g.87531184T>A	T/T genotype (n=29)	18 (62.1)	11 (37.9)	Reference	
	T/A genotype (n=21)	11 (52.4)	10 (47.6)	1.49 (0.48~4.64)	0.494
	A/A genotype (n=10)	1 (10)	9 (90)	14.73 (1.64~132.64)	**0.016**
	T wild allele (n=79)	47 (59.5)	32 (40.5)	Reference	
	A mutant allele (n=41)	13 (31.7)	28 (68.3)	3.16 (1.43~7.02)	**0.005**
NV3: g.186784C>T	C/C genotype (n=22)	3 (13.6)	19 (86.4)	Reference	
	C/T genotype (n=38)	27 (71.1)	11 (28.9)	0.064 (0.016~0.26)	**<0.001**
	C wild allele (n=82)	33 (40.2)	49 (59.8)	Reference	
	T mutant allele (n=38)	27 (71.1)	11 (28.9)	0.27 (0.12~0.63)	**0.002**

SSNS: steroid-sensitive nephrotic syndrome; SRNS: steroid-resistant nephrotic syndrome; OR: odds ratio; CI: confidence interval. Statistically significant p-values are in bold

The analysis of the variants' genotype combinations and patient steroid responsiveness

Various genotypic combinations were tested to detect the association of a potential genotypic synergism with SRNS development. The combination of the NV1 heterozygous and NV2 mutant homozygous genotypes was associated with a higher risk of SRNS development [OR (95%CI): 12.39 (1.37~112.1), p=0.025; see Table 4]. Children who had the combination that included the NV3 C/C (wild) and the NV1 A/T (heterozygous) genotypes were more likely to develop SRNS compared to those who had the combination of the NV3 C/T and the NV1 wild (A/A) genotype [OR (95%CI): 12.46

(2.81~55.34), p=0.001]. Moreover, a higher risk of developing SRNS was associated with the combination that included the NV1 heterozygous, NV2 mutant homozygous, and NV3 wild genotypes compared to the reference combination that included the NV1 wild, the NV2 wild or heterozygous, and NV3 heterozygous genotypes [OR (95%CI): 17.25 (1.79~166.1), p=0.014].

Table 4. The distribution analysis of combined genotypes of different *MDR1* variants with respect to steroid responsiveness

The variant combinations	The genotype combinations	SSNS (n=30)	SRNS (n=30)	OR (95% CI)	p
NV1[a] and NV2[b]	NV1 [0] + NV2 [0] (n=36)	23 (63.9)	13 (36.1)	Reference	
	NV1 [0] + NV2 [1] (n=2)	0 (0)	2 (100)	8.7 (0.39~195)	0.286[W]*
	NV1 [1] + NV2 [0] (n=14)	6 (42.9)	8 (57.1)	2.36 (0.67~8.3)	0.181
	NV1 [1] + NV2 [1] (n=8)	1 (12.5)	7 (87.5)	12.39 (1.37~112.1)	**0.025**
NV1[a] and NV3[c]	NV1 [0] + NV3 [0] (n=31)	23 (74.2)	8 (25.8)	Reference	
	NV1 [0] + NV3 [1] (n=6)	0 (0)	6 (100)	35.94 (1.82~708.7)	**0.003**[W]*
	NV1 [1] + NV3 [0] (n=7)	4 (57.1)	3 (42.9)	2.16 (0.39~11.8)	0.376
	NV1 [1] + NV3 [1] (n=16)	3 (18.8)	13 (81.3)	12.46 (2.81~55.34)	**0.001**
NV2[b] and NV3[c]	NV2 [0] + NV3 [0] (n=37)	27 (73)	10 (27)	Reference	
	NV2 [0] + NV3 [1] (n=13)	2 (15.4)	11 (84.6)	14.85 (2.79~79.06)	**0.002**
	NV2 [1] + NV3 [0] (n=1)	0 (0)	1 (100)	7.86 (0.3~208.5)	0.22[W]*
	NV2 [1] + NV3 [1] (n=9)	1 (11.1)	8 (88.9)	21.6 (2.39~195.3)	**0.006**
NV1[a], NV2[b], and NV3[c]	NV1 [0] + NV2 [0] + NV3 [0] (n=31)	23 (74.2)	8 (25.8)	Reference	
	NV1 [0] + NV2 [0] + NV3 [1] (n=5)	0 (0)	5 (100)	30.41 (1.52~610.5)	**0.007**[W]*
	NV1 [1] + NV2 [0] + NV3 [0] (n=6)	4 (66.7)	2 (33.3)	1.44 (0.22~9.41)	0.705
	NV1 [0] + NV2 [1] + NV3 [1] (n=2)	0 (0)	2 (100)	13.82 (0.6~318.1)	0.091[W]*
	NV1 [1] + NV2 [0] + NV3 [1] (n=8)	2 (25)	6 (75)	8.63 (1.44~51.72)	**0.018**
	NV1 [1] + NV2 [1] + NV3 [0] (n=1)	0 (0)	1 (100)	8.29 (0.31~223.8)	0.216[W]*
	NV1 [1] + NV2 [1] + NV3 [1] (n=7)	1 (14.3)	6 (85.7)	17.25 (1.79~166.1)	**0.014**

SSNS: steroid-sensitive nephrotic syndrome; SRNS: steroid-resistant nephrotic syndrome; OR: odds ratio; CI: confidence interval. W: Woolf-Haldane correction (was applied by adding 0.5 to each cell count if a zero was in at least one cell of the 2*2 table.) * p-value by the Fisher's exact test. Statistically significant p-values are in bold. a: The zero in square brackets represents the wild genotype, while 1 represents the variant (heterozygous) genotype for the NV1 variant. b: The zero in square brackets represents the wild or heterozygous genotype, while 1 represents the homozygous mutant genotype for the NV2 variant. c: The zero in square brackets represents the variant (heterozygous) genotype, while 1 represents the wild genotype for the NV3 variant.

The analysis of linkage disequilibrium among the variants haplotypes

The Lewontin's coefficient (D') of linkage disequilibrium (LD) was generated for the detected variants' haplotypes by utilizing the SHEsis tool, which provided the LD block illustrations (also known as heat blocks). The NV3 variant exhibited a high LD with the NV1 variant (D' = 0.999, r2 =0.104), and with the NV2 variant (D' = 0.872, r2 =0.183). The LD analysis also found an intermediate LD between the NV1 and NV2 variants (D' = 0.583, r2 =0.147; Figure 1).

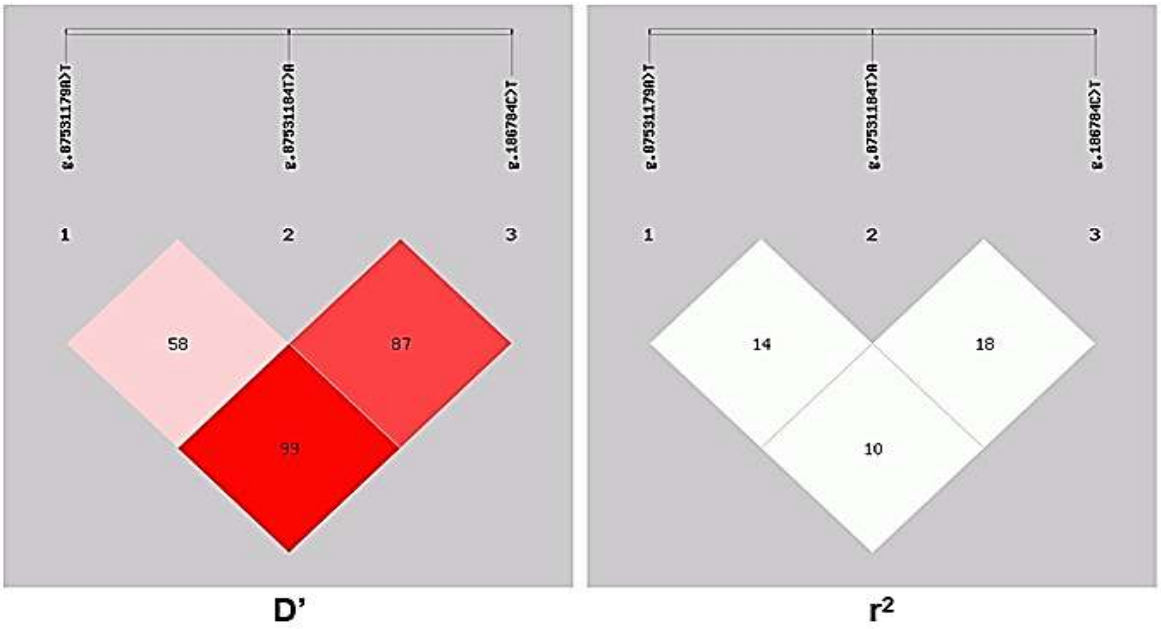

Figure 1. Linkage disequilibrium (illustrated as heat blocks) among the detected *MDR1* variants. Lewontin's coefficients (D') of linkage disequilibrium (LD) are illustrated on the left while the correlation coefficients (r^2) of LD are shown on the right.

Haplotype analysis with respect to patient steroid responsiveness

This study examined the three-loci haplotypes of the study patients, and six haplotypes for the NV1, NV2, and NV3 variants were found (Table 5). The ATT and ATC haplotypes had the highest frequency [n=36.55 (60.92%) and n=36.51 (60.85%), respectively] among the participants of both groups. The haplotype analysis demonstrated that the likelihood of developing SRNS was significantly higher among the AAC haplotype carriers [OR (95%CI): 2.55 (1.004~6.46), p=044]. However, a lower SRNS risk was significantly associated with the ATT haplotype [OR (95%CI): 0.24 (0.1~0.56), p<0.001; Table 5].

Table 5. Haplotype analysis for the detected variants with respect to steroid responsiveness

Three-loci Haplotypes [a]	SSNS (n=60)	SRNS (n=60)	χ2	OR (95% CI) [b]	p
A A C	8.17 (13.62)	16.77 (27.95)	4.03	2.55 (1.004~6.46)	**0.044**
A T C	17.83 (29.72)	18.68 (31.13)	0.07	1.11 (0.51~2.42)	0.797
A T T	27 (45)	9.55 (15.92)	11.43	0.24 (0.1~0.56)	**<0.001**
T A C	4.83 (8.05)	9.78 (16.3)	2.06	2.29 (0.72~7.28)	0.151
T T C	2.17 (3.61)	3.77 (6.3)	0.49	1.83 (0.33~10.14)	0.481
T A T	0 (0)	1.44 (2.4)	-	-	-

a: Haplotypes for three variants (NV1: g.87531179A>T, NV2: g.87531184T>A, NV3: g.186784C>T).
b: Haplotypes with a frequency of less than 3% in both groups have been dropped from the analysis. SSNS: steroid-sensitive nephrotic syndrome; SRNS: steroid-resistant nephrotic syndrome; χ2: chi-square; OR: odds ratio; CI: confidence interval. Statistically significant p values are in bold.

Discussion

The superiority of prednisolone to induce remission, along with its broad therapeutic window, was reasoned to justify the practice of an initial prednisolone trial in patients with NS. However, such practice predisposes children with SRNS to ineffective drug regimens with inadvertent toxicities [(33)]. Moreover, the arsenal of clinical practice is currently lacking the markers to aid in identifying SRNS. This study provided findings about three novel *MDR1* variants and their potential relevance to steroid resistance development in INS children. The results can be helpful to improve treatment safety and effectiveness by adjusting the steroid dosage and duration and prompting the earlier initiation of likely more effective immunosuppressing alternatives.

The *MDR1* variants detected by this work were not previously deposited under any reference number in the NCBI dbSNP database. This study uncovered the prevalence of these novel *MDR1* variants in a Middle Eastern population (specifically, in Iraq). About one-third of the INS patients had the minor alleles of the NV2

and NV3 variants (34.2% and 31.7%, respectively). Since the detected variants, to our knowledge, were not yet reported in a previous publication, a comparison of the variations' frequency rates with the results of other studies was not possible.

This study suggested that a higher risk of developing SRNS was significantly associated with the NV1 and NV2 polymorphisms in Iraqi nephrotic children. Despite the NV1 and NV2 variants being located in the intronic sequences, the relevance of the detected variants in this study to steroid responsiveness in INS children could be attributed to the fact that the variants might be in LD with other polymorphisms in the *MDR1* gene that invoke a change in the P-gp expression or function. Studies have reported the presence of LD between silent *MDR1* SNPs and missense variants [34,35]. This is also consistent with the LD analysis results of this study, which underlined a strong LD between NV1 and NV3 (an exonic variant) and between NV2 and NV3.

This investigation also found that the heterozygous genotype and the mutant allele of the NV3 variant were significantly related to a decreased SRNS risk. Moreover, the haplotype analysis in this study showed that a lower risk of developing SRNS was significantly associated with the ATT haplotype, which represents the wild alleles of the NV1 and NV2 variants and the mutant allele of the NV3 variant. The NV3 variant was identified in the coding sequences of exon 21 of the *MDR1* gene, which may explain the association of the NV3 variant with patients' responsiveness to steroid therapy. Interestingly, the combination of the NV1 and NV3 heterozygous genotypes was not associated with a significant risk of developing SRNS. The presence of the NV3 heterozygous genotype (protective) might likely have mitigated the risk that was associated with the NV1 heterozygous genotype. The possibility of a protective phenotype pertaining to an *MDR1* SNP genotype was reported previously in other studies. Cizmarikova et al. noted that the heterozygous genotype of the *MDR1* C3435T variant was significantly associated with a reduced risk of SRNS development in Slovakian nephrotic children [24]. Choi et al. studied the *MDR1* SNPs C1236T, G2677T, and C3435T and indicated a decreased risk of SRNS was associated with Korean children who had the TGC haplotype [36]. Though this is a novel variation, how exactly the NV3 variant affects P-gp activity and expression has not yet been investigated and warrants further study in future pharmacogenetic research.

When the combinations of the NV1, NV2, and NV3 genotypes were analyzed, the NV3 wild genotype alone or in combination with the NV1 heterozygous and the NV2 mutant homozygous genotypes was significantly associated with a higher risk of steroid resistance development. Moreover, the haplotype analysis identified a significant association between a higher risk of developing SRNS and the AAC haplotype, which represents the NV1 wild allele, the NV2 mutant allele, and the NV3 wild allele. These findings might partially be interpreted by the evident LD among the variants in this study. The association between different genotype combinations of the *MDR1* variants and steroid responsiveness was assessed by another pharmacogenetic study in Indian NS patients. Jafar et al. reported that the frequency of the combined mutant genotypes of the *MDR1* C3435T and C2677T variants was significantly higher in the SRNS group [22]. Parvin et al. also found a higher SRNS risk among nephrotic children with the CTC haplotype for the SNPs C1236T, G2677T, and C3435T in Bangladesh [35]. In contrast, inconsistent results were reported in other studies. The justification for the different findings among various studies might be attributed to the ethnic variations among the studied subjects [26,37]. Additionally, the variability among the patients might be due to different food or drug intake, considering the multi-ligand nature of the P-gp transporter [38].

Overall, this study underscored the link between the detected novel variants and developing steroid resistance in children with INS. The findings indicated that the NV1 and NV2 variants increased the risk of SRNS development, whereas the NV3 variant was associated with a lowered risk of a steroid-resistant outcome. Han et al. performed a systematic review and meta-analysis of several genetic association studies that included participants from European and Asian ethnic backgrounds. The *MDR1* SNP C1236T (a common *MDR1* polymorphism) was found to significantly increase the risk of developing SRNS in INS patients [26]. In Iraq, Abd Alridha et al. reported a similar finding that linked the *MDR1* variants (rs1128503 and rs2032583) to steroid resistance development in nephrotic children [30,39]. Nevertheless, this study provided a new insight about three novel *MDR1* variants in Iraqi children with INS. Furthermore, the findings discerned a relationship between the identified novel variants in the *MDR1* gene and the development of SRNS. Further research is necessary to validate the study findings and to investigate other variants (within the *MDR1* gene and in other genes) with possible ramifications for the steroid therapy of patients with NS. Considering the concern of multiple toxicities likely associated with the steroid regimens, such efforts can provide a crucial aid to elucidate the role of pharmacogenetics in developing an individual-tailored plan of treatment, which is of particular significance in the case of SRNS children.

A cautious interpretation of the findings is warranted on account of several limitations in this

study. A variability among the participants in terms of drug and food intake might be present, which could have influenced the study findings. A small patient sample was recruited from a single pediatric nephrology center while on ongoing medication therapy. Cohort-based studies of a larger sample of patients with the inclusion of multiple centers are vital to generalizing the study results. Moreover, this work was undertaken without prior podocyte-related genetic screening. However, this study excluded children who were less than one year old or had a family history. Future research should also assess additional pharmacogenes with possible involvement in mediating steroid resistance development in INS children.

Conclusion

This study revealed the presence of novel variants in the MDR1 gene among Iraqi children with INS and indicated the potential of their clinical relevance to developing SRNS. Children with the NV3 wild genotype alone or in combination with the NV1 heterozygous and the NV2 mutant homozygous genotypes tend to exhibit a prednisolone-resistant phenotype and may need treatment with an alternative therapeutic strategy. Further pharmacogenetic investigations are required to examine the implications of other variants for steroid therapy, which would optimize the efforts of personalizing medication therapy in children with NS.

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Conflicts of Interest

None to declare

Funding

None to declare

Ethics Statements

This study was approved by the Research Ethics Committee at the College of Pharmacy – University of Baghdad (Ref. No.: RECAUBCP17102021A, Decision Date: 17/10/2021) and the Research Committee of Babylon Health Directorate (Decision No.: 44, Decision Date: 28/03/2022). This study was compliant with the principles of medical research stated in the Declaration of Helsinki. Informed consent was acquired from all participants (their parents or caregivers) prior to their participation in this study.

Author Contribution

The authors confirm contribution to the paper as follows: study conception and design: AMAA and DJK; data collection: AMAA and AHAA; analysis and interpretation of results: AMAA and DJK; AMAA conducted the laboratory work regarding DNA extraction, PCR, and gel electrophoresis; draft manuscript preparation: AMAA. All authors reviewed the results and approved the final version of the manuscript.

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متباينات جينية جديدة في جين المقاومة للأدوية المتعددة لها المقدرة على تمييز الاستجابة للأدوية الستيرويدية لدى الأطفال العراقيين المصابين بمتلازمة التنادر الكلوي المجهولة السبب

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الخلاصة

تعد متلازمة التنادر الكلوي مجهولة السبب والمقاومة للأدوية الستيرويدية سببًا رئيسيًا لمرض للفشل الكلوي لدى الأطفال، مع سمات مرضية نسيجية مختلفة بالإضافة الى صعوبة التشخيص. تم اقتراح أن هذه المتلازمة قد تحدث بسبب متغايرات جينية في جين المقاومة للأدوية المتعددة ١، وهناك نتائج متفاوتة من دراسات مختلفة. هدفت هذه الدراسة الى التحري عن وجود متغايرات جينية جديدة في جين المقاومة للأدوية المتعددة ١ وتقييم ارتباطها بالاستجابة للأدوية الستيرويدية لدى الأطفال العراقيين المصابين بمتلازمة التنادر الكلوي مجهولة السبب. تم إجراء هذا البحث في استشارية أمراض كلى الأطفال في مستشفى بابل للنسائية و الأطفال وبالاعتماد على تصميم دراسة الحالات والشواهد واشتملت الدراسة مجموعة من الأطفال الذين يعانون من المتلازمة المقاومة للأدوية الستيرويدية (٣٠) ومجموعة من الأطفال المصابين بمتلازمة التنادر الكلوي المستجيبة للأدوية الستيرويدية (٣٠). تم جمع البيانات خلال مدة ثلاثة أشهر (من آذار الى حزيران ٢٠٢٢). تم استخلاص الحامض النووي الجينومي من عينة دم لكل مشارك باستخدام الطريقة الكيميائية الملحية. ثم تم فصل الحامض النووي الجينومي بواسطة عمود سيليكا تحت ظروف ملحية عالية. وقد استخدمت بواديء تم تصميمها خصيصا لمضاعفة جين المقاومة للأدوية المتعددة ١ من خلال تفاعل البلمرة التسلسلي. بعدها تم التنميط الجيني باستخدام تحليل تسلسل الحامض النووي لسانكر. وقد كشفت هذه الدراسة عن ثلاثة متغايرات جينية جديدة وهي كل من [g.87531179A>T (المتغايرة الأولى)، و g.87531184T>A (المتغايرة الثانية)، و g.186784C>T (المتغايرة الثالثة)]. حيث ارتبط خطورة الاصابة بالمتلازمة المقاومة للأدوية الستيرويدية بشكل ملحوظ بالنمط الجيني A/T للمتغايرة الجديدة الاولى [نسبة الأرجحية = ٣,٢٩ ومدى ثقة ٩٥٪ مقداره (١,٠٩ - ٩,٩٥)، p=0.035] والنمط الجيني A/A للمتغايرة الجديدة الثانية [نسبة الأرجحية = ١٤,٧٣ ومدى ثقة ٩٥٪ مقداره (١,٤٦ - ١٣٢,٦٤)، p=0.016] مقارنة بالطراز الوراثي غير المتغاير لكل من المتباينتين. وعلى النقيض من ذلك، فقد إرتبط الطراز الوراثي C/T للمتغايرة الجديدة الثالثة بإنخفاض ملحوظ لخطر الإصابة بالمتلازمة المقاومة للأدوية الستيرويدية مقارنة بالطراز الوراثي الغير متغاير لهذه المتباينة الجينية [نسبة الأرجحية= ٠,٠٦٤ ومدى ثقة ٩٥٪ مقداره (٠,٠١٦ - ٠,٢٦)، p<0.001]. بالإضافة الى لك، اشار تحليل الطرز الفردية الى أن حدوث المتلازمة المقاومة كان اقل بشكل ملحوظ لدى الأطفال ذوي الطراز الفردي (ATT) والذي يمثل الاليل الطبيعي لكل من المتباينة الأولى والثانية والاليل المتغاير للمتباينة الثالثة [نسبة الأرجحية= ٠,٢٤ ومدى ثقة ٩٥٪ مقداره (٠,١ - ٠,٥٦)، p<0.001]. وقد إستنتجت هذه الدراسة بأن الاطفال الذين لديهم النمط الوراثي الغير متغاير من المتباينة الجديدة الثالثة لوحده أو بالإضافة الى تواجده بمعية الطرز الوراثية المتغايرة للمتباينات الجديدة الاولى والثانية هؤلاء الأطفال كانوا أكثر عرضة للإصابة بمتلازمة التنادر الكلوي المقاومة للأدوية الستيرويدية، وقد يكونوا بحاجة الى مثبطات مناعية بديلة. كما وأن إجراء المزيد من البحوث ضروري لتفسير تأثير التباين الجيني على الاستجابة للأدوية الستيرويدية والذي من شأنه أن يكشف المزيد من المعلومات المهمة لتحسين الخطة العلاجية المتخصصة بكل فرد من الأطفال المصابين بمتلازمة التنادر الكلوي.

الكلمات المفتاحية: العراق، جين المقاومة للأدوية المتعددة ١، الأطفال المصابين بالتنادر الكلوي، متباينة جينية جديدة، المقاومة للأدوية الستيرويدية.