Original Article (Galley Proof Copy)

Genetic Variations in the NPHS2 Gene in Children with Nephrotic Syndrome:

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ABSTRACT
Introduction: Children with various genetic backgrounds, including those carrying the NPHS2 gene, which encodes the protein podocin necessary for the maintenance of the glomerular permeability barrier, are more likely to develop idiopathic nephrotic syndrome (INS), which is the most prevalent glomerular disease. Variations in this gene may influence the prognosis and steroid responsiveness in children with INS.
Aim of the study: Our objective was to investigate the association of NPHS2 genetic variants rs61747728 and rs7415347 with susceptibility to INS in children and their response to steroid therapy.
Methods: Fifty children with INS, 25 with steroid-sensitive (SSNS), 25 with steroid-resistant (SRNS), and 50 healthy controls of the same age and gender participated in a cross-sectional study. By the Real-Time Polymerase Chain Reaction (RT-PCR) approach, all participants were tested for NPHS2 (c.686G>A; rs61747728) and (c.538G>A; rs7415347) SNVs.
Results: In the current study, all patients (SSNS and SRNS) and the control group had the homozygous common genotype (GG). In addition, when it came to NPHS2 686G>A and 538G>A, all patients and controls had the G allele, and no one had the harmful A variant
Conclusion: We conclude that *NPHS2* (c.686G>A; rs61747728) & (c.538G>A; rs7415347) variations do not influence the susceptibility and response to steroids in INS patients involved in our study.

Keywords: Idiopathic nephrotic syndrome; *NPHS2*; c.686G>A; c.538G>A Steroid-resistant; Steroid-sensitive.

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INTRODUCTION

One of the most common primary kidney diseases that can progress to chronic kidney disease is idiopathic nephritic syndrome (INS) [1]. INS is caused by a glomerular filtration barrier injury and manifests clinically as heavy range proteinuria, decrease serum albumin, edema (puffiness of eyelids then generalized), and hyperlipidemia. Approximately 10% of children with SRNS have a poor prognosis [2].

Mutations in the genes encoding nephrin (NPHS1), podocin (NPHS2), alpha-actinin4 (ACTN4), CD2-associated protein (CD2AP), Wilms' tumor 1 gene (WT1), transient receptor potential cation channel 6 (TRPC6), and Laminin-beta-2 (LAMB2) are well known to cause INS [3]. The first mutations discovered were NPHS2 variations in children with SRNS who developed end-stage renal disease during their first decade [3]. The p.R229Q (c.686G>A; rs61747728) variant among the previously examined NPHS2 SNVs needs special attention. Exon 5 position 686's G to A nucleotide mutation causes non-conservative amino acid exchange from Arginine to Glutamine. Podocin binding to nephrin in vitro is significantly reduced as a result of the encoded peptide's changed biological characteristics [4]. Additionally, consideration must be given to the V180M (c.538G>A; rs74315347) variant, which results in an amino acid exchange of valine to methionine and a structural alteration in the ensuing podocin [5].

The selection of these two variants was dependent on these are exonic missense mutations causing change in amino acid composition of the protein podocin resulting in structural and functional alteration of podocin protein. According to Clinvar data base [6], both variants were categorized as pathogenic variants as denoted in many literatures; For the V180M (c.538G>A; rs74315347) variant it was interpreted as pathogenic as in Al-Hamed et al. [5] , Mbarek IB et al. [7] , Mubarek M et al. [8]. As regards the pathogenicity of the p.R229Q (c.686G>A; rs61747728) variant showed conflicting interpretation of pathogenicity from pathogenic as interpreted in Zhou Q et al. [9], Lu et al. [10], likely pathogenic as denoted in Santin S et al.[11], to uncertain significance. So the choice of these variants to study the effect of them on the selected population of Egyptian children was done, but limited financial resources met us to elevate the number of participants in the study.

Aim of the study: We attempted to know whether the NPHS2 genetic variations rs61747728 & rs7415347 were related to INS susceptibility in our examined sample of children.

METHODS

The current study was a cross-sectional study that was performed at the pediatric nephrology clinic of our institute Children's Hospital. After institutional review board approval (no. I-331016) and guardian consent, 100 participants were included.

There were two groups of participants: patients and controls.

The patient’s group was composed of 50 patients with INS which was described as edema, protein/ creatinine ratio > 2 mg/mg creatinine plus

hypoalbuminemia (KDIGO, 2012) [12]. We excluded patients with nephrotic syndrome secondary to autoimmune diseases, diabetes, or viral infections. This group is further divided into the following subgroups according to their response to steroid therapy:

- SSNS: children developed complete remission in both edema and proteinuria (urine protein to creatinine ratio (UPCR) based on a first morning void or 24-hour collection of ≤20 mg/mmol or 0.2 mg/mg or Urine dipstick with negative or trace protein in three successive days) within the first 4 weeks of steroid therapy [13].
- SRNS: children did not achieve complete remission after four weeks of prednisone therapy daily with a dose of 60mg/m2 treatment [14].

The control group included 50 healthy volunteers of the same age and gender ranging in age from 1 to 12 years old, without a history of nephrotic syndrome or any autoimmune disease, 52% (n = 26) of whom were females with an average age of 5.59 ± 3.6 years.

A comprehensive case history, clinical examination, and laboratory workup including blood albumin, kidney functions, serum cholesterol, and urinary protein/creatinine ratio were performed on all subjects. Routine chemistry investigations were analyzed on Bechman Synchron CX9 PRO (Bechman Coulter, Boulevard, Brea, USA). DNA was extracted and analyzed for NPHS2 (c.686G>A; rs61747728) and (c.538G>A; rs7415347) SNVs analysis using RT- PCR method.

Sample collection and storage: Each participant had five milliliters of venous blood collected, which was divided into two tubes as follows: two ml of blood was placed in a plain vacutainer with EDTA to perform DNA extraction and stored at -70 C till the time needed to extract the DNA. Three milliliters of blood were placed in plain tubes to separate serum and use that serum for regular laboratory tests.

Analysis of *NPHS2* (c.686G>A; rs61747728) and (c.538G>A; rs7415347) variations by RT-PCR Technique: RT-PCR allelic discrimination was operated by Taq-Man SNV Genotyping Assays was done on StepOne™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) by the fluorogenic 5′ nucleases with TaqMan minor groove binder (MGB) probes to detect the *NPHS2* gene SNVs (rs61747728; c.686GA; R229Q) and (rs74315347; c.538GA; V180M). The VIC labeled (Allele 1) TaqMan MGB probe represented the wild-type and the FAM-labeled (Allele 2) was the mutant probe. Each reaction contained was 20 µL; 10 µL of them TaqMan Universal PCR Master Mix (2X), 0.5 µL assay mix (20X) containing primers and probes, and 3 µL extracted DNA, the rest of the volume completed by 6.5 µL nuclease-free water. One reaction with no DNA template was included as a negative control to be sure that no contaminated DNA was amplified. The initial hold step of the amplification reaction was at 95 °C for 10, then 50 cycles followed to allow the three-step PCR to occur including the denaturation step at 92°C for 15 sec, the annealing step at 60 °C for 1 min, and finally the extension step at 60 °C for 1 min. Digestion of the probe produces the fluorescent reporter dye (either FAM or VIC) from the quencher dye **(Figure 1)**.

Statistical Methods

For parametric data, the mean ± standard deviation was used. Non-parametric data, the median was the choice. When appropriate, we used frequencies and percentages for quantitative data.

We chose the student t-test for independent samples when comparing the two groups' numerical variables. When comparing more than two groups, one method analyzing variance (ANOVA) test was used. The Chi-square test was applied when comparable categorical data were needed. In the state of the expected frequency of less than 5, an exact test was applied. When the P value was less than 0.05 statistical significance was defined. SPSS (Statistical Package for the Social Science; SPSS Inc., Chicago, IL, USA) version 22 was applied for all statistical calculations.

RESULTS

The current study included two groups

A) The patient group consisted of 50 patients, their ages ranging from 1 to 12 years. Demographic Characteristics of groups studied **(Table 1)**.

B) The control group consisted of 50 age and gender-matched healthy volunteers with no history of nephrotic syndrome or any autoimmune disease.

On comparing the SSNS and SRNS groups with the control group, it was found that there was a highly significant statistical difference in urinary protein/creatinine ratio, serum cholesterol, serum Albumin, and kidney function tests *(P= 0.00)*.

By comparing the SSNS and SRNS groups, we found that there were statistically significant differences in the urinary protein/creatinine ratio, total cholesterol, serum urea, and serum creatinine in the SRNS participants compared to the SSNS subjects, but not in the serum albumin levels **(Table 2)**.

Regarding *NPHS2 686G>A and 538G>A* variation genotyping assay, three possible genotypes could be determined for each SNV; GG is the wild (common) genotype, and GA is the heterozygous, and AA is the mutant (alternative) genotype.

In the current study, SSNS subjects, SRNS patients, and the control group population genotyping assay showed the common homozygous genotype (GG). So, no statistically significant differences were demonstrated between the INS cases and the control group **(Table 3)**. Accordingly, NPHS*2 686G>A* and *538G>A* allelic frequency (G allele and the risky A allele) showed no significant differences.

Table 1: Distribution of age (mean ±standard deviation) and gender in the examined groups

		SSNS (n=25)	SRNS (n=25)	Control (n=50)
Age (years)		7.60± 2.817	8.30± 3.547	5.59± 3.62
Gender	Females	14 (56.0%)	14 (56.0%)	26 (52.0%)
	Males	11 (44.0%)	11 (44.0%)	24 (48.0%)

SSNS: steroid-sensitive nephrotic syndrome; SRNS: steroid-resistant nephrotic syndrome

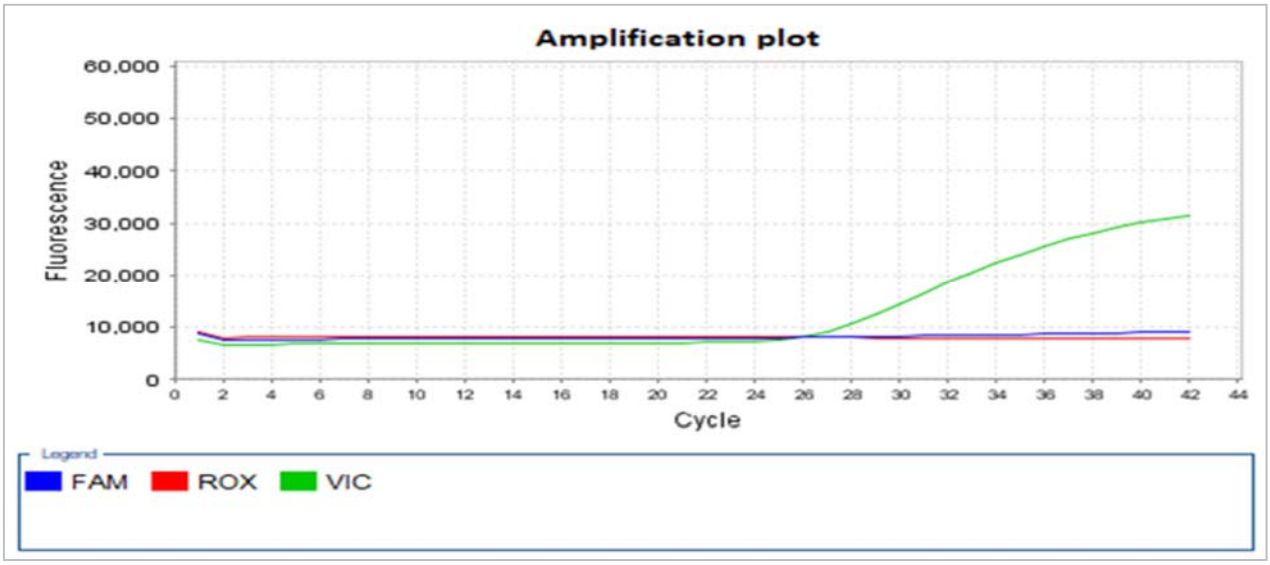

Figure 1: An Amplification plot of a case of SSNS regarding *NPHS2* 686G>A, showing the G allele *(VI labeled)* representing the common type versus the A allele *(FAM-labeled)* representing the alternative variant. As shown in this plot, this case was homozygous wild representing the GG genotype.

Table 2: Comparison of biochemical parameters between SSNS and SRNS groups

	SSNS (n=25)	**SRNS (n=25)**	***P*-value**
Urinary protein/creatinine ratio (mg/mg creatinine)	3.31 ± 1.04	5.51 ± 1.18	0.00*
Total cholesterol (mg/dl)	364.84 ± 117.05	437.4 ± 113.47	0.03*
Serum urea (mg/dl) **	10 (4-27)	21 (6-52)	0.00*
Serum creatinine (mg/dl) **	0.5 (0.2-0.7)	1(0.2-3.2)	0.00*
Serum albumin (g/dl) **	2 (1.1-3.2)	1.8 (0.8-3)	0.45

* P-value < 0.05* is statistically significant; ** median (25th - 75th percentile); SSNS: steroid-sensitive nephrotic syndrome; SRNS: steroid-resistant nephrotic syndrome.

Table 3: Frequency distribution of NPHS2 686G>A & 538G>A genotypes among cases & control groups

	Cases n=50		**Controls** n=50	
NPHS2 686G>A				
GG (homozygous wild)	50	100%	50	100%
GA (heterozygous mutant)	0	0%	0	0%
AA (homozygous mutant)	0	0%	0	0%
NPHS2 538G>A				
GG (wild)	50	100%	50	100%
GA (heterozygous mutant)	0	0%	0	0%
AA (homozygous mutant)	0	0%	0	0%

Discussion

INS is considered the commonest glomerular disease in pediatric patients. Depending on geography and ethnicity, the estimated incidence ranges from 1 to 16 patients per 100,000 children [15, 16].

It is well recognized that a considerable proportion of INS patients, particularly SRNS, have nephrotic syndrome caused by a single gene [17]. NPHS2, located on the long arm of chromosome 1 and coding for the Podocin, is one of the most analyzed genes

[18].This podocin interacts with nephrin to stabilize the permeability unit of podocytes, therefore, different variations of NPHS2 (missense, nonsense, and frameshift variations) can play a key role in the disruption of the glomerular filtration barrier [19].

In the ongoing study, there was no statistically significant difference between the INS and the control groups, nor between the SRNS and the SSNS groups, when it came to NPHS2 686G>A; p. R229Q genotype variations. The GG genotype (wild) was found in all our participants, but the GA (heterozygous mutant) and AA (homozygous mutant) variations were not found in any of the groups. Our results were in agreement with a previous study done by Fotouhi et al. who did not find the R229Q variation in any of their subjects from Iranian-Azeri population [20]. Another study showed similar results to ours was Ruf et al. who where variation R229Q was observed for 13 of 190 patients with SRNS (7%), six of 124 patients with SSNS (6%), and nine of 80 healthy control subjects (11%). No significant difference among those groups could be noted [21].

On the contrary Zou et al. found that p.R229Q play an important role in enhancing susceptibility of SRNS and Focal segmental nephrotic syndrome particularly in ethnicity of Caucasian and age of early onset patients by meta analysis of 21 studies composed of 2489 patients and 6oo4healthy controls (allelic OR 1.9, 95%CI =1.45-2.98, P=0.009) [9]. Additionally, Tory et al. discovered that NPHS2 686G>A; allele had a pthogenicity depends on the trans-associated mutation with a disease phenotype only when it is coupled specifically with certain 3′ NPHS2 mutations because of changes occurs in heterodimerization and mislocalization of the encoded p.R229Q podocin [22].

Regarding 538G>A; V180M the NPHS2 genotype frequencies: GG the homozygous wild (common) genotype , GA the heterozygous mutant (alternative) genotype and AA the homozygous mutant (alternative) genotype, the results of the present study showed that there was no statistical significant difference between the INS group and the control group, nor between the SRNS versus the SSNS group. All our studied subjects showed the GG genotype, while the GA and AA variants were not detected in any of the studied groups.

These results are in agreement with a study done by Baylarov et al. which was conducted on twenty-nine children of the Azerbaijani population with different types of nephrotic syndrome caused by chronic glomerulonephritis. He found the V180M in homozygous state in only 1% of the patients and from this result he concluded that this SNV is of uncertain significance [23].

Also in agreement with our study, Abid and his associates, published a study that was conducted on sixty-four NS patients. Whole exome sequencing of the *NPHS2* gene did not detect the V180M variation in any of the patients included in the study [24].

In contrast to our results, a study was conducted by Al-Hamed et al. who did a molecular genetic analysis on a cohort of Saudi-Arabian childhood with NS. He detected the p.V180M variation in homozygous state (AA) in 1% of his patients, yet he suggested its pathogenicity because all cases with this variation presented with childhood SRNS [5]. In addition, Lu et al. did a meta-analysis

investigation on the influence of podocin protein amino acid changes, with 16 probable variants included in their study [25].

Bouchireb et al. studied 67 NPHS2 polymorphic variants were detected among these variations, the two variant of our concern R229Q (c.686G>A; rs61747728) and V180M (c.538G>A; rs74315347) were detected as pathogenic. They also found that not all ethnic groups showed similar frequency of mutations [26]. Their research found a statistically significant link between possessing the 538G>A; p. V180M variant and the likelihood of proteinuria. Genetic differences among the analyzed groups because of ethnic and environmental factors could explain the gap between current results and the results of different investigations. Furthermore, while NPHS2 686G>A; R229Q and 538G>A; V180M variations alone did not appear to influence illness progression in INS patients in many investigations, other changes may be present concurrently in many cases for INS to occur.

From the results of present study, we recommended to increase the number of participants to demonstrate the prevalence of such mutant genes in our population.

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AUTHORS' CONTRIBUTIONS

The submitted manuscript is the work of the author & co-author.
All authors have contributed to authorship, and have read and approved the manuscript.
Conception and design of the study: all authors
Acquisition of data: 3rd author
Analysis and/or interpretation of data: first and second authors
Drafting the manuscript: all authors
Revising the manuscript critically for important intellectual content: 1st and last author
Approval of the version of the manuscript to be published: all author

STATEMENTS

Consent for publication

The attached manuscript its contents and materials have not been previously reported at any length or being considered for publishing elsewhere.

Ethics approval

The study was ethically conducted in compliance with the World Medical Association's Helsinki

Declaration. This study protocol and the consent were approved and deemed sufficient by the
Ethical Committee of Cairo University and the Institutional Review Board of Cairo University approved the study protocol (Code no. I-331016). Informed written consent was obtained from the legal guardians.

Availability of data

The authors have indicated that the data and material are factual and genuine.
The corresponding author, [YR], may provide the data that back up the study's conclusions upon request.

Conflict of interest

The authors have indicated they have no potential conflicts of interest to disclose.

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