

# Analysis of *NPHS2* Gene Mutations in Egyptian Children with Nephrotic Syndrome

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## Abstract

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**Keywords:** *NPHS2* mutation; Steroid resistant nephrotic syndrome; R229Q polymorphism

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**BACKGROUND:** Mutations in the *NPHS2* genes are the main aetiology of early-onset and familial steroid-resistant nephrotic syndrome (SRNS). The pathogenic *NPHS2* mutation together with the p.R229Q variant has been less described among Egyptian children.

**AIM:** This study aims to determine the mutation of *NPHS2* in children with NS and discover the role of p.R229Q variant in SRNS

**METHODS:** The study included 53 children with NS, and 53 healthy volunteers matched in age and sex controls. The median age at disease onset was 7.3 years. Among NS cases, 31 cases had steroid-sensitive nephrotic syndrome (SSNS) and 22 children with steroid-resistant nephrotic syndrome (SRNS). Polymerase chain reaction amplification of the whole coding region of *NPHS2* gene was carried out for its mutational analysis. Restriction digestion testing was carried out after PCR to determine the presence of R229Q polymorphism. Randomly selected samples were re-genotyped by two independent technicians for assessment of Quality control

**RESULTS:** NS patients showed a significant higher frequency of heterozygous genotype GA (89.5%) compared to control group (10.5%) with increased risk of NS (OR, 12.04; 95% CI, 2.61 to 55.38;  $p < 0.0001$ ). Moreover, SRNS showed a significant higher frequency of GA genotype (68.2%) than the SSNS group (6.5%). The GA genotype was associated with increased risk of SRNS (OR, 31.1; 95% CI, 5.73 to 168.48;  $P < 0.001$ ) and the A allele was associated with increased risk of SRNS (OR, 15.52; 95% CI, 3.325 to 72.422;  $P < .001$ ).

**CONCLUSION:** R229Q polymorphisms are associated with SRNS, and any child with SRNS should be searched for mutations in the *NPHS2* gene.

## Introduction

The most common primary glomerular disease in children is nephrotic syndrome (NS). Nephrotic syndrome in children is classified as having steroid-sensitive (SSNS) or steroid-resistant (SRNS) [1].

*NPHS2* gene mutation has been reported in 10–30% of children with sporadic SRNS children [2]. The frequency of SNP (p.R229Q) which is the most

frequently reported polymorphism among SRNS is 5% in the European population as compared to healthy one [3]. Progressive damage of the glomerular filtration barrier occurs in these patients resulting in the development of end-stage renal disease (ESRD) [4]. An autosomal recessive form of SRNS with an early onset of the disease occurs due to mutations in the *NPHS2* gene. One of the major glomerular diseases in Egyptian children is idiopathic NS and about 30% of these NS children is resistant to treatment by steroids [5].

In contrary, in other parts of the world ,

resistance to steroid therapy is prevalent in 10–20% of cases of NS [6]. Therefore, the aim of this study is to analyze the mutation of *NPHS2* gene in Egyptian children by applying next generation sequencing (NGS) and determine the role of SNPs in Egyptian children, concentrating our analyses on SRNS children carrying the p.R229Q variant.

## Subjects and Methods

The current case control study was conducted on 53 patients with nephrotic syndrome (NS) and 53 healthy volunteers with matched age and sex during the period from January 2017 to June 2018. The patients were referred from the Pediatric Nephrology Units, Al Zahra and Al – Hussein hospitals, Al-Azhar University, Cairo, Egypt. The clinical records of all the subjects were reviewed for age at onset, gender, family history, treatment modalities and response to therapy.

All patients were diagnosed as nephrotic syndrome according to their clinical presentation and laboratory studies. The patient's group was categorized into 2 groups: 21 children with SRNS and 32 children with SSNS. Steroid responsive NS (SSNS) was regarded as complete remission achieved with steroid therapy. Steroid-resistant NS (SRNS) was regarded as failure to achieve remission following 4-week prednisone 60 mg/m<sup>2</sup> followed by three methylprednisolone pulses [7], [8].

The informed consent forms were given by caregiver of patients.

The study protocol was approved by the medical Research Ethical Committee of AL-Azhar University.

Genomic DNA was extracted from peripheral leukocytes of whole-blood samples using standard laboratory protocols.

The 5 mL blood samples were drawn into tubes containing EDTA. Genomic DNA was extracted from peripheral leukocytes using standard techniques. *NPHS2* exon 5 was polymerase chain reaction (PCR)-amplified using the following primers: F 5'-AGGATTTACCACAGGATTAAGTTGTGCA – 3' and R 5'-TAGCTATGAGCTCCCAAAGGGATGG – 3'. Three microliters of unpurified PCR product were diluted to 10  $\mu$ L in recommended restriction buffer containing 5 U of *Cl*I and digested at 37°C overnight. The PCR products were visualized by electrophoresis in a 3% agarose gel with ethidium bromide and stored in digital form. Quality control for these assays was assessed by randomly selecting 50 samples to be re-genotyped by two independent technicians. Each batch of restriction digestion contained a positive control (confirmed G allele) to avoid mistyping. 10% of the

samples were randomly picked and re-genotyped to give consistent results. The products were resolved on 2% agarose gel and viewed in a gel documentation unit (Bio-Rad). Quality control for these assays was assessed by randomly selecting 30 samples to be re-genotyped by two independent technicians.

## Statistical Analysis

The SPSS version 21 was used in this research. Chi-square test ( $\chi^2$ ) was used for comparison of frequencies between patients and controls and t test was used for comparing means. The association between case-control status and each polymorphism, measured by the odds ratio (OR) and its corresponding 95% confidence interval (CI). Hardy-Weinberg equilibrium was checked using  $\chi^2$  test to compare the observed genotype frequencies with the expected frequencies among the case and control subjects.

## Results

Table 1 shows distribution of genotypes of R229Q polymorphism in NS patients compared to control group. NS patients showed significant higher frequency of heterozygous GA genotype (89.5%) compared to control group (10.5%); ( $\chi^2 = 14.43$ ;  $p < 0.001$ ), and increased risk of NS (OR, 12.04; 95% CI, 2.61 to 55.38;  $p < 0.0001$ ).

**Table 1: Genotypes distribution of R229Q polymorphism in NS cases and controls**

Genotype	NS (n = 53)	Controls (n = 53)	Odds ratio (95% Confidence Interval)	P value
GG	36 (41.4%)	51 (58.6%)	1	
GA	17 (89.5%)	2 (10.5%)	12.04 (2.61-55.38)	< 0.0001
AA	0	0	--	--
$\chi^2 = 14.43$ ; $P < 0.001$				

Table 2 shows comparison of the genotype's distribution of R229Q polymorphism between SRNS and SSNS patients. Significant increase of heterozygous genotype (GA) was observed in SRNS (68.2%) as compared to SSNS patients (6.5%)  $\chi^2 = 22.5$ ;  $P < 0.001$ .

**Table 2: Genotypes and allele frequency distribution of R229Q polymorphism in children with SRNS and SSNS**

Genotype	SRNS (n = 22)	SSNS (n = 31)	Odds ratio (95% Confidence Interval)	P value
GG	7 (31.8%)	29 (93.5%)	1	
GA	15 (68.2%)	2 (6.5%)	31.1 (5.73-168.48)	< 0.0001
AA	0	0		
$\chi^2 = 22.51$ $p < 0.001$				
Allele				
G	29 (32.9%)	60 (67%)	1	
A	15 (88.2%)	2 (11.8%)	15.52 (3.325-72.422)	< 0.0001

The GA genotype was associated with increased risk of SRNS (OR, 31.1; 95% CI, 5.73 to 168.48;  $P < 0.001$ ) and the A allele was associated with increased risk of SRNS (OR, 15.52; 95% CI, 3.325 to 72.422;  $P < 0.001$ ).

Amplified PCR products in 6 healthy subjects with wild genotype (GG) were illustrated in Figure 1(left). Moreover, PCR product of exon 5 of *NPHS2* gene for three patients and two healthy controls with wild genotype (GG) of R229Q and for one patient with the heterozygous genotype (GA) of R229Q was illustrated in Figure 1(right).

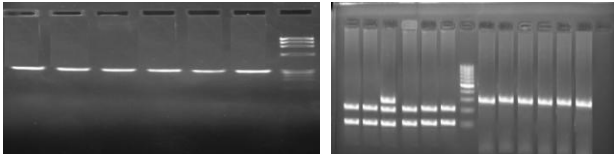


Figure 1: Amplified PCR products in 6 healthy subjects (left); Agarose gel stained with ethidium bromide illustrating PCR product of exon 5 of *NPHS2* gene before and after digestion with *Cla*I endonuclease enzyme for patients and healthy controls with wild genotype (GG) of R229Q and for one patient with the heterozygote genotype (GA) of R229Q (right)

Figure 2(left) shows sequence chromatogram of exon 5 of *NPHS2* gene, illustrating wild pattern GG. Figure 2(right) shows sequence chromatogram of exon 5 of *NPHS2* gene and heterozygous pattern (p.Arg229 Gln) (c.686G > A). Site of mutation is denoted by the arrow.

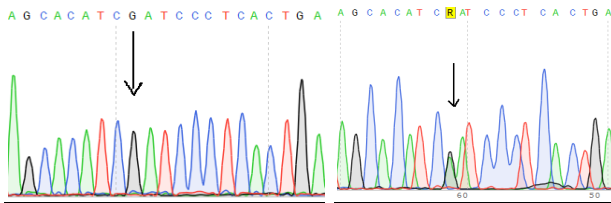


Figure 2: Sequence chromatogram of exon 5 of *NPHS2* gene showing wild pattern (p.Arg 229Gln) (c.686G > A). Site is denoted by the arrow (left); Sequence chromatogram of exon 5 of *NPHS2* gene showing heterozygous pattern (p.Arg229 Gln) (c.686G > A). Site of mutation is denoted by the arrow (right)

## Discussion

The R229Q polymorphism (c.686G > A; rs61747728) is considered a non-neutral polymorphism and it has also been associated with glomerular disease. It is a podocin variant resulting in an amino acid substitution from arginine to glutamine in ~1 – 2% of European populations; the R229Q variant is being present and is associated with the development of microalbuminuria. The adult-onset FSGS is the result of compound heterozygosity of the R229Q variant with a pathogenic podocin mutation [9]. The role of *NPHS2* mutations in adult onset disease

have limited confirmation because of identification of few cases [10], [11], [12]. Few studies reported the effect of p.R229Q and p.P20L in causing disease in European, North American Caucasian and South American populations [13], [14]. A non-conservative substitution was caused by p.P20L in a previous study [15]. In the Czech population, the highest frequency of p.R229Q has been reported (12%) [16]. An increased (2 – 3 folds) risk of micro albuminuria with progression gradually to ESRD at the age of thirty and forty years is caused by p.R229Q, with tendency to have later-onset disease (i.e. typically FSGS) [17]. The single heterozygous mutation (p.R229Q) could not by itself be considered as a causative mutation because a previous study predicted 2 *NPHS2* gene SNPs, a heterozygous 1082T > C and a homozygous 954T > CA [4]. A low prevalence of the two genes in Japanese and Chinese NS patients was reported in several studies [18], [19]. There are a number of other genes make a significant contribution to the spectrum of disease-causing mutations beside the *NPHS1* and *NPHS2* genes. In familial autosomal-recessive steroid-resistant nephrotic syndrome *NPHS2* mutations was detected for the first time by [20]. Also, in sporadic cases of steroid-resistant nephrotic syndrome [2], [15], [21] and late-onset focal segmental glomerulosclerosis (FSGS) [12], *NPHS2* mutations were identified thereafter. Individuals with the same genotype revealed a wide range of phenotypic variability [19]. In a previous study the mutation detection rate in familial autosomal-recessive and sporadic steroid-resistant nephrotic syndrome was 43% and 10.5% respectively [22]. The *NPHS2* mutations were present in patients of Italian, French, German, and Israeli-Arab origin, but it is not present in children of Israeli-Jewish and Japanese origin [2], [15], [21].

In conclusion, *NPHS2* mutations were observed in the studied Egyptian children with nephrotic syndrome and significant higher frequency was detected in SRNS cases. Therefore, every child with SRNS should be searched for mutations in *NPHS2* gene by target-oriented next generation sequencing analysis. Moreover, mutational analysis of *NPHS2* genes should be included in the diagnosis of NS among Egyptian patients.

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