

Study of Genetic Variation in Podocin Gene Associated with Idiopathic Nephrotic Syndrome

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Abstract

Background: Nephrotic syndrome (NS) is a kidney disease predominantly present in children with idiopathic condition; final stage of the disease progresses into end-stage renal disease. Generally, NS is treated using standard steroid therapy, however; most of the children are steroid sensitive and about 15–20% are non-responders (SRNS). In SRNS patients, the most common histopathological subtype is focal segmental glomerulosclerosis (FSGS). Mutations in several genes including NPHS2 have been implicated in SRNS. Gene R229Q polymorphism (p.R229Q) of NPHS2 is associated with adolescent- or adult-onset SRNS in European and South American populations. The present work aimed to study the effect of NPHS2 R229Q genetic variations on the susceptibility to idiopathic NS and the treatment response in NS children from Benha University Hospital. **Methods:** Mutation analysis was carried out by Taqman allele discrimination of the NPHS2 gene R229Q polymorphism (rs61747728) using specific primers and probes in 40 INS (20 MCD and 20 FSGS) children and 20 healthy controls. The allele and genotype frequencies of NPHS2 gene were calculated for both cases and controls. **Results:** The wild allele and the wild genotype frequencies of rs61747728 were 100% for both nephrotic syndrome and control children. The mutant allele could not be detected in the population included. **Conclusion:** Only the wild allele and genotype were present in the population of this study (both nephrotic syndrome and control subjects).

Keywords

- NPHS2 R229Q
- rs61747728,
- Minimal change disease
- Focal segmental glomerulosclerosis
- Taqman allele discrimination

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Introduction

In children, nephrotic syndrome (NS) is defined as protein excretion of more than 40 mg/m²/h or a first-morning urine protein/creatinine of 2-3 mg/mg creatinine or greater [1]. The childhood NS has a male predominance (74%). The mean age of disease onset was 4.43 ± 2.7 years, with patients having onset of ≤ 6 years constituting 81% of patients [2]. Idiopathic nephrotic syndrome (INS) is the most popular form of childhood NS, representing more than 90% of cases between 1 to 10 years of age and 50% after 10 years of age [3].

Subjects with minimal change disease (MCD) have a 95% response rate to steroids, however, 75% will relapse and 50% (frequent relapsers or steroid dependent subjects) will demand higher and prolonged doses of steroids thus increasing the risk of adverse effects [4]. SRNS defined as patients who fail to enter remission after 8 weeks of corticosteroid treatment they are at significantly higher risk for development of complications of the disease, as well as progression of the disease to chronic kidney disease (CKD) or end stage renal disease (ESRD) [5].

Three observations provide important clues to the primary pathophysiology of INS; first; Mutations in several podocyte proteins have been identified in families with inherited NS, highlighting the central importance of the podocyte. Second; a plasma factor may alter glomerular permeability, especially among patients with steroid-resistant nephrotic syndrome (SRNS). This factor nature still remains unknown but it seems to be derived from lymphoid cells. The association of NS with primary immunological disorders as lymphoma,

leukaemia, thymoma, Kimura's disease, and Castleman's disease, and therapeutic agents like interferon favors this hypothesis. Altered T-lymphocyte responses could result in the production of a permeability factor that interferes with the expression, function, or both, of key podocyte proteins to cause proteinuria. The podocyte target of such a putative factor is, however, unclear [6]. Third; a higher rate of certain gene polymorphisms among nephrotic patients than controls suggest the existence of disease susceptibility genes [7].

Podocin (NPHS2), the protein product of NPHS2 gene located on chromosome 1, is a member of stomatin protein family. Podocin is exclusively expressed in the podocytes and localizes at the insertion of the slit membrane. Due to its similarity to stomatin, it is believed that podocin forms a hairpin like structure with intracellular NH₂- and COOH-termini [8].

Several groups from European and Middle Eastern populations reported that near 10–30% of sporadic SRNS was due to NPHS2 gene mutations [9]. The incidence of NPHS2 mutations was 25% in cases of sporadic SRNS in an Egyptian study [10]. For most patients, no curative treatment is available. The most frequent renal histologic feature of SRNS is focal segmental glomerulosclerosis (FSGS). Moreover, in patients with FSGS, the risk of recurrence after kidney transplantation is estimated to be about 11% - 50%, an event that again leads to terminal renal failure [11]. FSGS associated with NPHS2 mutation is uniformly steroid resistant and generally shows poor response to cyclosporine A (CSA) [12].

Pasini et al. decided to adopt the same dosage (body surface area dosing) for all our patients in order to avoid any hypothetical bias when evaluating the results of therapy. They suggested that prednisolone (PDN) could be given at 60 mg/m²/day, with a maximum dose of 60 mg/day [13].

Calcineurin inhibitors (CNIs) are the preferred drugs for treatment of childhood steroid-resistant nephrotic syndrome (SRNS) who are also resistant to cyclophosphamide (CYC). Although few studies have shown a benefit of one over the other, efficacy and safety of either CNIs [tacrolimus (TAC) or CSA] in this special population remained to be assessed in long-term studies. The long-term outcome of renal function was significantly better in patients who were treated with TAC as compared to CSA [14]. The present work aimed to study the effect of NPHS2 R229Q genetic variations on the susceptibility to idiopathic NS and the treatment response in NS children.

Subjects and methods:

Subjects:

The study group included 40 children with INS (22 male and 18 female) and 20 healthy controls (10 male and 10 female) without a familial history of renal disease attending the Pediatric Nephrology Clinic, Pediatrics Department, Benha University Hospital. It was a prospective study for 8 months from December 2016 till July 2017. The mean age of onset for INS was 8.37 years and for controls was 4.7 years. All cases fulfilled the criteria of the International Standard of Kidney Disease in Children (1981) for diagnosis of NS. Of the 40 INS children, 20 children were steroid-resistant

and biopsy confirmed FSGS and the rest were steroid-sensitive NS (SSNS). All these children had massive proteinuria [40 mg/m²/day or 50 mg/kg body weight/day] or a random sample of urinary protein-to-creatinine ratio exceeding 2 mg/mg which effectively resulted in severe hypoalbuminemia of serum albumin < 2.5 g/dl.

Inclusion criteria: patients with INS, patients aged 1 to 16 years and both sexes were included.

Exclusion criteria: patients who have secondary NS, other chronic diseases, steroid therapy for diseases other than nephrotic and malignancy. Ethical approval from the Committee of Research Ethics, Faculty of Medicine, Benha University was taken before the start of study. Written informed consents from the parents of children were taken prior inclusion in the study.

All these children had initially a course of prednisolone for 4- 6 weeks. Children, found to be resistant, had a course of either IV cyclophosphamide or oral cyclophosphamide, followed by either oral cyclosporine or oral tacrolimus and then mycophenolate mofetil along with steroids. None of the children had progressed into chronic kidney disease stage 2 or more during the study period.

Sampling:

Two milliliters (2 ml) venous blood samples on ethylene-diamine tetra-acetic acid (EDTA) were collected from each subject mixed well and aliquoted into 2 sterile eppendorff tubes to be stored at -80°C for further study of gene polymorphism. The other laboratory DATA were collected from the patient records.

Mutation and genotyping analysis

Extraction: Genomic DNA was extracted from 200µl blood sample; using Purelink® Genomic DNA minikit Catalog No. K1820-01 (Invitrogen, Life Technologies). The DNA optical density was measured at wavelengths 260 and 280 nm by nanodrop 2000 UV spectrophotometer (Thermo-Fisher Scientific, Wilmington, USA). OD260 measured the concentration; a reading of 1 equals 50µg/ml. Pure DNA had an OD260/OD280 ratio of 1.7-2 [15]. The analysis was done at Medical Biochemistry Department and Molecular Biology and Biotechnology Unit, Faculty of Medicine-Benha University.

Allele Discrimination: Detection of NPHS2 [R229Q (rs61747728)] gene polymorphism was done by 5' Nuclease Taqman SNP Genotyping Assay Technology. In 20 µl reaction, genomic PCR amplification was done using Taqman 5' allele discrimination assay for human single nucleotide polymorphism (SNP) (Applied Biosystem, Foster City, California, USA). The Taqman assay supplied for NPHS2 rs61747728 SNP was 40X. NPHS2 rs61747728 contained sequence specific primers for both alleles (G and

A) of the SNP and 2 Taqman probes; one probe labeled with VIC dye detects the G allele and the other labeled with FAM detects the A allele. Amplification was done in Stepone Real Time PCR System (Applied Biosystem, Foster City, USA). The following thermal cycling conditions were run during which the amount of DNA in each sample was quantified; pre-PCR read for 1 cycle (hold for 15 sec. at 60°C), then Amplitaq Gold enzyme activation for 1 cycle (hold for 10 min. at 95°C) to be followed by 40 cycles of PCR amplification (denaturation; 15 sec. at 92°C and annealing/extension for 1 min. at 60°C. finally post-PCR read was done (hold for 15 sec. at 60°C). Two no template controls (NTCs) using DNase free water were essentially done in each run to enable detection of DNA contamination. Multicomponent plot of R229Q SNP of podocin (NPHS2) gene was represented by figure (1). It showed amplified G allele detected by VIC-labelled probe (green curves) but non-amplified A allele detected by FAM-labelled probe (blue lines). The passive ROX dye of the Stepone apparatus appeared as red lines. The success rate for this genotyping was 100%.

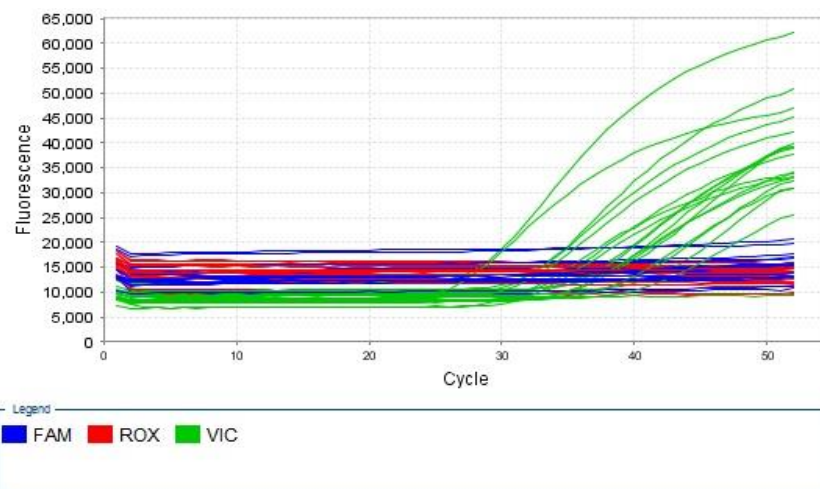


Figure (1): Multicomponent plot of R229Q SNP of podocin (NPHS2) gene

Statistical analysis: The data were coded, entered and processed on computer using SPSS (version 18). The results were represented in tabular and diagrammatic forms then interpreted. Mean, standard deviation, range, frequency, and percentage were used as descriptive statistics. Chi-Square test (X^2) was used to test the association variables for categorical data. Student's t-test was used to assess the statistical significance of the difference between two population means in a study involving independent samples. P value was considered significant as $p \leq 0.05$.

Results:

Patient characteristics: NPHS2 gene mutation screening was performed in 40 INS patients and 20 healthy children of Egyptian population without a familial history of renal disease as healthy controls. The patients were divided into SRNS (20 patients) and SSNS (20 patients). The age of onset

was 1-16 years with the mean age of 8.37 ± 4.17 years for INS children and 4.7 ± 2.23 years for healthy controls. There was a predominance of male children (55%) and 45% for females between INS and controls while among 20 children with SRNS a similar gender distribution was seen. Mean SBP of INS children's 128.83 ± 14.52 mmHg and 93 ± 20.03 mmHg for healthy controls. Regarding DBP of INS mean was 81.55 ± 11.03 mmHg and 60.0 ± 5.38 mmHg for controls.

All the 20 children with SRNS were biopsy proved FSGS.

Table (1) showed that the mean values of potassium and sodium were non-significant while pyuria, hematuria, albumin in urine, urine 24h protein, urine protein/creatinine ratio, cholesterol, urea and creatinine were significantly higher among cases than controls, but serum albumin was significantly lower among cases than controls.

Table (1): Laboratory investigations of idiopathic nephrotic syndrome (INS) cases and controls

Variables	Cases (n.=40)	Controls (n.=20)	test	p
	n. (%) or Mean \pm SD			
Pyuria (>10 WBCs/HPF)	22 (55)	3 (15)	8.78 [†]	0.003 ^{**}
Hematuria (>5 RBCs/HPF)	17 (42.5)	0 (0)	11.86 [†]	0.001 ^{**}
Albuminuria				
+	5 (12.5)	5 (25)	48.75 [†]	0.00 ^{**}
++	12 (30)	0 (0)		
+++	23 (57.5)	0 (0)		
Urine 24h protein (mg/m²/h)	2746.8 \pm 1375.1	955.5 \pm 116.6	32.52 [#]	0.00 ^{**}
Urine protein / creatinine ratio (mg/mg)	4.96 \pm 1.79	0.381 \pm 0.088	31.52 [#]	0.00 ^{**}
Serum albumin (g/dl)	2.54 \pm 0.429	3.22 \pm 0.233	7.41 [#]	0.009 ^{**}
Serum cholesterol (mg/dl)	488.1 \pm 118.73	205 \pm 26.7	33.93 [#]	0.00 ^{**}
Serum creatinine (mg/dl)	0.73 \pm 0.266	0.65 \pm 0.121	3.99 [#]	0.049 [*]
Serum urea (mg/dl)	65.45 \pm 11.1	51.82 \pm 19.27	7.2 [#]	0.009 ^{**}
Serum potassium (mmol/l)	4.64 \pm 0.76	4.01 \pm 0.545	7.2 [#]	0.123
Serum sodium (mmol/l)	138.5 \pm 6.98	138.55 \pm 3.54	9.18 [#]	0.98

WBCs: white blood cells, RBCs: red blood corpuscles, HPF: high power field, *: high significant, †: Chi square, #: Student t test

Table (2): Laboratory investigations of steroid sensitive and steroid resistant nephrotic syndrome cases

Variables	SSNS (n.=20)	SRNS (n.=20)	test	p
	n. (%) or Mean \pm SD			
Pyuria (>10 WBCs/HPF)	9(45)	13(65)	1.62 [†]	0.2
Hematuria (>5 RBCs/HPF)	7(35)	10(50)	0.92 [†]	0.34
Albuminuria			7.46 [†]	0.02 [*]
+	5(25)	0(0.0)		
++	7(35)	5(25)		
Albuminuria				
+++	8(40)	15(75)		
Urine 24h protein (mg/m²/h)	2222.6 \pm 1246.73	3271.05 \pm 1322.45	1.02 [#]	0.32
Urine protein / creatinine ratio (mg/mg)	4.22 \pm 1.71	5.71 \pm 1.58	0.31 [#]	0.58
Serum albumin (g/dl)	2.67 \pm 0.45	2.4 \pm 0.37	0.98 [#]	0.76
Serum cholesterol (mg/dl)	472.2 \pm 123.13	504 \pm 115.07	0.06 [#]	0.61
Serum creatinine (mg/dl)	0.73 \pm 0.222	0.73 \pm 0.235	0.09 [#]	0.018 [*]
Serum urea (mg/dl)	49.6 \pm 18.03	54.05 \pm 20.66	0.26 [#]	0.8
Serum potassium (mmol/l)	4.55 \pm 0.577	4.72 \pm 0.915	6.06 [#]	0.018 [*]
Serum sodium (mmol/l)	138.75 \pm 6.82	138.25 \pm 7.31	0.06 [#]	0.8

WBCs: white blood cells, RBCs: red blood corpuscles, HPF: high power field, ^{*}: significant, [†]: Chi square, [#]: Student's t test

Table (3): Genotype and allele frequencies among the study groups

NPHS2 R229Q	Controls (n.=20)	SSNS (n.=20)	SRNS (n.=20)
	n. (%)		
GG genotype	20 (100)	20 (100)	20 (100)
GA genotype	0 (0)	0 (0)	0 (0)
AA genotype	0 (0)	0 (0)	0 (0)
G allele	40 (100)	40 (100)	40 (100)
A allele	0 (0)	0 (0)	0 (0)

Table (2) showed that the mean value of serum potassium was significantly lower among steroid responders (minimal change disease) and that albumin in urine was significant higher among non-responders (focal segmental glomerulosclerosis) while pyuria, hematuria, urine 24h protein, protein/ceatinine ratio, serum sodium, albumin, cholesterol, urea and creatinine were non-significant.

Table (3) showed that the genotype and allele frequencies of NPHS2 R229Q were 100% wild with no mutation detected among controls and

idiopathic nephrotic syndrome cases [steroid sensitive (MCD) or steroid resistant (FSGS)].

Discussion:

Nephrotic syndrome (NS) is defined by proteinuria, peripheral edema, and hypoalbuminemia. Depending on the response to standard steroid therapy, it is subclassified to steroid-sensitive NS (SSNS) and steroid-resistant NS (SRNS). Steroid-resistant NS is also resistant to other immunosuppressive agents and have a tendency to progress to end-stage renal disease (ESRD) [16].

Positional cloning identified NPHS2, encoding podocin, as a possible etiological genetic factor in autosomal recessive SRNS, including FSGS [17]. NPHS2 expression is restricted to podocytes as determined by insitu-hybridization studies. About 50 NPHS2 gene variants and/or nonsilent SNPs have been reported and recognized as potentially being involved in proteinuria. The R229Q SNP is one of the most commonly reported podocin sequence variations [18].

This study showed that all cases and controls had 100% frequencies for GG genotype and G allele as no gene mutation was detected among all subjects.

Our results were matching with Reiterova et al., [19] as The R229Q polymorphism has a lower frequency among Africans, African-Americans, and Asians (zero to 1.5%). According to Machuca et al., [20] the highest frequency of R229Q has been reported in Chileans and Argentineans (7.3%). Also, Lipska et al., [21] reported that NPHS2 mutations were found to be in of higher prevalence among SRNS patients of the Polish population. Tryggvason et al., [22] proposed that R229Q, found in around 4% of European populations, is associated with an increased risk of microalbuminuria. In contrast to our results, Dhandapani et al., [23] proposed that, detection of these NPHS2 mutations is of clinical importance as the preponderance of these mutations in SRNS not only confirms genetic heterogeneity in SRNS but also underscores molecular defect leading to the non responsiveness of these patients to steroid therapy. Failure to response to steroid

treatment has an important ramification for the risk of developing progressive renal failure later in life leading to ESRD. The higher frequency of these mutations (18%) observed in SRNS is of noteworthy, though not significant, confirming the previous findings that mutations in the NPHS2 is known to cause SRNS, occurring in both sporadic and familial cases of SRNS [24]. Coming to the genetic results found in this study, the polymorphism R229Q was found in 0% of cases. Several studies did not report any polymorphism in SRNS [18], however, higher percent of this polymorphism was found by many other studies [18]. In addition, The polymorphism R229Q was absent among SSNS and SRNS subcategories a finding which was detected by Landau et al. [25] who did not find any polymorphisms in SSNS, as well as Lahdenkari et al., [26] and Caridi et al., [27]. Our results concerning the absence of the NPHS2 R229Q variant in the present study could be also explained by the small sample size of the study and different ethnic population included (Egyptian subjects).

RECOMMENDATION:

From the results of this study we recommend additional studies with larger sample size, and preferably with the cooperation of multiple centers to further evaluate the response of children resistant to steroid, as regards various available modalities of therapy so as to reach final conclusion to the important question; are we justified to stop any further medications in cases having such mutations, to minimize side effects?

CONCLUSION

Only the wild allele and genotype were present in the population of this study (both nephrotic syndrome and control subjects).

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Compliance with ethical standards

Conflict of interest: The authors report no conflicts of interest and they are responsible for the content and writing of this article.

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