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CLINICAL STUDY

NPHS2 gene sequencing results in children of the Azerbaijani population with different types of nephrotic syndrome caused by chronic glomerulonephritis

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ABSTRACT
OBJECTIVES: The aim of the study was to determine the mutation of the podocin gene (*NPHS2*) in children with minimal changes diseases (steroid sensitive nephrotic syndrome (NS)) and steroid resistant NS.
BACKGROUND: Despite the fact that the role of genetic factors is a well-known phenomenon, in NS there are still unknown aspects that are yet to be discovered. NS, type 2 is caused by *NPHS2* gene and is characterised with proteinuria, minimal change disease on renal biopsy, poor response to steroid treatment, etc.
METHODS: Twenty-nine children (65.5 % male, 34.5 % female) with nephrotic syndrome caused by chronic glomerulonephritis were examined and patients were tested for *NPHS2* gene with Sanger technique.
RESULTS: The average age was 7.2 ± 2.65 years. 82.8 % of patients had NS with minimal changes, 17.2 % had a steroid resistant NS. The analysis of the *NPHS2* gene revealed a likely pathogenic (Arg168His), uncertain significance (Pro20Ley, Leu169Pro, Val180Met, Arg229Gln, Val290Met) and benign (Gly34Gly, Ala318Ala) variants. No novel variants were detected.
CONCLUSION: This is the first study investigating the nephrotic syndrome related to *NPHS2* gene in Azerbaijani population. The high prevalence of uncertain significance variants emphasises the importance of population studies in this region as such data are necessary for classifications of the detected genetic variants *(Tab. 1, Ref. 25)*. Text in PDF *www.elis.sk.*
KEY WORDS: Azerbaijanian children, nephrotic syndrome, chronic glomerulonephritis, mutation, *NPHS2.*

Introduction

Nephrotic syndrome (NS), which is characterized by proteinuria, hypoalbuminemia and pronounced oedema, is a pathological condition that affects the glomerular apparatus of the kidney in children. According to the literature, the frequency of NS is 0.5 per 10,000 children (1–3). It is believed that the NS can be a manifestation of glomerulonephritis (1, 2). NS is a common glomerulopathy among children, while its incidence ranges from 12–16 per 100,000 (4). According to studies conducted in New Zealand (2001–2004) and Netherlands (2003–2006), the annual incidence of idiopathic NS was between 1.15 and 2.10 cases per 100,000 children, respectively (5, 6).

It has been established that genetic factors play an important role in the etiology and pathogenesis of NS (1). To study the role of these factors in the etiology and pathogenesis of NS, an approach based on the study of polymorphic markers of related genes, one of which is the podocin gene (*NPHS2*, OMIM *604766), is used (7). It is known that podocin is a transmembrane protein that is important in the processes of glomerular filtration, and it is expressed in podocytes. Mutations in the *NPHS2* gene lead to a change in the corresponding protein, which disrupts the functioning of the slit diaphragm. This condition then manifests as type 2 of nephrotic syndrome (OMIM # 600995) inherited in an autosomal recessive pattern. The protein molecules without a functional slit diaphragm pass through the glomerular basement membrane and are excreted in the urine. This then leads to a gradual deterioration in kidney function and development of the terminal stage of renal failure.

In 1995, the suspected gene was mapped by A. Fuchshuber et al. (8) on chromosome 1q25-q31 in autosomal recessive NS. For the first time, mutations in the *NPHS2* gene were detected in children aged 3-5 years with a manifestation of a steroid resistant NS (SRNS) (9). Subsequently, mutations of the *NPHS2* gene were identified in sporadic cases of SRNS with focal segmental glomerulosclerosis in children and adults (10, 11). Currently, more than 100 pathogenic mutations and 25 polymorphic variants in the *NPHS2* gene have been identified in patients with familial and sporadic SRNS. Most mutations are represented by missense mutations (57.7 %), frameshift mutations (26.9 %), inframe deletions (3.9 %), nonsense mutations (7.7 %), and splice region mutations (3.9 %) (11).

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As a result of a large-scale screening analysis of mutations in the gene *NPHS2*, carried out in Europe on 901 patients, it was found that mutations were more frequent in patients with familial autosomal recessive SRNS than in sporadic cases, namely in 53 % and 10.3 % of cases, respectively (11). Homozygous and compound heterozygous *NPHS2* mutations, as well as a combination of heterozygous mutations and polymorph variant c.686G>A (p.Arg229Gln), were identified in 50 % of patients with familial autosomal recessive SRNS and only in 8.2 % of cases with sporadic SRNS. The main clinical features of sporadic cases of SRNS with homozygous and compound heterozygous mutations in the *NPHS2* gene included the facts that childhood age of manifestation of the disease was up to 10 years, and there was preferential development of focal segmental glomerulosclerosis (69.8 %), primary resistance to steroid therapy, and progression to terminal chronic renal failure during the first decade of life (10).

Based on the analysis of the expression of *NPHS2*, it was demonstrated that even in carriers of simple heterozygous polymorphous *NPHS2* variants, as well as in carriers of combinations of compound heterozygous mutations and polymorphic variants, there was a disturbance in the uniform distribution of podocin with preferential localization only in the bodies of podocytes or along the glomerular basement membrane (12). The explanations for the role of simple heterozygous mutations in the *NPHS2* gene may lie in the possible presence of other, yet unidentified, mutations in this gene, possibly involving regulatory sequences or mutations in non-coding regions of the gene. In addition, there may be a combination of several mutations in unknown genes that can produce an additional effect on the basis of the development of the disease.

The aim of the study was to determine the variants in coding and non-coding regions of the *NPHS2* gene in Azerbaijani children with steroid sensitive NS (minimal change disease (MCD)) and steroid-resistant nephrotic syndrome (SRNS).

Material and methods

Twenty-nine children; 19 (65.5 %) male and 10 (34.5 %) female child patients aged from 5 to 11 years (mean age 7.2 ± 2.65 years) with NS-associated chronic glomerulonephritis (CGN) were included into the study. Inclusion criteria were the age of less than 16 years and the diagnosis of NS. Patients with genetic syndromes, chromosomal aberrations, connective tissue diseases and vasculitis were excluded.

Clinical studies were conducted at the Azerbaijan Medical University, while molecular genetic studies were performed in the Genetic Diagnostic Center AFGEN (Biological Medicine, Baku) and Medical Faculty of the Aegean Medical University (Izmir, Turkey). Parents signed the informed consent. Study was approved by Ethics Committee of Azerbaijan Medical University.

In all patients, the diagnosis of NS was made based on the symptomatic complex characteristic of this disease, namely oedema, proteinuria over 3 g/24 h, hypoalbuminemia below 25 g/l, and hyperlipidaemia. The functional state of the kidneys was judged by the results of a dynamic examination of patients with the determination of the glomerular filtration rate (GFR), calculated by the G.J. Schwartz formula (13).

NPHS2 gene is evaluated by Sanger technique. Isolation of DNA from leukocytes after taking 200 µl of blood from the vein was performed by using a DNA Prep 200 DIAtom™ reagent kit. For sequencing, BigDye® Terminator V.3.1 Cycle Sequencing Kits (Applied Biosystems, USA) was used. The purification reaction was carried out using the BigDye X Terminator™ Purification Kit. The nucleotide chain of ABI3130xl of each exon of the *NPHS2* gene is read in the PCR system. The results were evaluated using SeqScape v.2.7 according to NM_014625.3 transcript ID and variants were classified as Benign (B), Likely Benign (LB), Variant of Uncertain Significance (VUS), Likely Pathogenic (LP), Pathogenic (P)) according to the American College of Medical Genetics (ACMG) and Genomics and the Association for Molecular Pathology 2015 guidelines (14).

Results

Of the 29 examined patients, 24 (82.8 %) were diagnosed with MCD and 5 (17.2 %) with SRNS. The observed sequence variants are described in Table 1.

Tab. 1. Nucleotide substitutions in the *NPHS2* gene in children with different types of NS (n=29).

Exon	Nucleotide change	Amino acid change	Heterozygote/Homozygote	Classification	Patients, n (%)	MCD (n=24)	SRNS (n=5)
1	c.59C>T	p.Pro20Ley	Homozygote	Uncertain Significance	2 (6.9)	2	–
1	c.102A>G	p.Gly34Gly	Homozygote	Benign	19 (65.5)	15	4
4	c.503G>A	p.Arg168His	Homozygote	Likely Pathogenic	1 (3.4)	1	–
4	c.506T>C	p.Leu169Pro	Homozygote	Uncertain Significance	2 (6.9)	2	–
5	c.538G>A	p.Val180Met	Homozygote	Uncertain Significance	1 (3.4)	1	–
5	c.686G>A	p.Arg229Gln	Homozygote	Uncertain Significance	1 (3.4)	1	–
7	c.868G>A	p.Val290Met	Homozygote	Uncertain Significance	2 (6.9)	2	–
8	c.954C>T	p.Ala318Ala	Heterozygote	Benign	1 (3.4)	2	3
			Homozygote		12 (41.4)	6	2
5'UTR							
5'UTR-51G>T			Heterozygote	Uncertain Significance	2 (6.9)	–	2
Intron							
c.452-46C>T			Heterozygote	Benign	1 (3.4)	–	1
c.452-21C>T			Heterozygote	Benign	2 (6.9)	–	2

Sequencing analysis of the *NPHS2* gene showed complex variant combinations in 44.8 % of cases, and one type of mutation in 55.2 % of cases.

The sequencing of the *NPHS2* gene revealed variants in 5'-terminal untranslated region (5'-UTR) in 6.9 % of cases in a heterozygous form (Tab. 1). Also, four mutation combinations (homozygous variant c.102A>G, homozygous variant c.954C>T, a heterozygous variant 5'UTR-51G>T and c.452-46C>T and c.452-21C>T heterozygous variants in one SRNS-diagnosed male patient (3.4 %) and three mutation combinations (homozygous variant c.102A>G, heterozygous variant c.954C>T and 5'UTR-51G>T heterozygous variant) were detected in one SRNS-diagnosed female patient (3.4 %).

In 11 patients (37.9 %), two variant combinations were determined. At the same time, 10 patients (34.5 %) had a combination including homozygous mutation c.102A>G and heterozygous mutation c.954C>T, and in 1 (3.4 %) homozygous mutation c.954C>T and in IVS3-21C>T heterozygous mutation.

In 16 patients (55.2 %), only one variant was observed as follows: homozygous variant c.102A>G in 7 patients (24.1 %); homozygous variant c.59C>T in 2 patients (6.9 %); homozygous variant c.506T>C in 2 patients (6.9 %); homozygous variant c.868G>A in 2 patients (6.9 %); homozygous variant c.503G>A, homozygous variant c.538G>A and homozygous variant c.686G>A were observed in 1 patient, each (3.4 %). All observed variants are summarized in Table 1.

Two mutant combinations, namely homozygous mutation c.102A> G and heterozygous mutation c.954C>T were found in 8 patients with MCD.

All 16 patients with no mutations were diagnosed with MCD.

Discussion

This study is the first evaluation of *NPHS2* gene mutations in NS patients in Azerbaijan and thus the frequency and spectrum of *NPHS2* gene mutations in Azerbaijan are unknown.

There are few studies describing variants in untranslated regions and intronic positions. As can be seen from Table 1, in children with MCD, no mutations were detected in the intron and unencoded area, whereas in children with SRNS, such mutations occurred. There was found only one 5'UTR region variant -51G>T heterozygote, namely in two patients with SRNS in combination with other benign variants (c.102A>G and c.954C>T) and intronic variants (c.452-46C>T and c.452-21C>T). 5'UTR-51G>T variant classified as LB in ClinVar database despite the fact that there are resources classifying it as LP (15). According to Duca et al such variants in *NPHS2* promoter (-51T, -116T, and -535 insCTTTTTT(3)) downregulate gene expression and play a role in pathogenesis (16). For now, it is decided to be classified as VUS. Intronic variants c.452-46C>T and c.452-21C>T also have been previously reported and accepted as polymorphism in studies (15).

The variant c.59C>T has been seen in two patients with MCD. There is a contradiction in literature regarding this variant. While older studies showed this variant as being pathogenic (17, 18) the new ones accept it as being benign (19–23). Despite the fact that there are several studies predicting c.59C>T to have a disease-causing effect in European, North American, Caucasian and South American populations (24), in our study, it was decided to classify it as VUS while further segregation and detailed pedigree analyses were planned.

Interestingly, the classified LP variant c.503G>A is mostly reported in patients with SRNS, but in our research it was seen in one patient diagnosed with MCD, which shows the possible clinical variability caused even by the same pathogenic variant of the same gene depending on population.

Leu169Pro (21), Val180Met (25), Val290Met (21) and Arg-229Gln (21) were classified as VUS because of insufficient evidences and lack of segregation analysis. Nevertheless, they are thought to be the cause of clinical manifestation in patients.

Conclusion

Gene mutations are the most common molecular-genetic cause of NS. Genetic heterogeneity of NS indicates that screening strategies should continue to include multiple NS genes, including rare and newly discovered genetic causes, to ensure a high yield of molecular genetic diagnoses. Subsequently, this will lead to improvements in both accuracy of diagnosis and clinical management.

Identification of mutations is important because it can influence the decision of doctors as to how to treat patients while patients suffering from mutations can get rid of the side effects of immunosuppressive therapy and ultimately can be considered for kidney transplantation from a living donor.

Although, only one patient's result was thought to be explaining the clinical symptoms, the high prevalence of uncertain significance variants and some cases with benign variants indicate the need to continue the genetic study of podocyte-related genes. The variable clinical manifestation seen in this work one more time emphasises the importance of populational genetic studies.

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