Molecular Genetic Analysis of Steroid Resistant Nephrotic Syndrome, Detection of a Novel Mutation

Niloofer Serajpour,¹ Behnaz Karimi,² Pedram Khosravi,² Nakysa Hooman,³ Rozita Hosseini,³ Azadeh Shojaei¹-²

Introduction. Nephrotic syndrome is a heterogeneous disease in children, with nearly 10% categorized as steroid-resistant. In this study we evaluated disease related mutations within NPHS1, NPHS2 and new potential variants in other genes.

Methods. In the first phase of study, 25 patients with SRNS were analyzed by Sanger sequencing for NPHS1 (exon 2, 26) and all exons of NPHS2 genes. In the next step, whole exome sequencing was performed on 10 patients with no mutation in NPHS1 and NPHS2.

Results. WES analysis revealed a novel mutation in FAT1 (c.10570C > A; Q3524K). We identified 4 pathogenic mutations, located in exon 4 and 5 of NPHS2 gene in 20% of patients (V180M, P118L, R168C and Leu156Phe). Also our study has contributed to the description of previously known pathogenic mutations across WT1 (R205C) and SMARCAL1 (R764Q) and a novel polymorphism in CRB2.

Conclusion. It seems that NPHS2, especially exons 4 and 5, should be considered as the first step in genetic evaluation of Iranian patients. We suggest conducting WES after NPHS2 screening to identify the potential genes associated with SRNS, Further studies are required to examine more common genes in the first step and then designing native laboratory panels.

INTRODUCTION

Nephrotic syndrome (NS) is a common chronic glomerular disease in children¹ which is characterized by proteinuria, hyperlipidemia, hypo-albuminemia and edema.¹,² According to the patient’s response to the steroid therapy the disease can be divided to: resistant and sensitive. Nearly 10% of patients are not responsive to steroid therapy during four weeks who described as steroid-resistant nephrotic syndrome (SRNS).⁴ SRNS is considered as a poor prognosis disease and mostly required dialysis and transplantation.⁵ The most frequent renal histological feature associated with SRNS is focal segmental glomerulosclerosis (FSGS). Moreover minimal change nephrotic syndrome (MCNS), and diffuse mesangial sclerosis (DMS) have been identified.¹,⁶,⁷ SRNS is represent as isolated kidney disease or syndromic disorder.¹ The fenestrated endothelium, the glomerular basement membrane (GBM) and the podocytes form three layers of glomerular filtration barrier (GFB) which is impaired in NS and causes proteinuria.⁸ Tow major proteins of podocytes including Nephrin and Podocin, coded by NPHS1 and NPHS2 are considered to play an important role in GFB, respectively. Most cases of SRNS are sporadic representing both AR and AD inheritance. Mutations in these genes are the most common identified genes in AR form.

The aim of this study was to screen mutations
causing disease within NPHS1 and NPHS2, figuring out the most common mutations in Iranian children and comparing the prevalence of such mutations among different nations. Due to heterogeneity of this disease, WES was performed for 10 patients in pilot study to evaluate other related genes and exploring new potential mutations. Indeed, prevention of ineffective treatment with steroids and helping clinicians to properly predict post transplantation outcome may be facilitated via indicating the specific mutations.

**MATERIALS AND METHODS**

**Patients’ Description**

25 subjects were recruited from Ali-Asghar children’s hospital in Tehran, Iran. The enrollment of patients in this study is based mainly on the clinical diagnosis of SRNS and the age at the onset of disease varying from congenital to childhood. 3 of these children have been progressed into end-stage renal disease and 5 of patients are on dialysis. The informed consent forms were signed by parents of patients. The study was approved by the Ethical Committee of “Iran University of Medical Sciences, Faculty of Medicine”.

**Polymerase Chain Reaction (PCR) and Sequencing**

Four mL of whole blood was taken from Patients and transferred into tubes containing 200 μL EDTA for DNA isolation. DNA was isolated from peripheral blood of all samples by Yekta Tajhiz Azma (YTA) kit (Iran).

All exons of NPHS2 gene were screened (primers are available upon request). Exons 2 and 26 of NPHS1 gene were amplified using four primer pair (Supplementary data). Subsequently, to confirm the identified mutations in affected children, their parents were studied well.

Reaction were accomplished in a total volume of 25 μL containing 12.5μL Master Mix (Amplicon with 1.5mM MgCl2), 11 μl DEPC water, 20-40 ng template DNA and 10 pmol from each primer as well. After initial denaturation at 94 °C for 3 min, 35 PCR cycles were performed using Thermo fisher thermocycler (SimpliAmp™ Thermal Cycler 96-well, Applied Bio systems); each cycle included denaturation at 94°C in 30s, annealing temperature at 62 for exon 2, 26, extension at 72°C for 30s and final extension at 72°C in 5 min.

PCR products were subjected to electrophoresis on agarose gel (1.5%). Sequencing was done by MACROGEN Company in South Korea using classic Sanger method with ABI. Obtained sequences were aligned to the reference genome by chromas software and blast in Refseq in ncbi.

**Whole Exome Sequencing (WES)**

The second phase of study, WES was carried out for 10 patients by Colombia University Medical Center, IGM Institute for Genomic Medicine, Hemer Health Science.

**RESULT**

**Polymerase Chain Reaction (PCR) and Sequencing**

25 children with SRNS were referred to Ali-Asghar hospital in Tehran, Iran to be examined for mutational analysis. Their mean age at the onset of symptoms was (2.54 ± 3.24) years (congenital to 14 years Positive family history was detected in 4 patients (16%), while 21 patients were sporadic (84%) in this cohort. Renal biopsy of patients indicated four different conditions, including FSGS (44%), MCNS (28%), CNF (8%), MeSPGN (4%). Histological data of 3 patients were not available. Also 9 patients showed positive family history of kidney stone (Table 1).

We identified c.567.568insT known pathogenic frameshift mutation (L156fsx166) in 2 patients. Moreover c.502C  > T (p.168R  > C) pathogenic homozygous mutation was found in one patient. Both of these mutations were located in exon 4 of NPHS2 gene. Parents were screened for these mutations (Figure 1). Although no mutation causing disease was detected in the other studied exons of NPHS1 and NPHS2 genes by this method, benign or likely benign variants were detected in 15 patients (56%) within these regions (Table 2).

**Whole Exome Sequencing (WES)**

To investigate other related genes and confer higher detection rate, WES was performed for 10 patients, with negative findings in the first phase of study. Two pathogenic mutations in NPHS2 were found in exon 2 and 5. Furthermore, two other causative mutations were identified within WT1 and SMARCAL1 genes in 2 patients. Significantly, a novel mutation in FAT1 was detected. To predict the clinical significance of the found mutations,
3 different softwares were used, including SIFT, Polyphen, mutation assessor, which revealed the prediction score of 0.034, 0.044, and 2.1; respectively (Table 3). Moreover, the parents of these patients were sequenced by PCR for confirmation of the novel mutation (Figure 2). To amplify this region a specific primer pair was designed by primer3plus (supplementary data). Another novel variant were found in CRB2 predicted to be “benign” by the above-mentioned silico analysis software (0.058, 0.671) (Table 2).

**DISCUSSION**

Idiopathic nephrotic syndrome (INS) is a common
clinical condition displaying genetic heterogeneity and significant phenotypic variability.\textsuperscript{2,13-15} It can be caused by many single-gene mutations in both recessive (NPHS1, NPHS2, SMARCAL1, FAT1, and CRB2) and dominant inheritance forms (caused by genes such as WT1 and TRPC6).

Recessive mutations in NPHS1 and NPHS2 cause severe clinical features of early-onset NS and progress to ESRD, either during infancy or throughout childhood. Whereas, hereditary autosomal-dominant NS is rare, occurring mostly in juvenile and adult familial cases.\textsuperscript{1}

NPHS2 mutations have been reported as the most common cause of childhood onset in autosomal recessive SRNS.\textsuperscript{5, 16-17} The most frequent renal histological feature associated with SRNS is focal segmental glomerulosclerosis (FSGS).\textsuperscript{3,18} In present study, the pathological manifestation of FSGS was about 44% in patients in which 4 out of 11 patients with FSGS were showing NPHS2 mutation in a homozygous state.

Previous studies claim that incidence of NPHS2 mutations in children may vary according to ethnicity(19, 20). In 2013, Basiratnia et al\textsuperscript{21} showed that NPHS2 mutations were about 31%, responsible for 57% and 26% of sporadic and familial forms of SRNS. In our study, 5 of 25 (20%) carried NPHS2 mutations (40% familial and 60% sporadic); both studies among Iranian population are almost consistent with findings of studies performed among American,\textsuperscript{22} Turkish,\textsuperscript{23} Arab,\textsuperscript{24} and Mexican\textsuperscript{25} population. (26%, 24.7%, 22%, and 21%; respectively), while is in contrast to those findings among Far East population like Chinese (4.3%),\textsuperscript{26} Japanese (4%),\textsuperscript{27} and south Korean children (0%)\textsuperscript{28}. Due to all of these data, we suppose a hypothesis that the incidence of NPHS2 mutations decreases from northwest to southwest.

In present study we identified a known frameshift and a missense mutation in exon 4 of NPHS2 (Leu156Phe, R168C) in 3 patients.\textsuperscript{9,29} Previous investigations have been reported two other mutations within this exon among Iranian population (R168H, D160G).\textsuperscript{21,30} Although Otoukesh et al. indicated no mutation in exon 5 in 2009, later 3 pathogenic mutations (V180M, R238S, F185fsX186) in this region were found by Basiratnia and her colleagues in 2013,\textsuperscript{21} similarly V180M in exon 5 was identified in one of our patient. Moreover, in our study P118L mutation was detected in exon...
This missense mutation in podocin seems to be a relatively common NPHS2 mutation as it was found in several conducted studies.22,31,32 Although, Behnam et al. reported there are more than 65% hot spot mutations in exon 8 of NPHS2, no mutation was found in our study within this region. Despite the high rate of NPHS2 mutation, no hot spot mutations have been identified for this gene. But according to earlier21 and present study, we recommend NPHS2 especially exons 4 and 5 to be considered as first step genetic approach in children with SRNS. Our finding is consistent with the other nations indicating common presence of SNPs within exon 4 and 5.9,23,33,34

NPHS1 Mutations is another primary important gene associated with congenital nephrotic syndrome (CNS) that manifests within 90 days after birth with SRNS.35 So far more than 200 mutations in NPHS1 have been reported (http://www.hgmd.org/, accessed on 2017). Tow known fin minor and fin major mutations in NPHS1 (within exon 26 and 2, respectively) have been found in majority of children.36 A study conducted in northwest of Iran by Behbahan et al.37 in 2013 revealed 6 different mutations in 80% of SRNS children showed no mutation within these exons. Similar to Brazilian17 and polish38 studies, our result indicated pathogenic mutation neither within these exons among all patients nor in other exons studied by WES. Due to Behbahan’s findings37 and our study, we suppose that exon 2 and 26 of NPHS1 gene may not be causative exons for SRNS in Iranian children.

It is acknowledged that more than 53 genes are associated with SRNS in both recessive and dominant inheritance forms.15,39 Gemma Bullich et al. supposed that genetic testing using standard Sanger methods is costly and time consuming, even if only the most frequently mutated genes are analyzed2 however we believe that screening for pathogenic variants in some common genes by this method could be the first cost effective approach. Due to genetic heterogeneity of SRNS, Next step may be employing WES. This should take in to account that although WES has nearly 30% higher cost it leads to identification of new disease-causing mutations covering all genes associated with SRNS.40

In our study, through evaluation of WES data in patient number 16, known R764Q mutation was found in SMARCAL1 gene, a transcription factor expressed in podocyte.41 This mutation is related to a disorder known as Schimke immune-osseous dysplasia (SIOD) showing SRNS, short stature and immune deficiency.42,43 Finding a putative mutation using WES method in this case helped us diagnose a disease, which its symptoms overlap with SRNS.

Another gene involved in SRNS is FAT1, which Loss of function mutations in this gene result in decreased cell adhesion and migration in fibroblasts and podocytes.44 Most of previous studies describe the role of FAT1 heterozygous mutations in some cancers,45,46 whereas Heon Yung Gee and colleagues in 2016 reported 4 different homozygous variants as causative factors for glomerulotubular nephropathies such as NS.44 We identified a novel recessive variant Q3524K in FAT1 as SRNS cause in a 10 years old girl from consanguine parent. This potentially pathogenic variant was evaluated by some predictive tools including SIFT, Polyphen and Mutation Assessor. However to confirm its pathogenicity some functional and in vitro investigations are needed.

Mutations in the WT1 gene, encoding the Wilms’ tumor 1 protein, which typically lead to Denys-Drash syndrome or Frasier syndrome, can also cause SRNS Type 4.47-49 Exon 8 and 9 of this gene has been considered as one the most prominent implicated genes in SRNS.15,50 Clinical manifestation of patient number 18 showed an affected girl with congenital and sporadic CNS/SRNS. In this index, WES analysis revealed one homozygous R205C mutation in exon 7, which was a de novo mutation in a hot spot region.51 Our findings are in consistent with previous reports identifying WT1 mutation mostly in girls, within hot spot regions of WT1 gene (exons 5, 6, 7, 8 and 9) and often in de novo state.42,52

The low rate of mutation frequency in WT1 gene of our study (4%) is similar to some reports by Ruf and his colleagues,53 Cho et al.28 and Alharthi et al.54 (6.9%, 5.7%, and 5%; respectively) but is less than what reported in MUCKA study (8.9%)50 and more than the finding related to Indian children (1.7%). Although, mutation rate of WT1 is low but patients carrying WT1 mutations represent in early onset with more severe phenotype and congenital form of SRNS.

Overall, SRNS causes 15% of all chronic kidney disease. In our study, we noticed the interesting data that positive family history of kidney stone
were existed in 16 out of 25 (64%) patients. This finding triggered a hypothesis in our mind that this factor may increase the risk of NS significantly, although to prove its accuracy, more samples and further investigations should be performed.

CONCLUSION

In summary, this is the first and largest study among Iranian population with different ethnic origins that investigates causative variants associated with SRNS through screening both common genes (NPHS1 and NPHS2) and whole exome study. Among 25 patients who underwent for PCR sequencing for all exons of NPHS2, 5 patients carried a mutation causing disease, suggesting that NPHS2 especially exons 4 and 5 of this gene should be considered as the first step genetic approach in children with SRNS. For the first time in Iran 3 known variants were detected in WT1, SMARCAL1 and CRB2, significantly and a novel variant was identified in FAT1 gene.

Since the heterogeneous clinical and pathological spectrum, a molecular diagnosis based on sequencing is required. Identification of mutations causing SRNS is of importance, not only for therapeutic considerations but also for genetic counseling.

KEYNOTE

To detect common and potential mutation associated with SRNS, we performed sanger sequencing for NPHS1 (exon 2, 26) and all exons of NPHS2 genes and WES on 10 patients with no mutation in mentioned genes. Our finding manifested a novel mutation in FAT1 gene. Moreover we found exon 4 and 5 NPHS2 gene as the most common causative region. Additionally some known mutations were found in SMARCAL1 and WT1 genes.

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Correspondence to:
Azadeh Shojaei, MD
Iran University of Medical Sciences, Shahid Hemmat Highway, Tehran, 144961535, PO Box: 14665-354, Iran.
E-mail: a_shojaei2007@yahoo.com

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