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A MULTICENTER STUDY IN SAUDI ARABIA TO ASSESS CHILDHOOD NEPHROTIC SYNDROME AND ITS RELATIONSHIP TO NPHS1 GENE MUTATION

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ABSTRACT

Congenital nephrotic syndrome of Finnish type (*NPHS1*) is an autosomal recessive gene disorder characterized by severe proteinuria of intrauterine onset. Focal and segmental Glomerulosclerosis (FSGS), both are a primary idiopathic condition, which is a common pattern of glomerular injury, defined by segmental sclerotic lesions involving only a subpopulation of glomeruli. This study is to assess Childhood Nephrotic Syndrome and its Relationship to NPHS1 Gene Mutation. We sought to identify Fin_{Major} (L41fsX90, exon 2) and Fin_{Minor} (R1109X, exon 26) within the *NPHS1* gene. Seventy-four Saudi cases with nephrotic syndrome were collected from different pediatric hospitals in Makkah, Jeddah, Al-Hada, Al-Taif and Al-Riyadh. All nephrotic cases were classified as congenital and idiopathic. All genomic DNA samples were isolated from peripheral blood lymphocytes, and polymerase chain reactions (PCR) was used to amplify the *NPHS1* gene. To detect the mutations Fin_{Major} and Fin_{Minor} (*NPHS1* gene), specific restriction endonucleases were utilized using restriction fragment length polymorphisms (RFLP). Among 74 nephrotic cases, we identified no mutations due to Fin_{Major} and Fin_{Minor} within the *NPHS1* gene.

KEYWORDS: NPHS1, pediatrics, nephrotic symdrome, genes.

INTRODUCTION

The idiopathic nephrotic syndrome is a clinical pathologic entity occurring mainly in children and is characterized by massive proteinuria (loss of protein), hypoalbuminemia, hyperlipidemia, edema, and minimal glomerular changes. The degree of proteinuria in patients with renal disease correlates closely with the severity of damage to the glomerulus. It was noted that, in some instances, renal biopsy may show focal segmental glomerulosclerosis or diffuse mesangial proliferation[1].

Most patients with idiopathic nephrotic syndrome respond to steroid therapy and show a favorable outcome. However, 20% are steroid-resistant, with progression to end-stage renal failure in many cases. The nephrotic syndrome may recur after renal transplantation in such cases[2]. The *NPHS1* gene (MIM #602716) is located on chromosome locus 19q13.1 [3-4]. The gene spans about 26 kb of DNA sequence and contains 29 exons[5] and a recently found additional exon 271 bp in length located 3.7 kb upstream of the ATG translation initiation codon in exon 1. Thus, the originally reported exon 1 has been named exon 1A, and the additional exon, exon 1B [6]. The gene produces a 4.3-kb long mRNA, which is translated to a protein called nephrin.

Mutations in the *NPHS1* gene lead to idiopathic congenital nephrotic syndrome of the Finnish type (CNF)[7], which is an autosomal recessive renal disease [3,8] and is mainly found in Finland with an approximate incidence of 1/10,000 of live-births[9]. Over 90% of *NPHS1* mutations in the Finnish population is either Fin_{major} with an early stop codon in exon 2 or Fin_{minor} with a stop codon in exon 26. A variety of different mutations leading to various clinical manifestations has been described also in other

countries[10-12]. The clinical features of idiopathic nephrosis of the Finnish type (CNF; OMIM #256300) are massive proteinuria already *in utero*, edema, enlarged placenta and low birth weight[12-14]. Detection of high levels of -fetoprotein in the amniotic fluid and in the maternal circulation has been used for prenatal diagnosis of CNF [15]. However, after the finding of the causative NPHS1 gene defect, the diagnosis is not infallible without mutation analysis[16]. Interestingly, after transplantation, 20% of CNF patients manifested with a recurrence of the nephrotic syndrome, which has been shown to occur due the presence of nephrin-specific circulating auto-antibodies [17]. In earlier times, CNF led to death during the first months of life, but in 1984, a new treatment protocol was introduced. It consists of early nephrectomy and dialysis until the body weight rises to an acceptable level for transplantation[18]. This treatment removes all symptoms apparently with no later manifestations in other organs [19]. The predicted domain structure of nephrin classifies it as a member of the immunoglobulin (Ig) superfamily. Moreover, a new subfamily of nephrin-like (NEPH) molecules including (in addition to nephrin) NEPH1, NEPH2 and NEPH3/ filtrin has recently been described[20-21].

Response to treatment varies among patients and the cause of resistance to steroid in some patients is not well known. [22] Recent studies have determined the clinical course of children with idiopathic childhood nephrotic syndrome who received intravenous methylprednisolone following failure to achieve remission with standard oral prednisolone therapy [23]. Kurosu *et al.* (2009) have concluded that rituximab may be an effective treatment agent for resistant nephrotic syndrome and the peripheral

B cell count may be a useful marker in steroid-resistant patients for preventing disease relapse [24].

The objective of this study is to determine the relationship of steroid response to the NPHS1 gene mutations, the prevalence of the NPHS1 gene mutation in nephrotic syndrome among Saudi children, the frequency of family history and NS patients of steroid-resistant NS (SRNS) and steroid-sensitive NS (SSNS), and to compare the Saudi population to those reported from other ethnic groups.

MATERIALS & METHODS

The nephrotic patients (N= 74) were selected from pediatric nephrology clinic of different hospitals by consultants of Pediatric Nephrologists among different regions of Saudi Arabia (*e.g*., Makkah, Jeddah, Al-Hada, Al-Taif, Riyadh, Eastern regions) as having at least one affected child from each family based on clinical criteria at time of the study in 2011. The ages of the presenting children ranged from the neonates to 18 years. Healthy subjects (N= 50) were selected as a control group with matched ages and sex as the NS group. Thorough clinical examination and construction of pedigree was taken. Biochemical investigations include: serum creatinine (umol/l), and GFR (ml/min). Intravenous blood samples were drawn in 3.5-cm^3 sterile tubes containing ethylene diaminetetraacetic acid (EDTA) solution. The blood samples were transferred to the Molecular laboratories-Department of Medical Genetics, Faculty of Medicine in ice blocks in temperature around (1-10ºC) and stored in a freezer at –20ºC till use.

The DNA was isolated through Intravenous peripheral blood samples collected from all subjects (N= 120) on EDTA test tubes. All samples were frozen till use. The blood-EDTA samples were thawed and genomic DNA samples were isolated using Blood mini-Spin Column Kit (Qiagen, USA) as recommended by the manufacturer: Proteinase K (20 µl) was pipetted into the bottom of a 1.5-ml-microcentrifuge tube. A whole blood sample (200-µl) was added to the microcentrifuge tube. We use up to 200 µl whole blood, plasma, serum, buffy coat, or body fluids, or up to $5x10^6$ lymphocytes in 200 µl phosphate-buffered saline (PBS). Lysis buffer (AL) (200 µl) was added to the sample and incubated at 56°C for 10 min. for complete hemolysis of the RBCs, lyses of the cell pellet and digestion of the proteins. Absolute ethanol (200-µl) was mixed with the sample to precipitate the DNA. The sample-ethanol was carefully applied to the QIAamp spin column (QIAGEN, USA), and then the mixture was centrifuged at 8000 rpm for 1 min. The filtrate was discarded. The column was carefully washed with the washing buffer (AW1) (500-µl). The tube was centrifuged at 8,000 rpm. Another 500-µl of the washing buffer (AW2) was added and again centrifuged at full-speed for 2 min. The column was opened and 200-µl buffer AE (elution buffer) were added, incubated at room temperature for 1min and then centrifuged at full-speed for 1 min. The highly pure DNA sample was refrigerated at 4°C until use, −20°C for longer time or −70°C forever.

Genomic DNA was added to a 25 ml reaction volume of 0.5 mM of each primer, 200 mM of each dNTP, 67 mM Tris–HCl, 16 mM $(NH_4)_2SO_4$, 0.01% Tween-20, 1 mM $MgCl_2$, and 0.15 units *Taq* DNA polymerase. The samples were then subjected to 30 cycles on PCR Engine Dyad (Bio-Rad Laboratories Inc., Hercules, CA). For PCR-RFLP, the target DNA in exon 2 (Fin$_{Major}$ site) was amplified using primers 5`-TTC TGG GCC CTG CCT GAA ATC-3` and 5`-CAT CTT TGG CCC ATT GCA C-3`, and the target DNA in exon 26 (Fin$_{Minor}$ site) was amplified using primers 5`-GGG GCT TGC ATA GGG TCA CT-3` and 5`-AGT GTC CCG CTC TCC TGT CC-3`.

The cycling protocol included an initial temperature cycle of 95°C for 5 min, 58°C for 30 seconds, and 72°C for 45 seconds. 30 cycles were performed in total, with final extension for 10 min at 72 °C. For detection of the Fin$_{Major}$ mutation by RFLP, 20 ml of a restriction enzyme mixture containing 3 units of *Taq*I enzyme (Promega), 0.1 g/L acetylated bovine serum albumin (BSA) and 13 Buffer E (1 mmol/L dithiothreitol, 100 mmol/L NaCl, pH 7.5, 6 mmol/L $MgCl_2$, and 6 mmol/L Tris-HCl) was added directly to the PCR reaction mixture containing the 103-bp PCR product, and the mixture was incubated at 65 °C for 1 h. For Fin$_{Minor}$ mutation detection, 20 mL of restriction enzyme mixture containing 3 units of *Ava*II enzyme (Promega), 0.1 g/L acetylated BSA and 13 Buffer C (10 mmol/L Tris-HCl, pH 7.9, 50 mmol/L NaCl, 10 mmol/L $MgCl_2$, and 1 mmol/L dithiothreitol) was added to the PCR reaction mixture containing the 239-bp PCR product, and the mixture was incubated at 37°C for 2 h. The analyses were completed by electrophoresis in a 3% MetaPhor agarose gel, after the digestion. Positive results were confirmed the R229Q mutation by DNA capillary sequencing analysis using BigDye terminator method (Applied Biosystem Inc., Model 5300, USA).

RESULTS

The selected 74 Saudi children, whom were suffering from nephrotic syndrome (NS) regardless of their causative factors, were classified according to age of onset, family history and their steroid respond. The total of steroid-resistant NS (SRNS) was 34.3% and 65.7% were steroid-sensitive NS (SSNS). Only 27% of SRNS patients represented positive family history and the remaining 73% cases stuck to negative family history (Figure 1). In the same manner, 19% cases had a positive family history, while 81% cases of negative family history were linked to the SSNS patients.
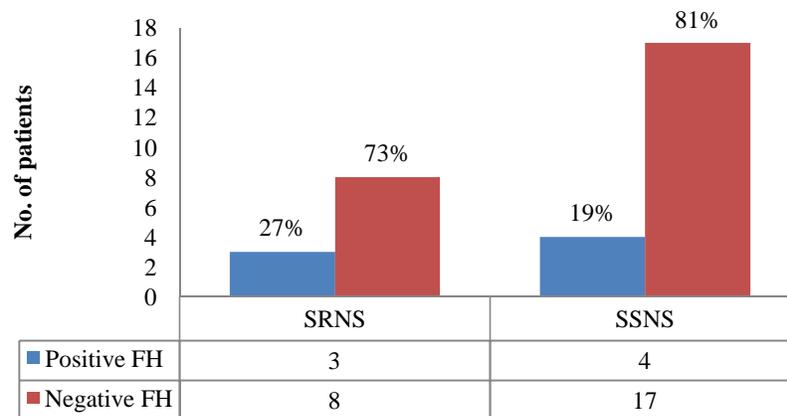
FIGURE 1. The frequency of family history and NS patients of SRNS and SSNS.

As for the *NPHS1* gene, the PCR-RFLP analyses for the detection of the NPHS1 Fin$_{Major}$ and Fin$_{Minor}$ mutations were performed. Among 74 nephrotic cases, we found no Fin$_{Major}$ mutation due to a 2-bp deletion in exon 2, or a Fin$_{Minor}$ nonsense mutation in exon 26 within the *NPHS1* gene.

DISCUSSION

Congenital nephrotic syndrome (CNS) of the Finnish type (CNF), a rare autosomal recessive disorder, was found to be caused by NPHS1 gene. CNS is characterized by a large placenta, massive proteinuria, and the onset of nephrotic syndrome shortly after birth [13,25]. To date, in both Finnish and non-Finnish patients, more than 70 different mutations including insertions, deletions, missense, nonsense, splice site and promoter mutations have been reported[11,16,26]. After reporting homozygous mutations in the NPHS1 gene of Japanese CNS patients in 2000 [27] in 2005 in another Japanese study only 2 out of 13 CNS patients in the NPHS1 gene had homozygous mutations, infering that mutations of NPHS1 was not a major cause of CNS in Japanese patients [28]. In contrast, two mutations Fin$_{Major}$ (L41fsX90) and Fin$_{Minor}$ (R1109X) were identified as the predominant cause of CNS in the Finnish population. [13] These mutations have been found in over 90% of Finnish patients[13] and mutations of the NPHS1 gene in both alleles were found in 11 out of 15 Italian patients.[16]

Unfortunately, we did not find any mutations within the NPHS1 gene due to L41fsX90 or R1109X for the cases with congenital or idiopathic nephrotic syndrome.

Limitations & Recommendations

Screening more genetic loci within the NPHS1 gene. Other responsible genes, *e.g.* NPHS2, NPHS3, WT1, ACTN4 genes might be investigated for this purpose.

CONCLUSION

Based on our molecular results of this unreeled study, we could not find any mutations due to Fin$_{Major}$ (L41fsX90) and Fin$_{Minor}$ (R1109X) within the NPHS1 gene. We identified a R229Q mutation in a lower frequency compared with other ethnic populations, but the R291W mutation is absent.

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