Mutations in \textit{NPHS}_2 Encoding Podocin Are a Prevalent Cause of Steroid-Resistant Nephrotic Syndrome among Israeli-Arab Children

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Abstract. Steroid-resistant nephrotic syndrome (SRNS) represents a heterogeneous group of kidney disorders that are often resistant to other immunosuppressive agents and tend to progress to end-stage renal failure. Mutations in the gene \textit{NPHS2} that encode a protein named podocin have recently been found in a recessive form of SRNS. Ten children from two inbred families of Israeli-Arab descent presented with SRNS. Renal histologic findings were of diffuse mesangial proliferation. Six patients reached end-stage renal failure, but nephrotic syndrome did not recur after renal transplantation. Mutation analysis of \textit{NPHS2} revealed that they were homozygous for the C412T mutation (R138X). Eighteen children were subsequently analyzed with SRNS due to biopsy-proven focal segmental glomerulosclerosis (FSGS) from unrelated families of Israeli-Arab descent. Analysis disclosed six additional patients (33%) bearing the same mutation in a homozygous pattern. Three of them had no affected relatives, although they came from large families. Taken together, of the 27 patients tested (familial and nonfamilial), 15 patients (55%) were homozygous for the mutation (R138X). They all shared the same haplotype and were homozygous for the A1023G polymorphism, thus pointing to a possible founder effect. Thirteen children of Israeli-Jewish origin with FSGS and biopsy-proven FSGS and 15 children of both ethnic groups with steroid-responsive FSGS were tested, and none was found to have mutations in \textit{NPHS2}. The results of this study demonstrate that mutations in \textit{NPHS2} are a common cause of SRNS in Israeli-Arab children. Mutations in \textit{NPHS2} may cause SRNS in nonfamilial cases. The interethnic differences in the occurrence of \textit{NPHS2} mutations may explain, in part, the previous observation that Arab patients with FSGS in Israel have a worse prognosis as compared with Jewish patients, despite similar presenting symptoms and medical management. Identifying the causing mutation will enable clinicians to avoid unnecessary immunosuppressive therapeutic trials in newly diagnosed patients and to provide prenatal diagnosis to families at risk.

The term “nephrotic syndrome” is applicable to any condition with massive proteinuria, hypoalbuminemia, and edema. The glomerular capillary wall, which is composed of a basement membrane covered by fenestrated endothelium on the inner part and visceral epithelial cells (podocytes) on the outer part, is responsible for plasma ultrafiltration during urine formation. Dysfunction of the glomerular barrier results in leakage of plasma proteins and the development of nephrotic syndrome (1).

The exact pathogenesis of nephrotic syndrome in childhood remains elusive. Over the last three decades, much research has been directed toward the definition of linkage between the immune system and nephrotic syndrome (2–9). The accumulated knowledge, often circumstantial, has led to the successful use of steroids (and subsequently other immunosuppressive medications) to treat nephrotic syndrome in children. The role of immune mechanisms was substantiated with the detection of a glomerular permeability factor in a group of patients with focal segmental glomerulosclerosis (FSGS) who experienced recurrence of nephrotic syndrome after renal transplantation (10). The most common variety of nephrotic syndrome in children is characterized by minimal histologic changes in the glomeruli on light microscopy (minimal change nephrotic syndrome), and indeed, the majority of patients respond to steroids and have favorable long-term outcome.

Steroid-resistant nephrotic syndrome (SRNS) represents a heterogeneous group of kidney disorders that often are resistant to additional immunosuppressive agents and tend to progress to end-stage renal failure. The most prevalent histologic findings associated with SRNS in children are diffuse mesangial proliferation and FSGS. It has been suggested that a lack of therapeutic response to steroids is a better prognostic indicator of outcome than the underlying histology (11). The exact factors that determine steroid responsiveness have not been fully elucidated. Inter-ethnic differences in prevalence and outcome have been shown in the United States: children with
FSGS of African American or Hispanic descent have a worse prognosis compared with white children (12). We have previously shown in a study comparing Arab and Jewish children with FSGS in Israel that there is a much greater tendency toward progressive renal disease in Arab children despite similar presenting symptoms, medical management, and follow-up (13). The pathogenetic mechanisms underlying inter-ethnic differences in the course of the disease remains obscure.

In recent years, the molecular bases of several conditions leading to SRNS have been identified. The common denominator shared by the following entities is that they all stem from a structural defect in the glomerular barrier, thus explaining their unresponsiveness to immunosuppressive medications (14). The congenital nephrotic syndrome of the Finnish type is caused by mutations in the NPHS1 gene encoding a protein called nephrin that is exclusively expressed at the slit diaphragm joining the podocyte foot processes (15,16). Mutations in ACTN4, mapped to 19q13 and encoding α-actinin-4, an actin filament cross-linking protein, have been found in dominantly inherited FSGS (17). This gene is highly expressed in the podocyte and is upregulated during the evolution of nephrotic syndrome in animal models (18). More recently, the NPHS2 gene has been cloned and found to encode a protein, podocin, that is specifically expressed in the podocytes of fetal and mature kidney (19). It mapped to the 1q25-q31 locus, previously designated SRN1 (20), and was found to be mutated in families with recessive inheritance of SRNS.

The aims of this study were twofold: to confirm the genetic basis of familial SRNS in two kindreds, and to determine the existence of mutations in the NPHS2 gene in a large group of children with nonfamilial FSGS. Identification of the causing mutation will enable us to provide prenatal diagnosis and to avoid unnecessary immunosuppressive therapeutic trials in newly diagnosed patients.

Materials and Methods

Patients

Ten children from two heavily inbred families of Israeli-Arab descent were diagnosed with nephrotic syndrome and followed in the Division of Pediatric Nephrology at the Shaare Zedek Medical Center, Jerusalem, Israel. Nephrotic syndrome consisted of massive proteinuria (>40 mg/m² per hour), hypoalbuminemia (<2.5 g/dl), and edema. Remission of the nephrotic syndrome was defined as the disappearance of proteinuria (<4 mg/m²/h); steroid resistance was defined as the lack of response to daily orally administered prednisone treatment at 60 mg/m² for 1 mo.

We subsequently analyzed three additional groups of patients with SRNS who had no family history of kidney disorders: (1) 18 children of Israeli-Arab origin with SRNS and biopsy-proven FSGS; (2) 13 children of Israeli-Jewish descent with SRNS and biopsy-proven FSGS; and (3) 15 children of both ethnic groups with biopsy-proven FSGS and a favorable course (steroid-responsive nephrotic syndrome). The biopsy findings included mild mesangial proliferation, interstitial alterations, and 5 to 10% segmentally sclerosed glomeruli.

After informed consent for the genetic studies was obtained from the parents of all patients, blood samples were obtained from all patients for DNA analysis. The study was approved by the Shaare Zedek Medical Center Ethics Committee.

Homozygosity Mapping

Homozygosity mapping to the SRN1 locus on chromosome 1q25-q31 (20) was performed on the two kindreds with SRNS. The disease was assumed to be transmitted in an autosomal recessive mode with complete penetrance. Allele frequencies were assumed to be equal for each marker.

Mutation Analysis in the NPHS2 Gene

Genomic DNA was extracted from whole blood collected in ethylenediaminetetraacetate-containing tubes. It was performed by using conventional molecular biology techniques with the EZ-DNA kit (Genomic DNA Isolation Reagent; Biological Industries, Beit Ha’emek, Israel). PCR was used to amplify individual exons of the NPHS2 gene by using primers on the basis of published information regarding intron-exon boundaries (19). PCR primers are listed in Table 1.

PCR reaction was performed in a total volume of 25 μl containing 1.5 μl genomic DNA, 10 pg of each primer, 2.5 μl of deoxynucleotide triphosphates (final concentration of 200 μM), 2.5 μl of 10X reaction buffer, and 0.2 to 0.5 unit of thermostable DNA polymerase. DMSO was occasionally added to the reaction mixture.

DNA was denatured at 95°C for 1 min, followed by 39 cycles of denaturation of 10 s at 95°C, annealing for 10 s at 55°C, extension of 1.5 \( \mu \)l of deoxynucleotide triphosphates (final concentration of 200 μM), 2.5 μl of 10X reaction buffer, and 0.2 to 0.5 unit of thermostable DNA polymerase. DMSO was occasionally added to the reaction mixture.

DNA was denatured at 95°C for 1 min, followed by 39 cycles of denaturation of 10 s at 95°C, annealing for 10 s at 55°C, extension of 30 s at 72°C, and final extension of 5 min at 72°C. This protocol was implemented for the amplification of exons 1 to 4, 7, and 8. For exons 5 and 6, DNA was denatured at 94°C for 4 min, followed by 40 cycles of denaturation at 94°C for 10 s, annealing at 55°C for 30 s, with a final extension time of 5 min.

PCR products were electrophoresed on a mutation detection enhancement gel. Gels were visualized by silver staining (21).

Table 1. Primers for DNA sequence analysis

<table>
<thead>
<tr>
<th>Exon</th>
<th>Forward Primer (5'-3')</th>
<th>Reverse Primer (5'-3')</th>
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<tbody>
<tr>
<td>1</td>
<td>GCAGGGCTCCACAGGGACT</td>
<td>TCAGTGCTCTGCTTTGGGAT</td>
</tr>
<tr>
<td>2</td>
<td>AGGCAGTGAATACAGTGGAAG</td>
<td>GGCCCTAGGAAATTACCTA</td>
</tr>
<tr>
<td>3</td>
<td>TTCTGGAGTGATTTGGAAG</td>
<td>TGAAGAAATTGGCAAGTCAG</td>
</tr>
<tr>
<td>4</td>
<td>AAGGTGAAACCCAAACAGC</td>
<td>CGTQAOGTAGACATTGAAAA</td>
</tr>
<tr>
<td>5</td>
<td>CATAGGAAGGGAGCCCAAGA</td>
<td>TTCAGGGCATATTGGCCATTA</td>
</tr>
<tr>
<td>6</td>
<td>CTCCCACTGACATCTGA</td>
<td>AAATTTAAATGAAACAGAAA</td>
</tr>
<tr>
<td>7</td>
<td>CTAATACATGGCTGACACCAC</td>
<td>TTCTAAGGGCAAGTCCTGG</td>
</tr>
<tr>
<td>8</td>
<td>GGTGAAGCCTTCAGGGAATG</td>
<td>TTCTATGAGGAGCCCTTTA</td>
</tr>
</tbody>
</table>
Variants seen on the single-strand conformation polymorphism (SSCP) gel were directly sequenced by the fluorescence dideoxy terminator method (22). Fluorometric sequences were analyzed with the Applied Biosystem 3700 DNA Analyzer (Applied Biosystem, Foster City, CA) according to the manufacturer’s protocol.

Comparison of the tested nucleotide sequence with the published sequence of NPHS2 gene was performed (GenBank database). Screening of the R138X mutation in the NPHS2 gene was performed by amplifying genomic DNA by using primers that selectively amplify normal or altered DNA sequences, a technique known as amplification refractory mutation system. The primers used for the detection of the mutation in exon 3 were as follows: 5’-GGTTGTA-CAAGAGTAATGGAAAGAGTAATTATATTAT-3’ for the mutated DNA, and 5’-GGTTGTACAAGAGTAATGGAAAGAGTAATTA-TATTAC-3’ for the wild-type DNA. The reverse primer is detailed in Table 1. DNA was denaturated at 95°C for 2 min, followed by 38 cycles of denaturation at 94°C for 10 s, annealing at 54°C for 10 s, and extension at 72°C for 10 s. The mutated DNA fragment was amplified only when the first primer was used.

Results

Study of Familial SRNS

Ten children from two heavily inbred families of Israeli-Arab descent presented at the mean age of 1.6 yr with nephrotic syndrome. Four children originated from two related nuclear families, and the remaining six are the offspring of four consanguineous couples (Figure 1). Eight of the 10 children were biopsied within the first 3 mo after the diagnosis of SRNS. Biopsy was deferred in two young siblings of the proband in family 1B. The histologic findings on kidney biopsies were of diffuse mesangial proliferation without segmental sclerosis. In a few occasions, there were mesangial IgM deposits, C3 deposits, or both. The nephrotic syndrome was resistant to steroids in all children. In several individuals, other immunosuppressive agents had been attempted, including therapy with cyclophosphamide and cyclosporine, without response. Renal function has gradually deteriorated; six of the nine children reached end-stage renal failure at the mean age of 6.6 yr. Five patients underwent

Figure 1. Pedigrees of kindreds of Israeli-Arab origin with steroid-resistant nephrotic syndrome.
renal transplantation without recurrence of the nephrotic syndrome.

**Homzygosity Mapping**

The high rate of consanguinity enabled us to perform homozygosity mapping in these two families. Fifteen members, including four affected individuals from family B, and 23 members, including five affected children from family A, were enrolled in our study. The tenth affected patient died as a result of the disease before the initiation of this study, and a DNA sample was not available for analysis. All of the affected members studied were homozygotes for the following markers: D1S2790, D1S242, D1S218, and D1S416; this confirms linkage to the SRN1 locus harboring the recently cloned NPHS2 gene with a maximum log of odds score of 2.99 ($\theta = 0.0$).

**Mutation Analysis**

After linkage of SRNS was determined in both kindreds, the candidate gene NPHS2 was screened for mutations. The entire 8-exon coding region was screened by use of PCR-SSCP. SSCP analysis detected bandshift in exon 3 of one affected child from each family. Direct sequencing of the corresponding PCR product demonstrated that they all were homozygotes for the C412T nonsense mutation leading to R138X. This transition was found to be a heterozygous change in obligate carriers.

**Children with Biopsy-Proven Primary FSGS**

To ascertain whether mutations in NPHS2 may cause SRNS in nonfamilial cases as well, we analyzed three distinct groups of patients with biopsy-proven FSGS.

The first group included 18 children from unrelated families of Israeli-Arab descent who presented with SRNS at the mean age of 5.9 yr. Eight children were hypertensive at diagnosis of the nephrotic syndrome. All renal biopsies showed histologic findings consistent with FSGS. None of the patients responded to other immunosuppressive agents, and 12 (67%) of 18 children showed declining renal function; they reached end-stage renal failure at the mean age of 10 yr. Our analysis disclosed that six of 18 children in this group bore the same monohaplotypes, including three who had no affected relatives despite stemming from very large inbred families. In retrospect, each one of the remaining three had a sibling who had died as a result of a kidney disorder. SSCP analysis of NPHS2 in the remaining 12 children did not reveal any bandshift. After this study was completed, we identified massive proteinuria on the first day of life in a sibling of an affected child. Renal biopsy was consistent with minimal change nephrotic syndrome and homozygosity for the R138X mutation was confirmed.

The second group consisted of 13 children of Israeli-Jewish descent with SRNS secondary to biopsy-proven FSGS. They originate from 13 nonconsanguineous unrelated families, and none had an affected relative. Their mean age at presentation was 8.4 yr, and two were hypertensive at the time of diagnosis. Five children (38%) reached end-stage renal failure at the mean age of 13 yr. Screening the entire coding region of the NPHS2 gene with PCR-SSCP and direct sequencing, when indicated, did not reveal mutations in any of the affected children of this group.

Third, as a negative control, we tested 15 children (four Arab children and 11 Jewish children) with biopsy-proven FSGS who achieved long-lasting remission of their nephrotic syndrome with immunosuppressive protocols. As expected, none of them carried a mutation in the NPHS2 gene.

**Polymorphism in the NPHS2 Gene**

The SSCP analysis, followed by direct sequencing, disclosed a novel genetic polymorphism in exon 8 in both Arab and Jewish individuals. The A1023G transition did not result in a change in the amino acid sequence. All 15 affected individuals (both familial and nonfamilial cases) who were homozygous for the R138X mutation shared the same monohaplotypes and were homozygous for the A1023G polymorphism. We detected homozygosity for this polymorphism in 20% of healthy unrelated individuals of Arab or Jewish descent (80 chromosomes in each group were tested). This polymorphism was found in a homozygous pattern in only 9% of children with FSGS who did not carry a mutation in the NPHS2 gene. Because all patients are homozygotes for the same mutation, share a common monohaplotype, and belong to the same ethnic group, our findings point to a possible founder effect.

**Discussion**

We have detected the same homozygous mutation in the NPHS2 gene in 15 children with SRNS of Israeli-Arab origin. Twelve patients have affected siblings, and they originate from five unrelated kindreds. The remaining three are the first encounters in their respective families. The identification of a nonsense transition (C412T leading to R138X) segregating with the disease in families, and resulting in premature termination of the protein in affected individuals, confirms its causal role in SRNS. Podocin, encoded by the NPHS2 gene, is an integral structural protein of the podocyte, and a truncated protein will presumably result in a glomerular barrier dysfunction, leading to nephrotic syndrome. Although its exact role has not yet been fully delineated, it clearly explains why children bearing homozygous mutations fail to respond to immunosuppressive agents, but also why the nephrotic syndrome does not recur after renal transplantation.

Our affected population with the R138X mutation bear the same monohaplotype and belong to a distinct ethnic group residing in a confined geographic region—a phenomenon that points to a possible founder effect. This mutation has been previously identified in one consanguineous family from Egypt (19). This may be in favor of a Middle Eastern origin of this mutation. Nevertheless, we were unable to trace back the studied families’ origin to a common ancestor. Data concerning the origin of the Israeli-Arab population are lacking, and political events of many centuries may have resulted in population shifts. Further attempts are underway to define a founder and the age of this mutation.

We have noted a wide range of phenotypic variability between individuals with the same genotype in two respects: the
clinical characteristics and the histologic findings. Diagnosis of SRNS varied from the first day of life but more often was made between the ages of 1 to 4 yr. Podocin has been shown to be highly expressed in the fetal glomeruli (19), which may account for our novel observation of a pattern consistent with congenital nephrotic syndrome. It remains to be determined why most cases are manifested beyond the first 3 mo of life. We assume that the ultimate phenotype is governed by interactions between the NPHS2 gene and other genes, regarded as modifier genes (23), genetic polymorphisms, or both.

Our data confirm data of previous reports that demonstrated that the renal histologic findings in children with mutated NPHS2 might include the entire range of minimal change nephrotic syndrome, diffuse mesangial proliferation, and FSGS (19,20). Furthermore, we have occasionally encountered IgM, C3 mesangial deposits, or both in affected children with otherwise diffuse mesangial proliferation. We speculate that these are nonselective secondary inflammatory events in the course of declining renal function that may in fact result from massive proteinuria per se (24). It underscores the limitations of renal biopsy in predicting the natural history and therapeutic response in children with SRNS. It should be emphasized that the clinical and pathologic characteristics of patients with SRNS who were found to have NPHS2 mutations were indistinguishable from those who did not. Taken together, it confirms that the absence of therapeutic response is a better prognostic factor than the underlying histology.

To our knowledge, our data are the first to demonstrate that mutations in NPHS2 may cause SRNS in sporadic cases. The term “sporadic” may be a misnomer because this may represent the first case in a given family. It is unlikely that a homozygous mutation will occur de novo, and whenever tested, the parents were indeed found to be carriers of the mutation.

We have shown that the R138X mutation in NPHS2 is a prevalent cause of SRNS among children of Israeli–Arab descent occurring in 55% of the 27 patients tested. This observation has dictated our current policy of screening all newly diagnosed children with SRNS from this ethnic group for possible mutations in podocin, which is technically feasible because the gene contains only eight exons. Detection of a given mutation will avoid unnecessary (beyond 1 mo of daily steroids) immunosuppressive therapeutic trials that are useless but may bear significant morbidity. It will also prevent us from performing a renal biopsy. This molecular tool will enable us to provide genetic counseling, detect carriers, and propose prenatal diagnosis to couples at risk.

The inter-ethnic differences in the occurrence of NPHS2 mutations in our patient population may explain, in part, the corresponding differences in outcome. This observation should be substantiated in further studies performed in populations of common ancestry in neighboring countries as well as in other ethnic groups. It may well be that SRNS in different ethnic groups results from unidentified genetic perturbations in genes encoding various constituents of the glomerular capillary wall that serve as a barrier.

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References


