Rearranged NPHS2 (Podocin) Mutations Are Rare in Adult-Onset Idiopathic Focal Segmental Glomerulosclerosis

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Rearranged NPHS2 (podocin) mutations account for up to approximately 30% of steroid-resistant idiopathic FSGS in children and are associated with a reduced risk for disease recurrence after renal transplantation. R229Q, a missense variant that is present in 3.6% of the white population, has been implicated as a common disease-causing mutation. Given these clinical implications, we examined the role of NPHS2 mutations in a cohort of patients with adult-onset FSGS. We used denaturing HPLC to screen for heterozygous and homozygous gene variants in PCR-amplified DNA fragments that contained all exons and splice junctions of NPHS2. Bidirectional sequencing was performed to define all of the gene variants detected. With the use of the denaturing HPLC in a single-blind pilot study, 40 of 43 known NPHS2 mutations were detected from 22 pediatric patients with FSGS to establish a test sensitivity of 93%. This screen then was applied to 87 adult patients with idiopathic FSGS (15 steroid-sensitive, 63 steroid-resistant, and nine familial cases). In this latter cohort, compound heterozygous mutations were detected only in one patient with steroid-resistant nephrotic syndrome. This response in turn remains the best guide to long-term prognosis. By contrast, approximately 50% of patients who have FSGS and fail to respond to immunosuppressive treatment will progress to ESRD within 7 yr (8,9).

Idiopathic FSGS is a clinicopathologic syndrome that reflects a common pattern of renal injury as a result of immunologic and nonimmunologic causes (1–5). Recent epidemiologic studies have shown that the incidence of FSGS has increased by two to fourfold in the past two decades, although the reasons for this increase are unclear (6,7). There is a racial difference in this syndrome with a two to four times higher prevalence in black compared with white patients (7). Overall, it is the single most common primary glomerular disease, responsible for approximately 3% of ESRD in the United States (6,7). At the clinical level, up to 40% adults with FSGS can be expected to respond to a course of intensive immunosuppressive treatment with complete remission of the nephrotic syndrome. This response in turn remains the best guide to long-term prognosis. By contrast, approximately 50% of patients who have FSGS and fail to respond to immunosuppressive treatment will progress to ESRD within 7 yr (8,9). Currently, there are no reliable clinical predictors to identify patients who have FSGS and will respond to immunosuppressive treatment. Consequently, one expert has recommended that all patients with FSGS be treated with a minimum of 3 to 4 mo of high-dosage corticosteroids, which can be associated with significant risk for iatrogenic complications (9).

Recent studies have identified several disease genes or gene loci that are responsible for rare forms of familial nephrotic syndrome (10–17). With the exception of familial steroid-sensitive nephrotic syndrome (18,19), these disorders typically are steroid resistant and do not recur in the renal allografts (10–17). Nephrin (NPHS1) was the first disease gene identified as the cause of Finnish congenital nephrotic syndrome, a recessive disorder that is characterized by severe nephrotic syndrome at birth and ESRD within the first year of life (10). Podocin (NPHS2) was identified later as the disease gene for an autosomal recessive form of FSGS linked to chromosome 1q25-31, which is characterized by early childhood onset of steroid-resistant nephrotic syndrome and ESRD between 3 mo and 5 yr of age (11). Linkage studies of adult-onset familial FSGS have revealed genetic heterogeneity with at least three loci identified on chromosome 19q13, 1q25-31, and 11q21 (12–16). Using a positional-candidate gene approach, two research teams recently identified alpha-actinin 4 (ACTN-4) and transient receptor potential 6 (TRPC6) as the disease genes for the dominantly inherited familial FSGS linked to 19q13 and 11q21, respectively (12–14). Most patients with these two syndromes develop proteinuria and renal failure between 20 and 40 yr of age. Their renal pathology comprises a spectrum of findings that range from...
from FSGS, global sclerosis, collapsing lesion, mesangial proliferation, and minimal-change lesion (14,17,18). Finally, a splice site variant of CD2AP, an adapter protein that facilitates T cell adhesion to antigen-presenting cells, also has been implicated to cause sporadic FSGS (20).

From the perspective of molecular pathobiology, it is interesting to note that nephrin, podocin, CD2AP, TRPC6, and α-actinin 4 all localize to the glomerular podocyte (2,5,21–25). Nephrin is a key structural protein that forms the slit diaphragm by heterodimerization with at least two other proteins, NEPH-1 and NEPH-2 (22,22,23). Thus, recessive nephrin mutations are associated with the most severe form of nephrotic syndrome (10). Podocin acts as a scaffold protein to recruit nephrin and CD2AP to the lipid rafts at the slit diaphragm (2,22,23). Together with TRPC6, a nonspecific cationic channel protein (13,14), these proteins interact to activate a phosphoinositide 3-OH kinase-dependent AKT signaling pathway that controls complex cellular programs, including remodeling of actin cytoskeleton and cell survival (24,25). The interruption of the latter function has been implicated as a cause of podocytopenia and FSGS (2). In contrast, ACTN-4 mutations, which cause a more slowly progressive form of FSGS, is associated with increased affinity of α-actinin 4 to the filamentous actin in vitro (12).

Although recessive NPHS2 mutations initially were reported to cause familial steroid-resistant nephrotic syndrome in children with ESRD, occurring between 3 mo and 5 yr of age (11), recent studies have shown that they are associated with a broader clinical spectrum. Indeed, homozygous or compound heterozygous NPHS2 mutations have been documented in 30 to 46% of familial and 10 to 30% of sporadic steroid-resistant nephrotic syndrome in older children (26–30). Typically, FSGS is the most common pathology associated with these cases, although a spectrum of glomerular lesions, including mesangial proliferation and minimal-change lesion, also may be seen (27,28). To define further the clinical relevance of NPHS2 mutations, we undertook a comprehensive mutation screening study in 87 cases of adult-onset idiopathic FSGS.

Materials and Methods

Using the Toronto Glomerulonephritis Registry database and through chart review of patients at the offices of community and academic nephrologists in Toronto, we recruited 87 adult patients with idiopathic FSGS (15 steroid-sensitive, 63 steroid-resistant, and nine steroid-resistant/no remission). Patients who never received any immunosuppressive therapy with no remission were classified as steroid resistant. Patients who never received any immunosuppressive therapy were considered indeterminate.

NPHS2 Mutation Analysis

We amplified eight gene fragments by PCR covering all of the exons and splice junctions of NPHS2 using genomic DNA as template (Supplementary Table 1). Because the optimal fragment size for denaturing HPLC (dHPLC) screening is between 100 and 400 bp, we used Meta-PCR with four primers to link exons 3 (73 bp) and 6 (56 bp) together to increase the PCR fragment size (31). In addition, we modified some of the primers to increase the size or GC content of the PCR products for optimal dHPLC screening (Supplementary Table 1). We used dHPLC to screen for heterozygous gene variants in the PCR-amplified fragments of NPHS2 (32,33). To detect homozygous gene variants, we repeated the screen by mixing the test DNA with normal control DNA in a 1:1 ratio. For increasing the mutation detection rate, each PCR fragment was screened by at least two different temperatures. Bidirectional sequencing was performed to define all of the gene variants detected.

To determine whether the two mutations (R229Q in exon 5 and Q285fsX302 in exon 7) that were found in patient TOR2679 were arranged in cis or in trans, we performed long-range PCR to amplify a 4.76-kb genomic fragment that spanned exons 5 and 7 of NPHS2, using the following primers: 5′-GGGACTCCGCCAAAGAGCCCCAAAGATCAA-3′ and 5′-AGGAAAGCAGGGGAAATG-3′ with an annealing temperature of 64°C. “Hot-start” PCR was performed with 100 ng of genomic DNA, 10 pmol of each primer, 10 mmol/L dNTP, and 0.75 U of HotStar Taq (Qiagen, Mississauga, ON, Canada) in a 25-μl reaction for 30 cycles. The long-range PCR product then was subcloned into the pCR2.1-TOPO vector and transformed in competent Escherichia coli using the TOPO TA cloning kit (Invitrogen, Burlington, ON, Canada) according to the manufacturer’s instructions. Purified genomic DNA from six individual clones was sequenced at both mutation sites using the forward primers for exons 5 and 7 (Supplementary Table 1).

Statistical Analyses

Continuous variables are expressed as mean and 95% confident intervals, and categorical data are expressed as proportions. Fisher exact test was used to test for differences in proportion between patient groups.

Results

Clinical Characteristics of Study Patients

The clinical characteristics of our study patients are shown in Table 1. Overall, 97% (84 of 87) of them were white and 58% (50...
of 87) were male. Seventeen percent (15 of 87) and 73% (63 of 87) of them had steroid-sensitive (SS-) and steroid-resistant (SR-) FSGS, respectively. The remaining 10% (nine of 87) had familial FSGS, and none of them experienced a partial or complete remission of their nephrotic syndrome at the last follow-up. However, because none of these cases received immunosuppressive drug treatment, their outcome was considered indeterminate. By contrast, patients with SR-FSGS received immunosuppressive drugs for a mean duration of 19 mo, making inadequate treatment an unlikely cause of their progressive renal disease. In general, patients with familial FSGS presented earlier but with lower levels of proteinuria than those with SS- and SR-FSGS. Nonetheless, their renal outcomes (CKD and ESRD) were similar to patients with SR-FSGS. As expected, the outcomes of patients with SS-FSGS were excellent with long-term renal preservation.

**Table 1. Clinical characteristics of study patients**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Steroid Sensitive (n = 15)</th>
<th>Steroid Resistant (n = 63)</th>
<th>Familial (n = 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at clinical presentation (yr)</td>
<td>41 (31 to 52)</td>
<td>38 (35 to 42)</td>
<td>23 (17 to 29)</td>
</tr>
<tr>
<td>Age at renal biopsy (yr)</td>
<td>43 (33 to 53)</td>
<td>42 (38 to 45)</td>
<td>27 (20 to 33)</td>
</tr>
<tr>
<td>Gender ratio (M:F)</td>
<td>11:4</td>
<td>37:26</td>
<td>4:5</td>
</tr>
<tr>
<td>Serum creatinine at clinical presentation (mg/dl)</td>
<td>1.1 (0.97 to 1.3)</td>
<td>1.4 (1.3 to 1.6)</td>
<td>1.1 (0.76 to 1.4)</td>
</tr>
<tr>
<td>Proteinuria at clinical presentation (g/d)</td>
<td>8.5 (5.5 to 12)</td>
<td>5.7 (4.1 to 7.3)</td>
<td>3.1 (1.1 to 5.2)</td>
</tr>
<tr>
<td>Serum creatinine at renal biopsy (mg/dl)</td>
<td>1.1 (0.95 to 1.3)</td>
<td>1.6 (1.4 to 1.8)</td>
<td>1.3 (0.9 to 1.4)</td>
</tr>
<tr>
<td>Proteinuria at renal biopsy (g/d)</td>
<td>9.0 (6.3 to 12)</td>
<td>6.5 (5.3 to 9.7)</td>
<td>4.5 (2.9 to 6.1)</td>
</tr>
<tr>
<td>Serum creatinine at last follow-up (mg/dl)</td>
<td>1.3 (0.99 to 1.6)</td>
<td>3.7 (2.92 to 4.5)</td>
<td>3.1 (0.8 to 6.3)</td>
</tr>
<tr>
<td>Proteinuria at last follow-up (g/d)</td>
<td>0.57 (0.19 to 0.95)</td>
<td>4.6 (3.6 to 5.6)</td>
<td>5.5 (0.6 to 10)</td>
</tr>
<tr>
<td>Duration (mo) of immunosuppressive treatment</td>
<td>31 (16 to 47)</td>
<td>19 (10 to 28)</td>
<td></td>
</tr>
<tr>
<td>Duration of follow-up (mo)</td>
<td>64 (31 to 97)</td>
<td>78 (62 to 94)</td>
<td></td>
</tr>
<tr>
<td>Patients with CKD (%)</td>
<td>0</td>
<td>29</td>
<td>33</td>
</tr>
<tr>
<td>Patients with ESRD (%)</td>
<td>0</td>
<td>40</td>
<td>22</td>
</tr>
</tbody>
</table>

aData are means and 95% confidence intervals or percentage. CKD, chronic kidney disease.

**NPHS2 Mutation Analysis**

To define the sensitivity of our dHPLC screen, we performed a single-blind pilot study in 22 pediatric patients with SR-FSGS, in whom 43 pathogenic NPHS2 mutations were identified previously (29). Using the conditions established in this study, we identified 40 of 43 known NPHS2 mutations in these patients to establish a test sensitivity of 93% (Supplementary Table 2). However, when we applied the dHPLC screen to our study patients, we found heterozygous mutations in only eight cases, apparent

**Table 2. Summary of NPHS2 mutations and gene variants detected in the study**

<table>
<thead>
<tr>
<th>NPHS2 Gene Variants</th>
<th>FSGS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Steroid Sensitive (n = 15)</td>
</tr>
<tr>
<td>Heterozygous mutations frequency nucleotide (AA) change</td>
<td>2</td>
</tr>
<tr>
<td>Compound heterozygous mutations frequency nucleotide (AA) change</td>
<td>2</td>
</tr>
<tr>
<td>Gene variants of unknown significance frequency nucleotide (AA) change</td>
<td>2</td>
</tr>
</tbody>
</table>

aN/D, none detected.

bPredicted to be benign by PolyPhen (http://www.bork.embl-heidelberg.de/PolyPhen/).
compound heterozygous mutations in one case, and no case of homozygous mutations (Table 2). We did not find any pathogenic NPHS2 mutations in our familial cases of FSGS. Overall, the R229Q variant accounted for 80% (eight of 10) of all of the putative mutant alleles detected in our study cohort (Table 2). Among white individuals, the allele frequency of this gene variant did not differ between patients with SR-FSGS and our normal control subjects (5 [4.0%] of 126 versus 3 [2.8%] of 108, respectively; two-tailed \( P = 0.73 \) by Fisher exact test). Although the allele frequency of R229Q was 10% (3 of 30) in our patients with SS-FSGS, the sample size for this estimate is small. We also found a highly conserved heterozygous missense variant (G124A; G42R) in a white patient with SR-FSGS. This variant was absent in 54 white control subjects and was predicted to be probably pathogenic by the software PolyPhen (http://www.bork.embl-heidelberg.de/PolyPhen/). In addition, we found several sequence variants of uncertain significance (A41G, E310K, E237Q, and IVS3 + 3InsA) as well as several previously reported neutral polymorphisms (G102A, G34G, C288T, S96S, A242V, T954C, A318A, A1038G, and L346L) (27–30,32).

Among the entire study cohort, we found recessive mutations in only one patient. This patient (TOR2679) with compound heterozygous mutations (R229Q in exon 5 and Q285fsX302 in exon 7) unexpectedly had SS-FSGS (Figure 1). She presented at 38 yr of age with an abrupt onset of pedal edema, weight gain, and proteinuria of approximately 10 g/d. After treatment with a course of prednisone (at 1 mg/kg per d), she underwent complete remission of her proteinuria and remained well at her last follow-up >2 yr later (Figure 2). Given her clinical response, our findings were unanticipated. To eliminate the possibility of a sample mix-up, we reconfirmed our findings by direct sequencing of her resampled DNA. Because both of her parents were not available and to confirm that she truly had compound heterozygous mutations, we performed long-range PCR to amplify a genomic fragment that spanned both exons 5 and 7 and isolated six individual PCR clones and sequenced them at both sites of mutations. The results of these studies indicated that the heterozygous NPHS2 mutations in this patient indeed were arranged in-trans (Figure 3).

Figure 1. Sequence variants within exon 5 (A) and exon 7 (B) of NPHS2 in patient TOR2679. Aberrant denaturing HPLC elution profiles were detected in the patient (double peak) compared with a control subject (single peak) at both 58°C and 59°C (left). Direct sequencing of the PCR fragments identified two heterozygous mutations (G686A; R229Q and 855/6delAA; Q285fsX302; right).

Figure 2. The clinical course of patient TOR2679 is consistent with steroid-sensitive FSGS. Bx, renal biopsy; Rx, treatment with prednisone at a starting dosage of 1 mg/kg per d.
Recessive \textit{NPHS2} mutations are an important cause of childhood-onset FSGS, which accounts for approximately 75\% of steroid-resistant nephrotic syndrome in this age group (26–30). Indeed, two large pediatric studies recently documented homozygous or compound heterozygous \textit{NPHS2} mutations in 26 to 34\% and 11 to 19\% of familial and sporadic steroid-resistant nephrotic syndrome, respectively. These studies also concluded that patients with recessive \textit{NPHS2} mutations did not have an increased risk for disease recurrence after renal transplantation (29,30). The spectrum of \textit{NPHS2} mutations that have been reported to date ranged from nonsense and frameshift mutations to missense mutations that affect highly conserved amino acid residues (26–30). Whereas nonsense and frameshift mutations are predicted to cause a loss of function of the mutant protein, the effects of missense mutations are difficult to predict. Nonetheless, recent \textit{in vitro} and \textit{in vivo} studies suggested that pathogenic \textit{NPHS2} missense mutations may result in intracellular trafficking defects such that the misfolded mutant proteins are not expressed properly in the podocyte slit diaphragm (34–36). Therefore, consistent with the recessive nature of the disease, missense \textit{NPHS2} mutations also may result in a loss-of-function effect.

Using dHPLC with a high sensitivity for detecting pathogenic \textit{NPHS2} mutations, we found that homozygous or compound heterozygous \textit{NPHS2} mutations are rare in our study patients. Given a large sample size and full clinical spectrum of the study cohort, our findings indicated that recessive mutations of \textit{NPHS2} are not an important cause of adult-onset SR-FSGS. The age of clinical presentation seems to be the most important predictor for SR-FSGS that arises from recessive \textit{NPHS2} mutations. The mean age of clinical presentation in our study cohort was in the late third decade of life. By contrast, the mean age of clinical presentation in sporadic cases of SR-FSGS that were reported recently in older children generally was within the first decade of life (26–30).

Our results are consistent with the findings of Caridi et al. (37) in which only three putative heterozygous but no homozygous or compound heterozygous \textit{NPHS2} mutations were found in a cohort of 64 adult patients with SR-FSGS. By contrast, Tsukaguchi et al. (32) reported recessive \textit{NPHS2} mutations in 30\% (9 of 30) of familial and 12\% (11 of 91) of sporadic cases in an adult cohort of patients with FSGS. However, only compound heterozygous but not homozygous R229Q mutations were present in 66\% (6 of 9) and 100\% (11 of 11) of their familial and sporadic cases, respectively. They suggested that R229Q, a common variant that is present in 3.6\% of the white population, might function as a recessive disease allele. Specifically, this variant altered a conserved amino acid residue, segregated with the disease in several small families, and was associated with reduced binding with nephrin \textit{in vitro}. However, information on response to immunosuppressive treatment was not reported in this study, and steroid resistance in these cases was only
suggested. If R229Q is not a disease allele, then recessive \textit{NPHS2} mutations also are rare in this study as well. Our findings in patient TOR2679, who had compound heterozygous \textit{NPHS2} mutations and SS-FSGS, raise the intriguing possibility that R229Q variant may not be a recessive disease allele. Rather, this patient simply might be a carrier for a heterozygous \textit{NPHS2} mutation \textit{Q228R} (Q285fsX302) who happened to have SS-FSGS. Indeed, the R229Q allele frequency in our white patients with SR-FSGS was not significantly different from our white control subjects.

**Conclusion**

Our study suggests that recessive \textit{NPHS2} mutations are rare in white individuals with adult-onset FSGS. Therefore, we cannot recommend \textit{NPHS2} mutation screening in this patient population. Additional studies are needed to determine whether our findings can be extended to nonwhite individuals with adult-onset FSGS. By contrast, \textit{NPHS2} genetic testing may be useful in the treatment of children with FSGS (29,30,38). The clinical significance of R229Q as a recessive disease allele in SR-FSGS needs to be defined further by \textit{in vivo} functional studies. In patients with compound heterozygous mutations, the presence of R229Q may be coincidental because it is a common variant when the disease allele may reside elsewhere in \textit{NPHS2}. Given that multiple proteins (nephrin, podocin, \textit{NPHS1}, \textit{NPHS2}, \textit{etc}) co-localize to the glomerular podocyte slit diaphragm and are components of a macromolecular signaling complex (2,5,21–25), transheterozygous mutations of \textit{NPHS2} with another podocyte-specific gene also may provide an alternative mechanism for SR-FSGS (39). Indeed, digenic mutations involving two different genes within a macromolecular complex or signaling pathway have been shown to cause retinitis pigmentosa (RDS and \textit{ROM1}), Waardenburg syndrome (\textit{MITF} and \textit{TYR}), junctional epidermolysis bullosa (\textit{COL17A1} and \textit{LAMB3}), and autosomal dominant polycystic kidney disease (\textit{PKD1} and \textit{PKD2}) (40–43).

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**Disclosures**

None.

**References**

Readers interested in the genetics of adult-onset focal and segmental glomerulosclerosis should review the January issue of *NephSAP* on the genetics of proteinuric disorders (Genetic Diseases of the Kidney, *NephSAP* Vol. 5, Iss. 1, January 2007).