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

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Recessive 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-sensitive FSGS (R229Q and Q285fsX302) and no homozygous mutations. Overall, R229Q accounted for eight (80%) of ten of the putative mutant alleles that were detected in the study cohort. Contrary to the pediatric experience, recessive NPHS2 mutations are rare in this study population, suggesting that the pathogenesis of FSGS in adults may differ from that in children. These data do not support R229Q as a disease-causing mutation for steroid-resistant FSGS.

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teinuria and renal failure between 20 and 40 yr of age. Their renal pathology comprises a spectrum of findings that range 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 (2,22,23). 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 (2,21–23). Together with TRPC6, a nonspecific cationic channel syndrome (10). Podocin acts as a scaffold protein to recruit nephrin and CD2AP to the lipid rafts at the slit diaphragm (2,21–23). 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 familial cases) for this study. To assess the prevalence of specific missense gene variants in the normal population, we also recruited 54 unrelated healthy white individuals to serve as control subjects. After informed consent, all study participants provided a blood sample for genomic DNA extraction. The institutional review board at the University of Toronto approved the research protocol used in this study.

Clinical Assessment

We reviewed the clinical records of all of the study patients to confirm their renal pathologic diagnosis (1) and to exclude any secondary causes for FSGS (e.g., morbid obesity, single kidney, sickle cell disease, HIV infection). Using the database from the Toronto Glomerulonephritis Registry, we obtained the clinical and laboratory data of most study patients at the time of their presentation, during their clinical course, and at the last follow-up. Specifically, we obtained data on their renal function, proteinuria, immunosuppressive treatment, and the clinical outcomes (e.g., presence of chronic kidney disease (CKD) or ESRD at the last follow-up.

Definitions of Clinical Outcomes

CKD was defined as persistent doubling of normal serum creatinine value, and ESRD was defined as requirement of chronic dialysis or renal transplantation. Complete remission to immunosuppressive therapy was defined as reduction of proteinuria ≤0.3 g/d plus stable renal function, partial remission was defined as reduction of proteinuria by at least 50% from the peak value but >0.3 g/d and stable renal function, and no remission was defined as persistent proteinuria with variability within 50% of the peak value. Patients who responded to immunosuppressive drug treatment (prednisone, cytotoxic drugs, cyclosporine, or combination) with at least one complete remission were classified as steroid sensitive, whereas those who received at least 3 mo of immunosuppressive treatment 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 Metagene 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′-GGGTCCCGAAAGGAGCCCAAGATA-CAA-3′ and 5′-AAGGAACGCAAAGGGAAATG-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-/H11002) 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.

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

Table 1. Clinical characteristics of study patientsa

<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.

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

<table>
<thead>
<tr>
<th>NPHS2 Gene Variants</th>
<th>Steroid Sensitive (n = 15)</th>
<th>Steroid Resistant (n = 63)</th>
<th>Familial (n = 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heterozygous mutations frequency nucleotide (AA) change</td>
<td>2</td>
<td>6</td>
<td>N/D</td>
</tr>
<tr>
<td>G686A (R229Q)</td>
<td>G686A (R229Q)</td>
<td>G124A (G42R)</td>
<td></td>
</tr>
<tr>
<td>Compound heterozygous mutations frequency nucleotide (AA) change</td>
<td>2</td>
<td>N/D</td>
<td></td>
</tr>
<tr>
<td>G686A (R229Q)</td>
<td>855/6delAA (Q285fsX302)</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Gene variants of unknown significance frequency nucleotide (AA) change</td>
<td>2</td>
<td>3</td>
<td>N/D</td>
</tr>
<tr>
<td>C122G (A41G)</td>
<td>G709C (E237Q)</td>
<td>IVS3 + 3InsA</td>
<td></td>
</tr>
<tr>
<td>G928A (E310K)</td>
<td>1b</td>
<td>1b</td>
<td></td>
</tr>
</tbody>
</table>

aN/D, none detected.

bPredicted to be benign by PolyPhen (http://www.bork.embl-heidelberg.de/PolyPhen/).
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 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, E235Q, 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 \( \geq 2 \) 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).

Discussion

Recessive 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 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 NPHS2 mutations did not have an increased risk for disease recurrence after renal transplantation (29,30). The spectrum of 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 in vitro and in vivo studies suggested that pathogenic 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 NPHS2 mutations also may result in a loss-of-function effect.

Using dHPLC with a high sensitivity for detecting pathogenic NPHS2 mutations, we found that homozygous or compound heterozygous 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 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 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 NPHS2 mutations were found in a cohort of 64 adult patients with SR-FSGS. By contrast, Tsukaguchi et al. (32) reported recessive 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 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 NPHS2 mutations also are rare in this study as well. Our findings in patient TOR2679, who had compound heterozygous 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 NPHS2 mutation (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 NPHS2 mutations are rare in white individuals with adult-onset FSGS. Therefore, we cannot recommend 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, 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 in vitro 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 NPHS2. Given that multiple proteins (nephrin, podocin, NPHS1, NPHS2, etc.) co-localize to the glomerular podocyte slit diaphragm and are components of a macromolecular signaling complex (2,5,21–25), transheterozygous mutations of 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 ROM1), Waardenburg syndrome (MITF and TYR), junctional epidermolysis bullosa (COL17A1 and LAMB3), and autosomal dominant polycystic kidney disease (PKD1 and PKD2) (40–43).

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Disclosures
None.

References


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