Prevalence, Genetics, and Clinical Features of Patients Carrying Podocin Mutations in Steroid-Resistant Nonfamilial Focal Segmental Glomerulosclerosis

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Abstract. Podocin mutations (NPHS2 gene) are responsible for the autosomal recessive form of steroid-resistant nephrotic syndrome. As a result of a screening for these gene alterations in a cohort of Italian patients with nonfamilial nephrotic syndrome and histologic focal segmental glomerulosclerosis (FSGS), nine patients with NPHS2 gene homozygous or composite heterozygous mutations were found. In addition to the previously described defects, two novel mutations at exon 4 were identified (frameshift, L169P); four single nucleotide polymorphisms (SNPs) and one dinucleotide repeat were also identified. On the basis of haplotype analysis, a founder effect was suggested for the 419delG mutation, the most frequently observed in the patients studied. Patients carrying NPHS2 mutations and without a family history of nephrotic syndrome were indistinguishable from those with idiopathic FSGS on the basis of the clinical phenotype. Two of the nine patients had normal renal function at 3 and 10 yr of age, despite the presence of the nephrotic syndrome. The other seven had reached end-stage renal failure at a mean age of 9.6 yr (range, 4 to 17 yr) and had received renal allografts. In those presenting with end-stage renal failure, the clinical and laboratory features both before and after transplantation were similar, including the age at onset, the amount of proteinuria, and the absence of any response to steroids and other immunosuppressants. Finally, two children presented recurrence of mild proteinuria after transplantation, which promptly remitted after plasmapheresis combined with cyclophosphamide. These data demonstrate that podocin mutations in nonfamilial cases of steroid-resistant nephrotic syndrome are frequent and may be due in one case to a founder effect. The pretransplantation and posttransplantation outcomes in the group of patients with mutations of the podocin gene are similar to classical idiopathic FSGS, including the possibility of recurrence of proteinuria that is mild and responsive to plasmapheresis. These observations support a role of molecular screening of the podocin gene in patients with nephrotic syndrome before immunosuppressive treatment is started.

The recent discovery of mutations of two proteins exclusively expressed by the podocytes, podocin and α-actinin-4 (1,2), in familial forms of focal segmental glomerulosclerosis (FSGS) has shed new light on the pathogenetic mechanisms of the disease (3). Podocin is a 42-kD integral membrane protein with a short extracellular domain and a possible connecting function between the slit diaphragm and the podocyte cytoskeleton (4). These observations follow important advances in the study of podocyte molecular defects that cause proteinuria, including the discovery of the nephrin gene mutations (5) as the cause of nephrotic syndrome of the Finnish type. Other studies in proteinuric mice that lack the CD2-associated protein (6), which regulates podocyte adhesion, further support the concept that the integrity of the glomerular filtration barrier largely depends on normal podocyte and slit diaphragm structure.

The prevalence and the clinical outcome of patients with podocin mutations and FSGS without a recognized history of familial proteinuria are unknown. This information may have important implications for the clinical and therapeutic approach to patients with nephrotic syndrome unresponsive to steroids because, theoretically, ineffective and potentially harmful immunosuppression should be avoided in carriers of the mutations. The posttransplantation outcome of patients receiving a renal allograft remains to be determined.

The objective of this report is to describe the clinical features of nine patients with primary, nonfamilial FSGS who carried the molecular defect of podocin, seven of whom presented with end-stage renal failure and received renal transplants.
Materials and Methods

Patients

The study included 44 subjects who had received a clinical and pathologic diagnosis of FSGS. Clinical criteria included the presence of steroid-resistant nephrotic syndrome, the exclusion of congenital nephrotic syndrome of the Finnish and the Denys-Drash or Frasier types, and the exclusion of multiplex families that presented with a clear familial inheritance for proteinuria. For first-degree relatives of the patients included in the study, we were able to document normal urinalysis; for all the other relatives, the absence of proteinuria was inferred from an interview. Consanguinity was in all cases excluded with the exception of the parents of one patient (patient 9, Table 1), who were second order cousins. Relevant clinical parameters, including urinalysis and renal function, were normal in all relatives. The histologic hallmarks of FSGS were the presence of at least one area of segmental or global sclerosis with or without Ig deposits (IgM, IgG) in the native glomeruli. Mesangial expansion of both the cellular component and of extracellular matrix was a frequent but not ubiquitous finding. Mean patient age was 22 yr (range, 3 to 32 yr). Ten patients had normal renal function (creatinine, <1.3 mg/dl), 2 had chronic renal failure (creatinine, >1.3 mg/dl), 1 had terminal renal failure in dialysis, and 31 had received a renal allograft. The therapy for primary nephrotic syndrome consisted of long courses of prednisone according to standard protocols (7); all patients were also treated with cyclophosphamide for 2 mo at 2 mg/kg and, in a few cases, with cyclosporin A (CsA) at an initial dose of 4 to 5 mg/kg. Immunosuppressive therapy after renal transplantation followed standard protocols as described previously (8). After transplantation, the urinary parameters were carefully followed every day for 2 mo and then weekly for another 10 mo. Two patients who presented proteinuria greater than 1 g/L posttransplantation were treated following the same protocol used in recurrence of idiopathic FSGS, consisting in plasmapheresis plus cyclophosphamide. A renal biopsy was not performed after recurrence of proteinuria in these cases. Plasmapheresis was performed by removing 1 to 1.5 plasma volumes each cycle according to the patient’s weight for either six cycles on alternate days (patient 6) or for six cycles and then an additional four cycles performed weekly (patient 7). Fluid replacement consisted of fresh plasma or saline solution supplemented with 4% albumin. In those patients treated with cyclophosphamide (2 mg/kg for 2 mo), azathioprine, or mycophenolate mofetil were transiently discontinued until the suspension of cyclophosphamide.

Molecular Analysis of Podocin

DNA was isolated from fresh peripheral blood samples. Molecular analysis of podocin was performed by direct sequencing as described previously by Boute et al. (1). Exons were amplified by PCR using flanking intron primers and subjected to automated sequence analysis by dye-terminator reactions (Automated Sequencer ABI 377; Applied-Biosystem, Milan, Italy).

Generation of New Markers to the NPHS2 Gene

Novel polymorphic DNA markers were found by using a search strategy for microsatellite markers in electronic databases (9). Specifically, a text file containing 16 repeats of dinucleotides AC, AG, AT, and CG was used as a search query for the sequence comparison program BLAST (www3.ncbi.nlm.nih.gov). The queries were searched against sequences of the recently released Human Genome Sequence (www3.ncbi.nlm.nih.gov), with reference to the chromo-

Table 1. Clinical features in nine FSGS patients with mutations of the NPHS2 gene

<table>
<thead>
<tr>
<th>Patient</th>
<th>Gender</th>
<th>Age (yr)</th>
<th>Age at Onset of Proteinuria</th>
<th>Therapy of NS</th>
<th>Proteinuria on Day of Presentation</th>
<th>Proteinuria on Day after Therapy</th>
<th>Dialysis Age (yr)</th>
<th>Transplant Age (yr)</th>
<th>NPHS2 (AA change)</th>
<th>NPHS2 (Nt change)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>16</td>
<td>4.0</td>
<td>Ste</td>
<td>6 to 7</td>
<td>Unchanged</td>
<td>9</td>
<td>12</td>
<td>Frameshift</td>
<td>419delG</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>26</td>
<td>1.0</td>
<td>Ste</td>
<td>8 to 9</td>
<td>Unchanged</td>
<td>13</td>
<td>14</td>
<td>Frameshift</td>
<td>419delG</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>20</td>
<td>1.5</td>
<td>Ste</td>
<td>6 to 7</td>
<td>Unchanged</td>
<td>12</td>
<td>15</td>
<td>Frameshift</td>
<td>419delG</td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>19</td>
<td>14.0</td>
<td>Ste/CsA</td>
<td>5 to 6</td>
<td>Unchanged</td>
<td>17</td>
<td>19</td>
<td>Frameshift</td>
<td>467/8insT</td>
</tr>
<tr>
<td>5</td>
<td>F</td>
<td>10</td>
<td>8.0</td>
<td>Ste/Cyc/CsA</td>
<td>6 to 7</td>
<td>Unchanged</td>
<td></td>
<td></td>
<td>V180M</td>
<td>538G→A</td>
</tr>
<tr>
<td>6</td>
<td>M</td>
<td>12</td>
<td>1.0</td>
<td>Ste/Cyc/CsA</td>
<td>8 to 9</td>
<td>Unchanged</td>
<td>7</td>
<td>9</td>
<td>R138Q</td>
<td>413G→A</td>
</tr>
<tr>
<td>7</td>
<td>M</td>
<td>10</td>
<td>2.0</td>
<td>Ste/Cyc</td>
<td>8 to 9</td>
<td>Unchanged</td>
<td>4</td>
<td>5</td>
<td>R138Q</td>
<td>413G→A</td>
</tr>
<tr>
<td>8</td>
<td>F</td>
<td>11</td>
<td>1.0</td>
<td>Ste</td>
<td>5 to 6</td>
<td>Unchanged</td>
<td>5</td>
<td>6</td>
<td>R138Q</td>
<td>413G→A</td>
</tr>
<tr>
<td>9</td>
<td>M</td>
<td>4</td>
<td>2.0</td>
<td>Ste/Cyc/CsA</td>
<td>10 to 11</td>
<td>Decreased</td>
<td></td>
<td></td>
<td>R138X</td>
<td>412C→T</td>
</tr>
</tbody>
</table>

Mean 14.22 3.83 9.57 11.43 6.65 4.43 4.69 5.06

a FSGS, focal segmental glomerulosclerosis; NS, nephrotic syndrome; Ste, steroids; Cyc, cyclophosphamide; CsA, cyclosporin A; AA, amino acid; Nt, nucleotide.
some 1 working draft sequence segment NT_004470 containing the whole NPHS2 gene.

Primer pairs were designed to the sequence flanking the repeats. One primer (forward) for pairs was labeled with a fluorescent dye to use Automated Sequencer ABI 377. These repeat markers were analyzed for polymorphisms by using DNA samples from 50 to 70 unrelated control individuals and in a three-generation family to confirm the allelic segregation.

**Statistical Analysis**

The differences in clinical parameters between FSGS patient carriers and noncarriers of podocin mutations were evaluated with one-way ANOVA. Data are expressed as mean ± SD.

**Results**

**Podocin Mutations**

Among the group of 44 FSGS patients, nine nonfamilial patients carrying podocin mutations (Table 1) were found. Patients 4, 5, and 8 presented composite heterozygous mutations, whereas the remaining six were homozygous. Overall, we found the same mutations described by Boute et al. (1), with the exception of two novel mutations at exon 4 in position 506 resulting in a T to C transition (L169P) and in a T insertion in position 467/8, producing a frameshift (Figure 1). In comparison with 75 normal DNA sequences, we could exclude that L169P was a polymorphism.

**Markers to the NPHS2 Gene and Haplotype Analysis**

A set of new markers (1 dinucleotide repeat and 4 single nucleotide polymorphisms [SNPs]) was identified in the NPHS2 gene (Table 2) and used to genotype patients carrying the more frequent mutations 419delG, R138Q, and L169P (Table 3). The dinucleotide repeats marker located in intron 1 of NPHS2 gene presented 5 alleles from 301 to 309 bp, with a polymorphism information content value of 0.66. Of the four SNPs identified, the one in exon 2 (c288C>T: S96S) was already reported by Wu et al. (10), and the others, one in intron 7 (EX7+7A>G) and two in exon 8 (c951T>C: A317A; c1038A>G: L346L), are of new description.

Haplotype reconstruction for the nine affected patients was carried out by using all polymorphisms above. Three of the four SNPs (c288C>T, EX7+7A>G, and c1038A>G) showing low heterozygosity were not informative. On the basis of analysis of the two informative polymorphisms (Intron1 and c951T>C), a founder effect was suggested for the 419delG mutation. On the other hand, a common haplotype was identified in three of the six haplotypes carrying the R138Q mutations (Table 3).

**Clinical Features and Recurrence in Carriers of the Podocin Mutations**

The clinical features of each patient carrying mutations of podocin are reported in Table 1, including the therapy of nephrotic syndrome at onset and the clinical outcome relative to renal function. Of the nine patients, two had normal renal function and massive proteinuria at the ages of 3 and 10 yr. Seven had developed end-stage renal failure and had received a renal allograft. Major clinical characteristics were comparable in FSGS patients with podocin mutations and in those without, including pathologic features and outcome. Accordingly, children with podocin mutations developed nephrotic syndrome at 3.83 ± 4.43 yr, and those with idiopathic FSGS presented proteinuria at 5.25 ± 2.92 yr. In all cases, an oral steroid regimen of 2 mg/kg and in pulse (10 mg/kg), in combination with cyclophosphamide (2 mg/kg for 2 mo) failed to induce remission. Two patients (patients 5 and 9) who still had their native kidneys and normal renal function at the ages of 10 and 4 yr, respectively, had been treated with CsA at an initial dose of 4 to 5 mg/kg and successive reduction to 2 to 3 mg/kg.
The response to the drug differed; in patient 5, the treatment failed to induce any change, whereas in patient 9, the only subject with the L169P mutation, we observed a stable reduction of proteinuria below the nephrotic range. In the remaining population of podocin mutation carriers, the mean age at which end-stage renal failure developed was 9.57 ± 4.69 yr, which was comparable to the 10.2 ± 5 yr in those with idiopathic FSGS. After renal transplantation, two patients (patients 6 and 7) presented a mild recurrence of proteinuria (2 to 3 g/d) after at least 10 d of normal urinalysis and normal renal function. In one case (patient 6), proteinuria occurred after 10 d, and in the other (patient 7) after 300 d. The therapy followed established protocols based on plasmapheresis and cyclophosphamide, which induced in both cases prompt remission of proteinuria. Renal function and urinalysis after 6 and 8 mo after the short course of plasmapheresis were normal.

**Discussion**

Podocin is a 42-kD transmembrane protein of podocytes that consists of a short extracellular domain that is followed by a transmembrane-spanning region and a long cytoplasmic tail. Podocin presumably interacts with the cytoskeleton on the one hand and with the slit-diaphragm nephrin on the other, thus performing a mechanotransduction function and a role in the stabilization of the permeability unit of podocytes (1,4). Podocin mutations have been implicated in the pathogenesis of corticoresistant nephrotic syndrome with autosomal recessive inheritance (1,11).

Our study contributes to descriptions of novel mutations of podocin and completes, to some extent, the seminal article by Boute et al. (1), which first showed podocin as the cause of familial nephrotic syndrome and described the most frequent mutations. The first of the two new mutations described in the present study was an insertion of thymidine that caused a frameshift from amino acid 156 and introduced a stop codon of ten amino acids downstream. The second mutation affected codon 169, determining a leucine to proline amino acid substitution. Several lines of evidence indicate that these mutations were pathologic. First, each sequence change was absent in 75 unaffected, unrelated control individuals. Second, with regard to the L169P substitution, alignment analysis of the human podocin and mouse-translated EST (Genbank accession...
no. AW106985) showed conservation of this residue. Finally, the substitution of leucine with proline would likely induce a secondary structure alteration. This possibility is even more probable in the case of the frameshift.

One mutation, 419delG, was identified in three homozygous patients from southern Italy. Haplotype reconstruction in these patients, using polymorphic markers localized at the NPHS2 locus, suggests common ancestry. In the case of the second most frequent mutation (R138Q), which was observed in two homozygous patients and in two heterozygous patients, a common origin could be hypothesized in only three of the six haplotypes.

The primary finding of this study is that mutations of podocin are frequent in nonfamilial cases of nephrotic syndrome in children who have been classified as idiopathic FSGS on the basis of clinical characteristics and pathologic features. In particular, in our patients, quantity and age at onset of proteinuria as well as unresponsiveness to steroids and progression to chronic renal failure mimicked idiopathic FSGS. In the only patient who carried the homozygous T506C transition (patient 9), we observed a stable reduction of proteinuria after cyclosporin therapy, a singular finding not explainable on the basis of current knowledge. Only a molecular approach is diagnostic in this setting, and we propose that all cases with heavy proteinuria that is resistant to steroids and suggestive of FSGS should undergo genotyping of NPHS2 before planning therapeutic regimens with steroids and other immunosuppressive drugs. Although the mutations in our series were studied in a pediatric age group, it is not possible to exclude adult onset of the disease, and thus the molecular approach should not be confined to children. With respect to the typical NPHS2 familial cases that are reported in the literature (12) in whom proteinuria occurred in the first years of life, in two patients of our series (patients 4 and 5), proteinuria started at the ages of 14 and 8 yr, respectively. Also, progression to end-stage renal failure was slower than the previously described cases (12).

Most of our patients received a renal allograft with a good posttransplantation outcome, including normal urinalysis and normal renal function after a mean follow-up of 59.15 mo. We observed the recurrence of proteinuria in 2 patients after 10 and 300 d that was mild and promptly responded to plasmapheresis. The good outcome and the rapid resolution of the proteinuria after plasmapheresis and cyclophosphamide therapy probably excluded the possibility of de novo disease and the necessity of allograft biopsy.

In summary, we report that carriers of mutations of podocin are frequent in sporadic cases of cortico-resistant nephrotic syndrome and present with a clinical outcome similar to primary FSGS of unknown origin. A molecular approach is necessary to distinguish between the two different entities also in consideration that they require a different therapeutic approach. Two new podocin mutations producing a frameshift and an L169P were also described. Finally, the posttransplantation outcome was good in our patients, although two presented with recurrence of proteinuria that was defined as mild on the basis of quantity and prompt remission after plasmapheresis.

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