Broadening the Spectrum of Diseases Related to Podocin Mutations

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Abstract. A total of 179 children with sporadic nephrotic syndrome were screened for podocin mutations: 120 with steroid resistance, and 59 with steroid dependence/frequent relapses. Fourteen steroid-resistant patients presented homozygous mutations that were associated with early onset of proteinuria and variable renal lesions, including one case with mesangial C3 deposition. Single mutations of podocin were found in four steroid-resistant and in four steroid-dependent; five patients had the same mutation (P20L). Among these, two had steroid/cyclosporin resistance, two had steroid dependence, and one responded to cyclosporin. The common variant R229Q of podocin, recently associated with late-onset focal segmental glomerulosclerosis, had an overall allelic frequency of 4.2% versus 2.5% in controls. To further define the implication of R229Q, a familial case was characterized with two nephrotic siblings presenting the association of the R229Q with A297V mutation that were inherited from healthy mother and father, respectively. Immunohistochemistry with anti-podocin antibodies revealed markedly decreased expression of the protein in their kidneys. All carriers of heterozygous coding podocin mutation or R229Q were screened for nephrin mutation that was found in heterozygosity associated with R229Q in one patient. Finally, podocin loss of heterozygosity was excluded in one heterozygous child by characterizing cDNA from dissected glomeruli. These data outline the clinical features of sporadic nephrotic syndrome due to podocin mutations (homozygous and heterozygous) in a representative population with broad phenotype, including patients with good response to drugs. The pathogenetic implication of single podocin defects per se in proteinuria must be further investigated in view of the possibility that detection of a second mutation could have been missed. A suggested alternative is the involvement of other gene(s) or factor(s).

The discovery of molecular defects of podocyte components (1–4) causing familial forms of nephrotic syndrome (NS) suggested the role of podocytes as the site of permselectivity in the kidney (5,6). Since then, knockout models of podocyte components and further molecular genetic screenings definitively consolidated this concept (7). Nephrin (NPHS1) was the first podocyte protein to be found mutated in association with a rare form of congenital NS with autosomal recessive inheritance (CNF) (1,2). Patients with CNF present massive proteinuria starting in utero and progress to end-stage renal failure within a few years (8,9). Renal histopathologic changes include immature glomeruli with fusion of foot processes and pseudocystic dilation of the proximal tubules (10). Podocin (NPHS2) was the second recognized protein to cause proteinuria in familial cases with recessive inheritance and in sporadic patients (3,11,12). The clinical picture of nephrotic syndrome caused by podocin mutations ranges from an early onset, thus resembling CNF, to a late onset in the second decade of life, resembling idiopathic focal segmental glomerulosclerosis (FSGS). Finally, α-actinin 4 was the most recently recognized structural component of the podocyte causing proteinuria in rare cases of dominant NS in humans (4). Recent data on nephrin/podocin/α-actinin 4 interaction underline the putative importance of protein-protein interactivity within this network and suggest a structural model in which nephrin represents the repulsion site within the slit diaphragm and is anchored by podocin to the cytoskeleton where α-actinin 4 is localized.

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(13,14). The coexistence of mutations affecting both nephrin and podocin in three patients with early-onset NS (15) reinforces this concept and provides further evidence for a digenic inheritance model in which mutation hints occur in one gene, with the second mutant acting as a modifier.

The initial studies on podocin mutations in sporadic NS (11,12) were conducted on selected populations with particular clinical pictures characterized by early onset and malignant outcome, mimicking familial cases. It is clear that determination of the actual impact of podocin mutations on sporadic NS requires that we extend screening in patients with variable phenotype and increase the number of observations. In this article, we report the results of a screening for podocin mutations in a sufficiently vast cohort of children with sporadic NS and different phenotype, including a variable response to drugs (from steroid sensitivity to strict resistance). In parallel, we also screened our patients for nephrin and for the exon 8 hot-spot α-actinin 4 mutations.

Materials and Methods

Patients

We enrolled 179 nephrotic children for the screening of podocin mutations. All were presenting or had presented proteinuria from moderate to severe before age 18 yr and had received (or were receiving) modular therapies according to their sensitivity to different drugs. As a rule, the therapeutic approach started with steroids following consolidated schemes (16) (2 mg/kg for 30 to 60 d); in case of unresponsiveness (partial or global), steroids were associated or substituted with cyclophosphamide (2 mg/kg for 60 d) and/or with cyclosporin (5 mg/kg starting dose, followed by tapering to reach the minimum dose required for maintaining cyclosporin serum levels between 50 and 100 ng/ml). In case of persistent steroid-cyclosporin resistance, methyl-prednisolone was given in pulses (10 mg/kg, 6 cycles). According to the scheme above, patients were subdivided in corticoresistant (n = 120) and/or corticodependent/frequent relapers (n = 59). The relevant clinical and pathologic features (i.e., gender, age at onset of proteinuria, evolution toward renal failure, renal transplant) are reported in Table 1. Renal histology was available in 128 cases. Overall, 91 children had a diagnosis of FSGS based on the histologic evidence of at least one segmental area of glomerulosclerosis; 22 presented mesangial IgM deposition, and 15 minimal change nephropathy. In two cases with podocin molecular defects, the pathologic features were not specific for FSGS and were therefore further investigated with electron microscopy. Most FSGS cases presented strict resistance to corticosteroids following the scheme reported above, but other FSGS cases were also observed who presented steroid sensitivity or belonged to the variant with corticodpendence or with frequent relapses of proteinuria (more than three episodes of proteinuria in 1 yr). Podocin/nephrin and α-actinin mutations were also evaluated in 100 normal controls (45 male controls, 55 female controls; age, 3 to 40 yr) enrolled among the blood donors in our hospital.

Mutational Analyses

With the informed consent, we obtained peripheral blood samples for genetic analyses from the enrolled patients and from selected parents and siblings. Genomic DNA was extracted according to standard procedures. Molecular analyses of podocin and nephrin were performed by direct sequencing as already described (1,3). Primer sequences for podocin were selected on the basis of what is already reported in the literature (3,12). For exons 2 and 6, primer design followed Karle et al. (12) to avoid the presence of a recognized SNP. Exons were amplified by PCR using flanking intronic primers and

| Table 1. General features and clinical details of 179 children with sporadic nephrotic syndrome subdivided in two subgroups according to the response to steroids | n | Gender (F/M) | Age at Proteinuria (mo) | Follow-up from Onset of Proteinuria (mo) | Cyclosporin Sensitivity n (Sens/Res/n.d.) | Renal Histology | ESRF n | NPHS2 Mutations |
|---|---|---|---|---|---|---|---|---|---|
| Nephrotic syndrome | 179 | 75/104 | 70 (1 to 216) | 108 (2 to 492) | 47/44/88 | MCN 15 | 59 | n.d. | Hom Het R229Q |
| Corticodependence/frequent relapses | 59 | 22/37 | 40 (3 to 147) | 74 (2 to 233) | 25/2/32 | MCN 4 | 1 | 0 | 4 | 5 |
| Corticoresistance | 120 | 53/67 | 71 (1 to 216) | 125 (1 to 492) | 22/42/56 | MCN 11 | 58 | 14 | 4 | 10 |

* 120 patients presented corticoresistance, 59 being treated with cyclosporin with variable response; 59 presented corticodependence and/or frequent relapses of proteinuria. MCN, minimal change nephropathy; Mes IgM, mesangial proliferation with IgM deposition; FSGS, focal segmental glomerulosclerosis; ESRF, end stage renal failure; Hom, homozygous; Het, heterozygous; n.d., not determined.
subjected to automatic sequence analysis by dye-terminator reaction (Automated sequencer ABI 3100; Applera, Milan Italy). For α-actinin 4, the hot-spot A682G and C695T mutations at exon 8 were detected by restriction site analyses as described by Kaplan et al. (4).

**Glomerular cDNA for Molecular Analyses**

After biopsy, the renal fragment was immediately placed at 4°C in an RNase inhibitor solution (Vanadyl ribonuclease complex, 20 mM; Life Technologies BRL) and then transferred to a micro-dissecting dish and cooled at 4°C, in which glomeruli were separated from tubules. After micro-dissection, isolated glomeruli were washed and transferred to a PCR tube that contained a human placental RNase inhibitor 40 U. Microdissected glomeruli were permeabilized immediately before RT in a mixture containing 0.2% Triton X-100, 40 U of RNase inhibitor, and 5 mM dithiothreitol (Sigma-Aldrich, St. Louis, Missouri). RT was performed utilizing a cDNA kit, according to the manufacturer’s instructions (Roche Diagnostics GmbH, Mannheim, Germany) for 90 min at 37°C. cDNA amplification was performed as described by Boute et al. (3).

**Anti-Podocin Antibodies**

Polyclonal anti-podocin antibodies were raised in rabbits immunized with the peptide GPEPSGSGRAGTP covering the amino acid sequence from 42 to 55 of podocin. This region is human-specific, with only 30% homology versus rat and mouse. Specificity of antibodies was controlled by two-dimensional electrophoresis (2-D) and immunoblot (Figure 1) of human podocyte (kindly provided by Prof. Giovanni Camussi) extracts in β-hexyl-glucopyranoside. Two-dimensional electrophoresis, preparation, and rehydration of immobilized gradients (IPG) and polyacrylamide gels have been described in detail elsewhere (17). Briefly, the IPG strips were rehydrated overnight at 4°C in 9 M urea, 2% wt/vol CHAPS, 0.6% wt/vol carrier ampholytes (IPG; Amersham Pharmacia Biotech), and a trace of bromophenol blue. Proteins, 30 μg, were solubilized with a solution containing 9 M urea, 4% wt/vol CHAPS, and 40 mM Tris.

Isoelectric focusing was performed at 18°C. SDS-PAGE in the second dimension was performed following the original technique described by Bjellqvist et al. (18). The applied voltage for electrophoresis was increased from 300 to 3500 V during the first 5 h, followed by 5000 V for a total of 100 kVh. Before the 2-D run, IPG strips were equilibrated within the strip tray for 30 min with a solution of 0.05 M Tris-HCl buffer, pH 6.8, 6 M urea, 30% vol/vol glycerol, 2% wt/vol SDS, and a trace of bromophenol blue. The second dimension was performed on 180 × 160 × 1.5-mm slabs of polyacrylamide gradient gels (%T, 8 to 16) using piperazine diacrylamide (PDA) as a cross-linking agent. The gels were run at 45 mA/gel constant current and maintained at a temperature of 12°C.

For Western blot, proteins were transblotted to Hybond nitrocellulose membranous (Amersham Pharmacia Biotech) with a Novablot semidry system using a continuous buffer system with 38 mM Tris, 39 mM glycine, 0.035% SDS, and 20% methanol. The transfer was achieved at 1.55 mA/cm² for 3.5 h.

**Immunofluorescence**

Podocin expression in the kidney was studied by indirect immunofluorescence in a few renal biopsies of patients carrying the R229Q. Cryosections were fixed in cold acetone, and sequentially incubated with the primary rabbit polyclonal antibody against podocin, followed by FITC-labeled goat anti-rabbit secondary antibody (Zymed; Histoline, Milan, Italy). Specificity of labeling was demonstrated by the lack of staining after substituting phosphate-buffered saline (PBS) and proper control immunoglobulins (Zymed) for the primary antibody. Control kidneys represented by normal portion of nephrectomy for cancer were mounted on the same slide and processed in parallel.

**Microalbuminuria Assay**

Microalbuminuria was determined with Albumin Kit purchased from Roche (Roche Diagnostic, Milan, Italy).

**Statistical Analyses**

Age at onset of proteinuria in subgroups was compared using the one-way ANOVA. Data on haplotype frequency in controls and nephrotic patients and in different pathologic subgroups were compared with the χ² test. Data are given as mean ± SEM.

![Figure 1. Two-dimensional electrophoresis and immunoWestern with polyclonal anti-podocin antibodies of cellular extracts from human podocytes. These antibodies recognized microheterogeneous spots with a molecular weight of 42 kD and pH around 7 that correspond to podocin.](image-url)
Results

Confirmation of High Incidence of Homozygous Podocin Mutations of Podocin in Sporadic NS

After our original observation (11) of a high incidence of homozygous podocin mutations in a small cohort of children with FSGS and renal failure (44 patients), we extended the screening to further 135 children (overall 179 patients studied) with NS and a more benign phenotype that varied from steroid-dependence or frequent relapses after steroid withdrawal to persistent steroid resistance (Table 1). Our results confirmed that homozygous podocin mutations occur frequently in patients with sporadic NS, and we extended the original observation to five new cases, the clinical data for whom are reported in Table 2. The spectrum of histopathology features in patients with homozygous podocin mutations was variable. A renal biopsy was available in 12 patients; the most frequent picture was consistent with FSGS (9 of 12); two children presented MCN and mesangial proliferation with IgM deposition, respectively. In one case, carrying a composite R138Q-V180M mutation, renal lesions were not reminiscent of a well-defined pathologic entity. Glomeruli (n = 18) appeared normal; at immunofluorescence, he presented diffuse mesangial deposits of IgG(++) and C3(++), and C1q(++). This picture was considered nonspecific, although indicative for a diffuse immunocomplex glomerulonephritis. Electron microscopy confirmed the presence of electron-dense deposits in mesangium with extension to subendothelial and subepithelial spaces (not shown).

Single Podocin Mutations

In eight patients, a single mutation of podocin was found; clinical features are reported in Table 2. As shown in Figure 2, three new mutations were found (R291Q, 555delT, A242V). With the exception of P20L, none of the mutations found in

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<th>Histology</th>
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<th>Age at ESRF (mo)</th>
<th>Renal Tx</th>
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* MCN, minimal change nephropathy; IgM, mesangial proliferation with IgM deposition; FSGS, focal segmental glomerulosclerosis; ESRF, end-stage renal failure; n.d., not determined.
Figure 2. Electropherogram of three podocin (NPHS2) and one nephrin (NPHS1) new mutations described in this study. They occurred in heterozygosity in four sporadic children who presented variable clinical outcome. Mutated nucleotide and altered amino acids are reported.
heterozygosity have ever been observed in homozygous patients. The P20L was found in five children. A relevant clinical characteristic of this cohort of patients was that most had sensitivity to drugs (5 of 8) and are currently presenting a normal renal function after a long follow-up (Table 2). From a genetic point of view, renal lesions due to podocin mutations should be inherited following a recessive model that should produce an evident phenotype only in homozygosity. We cannot therefore exclude the possibility of missing a second mutation in noncoding or regulatory regions of podocin. Alternative possibilities are that a second mutation involves another podocyte gene that interacts with podocin or focal loss of heterozygosity of podocin in glomeruli. This last possibility was excluded by sequencing podocin cDNA in microdissected glomeruli in one child presenting A242V heterozygous mutation and found persistence of the expression of the normal allele (data not shown). All heterozygous carriers of podocin mutation and R229Q were screened for nephrin. A new nephrin mutation (A907T) was found in one case associated with the R229Q variant of podocin; this patient developed proteinuria with steroid resistance at the age of 17 mo but afterwards responded to cyclosporin and actually has a normal urinalysis at the age of 197 mo. However, the mother of this patient presented the same association and is healthy at the age of 55 yr. Two patients presented a nephrin mutation determining a nucleotide change in the promoter (~489delGA). In the literature, the presence of the above nucleotide change in the promoter is associated with nephrotic syndrome occurring before 5 yr and is actually considered a pathogenic mutation; however, we performed a screening of 50 controls and found this nucleotide change in heterozygosity in 4. Moreover, the same ~489delGA was found in association with a complex trait for podocin in a simple family (see below, and Figure 3). They had inherited this variant from their healthy father who was homozygous for it. This observation supports the idea that this is indeed a polymorphism.

Podocin R229Q Variant

Podocin R229Q is a polymorphism that appears to enhance susceptibility to FSGS in association with a second mutant NPHS2 allele (19). We found heterozygous R229Q in 5 of 100 normal controls (allelic frequency 2.5%) and in 12 nephrotic patients, 5 of whom had steroid sensitivity. It was moreover associated with a mutant nephrin in one case (see above) and in one child homozygous R229Q was associated with homozygous R138X to give an overall frequency 4.2% in our cohort of nephrotic patients. In a simple family (that was studied on an anecdotal basis), two siblings presented an association of R229Q with the A297V mutation on the other allele (inherited from the mother and the father, respectively) (Figure 3); moreover, as a part of a complex trait, these siblings also presented the ~489delGA variant of the nephrin promoter that they inherited from a homozygous healthy father. The glomerular expression of podocin was evaluated by polyclonal anti-podocin antibodies in one of the two siblings for whom a renal biopsic fragment was available. Immunofluorescence demonstrated the absence of podocin in glomeruli (Figure 4).

**Figure 3.** Simple family consisting in two siblings with R229Q associated with A297V podocin mutation. They also presented the ~489delGA of nephrin in heterozygosity. They inherited the A297V and the ~489delGA from the healthy father and the R229Q from the healthy mother.

**Heterozygous Podocin Mutations Occurring in Relatives of Nephrotic Patients**

Proteinuria and microalbuminuria were determined in parents and siblings of three nephrotic patients who had been previously characterized: one carrying a composite 460/8insT associated with V180M mutation, the other presenting R138Q in homozygosity and the mother of a child with the P20L. In the first case, the proband inherited 460/8insT from the mother, while the father and two siblings were carriers of the second mutation. All the obligatory carriers had a normal urinalysis, including levels of albuminuria < 15 mg/L, and their renal function was normal.

**Exclusion of Mutations at Exon 8 of α-Actinin 4**

Mutational analyses at two hot-spot mutations at exon 8 of α-actinin 4 (4) (uniquely reported in the literature) showed no relevant alterations in our patients.

**Discussion**

The discovery of the podocyte structural components that determine glomerular permeability and their involvement in familial NS (1–4) represented a fundamental event in the rapidly evolving research area on the mechanisms of proteinuria. Nephrin (NPHS1), podocin (NPHS2), and α-actinin are the three proteins implicated in familial forms of NS in humans, with recessive and dominant inheritance. In familial cases, the clinical picture ranges from congenital and often malignant outcome in the case of nephrin mutations to more benign, albeit variable, forms in the case of podocin mutations. These latter have also been reported in sporadic patients with NS (11,12), with a clinical picture resembling idiopathic
ment to other patients with slower progression, we described a marked malignancy of the disease. By extending the enrollment of renal function had been enrolled, on the basis of assumption of that in previous studies only patients with rapid deterioration of pathologic description. The main reason for this discrepancy is our group (11) and others (12) and complete the clinical-gous podocin mutations than reported in previous studies by homozygous podocin mutations in sporadic NS, with an incidence of 12% in our cohort of children with steroid resistance. The results presented here confirm the occurrence of homozygous podocin mutations in sporadic NS, with an incidence of 12% in our cohort of children with steroid resistance. The phenotype consisted in all cases of severe proteinuria occurring in early childhood and of progression to end-stage renal failure, generally occurring within the second decade of life, i.e., later than in patients with idiopathic FSGS. Even the pathologic picture was variable, with two children showing MCN and mesangial deposits of IgM, respectively, and one case in which renal lesions more resembled an immunodeposit glomerulonephritis. These results show a different incidence of homozygous podocin mutations than reported in previous studies by our group (11) and others (12) and complete the clinical-pathologic description. The main reason for this discrepancy is that in previous studies only patients with rapid deterioration of renal function had been enrolled, on the basis of assumption of a marked malignancy of the disease. By extending the enrollment to other patients with slower progression, we described five new patients. Besides this confirmatory finding and the description of clinical features in carriers of homozygous podocin mutations (including atypical pathologic features), we observed an unexpected fact, that is a high incidence of carriers of single podocin mutations (8 of 165). Finally, we are also reporting a frequency of the R229Q variant higher than in normal people, a finding that confirms recent data by Tsukaguchi et al. (19), who associated this variant with late-onset FSGS. The same authors demonstrated a biologic difference between the R229Q and the wild-type peptide, characterized by an altered binding to nephrin of the former. This key observation gives functional support to the idea that podocin resulting from R229Q is biochemically altered. The meaning of all these data are that we probably miss a second NPHS2 mutation in heterozygous and R229Q patients due to the methodological approach (e.g., mutation in noncoding or regulatory regions). Tsukaguchi et al. (19) identified an associated podocin mutation in two patients with late-onset FSGS, and we found a second NPHS2 mutation in two siblings described anecdotally in this paper and could show markedly decreased expression of podocin in their glomeruli. A second possibility supporting a causative effect of heterozygous podocin mutations is loss of heterozygosity in glomeruli, a mechanism that has been documented in other renal diseases such as polycystic kidney (20). In this light, we sequenced a transcript cDNA purified from a bioptic renal specimen obtained from a heterozygous carrier of podocin mutation and found the expression of the normal allele, thus excluding this possibility.

So far, the association of steroid-resistant NS and heterozygous mutations of podocin has been reported in one family and two sporadic patients by Karle et al. (12). Moreover, five patients with heterozygous nephrin mutations and congenital nephrotic syndrome have also been described by Lenkkeri et al. (19). Nine fetuses with heterozygous nephrin mutations diagnosed in utero and with histopathologic features of Finnish NS have been recently reported by Patrakka et al. (8).

The novelty of our findings is that they describe the association of single mutations of podocin with less severe phenotypes than those already reported for homozygous patients. On the other hand, the mutations they display are different from those associated with the poor phenotypes and do not include putative severe loss of function defects. This raises the possibility that the encoded peptide may retain some functional aspects and determine a mild phenotype. On the other hand, one could also speculate that heterozygous coding podocin mutations contribute to proteinuria in association with other factors rather than directly determining it. Possible candidates are circulating plasma factors, the nature of which we (21–23) and others (24,25) are currently investigating; in this light, molecular defects of podocin would act as modifiers of the phenotype. Recent data by Le Berre et al. (26) on spontaneously proteinuric Buffalo/Mna rats support this possibility. Genetic analysis in Buffalo/Mna rats demonstrated that two recessive genes are implicated in the development of proteinuria and that one of them (Pur 1 on chromosome 13) is syntenic to the podocin-containing region of chromosome 1 in humans. Proteinuria and FSGS develop spontaneously in this strain of

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**Figure 4.** Immunofluorescence with anti-human podocin antibodies of glomeruli from (A) normal portion of a nephrectomy for carcinoma and (B) one of the two siblings presenting R229Q associated with A297V podocin mutation.
rats at the age of 2 mo, suggesting the implication of inherited causes. Le Berre et al. (26) showed that proteinuria occurred when normal LEW.1W kidneys were transplanted in Buffalo/Mna rats, whereas it relapsed when Buffalo/Mna kidneys were transplanted into LEW.1W, suggesting the existence of cofactors in spontaneously proteinuric rats. It must be noted that this is highly reminiscent of what happens in humans with idiopathic FSGS who develop rapid recurrence after renal transplant. Studies on this topic are currently in progress. Preliminary data on recurrence of proteinuria in patients with homozygous podocin mutations who underwent renal graft support its possible relationship with high serum permeability activity (27) and extends to humans the concept that podocin mutation may act in synergy with other actors of a multifactorial system.

In conclusion, the data reported here confirm the high incidence of podocin mutations in children with sporadic NS and contribute to outline the clinical and pathologic features in these patients. They also demonstrate a considerable incidence of podocin mutations in a subset of proteinuric patients. They also demonstrate a considerable incidence of podocin mutations in children with sporadic NS and support its possible relationship with high serum permeability activity (27) and extends to humans the concept that podocin mutation may act in synergy with other actors of a multifactorial system.

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