Patients with Mutations in *NPHS2* (Podocin) Do Not Respond to Standard Steroid Treatment of Nephrotic Syndrome

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Abstract. Nephrotic syndrome (NS) represents the association of proteinuria, hypoalbuminemia, edema, and hyperlipidemia. Steroid-resistant NS (SRNS) is defined by primary resistance to standard steroid therapy. It remains one of the most intractable causes of ESRD in the first two decades of life. Mutations in the *NPHS2* gene represent a frequent cause of SRNS, occurring in approximately 20 to 30% of sporadic cases of SRNS. On the basis of a very small number of patients, it was suspected that children with homozygous or compound heterozygous mutations in *NPHS2* might exhibit primary steroid resistance and a decreased risk of FSGS recurrence after kidney transplantation. To test this hypothesis, *NPHS2* mutational analysis was performed with direct sequencing for 190 patients with SRNS from 165 different families and, as a control sample, 124 patients with steroid-sensitive NS from 120 families. Homozygous or compound heterozygous mutations in *NPHS2* were detected for 43 of 165 SRNS families (26%). Conversely, no homozygous or compound heterozygous mutations in *NPHS2* were observed for the 120 steroid-sensitive NS families. Recurrence of FSGS in a renal transplant was noted for seven of 20 patients with SRNS (35%) without *NPHS2* mutations, whereas it occurred for only two of 24 patients with SRNS (8%) with homozygous or compound heterozygous mutations in *NPHS2*. None of 29 patients with homozygous or compound heterozygous mutations in *NPHS2* who were treated with cyclosporine A or cyclophosphamide demonstrated complete remission of NS. It was concluded that patients with SRNS with homozygous or compound heterozygous mutations in *NPHS2* do not respond to standard steroid treatment and have a reduced risk for recurrence of FSGS in a renal transplant. Because these findings might affect the treatment plan for childhood SRNS, it might be advisable to perform mutational analysis of *NPHS2*, if the patient consents, in parallel with the start of the first course of standard steroid therapy.

Nephrotic syndrome (NS) is defined as the association of proteinuria, hypoalbuminemia, edema, and hyperlipidemia. It constitutes one of the most common diagnoses in pediatric nephrology. Approximately 80% of all children with sporadic NS respond to steroid treatment. For decades, NS has been separated into two broad categories on the basis of the response to standard steroid therapy, *i.e.*, steroid-sensitive NS (SSNS) and steroid-resistant NS (SRNS) (1,2). In SRNS, approximately 75% of patients exhibit renal histologic features of FSGS and 20% demonstrate minimal-change NS (MCNS). Conversely, in SSNS, renal histologic features indicate MCNS in 80% of cases and FSGS in 20% (3). The pathogenesis of NS has been elusive, despite decades of research on its renal histologic and protein biochemical features. Protein biochemistry approaches have been applied to the study of the pathogenesis of FSGS, with some indicating a circulating "FSGS factor" (4–6). The most prominent hypothesis regarding the pathogenesis of SSNS was an immunopathogenetic concept. This was based on the observations of abnormal T lymphocyte function, the fact that remission is sometimes induced by measles, the susceptibility of patients to pneumococcal and other bacterial infections, the association of NS with Hodgkin's disease, and the response of NS to treatment with steroids and cyclophosphamide (CP) (7). Familial cases of SRNS and SSNS have been described; this strongly suggests the presence of monogenic variants of NS (3,8). Positional

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cloning revealed defects in four different genes as monogenic causes of SRNS in familial cases. Recessive mutations in *NPHS1*, encoding nephrin (OMIM no. 602716), cause congenital NS of the Finnish type (9). Recessive mutations in *NPHS2*, encoding podocin (OMIM no. 604766), cause SRNS type 1 (10). Mutations in *ACTN4*, encoding α-actinin 4 (OMIM no. 604638), have been identified as an autosomal dominant cause of SRNS (11). An additional locus for an autosomal dominant form of NS has been mapped to chromosome 11q21–q22 (OMIM no. 603965) (12). Identification of candidate genes for monogenic forms of SRNS indicates the importance of genetic factors in the pathogenesis of NS. Through identification of these three causative genes for SRNS, their gene products (nephrin, podocin, and α-actinin 4) were identified as being important for the function of the glomerular slit membrane of podocyte foot processes, which constitutes the primary molecular sieve of glomeruli (13).

Familial SRNS has been described as a childhood onset of proteinuria, rapid progression to ESRD, resistance to standard steroid therapy, and an absence of recurrence after renal transplantation (8). Since the identification of the *NPHS2* gene encoding podocin, different groups have demonstrated that mutations in the *NPHS2* gene represent a frequent cause of SRNS, occurring in 20 to 30% of sporadic (*i.e.*, nonfamilial) cases of SRNS (14–17). In addition, mutations in the *NPHS2* gene were recently identified as a cause of an adult-onset form of FSGS (18). Initial reports suggested that children with *NPHS2* mutations might exhibit primary resistance to standard steroid treatment. Those data, however, involved only 14 patients (10). In addition, the risk for FSGS recurrence in the renal transplant seemed to be much lower than the 30% recurrence rate observed in the general FSGS population (19). Again, these findings involved very few patients (10,17).

We therefore sought to examine these potential genotype/phenotype relationships among a large number of patients. Specifically, we performed mutational analysis for all eight *NPHS2* exons among 190 patients with SRNS from 165 different families and, as a control sample, 124 patients with SSNS from 120 different families. Our primary goals were to determine, among patients with homozygous or compound heterozygous mutations in *NPHS2*, (*1*) whether the patients respond to standard steroid treatment and (*2*) whether the risk of FSGS recurrence in a kidney transplant is indeed lower than among patients without mutations in *NPHS2*. We demonstrate that patients with homozygous or compound heterozygous mutations in *NPHS2* always exhibited primary steroid resistance and were not observed in the group with SSNS. In addition, we demonstrate that the rate of FSGS recurrence in a renal transplant was only 8% (two of 24 patients) among patients with homozygous or compound heterozygous mutations in *NPHS2*, compared with 35% (seven of 20 patients) among patients without *NPHS2* mutations. Mutational analysis of *NPHS2* among patients with SRNS might therefore help direct long-term treatment plans for patients with *NPHS2* mutations.

Materials and Methods

Blood samples for mutational analysis (www.renalgenes.org), clinical data, and informed consent were obtained from patients or their parents. Genomic DNA was directly isolated from blood samples with standard methods (20). Ethics approvals were obtained from the ethics commission of the University of Freiburg (Freiburg, Germany) and the ethics commission of the University of Michigan (Ann Arbor, MI). Diagnoses of SRNS and SSNS were established by pediatric nephrologists at different pediatric nephrology centers, according to published criteria (1). For clinical evaluations, we used a standard questionnaire (www.renalgenes.org), as described previously (14). Characteristic features defining the clinical diagnosis were age of onset, response to steroid therapy, histologic features of the kidney biopsy, progression to ESRD, and recurrence of FSGS after renal transplantation (Table 1). Standard steroid treatment and responses to steroid treatment, as well as responses to cyclosporine A (CsA) and CP therapy, were defined according to the International Study of Kidney Disease in Children and Arbeitsgemeinschaft fur Pädiatrische Nephrologie guidelines (1,2). Nephrotic range proteinuria was defined as >40 mg/m^2 per h, and low-grade proteinuria was defined as >4 mg/m^2 per h and <40 mg/m^2 per h. Standard steroid therapy was defined as 60 mg/m^2 per d prednisone administered orally in three divided doses for 6 wk, followed by treatment with 40 mg/m^2 per d prednisone administered orally in three divided doses on alternate days for an additional 6 wk (1). Primary resistance to steroid treatment was defined as the absence of remission, *i.e.*, a trace of proteinuria on dipstick analysis or <4 mg/m^2 per h after the initial 6 wk of standard steroid therapy (1,2). A partial response was defined as the disappearance of edema, an increase in the serum albumin concentration to >35 g/L, and the persistence of proteinuria of >4 mg/m^2 per h (2). Six patients with congenital NS and two patients with low-grade proteinuria never received steroid treatment and were viewed as having primary steroid resistance on the basis of their presentation with congenital NS or low-grade proteinuria by the physicians. Patients were categorized as being steroid-sensitive if at least a partial response to steroids was observed. Patients who developed steroid resistance in a later stage of the disease were considered steroid-sensitive for this study. Congenital NS was defined as the presentation of NS within the first 2 mo of life. Calculation of the frequency of mutations in *NPHS2* was based on the number of different families, rather than the number of patients, because affected siblings bear identical mutations by descent. For the evaluation of clinical data, the number of patients rather than the number of families was used, because in familial cases of FSGS with more than one affected sibling the clinical data varied among the affected siblings. Ethnically, the patients were from Central Europe, Turkey, and India.

Mutational analysis was performed with direct sequencing of one strand of all eight exons of the *NPHS2* gene. The reverse primer for exon 1 and the forward primers for exons 2 to 8 were used for sequencing. Primers for the eight exons of *NPHS2* were used and sequencing was performed as described previously (14,21). Very high sequence quality was obtained. When the results were in doubt, the complementary strand was also sequenced. All mutations and sequence variants were confirmed with sequencing of the complementary strand. Known single-nucleotide polymorphisms within the primer sequence were avoided (http://genome.ucsc.edu/), because known single-nucleotide variants within the primer sequence can suppress amplification of one of the two alleles of the amplified product. To exclude the possibility of polymorphisms, 160 chromosomes from 80 healthy control individuals (from a control group with ages and ethnic backgrounds similar to those of the patients screened

Table 1. Clinical data for patients with mutations and sequence variations in *NPHS2*[a]

Group	Family-Individual	Gender	Mutation (Amino Acid Exchange)	Age of Onset (yr)	Initial Symptoms	Biopsy	Steroid Therapy	CP/CsA Therapy	ESRD (yr after Onset)	KTx (yr after Onset)	Relapse of FSGS after KTx
A	236-1[b]	F	G413A/R138Q (H)	2.5	AS	FSGS	SR	ND	Y (11.4)	Y (13.5)	N (3.7)
A	260-1[b]	M	G413A/R138Q (H)	4.3	AS	?	SR	CP (N)	Y (4.9)	Y (5.0)	N (10)
A	260-2[b]	M	G413A/R138Q (H)	1.0	AS	?	SR	CP (N)	Y (7.2)	Y (8.6)	N (10)
A	330-1[b]	F	G686A/R229Q (h)-C871T/R291W (h)	3.5	?	FSGS	SR	CsA (P)	Y (21.2)	N	N
A	330-2[b]	M	G686A/R229Q (h)-C871T/R291W (h)	3.0	AS	FSGS	SR	ND	Y (14.8)	Y (17.7)	AR (0.0)
A	348-1[b]	M	G413A/R138Q (H)	3.3	AS	FSGS	SR	ND	N		
A	348-2[b]	M	G413A/R138Q (H)	2.0	AS	FSGS	SR	ND	Y (?)	Y (13, 36)	CR (22.0)
A	370-1[b]	M	G686A/R229Q (h)-C851T/A284V (h)	24.0	P-AS	FSGS	SR	CsA + CP (N)	N (3)	N	N
A	370-2[b]	F	G686A/R229Q (h)-C851T/A284V (h)	9.0	P-AS	FSGS	SR	ND	N (8.0)	N	N
A	398-1[b]	F	G413A/R138Q (H)	2.8	AS	FSGS	SR	CsA (N)	Y (2.6)	Y (3.3)	N (6.7)
A	460-1[b]	F	G413A/R138Q (H)	2.5	AS	MCNS	ND	ND	Y (8.5)	Y (9.7)	N (2.9)
A	460-2[b]	M	G413A/R138Q (H)	5.4	AS	MCNS	SR	CP (N)	Y (5.7)	Y (6.4)	N (6.4)
A	489-1[b]	M	G686A/R229Q (h)-C851T/A284V (h)	11.8	AS	FSGS	SR	CsA (N)	N (4.4)	N	N
A	489-2[b]	F	G686A/R229Q (h)-C851T/A284V (h)	3.5	AS	FSGS	SR	CsA (N)	Y (7.1)	N	N
A	747-1[b]	M	G413A/R138Q (H)	1.8	AS	FSGS	SR	ND	Y (2.0)	Y (3.1)	N (3.7)
A	747-2[b]	M	G413A/R138Q (H)	3.8	AS	FSGS	SR	CsA (P)	N (2.0)	N	N
A	763[b]	F	G538A/V180M (H)	16.6	AS	FSGS	SR	CsA (?)	Y (3.5)	N	N
A	764[b]	F	G413A/R138Q (H)	?	AS	MCNS	SR	CsA (P)	Y (?)	N	N
A	789[b]	M	IVS4-1G>T (H)	5.0	AS	FSGS	SR	CsA (?)	N (0.5)	N	N
A	803[b]	F	C851T/A284V (H)	2.0	AS	FSGS	SR	ND	Y (8.0)	Y (8.8)	N (1.1)
A	833[b]	F	del AA 855/56/S302X (H)	9.1	AS	MCNS	SR	CsA + CP (N)	Y (1.6)	Y (3.4)	N (7.7)
A	836[b]	F	G587C/R196P (h)-G868A/V290M (h)	1.3	AS	FSGS	SR	CsA (N)	N (10.1)	N	N
A	861	F	G868A-V290M(h)-del T 948/L347X (h)	2.2	P-AS	FSGS	SR	CsA (P)	N (13.8)	N	N
A	873	M	G770A/G257E(H)	0.3	P-AS	MCNS	ND	ND	N (0.5)	N	N
A	888	M	G413A/R138Q (h)-IVS7+2T>A (h)	3.5	AS	FSGS	SR	?	N (1.4)	N	N
A	911	F	G686A/R229Q (h)-A929T/E310V (h)	4.0	AS	FSGS	SR	CsA (N), CP (N)	Y (7.9)	Y (8.3)	N (1.5)
A	975-1	M	G413A/R138Q (H)	4.3	AS	FSGS	SR	CP (N)	Y (1.5)	Y (4.1)	N (5.7)
A	975-2	M	G413A/R138Q (H)	1.5	AS	FSGS	SR	ND	Y (9.7)	N	
A	1005	F	G413A/R138Q (h)-IVS3+2T>A (h)	3.4	AS	MCNS	SR	CsA (N)	Y (4.0)	Y (7.8)	N (1.5)
A	1006	M	G413A/R138Q (h)-G503A/R168H (h)	4.9	?	FSGS	SR	?	Y (6.2)	Y (8.5)	N (3.4)
A	1023	M	G413A/R138Q (h)-ins T 460–467/V165X (h)	1.5	AS	FSGS	SR	CsA (N)	Y (6.0)	Y (7.5)	N (4.3)
A	1030	F	G413A/R138Q (H)	0.7	AS	FSGS	SR	CP (N)	Y (9.3)	Y (13.1, 19.1)	N (CR6, ?)
A	1032	F	G413A/R138Q (h)-G868A/V290M (h)	11.0	P	IgM	ND	ND	N (2.7)	N	N
A	1033	M	G413A/R138Q (H)	0.5	AS	ND	ND	ND	N (1.2)	N	N
A	1041	F	ins A 29/E69X (h)-G304A/E102K (h)	2.2	AS	MP	SR	CP (N)	N (2.5)	N	N
A	1045	M	ins T 460–467/V165X (H)	1.0	AS	FSGS	SR	CsA (N)	Y (9.0)	N	N
A	1059-1	M	C353T/P118L (H)	2.0	AS	FSGS	SR	ND	Y (4.4)	N	N

Table 1. Continued

Group	Family-Individual	Gender	Mutation (Amino Acid Exchange)	Age of Onset (yr)	Initial Symptoms	Biopsy	Steroid Therapy	CP/CsA Therapy	ESRD (yr after Onset)	KTx (yr after Onset)	Relapse of FSGS after KTx
A	1069	M	G686A/R229Q (H)	7.4	AS	FSGS	?	CP (N)	Y (5.8)	N	N
A	1077	M	T803G/V268G (H)	3.5	AS	FSGS	SR	CP (N)	N (0.5)	N	N
A	1083	F	C353T/P118L (H)	3.6	AS	DMS	SR	CP (N)	Y (1.5)	N	N
A	1139	F	del T 948/L347X (H)	0.7	AS	FSGS	SR	CsA (N)	Y (3.2)	Y (3.6)	Y (0.1)
A	1173	M	G686A/R229Q (H)	5.5	AS	FSGS	SR	CsA (N), CP (N)	N (3.1)	N	N
B	236-2[b]	M	G413A/R138Q (H)	0.1	AS	FSGS	SR	ND	Y (9.4)	Y (10.4)	N (6.7)
B	355-1[b]	M	G413A/R138Q (h)-ins T 460–467/V165X (h)	0.0	AS	FSGS	SR	CsA (N)	Y (5.7)	Y (6.2)	N (1.6)
B	355-2[b]	F	G413A/R138Q (h)-ins T 460–467/V165X (h)	0.0	AS	FSGS	ND	ND	N (4.0)	N	N
B	398-2[b]	F	G413A/R138Q (H)	0.0	AS	FSGS	SR	CsA (N)	Y (4.2)	Y (5.0)	N (3.0)
B	515-1[b]	M	G413A/R138Q (h)-419 del G/V180X (h)	0.0	AS	CNS	SR	ND	Y (?)	Y (7.8)	N (9.0)
B	515-2[b]	F	G413A/R138Q (h)-419 del G/V180X (h)	0.0	AS	ND	SR	ND	Y (?)	Y (14.5)	N (0.4)
B	859[b]	M	G413A/R138Q (H)	0.0	P	MCNS	SR	CsA (P)	N (3.3)	N	N
B	935	M	ins T 460–467/V165X (h)-T506C/L169P (h)	0.0	AS	MCNS	SR	ND	N (1)	N	N
B	942-1	M	G413A/R138Q (h)-G503A/R168H (h)	0.0	?	?	?	?	?	?	?
B	942-2	M	G413A/R138Q (h)-G503A/R168H (h)	0.0	?	?	?	?	?	?	?
B	1028	M	C353T P118L (h)-G413A/R138Q (h)	0.0	AS	MP	ND	ND	N (6.4)	N	N
B	1201	M	ins T 460–467/V165X (h)-C871T/R291W (h)	0.0	P	FSGS	SR	CsA (N)	N (7.3)	N	N
B	1221	M	G378T/K126N (h)-del T 948/L347X (h)	0.0	AS	FSGS	ND	ND	Y (6.2)	Y (6.6)	Y (?)
B	1233	F	G413A/R138Q (h)-ins T 460–467/V165X (h)	0.0	AS	FSGS	SR	ND	N (2.9)	N	N
C	376-1[b]	M	G413A/R138Q (h)	4.0	AS	FSGS	SR	CsA (P)	Y (8.0)	N	N
C	376-2[b]	M	G413A/R138Q (h)	7.0	AS	MCNS	SR	CsA (R)	N (2.8)	N	N
C	923	M	A983G/Q328R (h)	?	?	ND	?	?	?	?	?
C	1086	M	G709C/E237Q (h)	10.5	AS	MCNS	SR	CsA (R)	N	N	N
C	1104	F	C725T/A242V (h)	1.6	AS	FSGS	SR	CP (R)	N (1.4)	N	N
D	3908	M	G709C/E237Q (h)	5.7	AS	MCNS	SS	CsA (?)	N	N	N
D	4286	M	C59T/P20L (h)	?	AS	MCNS	SS	ND	N	N	N
D	3147	M	G413A/R138Q (h)	3.2	AS	ND	SS	ND	N	N	N
D	1172	F	C871T/R291W (h)	1.5	AS	FSGS	SS-SR	CsA (N)	N (0.7)	N	N

[a] Group A, patients with steroid-resistant nephrotic syndrome (NS) (SRNS) and mutations in both alleles of *NPHS2*; P-AS, intermittent low-grade proteinuria progressing to acute NS; AR, acute rejection; AS, acute symptoms of NS; group B, patients with SRNS, mutations in both alleles of *NPHS2*, and CNS; group C, patients with SRNS and a single heterozygous sequence variant; CNS, histology of congenital NS; CP, cyclophosphamide; CR, chronic rejection; CsA, cyclosporine A; group D, patients with steroid-sensitive NS (SSNS) and a single heterozygous sequence variant; DMS, diffuse mesangial sclerosis; (h), heterozygous; (H) homozygous; IgM, IgM nephropathy; KTx, kidney transplantation; MCNS, minimal-change glomerulonephritis; MP, mesangioproliferative glomerulonephritis; N, no; (N), no remission; ND, not done; P, intermittent low grade-proteinuria; (P), partial remission; (R), complete remission; SS, steroid-sensitive; SS-SR, steroid-sensitive progressing to steroid-resistant; SR, steroid-resistant; Y, yes; ?, no data.

[b] Mutations for these patients were previously reported ((14)).

for *NPHS2* mutations) were checked for novel mutations with direct sequencing, after informed consent was obtained. For sequence evaluation, the program Sequencher was used.

Results

Clinical Data for the Patients with SRNS and SSNS

A total of 314 patients with NS (from 285 different families) were included in the study; 190 patients (165 families) demonstrated primary steroid resistance (SRNS) (Tables 1 and 2) and 124 patients (from 120 different families) were initially steroid-sensitive (SSNS) (Table 2). Renal biopsy results for patients with SRNS were as follows: FSGS, 115 of 190 patients (61%); MCNS, 34 of 190 patients (18%); mesangioproliferative glomerulonephritis, five of 190 patients (2%); membranoproliferative glomerulonephritis, four of 190 patients (2%); diffuse mesangial sclerosis, three of 190 patients (1%); IgM nephropathy, one of 190 patients (1%); congenital NS, one of 190 patients (1%); no data or no biopsy performed, 27 of 190 patients (14%) (Tables 1 and 2). Renal biopsy results for patients with SSNS were as follows: FSGS, 26 of 124 patients (21%); MCNS, 22 of 124 patients (18%); membranoproliferative glomerulonephritis, five of 124 patients (4%); mesangioproliferative glomerulonephritis, two of 124 patients (1%); no data or no biopsy performed, 69 of 124 patients (56%) (Table 2). The small number of biopsies performed in SSNS is explained by the milder course of SSNS. The median age of onset was 3.5 yr for the patients with SRNS and 4.4 yr for the patients with SSNS (Table 2). Congenital NS was diagnosed for 21 of 190 patients (11%) with SRNS. The median age of onset of NS for the patients with SRNS was 4.0 yr, after subtraction of the patients with congenital NS (Table 2). Sixty-eight of 190 patients with SRNS (36%) exhibited progression to ESRD, with a median age of 10.1 yr (Table 2). Two of 124 patients with SSNS, from different families, exhibited progression to ESRD. Both patients were initially steroid-sensitive and became steroid-resistant in a later phase of the disease. To strengthen the statistical power of the study, clinical data for 52 different SRNS families that we described previously (14) were included in this study. Data for those families in Table 1 and Figure 1 are specified. Clinical data for those patients were updated where possible.

Frequency of NPHS2 *Mutations in SRNS* versus *SSNS*

Mutational analysis of the *NPHS2* gene was performed for 190 patients with SRNS from 165 different families and for 124 patients with SSNS from 120 different families (Table 2). For 43 of 165 families with SRNS (26%), homozygous or compound heterozygous mutations in *NPHS2* were observed (Tables 1 to 3). For four of 165 families with SRNS (2%), only a single heterozygous sequence variant was observed, which does not explain the phenotype for a recessive disease and is primarily considered a polymorphism (Tables 1 and 2). Twenty-seven of fifty-six patients with SRNS (48%) with homozygous or compound heterozygous mutations in *NPHS2* represented familial cases, whereas 29 of 56 patients (52%) represented sporadic cases. Of the total of 152 patients with sporadic SRNS, 29 (19%) had homozygous or compound heterozygous mutations in *NPHS2*. In contrast to the findings for SRNS, none of the 120 families with primary SSNS demonstrated homozygous or compound heterozygous mutations in *NPHS2* (Table 2). For four of 120 families with SSNS (3%), only a single heterozygous sequence variation in *NPHS2* was observed (Tables 1 to 3).

Types of NPHS2 *Mutations Observed*

In total, 23 different mutations in the *NPHS2* gene were observed; 15 were missense mutations, five were frameshift mutations leading to a premature stop codon, and three were splice site mutations (Figure 1, Table 1). Ten of the mutations were novel. Except for the sequence variants R229Q and P20L, all mutations (novel or previously described) were absent from 160 chromosomes from healthy control individuals. The frequencies of the different mutations are indicated in Figure 1.

In particular, the following novel mutations were observed (Figure 1, Table 1). (*1*) A G304A transition leading to the nonconservative amino acid exchange E102K, conserved in mice during evolution, occurred heterozygously in F1041. (*2*) A C353T transition leading to the nonconservative amino acid exchange P118L, conserved during evolution in mice, *Drosophila melanogaster*, and *Caenorhabditis elegans*, occurred homozygously in F1059 and F1083 and heterozygously in F1028. (*3*) A G378T transversion leading to the nonconservative amino acid exchange K126N, conserved in mice during evolution, occurred heterozygously in F1221. (*4*) A G503A transition leading to the conservative amino acid exchange R168H, conserved in mice during evolution, occurred heterozygously in F1006 and F942. (*5*) A G770A transition leading to the nonconservative amino acid exchange G257E, conserved during evolution in mice, *D. melanogaster*, and *C. elegans*, occurred homozygously in F873. (*6*) A T803G transversion leading to the conservative amino acid exchange V268G, conserved during evolution in mice, *D. melanogaster*, and *C. elegans*, occurred homozygously in F1077. (*7*) An insertion of adenine at position 29 in codon 10, inducing a frameshift and resulting in a stop codon at E69X, occurred heterozygously in F1041. (*8*) A deletion of thymine at position 948 in codon 346, inducing a frameshift and resulting in a stop codon at L347X, occurred homozygously in F1139 and heterozygously in F861 and F1221. (*9*) The obligatory splice site mutation IVS3+2T→A, involving the 5' donor splice site of intron 3, occurred heterozygously in F1005. (*10*) The obligatory splice site mutation IVS7+2T→A, involving the 5' donor splice site of intron 7, occurred heterozygously in F888.

Types of Single Heterozygous Sequence Variants/Polymorphisms

In four families with SRNS and four families with SSNS, only a single heterozygous sequence variant/polymorphism was observed (Table 1). All of those single heterozygous sequence variants/polymorphisms were absent from 80 healthy control individuals. In total, six different single heterozygous sequence variants/polymorphisms were observed. Two single heterozygous sequence variants/polymorphisms, G413A (R138Q) and C871T (R291W), occurred in compound het-

Table 2. Clinical data and *NPHS2* mutational analysis data for 190 patients with SRNS from 165 different families and 124 patients with SSNS from 120 different families[a]

Group	No. of Families (% of Total Families)	No. of Patients (Including Siblings)	Median Age of Onset of NS (yr)	Biopsy FSGS/MCNS/Other/ND (%)	CR/PR/NR after Treatment with CP/CsA (%)	ESRD	Median Age at ESRD (yr)	ESRD (yr after Onset)	Relapse of FSGS after KTX
SRNS									
mutations in both alleles	43/165 (26%)[b]	56/190[c]	2.0[d] (n = 54)	37/8/5/6 (66/14/9/11)	0/5/24 (0/17/83)	33/56 (59%)	10.0[e]	6.0	2/24 (8%)
absence of mutations	118/165 (72%)[f]	129/190[g]	4.8[h] (n = 111)	80/20/10/19 (62/16/8/14)	12/14/38 (19/22/59)	34/129 (26%)	10.5[i]	3.0	7/20 (35%)
single heterozygous sequence variants	4/165 (2%)	5/190	5.5 (n = 4)	2/2/0/1 (40/40/0/20)	3/1/0 (75/25/0)	1/5 (20%)	12.0	5.0	0/0 (0%)
total of families/ patients with SRNS	165[j]	190[k]	3.5[l] (n = 169)	115/34/14/27 (61/18/7/14)	13/17/56 (15/20/65)	68/190 (36%)	10.1[m]	5.2	8/44 (18%)
SSNS									
mutations in both alleles	0/120 (0%)	0/124	NA	NA	NA	NA	NA	NA	NA
absence of mutations	116/120 (97%)[n]	120/124[n]	4.4 (n = 99)	25/20/7/68 (21/17/6/56)	NA	2/120	NA	NA	NA
single heterozygous sequence variants	4/120 (3%)[o]	4/124[o]	3.2 (n = 3)	1/2/0/1 (25/50/0/25)	NA	NA	NA	NA	NA
total of families/ patients with SSNS	120	124	4.4 (n = 102)	26/22/7/69 (21/18/5/56)	NA	2/124	NA	NA	NA

[a] CNS, congenital NS; MCNS, minimal-change NS; KTX, kidney transplantation; CR, complete response; PR, partial response; NR, no response; NA, not applicable.
[b] Eleven of 43 families presented with congenital NS.
[c] Fourteen of 56 patients presented with congenital NS.
[d] Median was 3.4 yr after exclusion of the 14 patients with congenital NS.
[e] Median was 10.0 yr after exclusion of the patients with congenital NS and ESRD.
[f] Six of 118 families presented with congenital NS, of which five showed mutations in *NPHS1* on both alleles.
[g] Eight of 129 patients presented with congenital NS, of whom seven showed mutations in *NPHS1* on both alleles.
[h] Median was 5.0 yr after exclusion of the eight patients with congenital NS.
[i] Median was 11.5 yr after exclusion of the patients with congenital NS and ESRD.
[j] Seventeen of 165 families presented with congenital NS.
[k] Twenty-one of 190 patients presented with congenital NS.
[l] Median was 4.0 yr after substraction of the 21 patients with congenital NS.
[m] Median was 10.7 yr after substraction of the CNS patients with congenital NS and ESRD.
[n] At least seven patients developed secondary steroid resistance in the clinical course of SSNS.
[o] At least one patient developed secondary steroid resistance in the clinical course of SSNS.

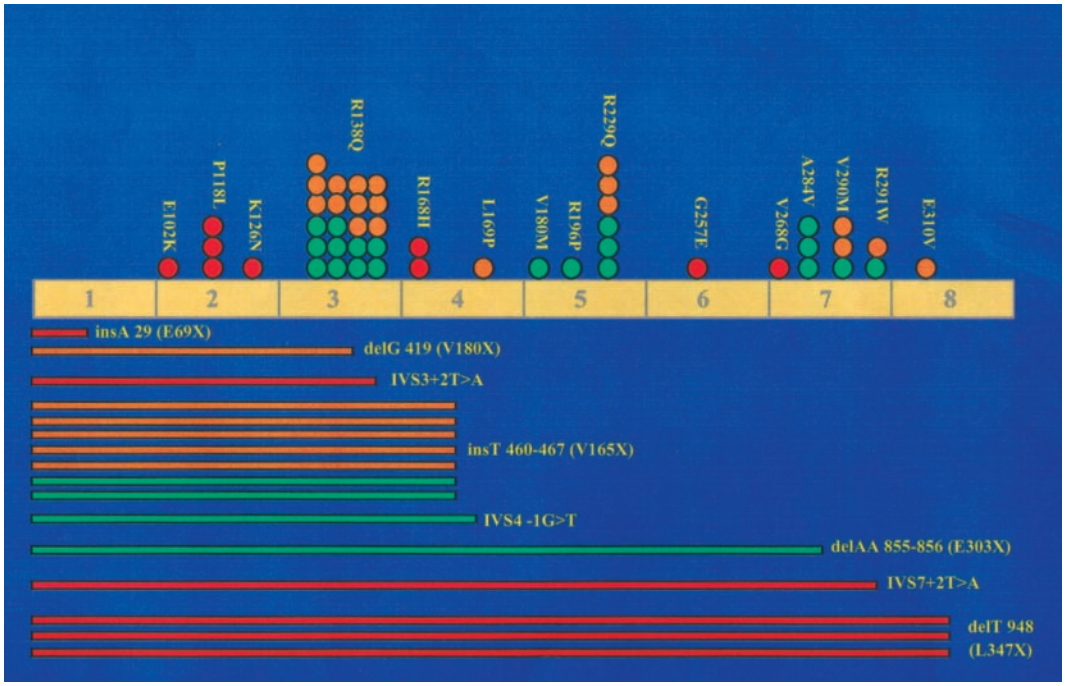

Figure 1. NPHS2 mutations observed in 165 families with steroid-resistant nephrotic syndrome (SRNS). The schematic diagram depicts the eight *NPHS2* exons. Missense mutations are indicated above the exon bar, as colored circles. The amino acid change in the podocin gene is indicated above the circles. Splice site and frameshift mutations are shown below the exon bar and are symbolized as colored bars, indicating the position of truncation. Mutations are color-coded; novel mutations not previously described are red, previously reported mutations observed again in the 165 families with SRNS are orange, and mutations previously described by our group (14) are green. The frequencies of the different mutations observed among the 165 families with SRNS are indicated by the different numbers of circles. Each circle or bar is equivalent to one mutation observed among 165 families with SRNS, corresponding to the data presented in Table 1.

Table 3. Distribution of mutations and heterozygous sequence variants in *NPHS2*[a]

	Homozygous Families/Patients	Compound Heterozygous Families/Patients	Heterozygous Sequence Variant Families/Patients
Group A + C, SRNS	22/27	12/15	4/5
Group B, SRNS + CNS	3/3	8/11	0
Group D, SSNS	0	0	4/4
Healthy control subjects	0	0	0

[a] CNS, congenital NS. Groups A to D correspond to Table 1.

erozygous or homozygous mutations in other families with SRNS (Figure 1, Table 1). Functional data demonstrated the relevance of these nucleotide variants (18,22). Three other single heterozygous sequence variants/polymorphisms, Q328R (F923), A242V (F1104), and E237Q (F1086), were not observed in compound heterozygous or homozygous mutations in other families with SRNS (Figure 1, Table 1). Two of the mutations were novel. (*1*) A G709C transversion leading to the conservative amino acid exchange E237Q, conserved in mice during evolution, occurred heterozygously in F1086 and F3908. (*2*) An A983T transversion leading to the nonconservative amino acid exchange Q328R, conserved in mice during evolution, occurred heterozygously in F923. Because no second mutation was observed in the recessive *NPHS2* gene, the significance of these single heterozygous sequence variants/polymorphisms for the disease phenotype is unclear. In one family (F4286), the amino acid exchange P20L, which was initially described by Boute *et al.* (10), was observed as a single heterozygous sequence variant. However, we consider this to represent an innocuous polymorphism, for the following reasons: (*1*) we observed three heterozygous nucleotide variants (P20L, R138Q, and R168H) in family F1006, (*2*) P20L occurred homozygously in two healthy control persons, and (*3*) the position is not conserved during evolution. The common polymorphism R229Q was observed for 13 of 190 patients with SRNS (7%), six of 124 patients with SSNS (6%), and nine of 80 healthy control subjects (11%). No significant difference among those groups could be noted. No other sequence variants affecting the coding protein sequence were detected among the 80 healthy control subjects.

Clinical Data for Patients with Mutations/Sequence Variations in NPHS2

We detected homozygous or compound heterozygous mutations in *NPHS2* for 56 of 190 patients with SRNS (29%) and 43 of 165 different families (26%) (Tables 1 and 2). For 14 of 22 patients with congenital NS (64%), homozygous or compound heterozygous mutations in *NPHS2* were observed. For seven of 22 patients with congenital NS (32%), homozygous or compound heterozygous mutations in *NPHS1* were observed (Ruf *et al.*, unpublished observations). For one patient with congenital NS, no mutation in *NPHS2* or *NPHS1* was observed. After exclusion of the patients with congenital NS, the median age of onset for patients with homozygous or compound heterozygous mutations in *NPHS2* was 3.4 yr (range, 0.3 to 24.0 yr), compared with 5.0 yr (range, 0.0 to 19.0) for the patients without mutations. Data on responses to treatment were available for 29 of 31 patients with SRNS and homozygous or compound heterozygous mutations in *NPHS2* who were treated with CsA or CP. None of those 29 patients achieved complete remission; five patients exhibited partial responses. For patients with homozygous or compound heterozygous mutations in *NPHS2*, the histologic findings from the kidney biopsies were as follows: FSGS, 37 of 56 patients (66%); MCNS, eight of 56 patients (14%); mesangioproliferative glomerulonephritis, two of 56 patients (3%); IgM nephropathy, one of 56 patients (2%); diffuse mesangial sclerosis, one of 56 patients (2%); congenital NS, one of 56 patients (2%); no data or no biopsy performed, six of 56 patients (11%) (Tables 1 and 2). For patients without mutations in *NPHS2*, the histologic findings were as follows: FSGS, 80 of 129 patients (62%); MCNS, 20 of 129 patients (16%); membranoproliferative glomerulonephritis, five of 129 patients (4%); mesangioproliferative glomerulonephritis, three of 129 patients (2%); diffuse mesangial sclerosis, two of 129 patients (2%); no data or no biopsy performed, 19 of 129 patients (14%). Therefore, renal histologic features did not differ for the groups of patients with and without the presence of homozygous or compound heterozygous mutations in *NPHS2*. Thirty-three of 56 patients with SRNS (59%) with homozygous or compound heterozygous mutations in *NPHS2* exhibited progression to ESRD, at a median age of 10.0 yr and at a median time of 6.0 yr (range, 1.5 to 21.2 yr) after the onset of disease symptoms. Thirty-four of 129 patients with SRNS (26%) without mutations in *NPHS2* exhibited progression to ESRD, at a median age of 10.5 yr and at a median time of 3.0 yr (range, 1.5 to 19.8 yr) after the onset of symptoms of NS. Kidney transplantation was performed for 24 patients with SRNS with homozygous or compound heterozygous mutations in *NPHS2* and for 20 patients without mutations in *NPHS2*. Seven of 20 patients with SRNS (35%) but without mutations in *NPHS2* developed recurrence of FSGS in their renal transplants, which is in accordance with data on FSGS recurrence published before mutational analysis of *NPHS2* was possible. In contrast, only two of 24 patients with SRNS (8%) with homozygous or compound heterozygous mutations in *NPHS2* developed FSGS recurrence. One of those two patients, F1139, demonstrated progressive proteinuria on day 7 after transplantation, which responded to immunosuppressive therapy. No biopsy was performed, and the function of the transplant is stable after 7 mo (Billing *et al.*, unpublished observations). No histologic evidence of the recurrence of FSGS has been noted to date. For four patients with SSNS (F3147, F3908, F4286, and F1172), a single heterozygous sequence variation in the *NPHS2* gene was observed. F3147 and F4286 experienced one episode of NS, which responded to steroid therapy. Biopsy results indicated MCNS for F4286, and no biopsy was performed for F3147. For F3908, two relapses of NS, which responded to steroid treatment, occurred after the initial steroid therapy. Histologic findings revealed MCNS. No clinical follow-up data were available. F1172 became resistant to steroids and CsA after initial steroid responsiveness. The histologic findings for the kidney biopsy indicated FSGS.

Discussion

On the basis of clinical data for <14 families, it was suspected that patients with mutations in *NPHS2* do not respond to steroid therapy and do not demonstrate recurrence of FSGS after kidney transplantation (10). To further evaluate these hypotheses with a number of patients sufficient to allow conclusions to be drawn, we performed mutational analysis of all *NPHS2* exons for 190 patients from 165 different families with primary SRNS and, as a negative control sample, 124 patients from 120 different families with SSNS. For 43 of 165 families with SRNS (26%), homozygous or compound heterozygous mutations in *NPHS2* were observed (Tables 1 and 2). For none of the 120 SSNS families were homozygous or compound heterozygous mutations in *NPHS2* identified. We therefore conclude that patients with homozygous or compound heterozygous mutations in *NPHS2* exhibit primary resistance to standard steroid treatment.

When familial and sporadic cases of SRNS were considered together, we observed homozygous or compound heterozygous mutations in *NPHS2* for 29% of patients from different families. When 14 familial SRNS cases were excluded from the total of 165, homozygous or compound heterozygous mutations in *NPHS2* were observed for 29 of 152 sporadic SRNS cases (19%). Caridi *et al.* (17) observed homozygous or compound heterozygous mutations in *NPHS2* for 14 of 120 patients with SRNS (12%). In contrast to those data, Maruyama *et al.* (23) performed mutational analysis for 36 Japanese children with SRNS without detecting any mutation in *NPHS2*. A different genetic background in the Japanese population could explain this finding. Variations in disease incidences among different ethnic groups have been described for other genetic diseases (24).

In contrast to the findings in SRNS, we did not detect any homozygous or compound heterozygous mutations in *NPHS2* among 124 patients with SSNS. Caridi *et al.* (17) performed mutational analysis of *NPHS2* for 59 patients with SSNS and Frishberg *et al.* (25) performed mutational analysis for 15 patients with SSNS without identifying any homozygous or compound heterozygous mutations in *NPHS2*. These data confirm that patients with homozygous or compound heterozygous mutations in *NPHS2* exhibit primary resistance to standard steroid treatment. Because methylprednisolone pulse therapy

was not evaluated in this study, no conclusion can be drawn regarding that mode of therapy.

We also provide data on a genotype/phenotype correlation regarding homozygous or compound heterozygous mutations in *NPHS2* and the recurrence of FSGS after kidney transplantation. On the basis of a small number of patients (<14 patients) with kidney transplants in the initial identification of the *NPHS2* gene (10), it was suspected that FSGS may not recur after kidney transplantation among such patients. No details regarding the number of patients who received kidney transplants were available at that time (10). We demonstrated that, whereas seven of 20 patients with SRNS (35%) without *NPHS2* mutations experienced FSGS recurrence in a renal transplant, only two of 24 patients with SRNS (8%) with homozygous or compound heterozygous mutations in *NPHS2* exhibited recurrence. Proteinuria was noted for one of the two patients with recurrence, which responded to steroid therapy (Table 2). No histologic evidence of FSGS recurrence has been noted to date. Our data thus demonstrate a significantly reduced risk of FSGS recurrence in a kidney transplant among patients with homozygous or compound heterozygous mutations in *NPHS2*, compared with patients without such mutations. This finding of a lower recurrence risk among patients with homozygous or compound heterozygous mutations in *NPHS2* could help direct the planning of living related donor transplants, which might be considered more readily on the basis of a lower recurrence risk. However, these data are in contrast to those of Bertelli *et al.* (26), who described FSGS recurrence for five of 12 patients (38%) with mutations in *NPHS2* (two of 12 with homozygous or compound heterozygous mutations in *NPHS2* and three of 12 patients with single heterozygous sequence variants in one allele of *NPHS2*) (26). The discrepancy can be explained by noting that (*1*) the recurrence of FSGS was proven by biopsy for only one of nine patients described by Bertelli *et al.* (26), whereas the second patient exhibited only a short period of proteinuria, with a prompt response to plasmapheresis, and (*2*) two patients with SRNS and FSGS recurrence described by Bertelli *et al.* (26) carried the single heterozygous sequence variant P20L. Our data indicate that this sequence variant most likely represents a polymorphism, because it occurred homozygously in a healthy control subject and the position is not conserved during evolution. Another patient described by Bertelli *et al.* (26) carried the single heterozygous sequence variant S211T. The functional relevance of this single heterozygous sequence variant remains unclear. Different pathogenic hypotheses have been postulated to explain the recurrence of FSGS. The most popular hypothesis suggests the involvement of one or more circulating factors altering renal permeability to proteins and causing proteinuria (4). Among patients with a molecular defect in both alleles of *NPHS2*, a defect in the protein podocin is considered to be the disease-causing mechanism. After kidney transplantation, the disease-causing mechanism should be cured. The occurrence of autoantibodies against podocin, as described for congenital NS, could explain the recurrence of disease. However, data reported by Bertelli *et al.* (26) render this hypothesis unlikely. Although our data indicate a reduced risk after renal transplantation for patients with homozygous or compound heterozygous mutations in *NPHS2*, recurrence of FSGS cannot be excluded in this population of patients. This indicates a pathogenesis of FSGS involving additional extrarenal mechanisms.

Thirty-one patients with homozygous or compound heterozygous mutations in *NPHS2* in our cohort were treated with CsA or CP or both. Data on the results of treatment were available for 29 patients. Complete remission after therapy was observed for none of those patients. No clinical response was observed for 24 patients (83%), and a partial response was noted for five (17%) (Tables 1 and 2). In comparison, 64 patients without mutations in *NPHS2* received CsA or CP treatment. Twelve of 64 patients (19%) demonstrated complete remission, 14 of 64 patients (22%) a partial response, and 38 of 64 patients (59%) no response (Table 2). Frishberg *et al.* (25) reported that several of 13 patients with homozygous or compound heterozygous mutations in *NPHS2* did not respond to immunosuppressive therapy, including CsA and CP; no clinical details were provided. Caridi *et al.* (17) reported on eight patients with homozygous or compound heterozygous mutations in *NPHS2* who received treatment with CsA without any response. Our data and the cited data on the responses to CsA and CP therapy among patients with homozygous or compound heterozygous mutations in *NPHS2* suggest that the patients with homozygous or compound heterozygous mutations in *NPHS2* might exhibit decreased responses to treatment. Different groups discussed an immunopathogenetic hypothesis for the pathogenesis of NS, including speculation regarding a circulating FSGS factor (4–7). This also explains the success of immunosuppressive therapy for NS. As mentioned above, the molecular defect in *NPHS2* and the resulting defect in the protein podocin are considered the disease-causing mechanism among patients with mutations in *NPHS2*. These defects are most likely not changeable with immunosuppressive therapy, as indicated by the nonresponsiveness of these patients to steroid therapy. However, five patients with molecular defects in *NPHS2* exhibited partial responses to CsA or CP therapy. These findings indicate a complex pathogenesis of FSGS, involving additional extrarenal mechanisms. The number of patients studied to date regarding responses to CsA or CP treatment is not sufficient to provide a statistically significant result to suggest a change in the therapeutic regimen for SRNS. Therefore, these data should be considered preliminary. Further studies with larger numbers of patients with SRNS will be important to delineate the influence of the presence of homozygous or compound heterozygous mutations in *NPHS2* on responses to CsA and CP treatment.

On the basis of the data on the lack of responses to standard steroid therapy among patients with homozygous or compound heterozygous mutations in *NPHS2*, we propose performing mutational analysis of *NPHS2* for every child (if consent can be obtained) immediately after presentation with the first episode of NS. Because the data are still based on a limited variety of ethnic and genetic backgrounds and because performance of the mutational analysis requires approximately 2 to 3 mo (www.renalgenes.org, www.genetests.org), the initial standard

steroid therapy should be administered. If the patient is carrying homozygous or compound heterozygous mutations in *NPHS2*, then a second trial of standard steroid therapy is not justified. It will be important to generate additional data on genotype/phenotype correlations for homozygous or compound heterozygous mutations in *NPHS2* and responses to CsA, CP, and methylprednisolone pulse therapies.

Although no disease-causing mutations were detected among patients with SSNS, four single heterozygous sequence variants were identified. The amino acid substitution E237Q, which occurred heterozygously in SSNS patient F3908, was observed only one more time, in SRNS patient F1086, as a single heterozygous sequence variant. No patients with homozygous or compound heterozygous mutations in *NPHS2* carrying this mutation have been identified by us or others (14,15,17,18). The single heterozygous nucleotide variant could still represent a very rare polymorphism. Clear functional relevance for the recessive R138Q mutation that occurred heterozygously in SSNS patient F3147 and the R291W mutation that was identified in F1172 has been demonstrated (18,22). Although the amino acid substitutions P20L and E237Q might be polymorphisms, functional relevance for these amino acid substitutions cannot be excluded, as recently demonstrated for the common polymorphism R229Q (17,18).

In three sporadic cases (F923, F1086, and F1104) and one familial case (F376) of SRNS, only a single heterozygous sequence variant of *NPHS2* was observed. Assuming a causative role of these single heterozygous sequence variants, a second mutation might have been missed or might be located in the promotor region or an intron. An interesting alternative would be the involvement of other genes in the pathogenesis of NS in these families, via the mechanism of "digenic disease" (27). The data provided in this study on mutational analyses for 285 different families with NS emphasize the relevance of mutational analysis of *NPHS2* in this cohort. They present clear evidence for the importance of genotypic information to guide further treatment for these patients.

From the data provided, we draw the following conclusions. (*1*) Because patients with homozygous or compound heterozygous mutations in *NPHS2* do not respond to standard steroid therapy for NS, we propose performing mutational analysis of *NPHS2* (if consent can be obtained) for every child immediately after presentation with the first episode of NS, thus avoiding an unnecessary second trial of standard steroid therapy. (*2*) Because patients with SRNS and homozygous or compound heterozygous mutations in *NPHS2* have reduced risks for recurrence of FSGS in a renal transplant, compared with children without mutations, living related donor transplantation might be considered more readily. (*3*) Additional studies with more patients will be required to delineate the genotype/phenotype correlations for homozygous or compound heterozygous mutations in *NPHS2* and responses to other forms of treatment, such as CsA, tacrolimus, CP, methylprednisolone pulse therapy, or mycophenolate mofetil. (*4*) The significance of single heterozygous sequence variants for four of 165 families with SRNS and 4 of 120 families with SSNS must be determined in functional studies.

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