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Novel Mutations in *NPHS2* Detected in Both Familial and Sporadic Steroid-Resistant Nephrotic Syndrome

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Abstract. Autosomal recessive steroid-resistant nephrotic syndrome (SRINS) belongs to the heterogeneous group of familial nephrotic syndrome and represents a frequent cause of end-stage renal disease in childhood. This kidney disorder is characterized by early onset of proteinuria, progression to end-stage renal disease, and histologic findings of focal segmental glomerulosclerosis, minimal change nephrotic syndrome, or both. A causative gene, *NPHS2*, has been mapped to chromosome 1q25-q31 and was recently identified by positional cloning. This study reports five novel *NPHS2* mutations: A284V, R196P, V290M, IVS4-1G→T, and 460-467insT in 12 (46%) of 26 multiplex families and in 7 (28%) of 25 single patients with the clinical diagnosis of a SRINS. Because *NPHS2* mutations were found in nearly 30% of these patients with "sporadic" SRINS, mutational analysis should also be performed in these patients. Besides better classification of the disease entity, identification of *NPHS2* mutations may save some of these patients from unnecessary steroid treatment and also permit the prediction of absence of disease recurrence after kidney transplantation.

Autosomal recessive steroid-resistant nephrotic syndrome (SRINS) belongs to the heterogeneous group of familial nephrotic syndrome. It is characterized by childhood onset of proteinuria, progression to end-stage renal disease, and histologic findings of focal segmental glomerulosclerosis (FSGS), or minimal change nephrotic syndrome (1). Several causative genes for familial SRINS are known. For a subgroup with an autosomal recessive trait, the responsible gene (*NPHS2*, formerly *SRN1*) has been mapped to chromosome 1q25-q31 (1) and recently identified by positional cloning (2). It encodes a 383–amino acid protein, podocin, that is almost exclusively expressed in glomerular podocytes. From database comparisons, podocin is predicted to be an integral membrane protein linking the plasma membrane and the cytoskeleton (2). Podocin is supposed to interact with nephrin, α-actinin-4, and CD2AP (3). Nephrin (*NPHS1*) causes the most severe form of nephrotic syndrome, the congenital nephrotic syndrome of the Finnish type. A less severe form of SRINS with autosomal-dominant inheritance and FSGS1, adult onset, and slow progression to end-stage renal failure has been described (4). *FSGS1* is caused by defects in the α-actinin-4 gene (*ACTN4*) located on chromosome 19q13 (5). A second autosomal-dominant FSGS gene locus (*FSGS2*) maps on chromosome 11q21-q22. It has not yet been identified (6).

In this study, we performed mutational analysis in families and single patients with SRINS to determine whether *NPHS2* mutations are also a cause of sporadic SRINS.

Materials and Methods

Patients

We analyzed 27 multiplex families (53 affected individuals were included) and 25 patients with "sporadic" SRINS originating mainly from Germany but also from other European countries. Parental consanguinity was not reported. For clinical evaluation, we used a standard questionnaire as previously described (7). The characteristic features defining the clinical diagnosis of SRINS included familial occurrence, age at onset in early childhood, resistance to steroid therapy, progression to end-stage renal disease within a few years, and absence of recurrence after renal transplantation (1).

Haplotype Analysis

Genomic DNA was extracted from leukocytes according to standard laboratory protocols. PCR was performed with five polymorphic markers spanning the critical region of *NPHS2* on chromosome 1q25-q31 (1,2,7). The respective order from the centromeric to the telomeric border was: cen, D1S416, D1S1640, D1S2791, NPHS2, D1S215, D1S2883, tel (flanking markers are underlined). In the multiplex families, we first performed haplotype analysis. In case of consistency with linkage to *NPHS2*, mutational analysis was performed.

Mutational Analysis

We performed mutational analysis by single-strand conformation polymorphism (SSCP) and direct sequencing of both strands as previously described (8) in 17 SRINS families compatible with linkage to the *NPHS2* gene locus on chromosome 1q21 and 25 patients with sporadic SRINS. The PCR of exons 3, 4, and 8 was carried out as previously described (2). For the remaining exons, the following primers and conditions were applied: exon 1: 5'-GCAGCGACTCCACAGGGACT-3' and 5'-TCCACCTTATCTGACGCCCC-3'; exon 2: 5'-AGAATTGGACCAACAGATGC-3' and 5'-AAGTGAGAATGGGCATGGTG-3'; exon

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Table 1. Clinical data of patients with steroid-resistant nephrotic syndrome[a]

Family No.	Individual	Gender	Symptoms at Disease Onset				Renal Biopsy (Age at Biopsy, yr)	Age at ESRF (yr)	Age at Transplant (yr)
			Age at Onset (yr)	PU (mg/m²/h)	HU	Creatinin (mg/dl)/GFR (ml/min)			
SRINS families									
INS11	II:1	M	3.3	>40	−	0.85/166	FSGS (11.5)	14.4	15.3; 38
	II:2	M	2	>40	−	0.9/130	FSGS (6.0)	14	14; 36
16	II:1	F	3	243	+	0.5/178	FSGS (3.0)	5.3	6.3
	II:2	F	0.5	313	+	0.2/194	FSGS (2.0)	4.2	5
18	II:1	M	3	ND	ND	ND	FSGS (7.5)	17.8	18.5
	II:2	F	3.5	>130	−	Normal	FSGS (8.0)	23	
28	II:1	M	4	404	+	0.7/105	FSGS (10.5)	13	
	II:2	M	7	153	+	0.4/172	MCNS (7.0)		
29	II:2	F	9	46[b]	−	0.44/116	FSGS (16.0)		
	II:1	M	13.4	282	−	0.9/96	FSGS (24.0)		
37	II:1	F	2.5	>40	−	Normal	FSGS (2.9)	13.9	15.8
	II:2	M	0.1	>40	−	ND	FSGS (6.0)	9.5	10.5
41	II:1	M	1	>40	+	Normal		8	8.5
	II:2	M	1	42[b]	+	0.7/normal		9.2	9.3
43	II:1	M	5.5	183	+	0.5/normal	MCNS (5.5)	11.2	11.8
	II:2	F	2.5	175[b]	−	0.2/normal	MCNS (2.6)	11	
46	II:1	M	11.8	81[b]	+	0.44/112	unspecific (13.8)		
	II:2	F	3.5	357	+	0.77/90	FSGS (3.8 and 8.8)	10.5	10.5
50	II:1	M	0.1	>40	−	Normal		<7.9	7.9
	II:2	F	0.1	>40	−	0.3/normal	FSGS (4.9)	10	
67	II:1	M	3.8	>40	+	0.1/normal	FSGS (5.75)		
	II:2	M	1.8	1583[b]	−	ND	FSGS (3.5)	3.8	4.9
92	II:1	F	0.1	96	−	Normal	FSGS (1.0)	6.1	7.2
	II:2	M	0.1	250[b]	−	0.2/130	FSGS (1.0)		
SporadicSRINSpatients									
72	II:1	M	16.6	217	+	0.5/90	FSGS (16.7)	20.1	
73	II:1	F	<7	625	−	0.3/201	MCNS (4.4)	7.4	
74	II:1	M	5	625[b]	+	0.9/62	FSGS (5)		
76	II:1	F	2	276	+	0.5/172	FSGS (8.6)	9	9.8
83	II:1	F	9.1	1208[b]	+	0.5/178	MCNS (7.9)	10.7	12.5
86	II:1	F	1.3	>417	+	Normal	FSGS (7.4)		
90	II:1	M	0.1	229	+	0.2/normal	MCNS (1)		

[a] INS, idiopathic nephrotic syndrome; PU, proteinuria; HU, hematuria; ESRF, end-stage renal failure; ND, not determined; normal, normal renal function, exact values not available. All patients presented with edema during initial clinical examination except INS 28 II:1/II:2, INS 29 II:1/II:2, INS 43 II:1/II:2, INS 92 II:1/II:2 and INS 72 II:1. Both siblings in INS11 received transplants twice because of rejection.

[b] Proteinuria indicated (mg/h).

5: 5'-AAAGGAGCCCAAGAATCAAG-3' and 5'-AAATATTTCAGCATATTGGCC-3'; exon 6: 5'-GTTTAGGCATGCTC TCCTC-3' and 5'-GATATGGCTATAGTACTCAGTG-3'; exon 7: 5'-GTCTGTGTGAAAGCTTTGGC-3' and 5'-GCAAAGGGGAAATGTTCTCC-3'; at annealing temperatures of 52°C (exon 5) and 60°C (exons 1, 2, 6, and 7). Because of the high CG content of exon 1, PCR was performed with *Taq* polymerase and Q solution according to manufacturer's instructions (Qiagen, Hilden, Germany**)**. To rule out polymorphism in 100 chromosomes of healthy individuals, we used SSCP for R138Q, SSCP and direct sequencing for V290M, digestion with *Hha*I (Amersham Pharmacia Biotech, Freiburg, Germany) for A284V and with *Cla*I (MBI Fermentas GmbH, St. Leon-Rot, Germany) for R229Q, polyacrylamide gel electrophoresis for 460-467insT (8), and allele-specific PCR for IVS-1 G→T (5'-TCCAAACTTTTTTCTGCCTAT-3' and 5'-AAATATTTCAGCATATTGGCC-3' [59°C]) and A196P (5'-GGAGATAGATGCCATTTGCTACTACCC-3' and 5'-TAAGTACCTTTGCATCTTGGGCGATGC-3' [62°C]). SSCP electrophoresis was performed for 6 h at 150 V with Power Pack P25 (Biometra, Göttingen, Germany). Direct sequencing of both strands was carried out as described previously (8).

Results

We report on 12 (46%) of 27 multiplex SRINS families and 7 (28%) of 25 single patients with SRINS with defects in the *NPHS2* gene (Table 1). All 31 patients showed initially proteinuria exceeding 40 mg/m² per hour and two patients (67 II:2 and 83 II:1) low-grade gross proteinuria exceeding 1000 mg/m² per hour. Edema was present in all of them, except

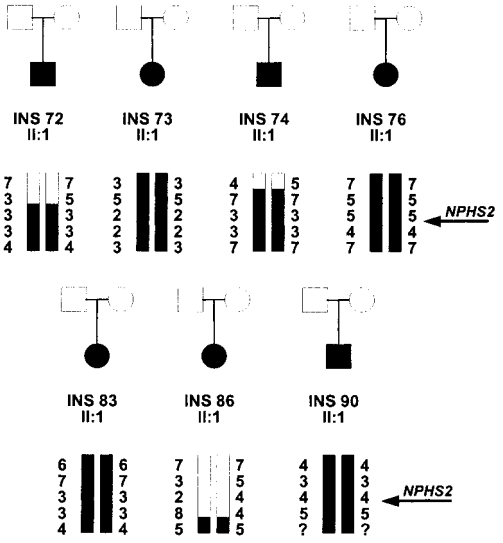

Figure 1. Pedigrees of seven patients with steroid-resistant nephrotic syndrome. Haplotypes were generated by using five consecutive microsatellite markers spanning the critical genetic *NPHS2* region on chromosome 1q25-q31. Affected individuals are indicated as black symbols. Male family members are shown as squares and female family members as circles. The differently shaded bars indicate heterozygous (white) and homozygous (black) haplotypes. The respective order from top to bottom was as follows: cen, D1S416, D1S1640, D1S2791, NPHS2, D1S215, D1S2883, tel (flanking markers are underlined). Note that in all families, haplotype data were compatible with homozygosity by descent, with the exception of INS 86. The haplotypes of the respective parents, analyzed in four patients (INS 72, 74, 86, and 90), were congruent with these findings (data not shown).

affected individuals of INS 28, 29, 43, 92, and 72, who had normal renal function. The age at onset varied from 0.1 to 16.6 yr (median, 3.0 yr; Table 1). Sixty-eight percent (21 of 31) of the patients progressed into end-stage renal failure within a median of 7.4 yr after diagnosis (range, 1.6 to 19.5 yr). No recurrence was reported in the 16 patients who underwent renal transplantation. The histologic findings in the 28 patients on whom renal biopsy was performed showed FSGS in 75% (21 of 28) and minimal change nephrotic syndrome in 21% (6 of 28). One biopsy result was unspecific (INS 46 II:1) (Table 1).

Haplotype Analysis

To determine consistency with linkage to *NPHS2* in the multiplex SRINS families, we first performed haplotype analysis followed by mutational analysis. All 12 families reported here were consistent with linkage to this chromosomal region (data not shown). To further evaluate potential consanguinity in the seven patients with apparently sporadic SRINS, which would give rise to homozygosity by descent (9) in the *NPHS2* gene, we carried out haplotype analysis in these patients too (Figure 1). Six of the seven tested patients showed homozygosity (INS 72, 73, 74, 76, 83, and 90) and one heterozygosity (INS 86) in the critical *NPHS2* interval. The haplotypes of the respective parents, analyzed in four patients (INS 72, 74, 86, and 90), were congruent with these findings (data not shown).

Mutational Analysis

A total of 51 people, 31 of whom were affected, were examined for mutational analysis. We detected 10 different missense, splice-site, and frameshift mutations. Of these, five mutations are novel (Table 2). Remarkably, we detected *NPHS2* mutations not only in familial cases but also in individual patients with SRINS. None of the mutations were found in at least 100 control chromosomes. In eight families and all seven patients, potential loss-of-function mutations in *NPHS2* were detected on both alleles; in four families, only one mutation was found (INS 18, 28, 29, and 46).

Missense Mutations

We identified three novel missense mutations affecting the carboxy-terminal cytoplasmic tail of podocin (Table 2) (Figure 2). The first, a C→T transition at position 851 leading to an alanine to valine substitution A284V, was found heterozygously in families INS 29 and 46, and homozygously in patient INS 76. The other two novel missense mutations were both identified in patients of family INS 86 in a heterozygous state: the 587G→C transversion affects the highly conserved arginine at position 196 (R196P), and the 868G→A transition replaces valine at position 290 (V290M).

We identified the frequently reported R138Q mutation homozygously in six families (INS 11, 16, 37, 41, 43, and 67) and two patients (INS 73 and 90); in three families (INS 28, 50, and 92) only the maternal allele was affected. Furthermore, we identified a homozygous V180M exchange in patient INS 72 and a heterozygous R291W substitution in family INS 18 segregating from the maternal side (Table 2).

Splice Site and Frameshift Mutations

We identified the first splice site mutation in *NPHS2*. IVS4-1G→T involves the 3' acceptor splice site of intron 4 and was detected in a homozygous state in patient INS 74 (Figure 2). Another novel mutation results in a 1-bp insertion at position 460 to 467 with a consecutive frameshift and premature stop codon T181X. This is the second potential loss-of-function mutation in family INS 92. In addition, we detected two further frameshift mutations due to deletions that have previously been described (2). In family INS 50, we found the paternal 419delG in both affected siblings in a heterozygous state. 855–856delAA was identified in the patient INS 83 in a homozygous state.

Polymorphisms

Aside from the potential loss-of-function mutations described, we detected a number of nucleotide exchanges that are probably without any influence on *NPHS2* function (Table 2). Some were silent without an amino acid exchange: 954T→C (A318A), 102G→A (G34G), 288C→T (S96S), and 1038A→G (L346L). In fact, one led to an amino acid exchange but was also detected in 3% of all chromosomes in 100 healthy controls: 686G→A (R229Q). Interestingly, the R229Q exchange was found in a heterozygous state in three of four families with only one mutation (INS 18, 29, and 46) whereby the respective missense mutations were on the opposite chromosome. Furthermore,

Table 2. NPHS2 mutations and polymorphisms detected in patients with steroid-resistant nephrotic syndrome[a]

Type of Mutation	Nucleotide Change	Effect on Coding Sequence	Exon/Intron	Mutation Status	Family INS No.	Patient INS No.	Ethnic Origin	Reference
Missense mutation	851C→T	A284V	7	Het.	29		AT	Novel
			7	Het.	46		PL	
			7	Hom.		76	CH	
	587G→C	R196P	5	Het.		86	G	Novel
	868G→A	V290M	7	Het.		86		Novel
	413G→A	R138Q	3	Hom.	11		CH	Boute *et al.*(2)
			3	Hom.	16		G	
			3	Het.	28		G	
			3	Hom.	37		G	
			3	Hom.	41		G	
			3	Hom.	43		NL	
			3	Het.	50		HY	
			3	Hom.	67		G	
			3	Het.	92		G	
			3	Hom.		73	G	
			3	Hom.		90	G	
	538G→A	V180M	5	Hom.		72	G	Boute *et al.*(2)
	871C→T	R291W	7	Het.	18		G	Boute *et al.*(2)
Splice site mutation	IVS4-1G→T	Splice site	IVS4	Hom.		74	G	Novel
Frameshift mutation	460–467insT	Frameshift	4	Het.	92		G	Novel
	419delG	Frameshift	3	Het.	50		HY	Boute *et al.*(2)
	855–856delAA	Frameshift	7	Hom.		83	CH	Boute *et al.*(2)
Polymorphism	686G→A	R229Q	5	Het.	18			Novel
			5	Het.	29			
			5	Het.	46			
	954T→C	A318A	8	Het.		92		Wu *et al.*(11)
			8	Hom.		74		Novel
	102G→A	G34G	1	Het.		83		Novel
	288C→T	S96S	2	Hom.		74		Wu *et al.*(10)
	1038A→G	L346L	8	Hom.		74		Wu *et al.*(11)

[a] het., heterozygous mutation; hom., homozygous mutation; AT, Austrian; PL, Polish; CH, Swiss; G, German; NL, Dutch; HY, Hungarian. Note that in families INS 18, 28, 29, and 46, there was only one heterozygous mutation identified.

none of the healthy control individuals carrying R229Q was homozygous for this exchange. A functional role of R229Q is unlikely; however, it cannot completely been ruled out. The polymorphisms 288C→T, 954T→C, and 1038A→G have recently been published by Wu *et al.* (10,11).

Discussion

This study reports the identification of 5 novel *NPHS2* mutations A284V, R196P, V290M, IVS4-1G→T, and 460-467insT in families and patients with sporadic SRINS with the clinical diagnosis of SRINS. None of the novels were found in at least 100 control chromosomes. In total, both potential loss of function *NPHS2* mutations were detected in eight SRINS families and seven patients with sporadic SRINS. In four families, only one heterozygous mutation was found, suggesting mutations in the promoter region in these patients.

The novel missense mutations are predicted to cause either conservative (A284V and V290M) or nonconservative amino acid substitutions (R196P) affecting the carboxy-terminal cytoplasmic tail of podocin. Furthermore, the latter R196P substitution is highly conserved among the stomatin-like protein family members (2). The absence of the mutations in at least 100 control chromosomes and the consistency with cosegregating in the families suggest a pathogenic role even for the conservative amino acid substitutions. In addition to the six R138Q mutations reported by Boute *et al.* (2), we observed 11 R138Q mutations within our cohort. A common haplotype did not emerge significantly from haplotype analysis in these families (data not shown) or in the patients (see INS 73 and 90; Figure 1). But because these patients originate mainly from northern Europe, we support a founder effect hypothesis in Europe (2). The novel splice site mutation IVS4-1G→T affects a guanine residue 100% conserved at splice acceptor sites of vertebrates. Because no renal specimen from the patient was

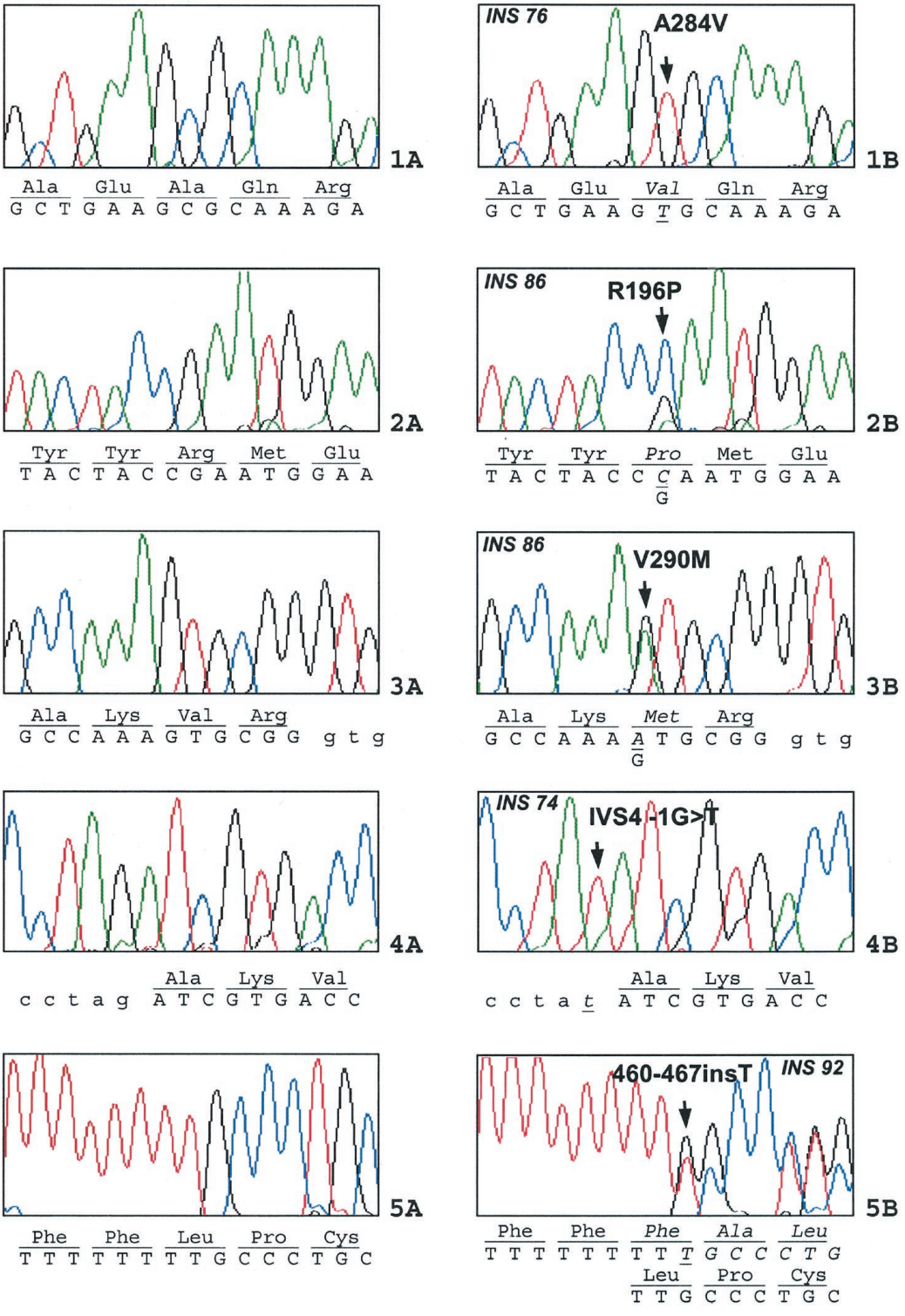

Figure 2. Detection of five novel *NPHS2* mutations by direct sequencing. The corresponding nucleotide sequence with the respective amino acids are shown at the bottom of each chromatogram. The left column exemplifies the respective chromatograms of healthy control individuals (1A to 5A). The right column shows the chromatograms of patients with steroid-resistant nephrotic syndrome with missense mutations (1B to 3B), a splice site mutation (4B), and a frameshift mutation (5B). In patient INS 76, we found a homozygous C→T substitution at position 851 leading to A284V (1B). Patient INS 86 showed two missense mutations in the heterozygous state. The first G→C substitution at position 587 leads to R196P (2B), whereas the G→A substitution at position 868 leads to V290M (3B). A homozygous splice site mutation IVS4-1G→T was detected in patient INS 74 (4B). A heterozygous frameshift mutation 460-467insT leading to a premature stop codon T181X was found in patient INS 92 (5B). The mutations cosegregated with the disease within the families, and none of them were found in at least 100 control chromosomes.

available, we could not clarify its effect in further RNA analysis. But it seems likely that this mutation results in either skipping of exon 5 or in activating a cryptic splice site. The novel frameshift mutation 460-467insT results in a premature termination at position T181X and will most likely lead to a premature truncation of the protein.

In four families (INS 18, 28, 29, and 46), only one heterozygous mutation was found. Assuming a causative role for *NPHS2* in these families and regarding the fact, that autosomal dominant inheritance is not likely (no more affected in the extended family pedigree), these patients may have mutations elsewhere in the promoter or in intron areas of the *NPHS2* gene.

All five novel *NPHS2* mutations reported here comprise missense, splice site, and frameshift mutation and confirm the crucial role of podocin in the function of the glomerular filtration barrier. We found *NPHS2* mutations not only in familial cases, but also in seven patients with sporadic SRINS. In six of the seven tested patients, homozygous mutations were found that suggested a common founder in these families. Haplotype analysis demonstrating homozygosity of additional flanking genetic markers corroborates this conclusion (Figure 1).

SRINS represents an important cause of end-stage renal disease in childhood. Because we found *NPHS2* mutations not only in familial cases but also in nearly 30% of patients with sporadic SRINS, we propose to perform mutational analysis in these patients as well. Besides better classification of the disease entity, identification of *NPHS2* mutations may prevent some of these patients from unnecessary steroid treatment and also permit the prediction of absence of disease recurrence after kidney transplantation. We are in the process of establishing systems to evaluate podocin function (*e.g.*, by testing protein-protein interactions of podocin with potential binding proteins such as nephrin, CD2AP, and ZO-1).

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See related editorial, "Not All in the Family: Mutations of Podocin in Sporadic Steroid-Resistant Nephrotic Syndrome," on pages 577–579.