Genetic screening in adolescents with steroid-resistant nephrotic syndrome

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Genetic screening paradigms for congenital and infantile nephrotic syndrome are well established; however, screening in adolescents has received only minor attention. To help rectify this, we analyzed an unselected adolescent cohort of the international PodoNet registry to develop a rational screening approach based on 227 patients with nonsyndromic steroid-resistant nephrotic syndrome aged 10–20 years. Of these, 21% had a positive family history. Autosomal dominant cases were screened for *WT1*, *TRPC6*, *ACTN4*, and *INF2* mutations. All other patients had the *NPHS2* gene screened, and *WT1* was tested in sporadic cases. In addition, 40 sporadic cases had the entire coding region of *INF2* tested. Of the autosomal recessive and the sporadic cases, 13 and 6%, respectively, were found to have podocin-associated nephrotic syndrome, and 56% of them were compound heterozygous for the nonneutral p.R229Q polymorphism. Four percent of the sporadic and 10% of the autosomal dominant cases had a mutation in *WT1*. Pathogenic *INF2* mutations were found in 20% of the dominant but none of the sporadic cases. In a large cohort of adolescents including both familial and sporadic disease, *NPHS2* mutations explained about 7% and *WT1* 4% of cases, whereas *INF2* proved relevant only in autosomal dominant familial disease. Thus, screening of the entire coding sequence of *NPHS2* and exons 8–9 of *WT1* appears to be the most rational and cost-effective screening approach in sporadic juvenile steroid-resistant nephrotic syndrome.

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Steroid-resistant nephrotic syndrome (SRNS) is a heterogeneous disorder caused either by dysregulation of the immune system or by genetic abnormalities affecting podocyte-specific proteins. Knowledge of the underlying pathology has major impact on the treatment and prognosis of the disorder. The genetic heterogeneity and phenotypic variability of SRNS mandates a rational, adapted approach to genetic screening.

The age of disease onset is an important predictor of the odds of finding an abnormality in a particular gene linked to SRNS. In recent years, several proposals for genetic screening paradigms have been put forward, which preferentially addressed congenital and infantile onset cases.[1–4] SRNS manifesting at adolescent age was addressed as a subject of minor interest in two recent reports only.[3,4] The current literature suggests that at least five genes should be taken into consideration in adolescent-onset SRNS: *NPHS2* in autosomal recessive (AR) and sporadic cases,[5,6] *WT1*[7] in autosomal dominant (AD) and sporadic cases, and *TRCP6*,[8] *ACTN4*,[9] and the recently identified *INF2*[10] in AD cases. In contrast, the occurrence of mutations in the genes *NPHS1*, *PLCE1*, *MYOE1*, and *PTPRO* in this age group is rather anecdotal.[11] Furthermore, *APOL1* variants are considered risk factors for focal segmental glomerulosclerosis (FSGS) in young adults, at least in the African-American population.[12]

Although most previous studies in SRNS focused on familial cases, the vast majority of adolescent SRNS cases are in fact sporadic. The PodoNet registry study collects clinical and genetic information as well as biomaterials from patients with SRNS across the pediatric age range. With almost 1500 cases from 66 pediatric nephrology centers in 21 countries compiled to date, PodoNet is the largest registry worldwide devoted to this rare disorder (Supplementary Material S1 online). Here, we utilized the PodoNet registry to perform comprehensive screening for genetic causes in an unselected population of 297 SRNS patients with disease onset in the second decade of life, including both sporadic and familial cases.

RESULTS

AR cases accounted for ~25% of Polish, Turkish, and Syrian patients enrolled in the study, whereas family history was positive in no more than 10% of patients from Western Europe and Latin America. A total of 38 (17%) patients (including members of 12 AR families) descended from consanguineous marriages; all of these were from Turkey or the Middle East.

The distribution of age at onset and degree of proteinuria was similar in the familial and sporadic forms. On biopsy, patients with familial SRNS showed less frequently minimal change histology (3% vs. 16%, $P=0.05$) and more commonly mesangioproliferative glomerulonephritis (24% vs. 9%, $P<0.05$) than patients with sporadic disease, whereas the proportion of cases with FSGS was similar (63 and 73%, not significant). The fraction of sclerosed glomeruli did not differ significantly in patients with familial (median 36, interquartile range 15–50%) and sporadic FSGS (median 18, interquartile range 10–50%).

Autosomal dominant SRNS

None of the AD patients was found to have a mutation in *TRCP6* or *ACTN4*. One patient was found to have an intronic mutation in *WT1* and two patients were positive for an *INF2* mutation located in its *hot spot* region (exon 4). Details regarding clinical presentation are given in Tables 1 and 2.

Sporadic and autosomal recessive SRNS

NPHS2 screening. In all, 5/38 (13%) AR patients and 11/179 (6%) sporadic cases were found to have podocin-associated SRNS. *NPHS2*-positive patients did not differ from the other adolescents with respect to age at first manifestation, time to end-stage renal disease, histopathology, and degree of proteinuria. FSGS was present in 10 cases, mesangioproliferative glomerulonephritis GN in 2 cases, and minimal change nephropathy and global glomerulosclerosis in 1 case each.

Homozygous mutations in *NPHS2* were found in 5 (all sporadic) patients and compound heterozygous mutations in 11 patients (Table 3). The most common mutations were

Table 1 | Clinical characteristics of patients with *WT1* mutation

No.	Family history	Mutation	Conventional (previous) nomenclature	Gender	Age at onset (years)	Time to ESKD (years)	Histopathology	Initial proteinuria (g/m^2/day)	Wilms' tumor	Urogenital abnormalities	Disorder of sex development	Response to immuno-suppression
1	AD	c.1432+4C>T	IVS9+4C>T	F	20	9	NA	NA	No	Horseshoe kidney	No	No response
2	No data	c.1432+4C>T	IVS9+4C>T	F 46,XY	11.5	– (2.2 obs)	FSGS	1.5	No	No uterus, vaginal rest, hypoplastic gonads	Yes	No response
3	No data	c.1432+4C>T	IVS9+4C>T	F 46,XY	13.0	0.75	FSGS	6.5	No	Female habitus bilateral ovarian dysgerminoma	Yes	Not treated
4	*De novo*	c.1432+5G>A	IVS9+5G>A	F 46,XY	10.0	7.7	FSGS	4.9	No	Rudimentary uterus and vaginal rest, hypoplastic gonads	Yes	No response
5	*De novo*	p.(Arg355*)	p.R287X	F 46,XX	15.6	2.5	NA	3	At age 3.0 years	None	No	Not treated
6	*De novo*	p.(Arg430*)	p.R362X	F 46,XX	12.2	4.4	FSGS	8.5	At age 1.2 years	None	No	Not treated
7	*De novo*	p.(Arg458*)	p.R390X	F 46,XX	15.3	– (0.7 obs)	FSGS	3.9	At age 0.8 years	Ovarian cyst	No	Partial response to CsA
8	No data	p.(Arg458*)	p.R390X	M 46,XY	18.5	– (9.3 obs)	FSGS	8	No	Penile hypospadia, hypoplastic scrotum, bilateral abdominal cryptorchidism, rudimentary uterus and vaginal rest, hypoplastic testes	Yes	Not treated

Abbreviations: AD, autosomal dominant; CsA, cyclosporin A; ESKD, end-stage kidney disease; F, phenotypic female; FSGS, focal segmental glomerulosclerosis; NA, not available; M, phenotypic male; obs, clinical observation.

Table 2 | Clinical and demographic characteristics of the patients with known mutations or novel sequence variants in the *INF2* gene

INF2 variant	Type of mutation	No. of patients	Origin of mutation	Ethnicity	Consanguinity	Age at diagnosis (years)	Proteinuria at diagnosis (g/m^2/day)	Histopathology on last examination	ESKD	Duration of clinical observation (years)
c.653G>A p.(Arg218Gln)	Known mutation	1	Paternal (AD trait)	Italian	No	16.8	1.0	FSGS	No	12.2
c.658G>A p.(Glu220Lys)	Known mutation	1	Probably maternal[a] (AD trait)	Italian	No	13.7	1.8	MCN	Yes	7.0
c.1736-6C>T	Novel	1	Paternal	Turkish	Yes	10.3	2.6	GGS	No	3.5
c.2053A>G p.(Ile685Val)	Novel	1	Paternal	Turkish	No	14.8	5.0	FSGS	No	4.9
c.2630G>A p.(Arg877Gln)	Novel	2	*De novo*/maternal	Turkish	No	10.6–14.8	1.0	FSGS	No	3.7–4.9

Abbreviations: AD, autosomal dominant; ESKD, end-stage kidney disease; FSGS, focal segmental glomerulosclerosis; GGS, global glomerulosclerosis; MCN, minimal change disease.
[a]Mother died at age 27 years for post-partum nephritis; no biological sample is available for mutational analysis.

as follows: p.Val180Met present in three cases from different ethnic backgrounds (Germany, Italy, and Turkey); p.Ala284Val found in two Chilean and one Portuguese patient; and c.1032delT reported in three adolescents from Northern Poland. No other significant findings with respect to allele distribution was observed in the PodoNet cohort.

The patients with homozygous *NPHS2* mutations were slightly younger at first manifestation than the compound heterozygous cases (12.2 vs. 14.6 years). Also, the course of disease was more severe among the homozygous *NPHS2* cases, 4/5 of whom reached end-stage renal disease within 5 years from initial diagnosis, whereas 55% of compound heterozygous cases were still treated conservatively at this time point.

Of the 16 patients with *NPHS2*-related SRNS, 9 carried the nonneutral p.Arg229Gln (commonly referred to as p.R229Q) polymorphism. Two additional subjects were homozygous for the p.R229Q polymorphism; SRNS in these cases was not considered to be caused by this common podocin variant. p.R229Q carriership did not affect age at disease onset or histology in this adolescent cohort; however, proteinuria at disease onset was significantly less marked in p.R229Q carriers than in patients with other mutations (3.5 vs.

Table 3 | Clinical and demographic characteristics of the patients with podocin-related SRNS

NPHS2 mutation(s)	Type of mutation	No. of patients	Ethnicity	Consanguinity	Age at diagnosis (years)	Proteinuria at diagnosis (g/m^2/day)	Time to ESKD (years)	Histopathology on last examination
(p.Trp122*);(p.Arg238Ser)	Known mutations	1	Turkish	No	15.9	NA	– (0.8 obs)	No data
(p.Arg138Gln);(p.Arg138Gln)	Known mutation	1	German	No	10	6.9	– (3.0 obs)	FSGS
(p.Arg138Gln);(p.Val180Met)	Known mutations	1	Italian	No	13.6	5.2	3.5	FSGS
(p.Leu169Pro);(p.Leu169Pro)	Known mutation	1	Turkish	No	11.8	6.9	4.4	FSGS
(p.Val180Met);(p.Val180Met)	Known mutation	2	Turkish,	No	10.6	8.0	2.4	FSGS
			German		16.6	6.0	3.5	FSGS
(p.Arg229Gln);(c.451+3A>T)	Novel mutation	1	German	No	14.8	NA	– (4.7 obs)	MCN
(p.Arg229Gln);(p.Ala284Val)	Known mutation	2	Portuguese,	No	10.3	1.6	– (4.7 obs)	MesPGN
			Chilean		11.9	NA	1.3	FSGS
(p.Arg229Gln);(p.Ala297Val)	Known mutation	1	Polish	No	13.5	2.4	3.6	MesPGN
(p.Arg229Gln);(p.Glu310Lys)	Novel mutation	1	Turkish	No	13.1	1.5	3.2	No data
(p.Arg229Gln);(p.His325Tyr)	Known mutation	1	Italian	No	18.8	5.8	2.4	FSGS
(p.Arg229Gln);(c.1032delT)	Known mutation	3	Northern	No	14.9	3.3	– (10 obs)	GGS
			Polish	No	15.7	6.5	– (2.7 obs)	FSGS
				no	17.6	3.3	– (6.4 obs)	FSGS
(p.Ala284Val);(p.Ala284Val)	Known mutation	1	Chilean	No	12.4	NA	0.8	FSGS

Abbreviations: ESKD, end-stage kidney disease; FSGS, focal segmental glomerulosclerosis; GGS, global glomerulosclerosis; MCN, minimal change disease; MesPGN, mesangioproliferative glomerulonephritis; NA, not available; obs, clinical observation; SRNS, steroid-resistant nephrotic syndrome.

6.4 g/m^2/day; $P = 0.03$). The 5-year kidney survival was 64% in compound heterozygous patients carrying the p.R229Q polymorphism versus 23% for patients without the polymorphism ($P = 0.07$).

WT1 screening. *WT1* mutations were found in 4.1% (7/169) of the sporadic cases (including the previously diagnosed syndromic case of *WT1* nephropathy with ambiguous genitalia). The patients encompassed a wide spectrum of clinical features, including Frasier syndrome, Wilms' tumor survivors, and isolated SRNS (Table 1). Three patients diagnosed with Wilms' tumor at infant age gradually developed mild proteinuria that, more than 10 years after cancer diagnosis, progressed to overt nephrotic syndrome and eventual diagnosis of the *WT1* mutation. One of them required extended genetic testing of the entire *WT1* gene in order to find the mutation p.(Arg355*) lying outside of the generally acknowledged *hot spot* for nephrotic syndrome-related mutations, but still within the region associated with simplex Wilms' tumor.

Within the sporadic SRNS cohort, the *WT1* cases did not differ from the *NPHS2*-related cases and those without detectable genetic abnormalities with respect to age at first manifestation, histopathology, and prospective renal survival. However, proteinuria at the time of diagnosis was significantly higher in *WT1* cases (5.7 vs. 3.4 g/m^2/day; $P = 0.04$).

INF2 screening. No known mutations in *INF2* were detected in the sporadic SRNS; however, three novel sequence variants were found in heterozygous state: two nonsynonymous single-nucleotide substitutions (p.(Ile685Val) and p.(Arg877Gln)), and one intronic putative splice site mutation (c.1736-6C>T) (Table 2). The variant carriers did not differ from the remaining screened cohort with respect to age at disease onset, clinical manifestation, response to drugs, time to end-stage renal disease, and histopathology.

Because of insufficient structural data, it was difficult to speculate on a potential effect of the detected novel missense variants on the structure and function of the INF2 protein (Table 4; Supplementary Material S2 online). Although p.(Ile685Val) lies in a region of high homology (90% similarity across chordates), it is a moderately evolutionarily conserved residue. The residue 877 is even less conserved, with only 12/25 orthologs expressing arginine; besides, the similarity rate among the orthologs for this fragment of the protein does not exceed 50%. Both variants lie within the formin homology 2 domain. A three-dimensional model based on the crystal structure of its closest homolog, that is, mDia mouse protein, was used to evaluate the probability of the variants to affect protein structure.[13] The exchanges were found unlikely to disturb the helical structure or to change its hydrophobicity profile sufficiently to cause displacement. Besides, no effect of the mutations on mRNA splicing was observed (Supplementary Material S2 online).

DISCUSSION

The compilation of a large unselected series of consecutive sporadic and familial cases of juvenile-onset SRNS in the international PodoNet registry allowed us to assess the prevalence of genetic abnormalities in known disease-causing genes in this age group. Whereas most previous genetic studies in patients with adolescent disease onset focused on individual SRNS-associated genes, we tested a panel of genes in order to establish a rational screening paradigm for genetic testing in this age group. *NPHS2*,[5,6,14] *WT1*,[7,15] *TRCP6*,[8] *ACTN4*,[9] and *INF2*[10] were chosen as the genes considered most relevant in adolescent-onset disease. For recessive familial traits and sporadic cases, certain mutations in *NPHS2* have been associated with late disease onset.[6,14,16–19] The podocyte genes with AD transmission are generally characterized by a milder disease course with typically late disease onset, and may also occur sporadically due to *de novo* mutations.[15,20–23] As previous works suggested a very low (<5%) incidence of *TRPC6* and *ACTN4* in sporadic SRNS

Table 4 | Summary of bioinformatic analyses of the detected novel sequence variants

Novel variant	Protein change	Protein domain	Conservation	MAF[a]	Human splicing finder[36]	ESE finder[37]	PolyPhen[32]	SIFT [33]	Prevalence in control population
NPHS2									
c.928G>A	p.(Glu310Lys)	Stomatin	High	Not reported	New biding site for 9G8, Tra2β enhancer proteins new site for hnRNP A1 binding	SF2/ASF binding site broken	Probably damaging	Tolerated	0
c.451+3A>T	—	—	—	Not reported	Binding site for Tra2β enhancer protein broken New binding site for IIE silencer protein	New sites for SC35, SRp40, and SRp55 binding	—	—	0
WT1									
c.1063A>T	p.(Arg355[a])	N-terminal	Moderate	Not reported	Exonic enhancer sites (EIE, ESE) broken	New site for SRp55 binding	—	—	0
INF2									
c.1736-6C>T	—	—	—	Not Reported	No significant motif modifications	SF2/ASF and SRp40 binding site modification	—	—	0
c.2053A>G	p.(Ile685Val)	FH2	High	<0.1%	No significant motif modifications	SF2/ASF binding site modification	Unknown	Tolerated	0
c.2630G>A	p.(Arg877Gln)	FH2	Low	1.5%	New site for hnRNP A1 binding	SRp40 binding site modification	Unknown	Tolerated	1%

Abbreviations: EIE, exon-identity element; ESE, exonic splicing enhancer; FH2, formin homology 2; hnRNP, heterogeneous nuclear ribonucleoprotein; IIE, intron-identity element; MAF, minor allele frequency; NHLBI, National Heart, Lung, and Blood Institute; SF2/ASF, splicing factor 2/alternative splicing factor.
[a]MAF estimation based on data of 2168 individual genomes cataloged by 1000 Genomes Project and 12,101 individuals collected at NHLBI Exome Sequencing Project (accessed 13 December 2012).

cohorts,[20,21] we restricted screening of these genes to familial cases. However, we explored the entire sporadic disease cohort for *WT1* mutations and assessed the full coding sequence of *INF2* in a randomly chosen subset of the sporadic cohort.

In our cohort, 79% of cases occurred sporadically, 17% showed AR, and 4% AD inheritance. A genetic cause was identified in 30% of the AD, 13% of the AR, and 10% of the sporadic cases. Taken together, the overall mutation detection rate was 11%. This figure is in keeping with previous screening studies in SRNS patients suggesting a steep inverse relationship of the rate of genetic diagnoses with age. Reported prevalence figures of genetic causes were 81–100% for congenital nephrotic syndrome[1,2,4] and 44% for 4–12 months.[2] The chances of finding a mutation drop considerably in older children as shown in a Spanish national study with detection rates of 24% for toddlers (1–5 years), 36% for school children (6–12 years), 25% for adolescents (13–17 years), and 14% for adults.[4] A few other single population–based studies (Japan, Tunisia, and Belgium) showed similar findings (reviewed in Benoit *et al.*[3]). The only population study performed in an adult cohort reported a mutation rate of 8%.[16]

NPHS2 mutations explained ~7% of all cases (13% of AR and 6% of sporadic) in this large and ethnically diverse population. Published reports suggest a role of *NPHS2*, and its nonneutral p.R229Q polymorphism in particular, in late-onset pediatric SRNS, especially among European and Latin American populations (reviewed in Machuca *et al.*[6]). Compound heterozygotes harboring *NPHS2* mutations and p.R229Q polymorphism were also found in 23–25% of Caucasians with adult-onset AR familial disease,[5] whereas the prevalence of *NPHS2* anomalies is much lower in adults with sporadic disease, ranging from 0–1% (0/64 [17], 1/87 [18], 0/265 [19]) to 10–11% of cases.[6,14]

The allele frequency of p.R229Q varies between 1 and 8% in selected populations, with an average of 3–5% for Europeans [6] (www.1000Genomes.org). Well in line with the reported figures, the minor allele frequency of p.R229Q was 6.3% in this ethnically diverse cohort. The allele distribution was consistent with the Hardy–Weinberg equilibrium, providing further evidence for p.R229Q to be a disease-associated variant rather than a causative change.

In view of the common involvement of p.R229Q, a two-step screening algorithm has been proposed for late-onset SRNS, limiting full sequencing of the *NPHS2* gene to carriers of the p.R229Q polymorphisms.[6,14] Our findings do not support this approach as only 56% (9/16) of adolescents with *NPHS2*-associated disease were compound heterozygous for a mutation combined with p.R229Q. Hence, selective screening of p.R229Q-positive patients only would fail to identify a significant fraction of the patients with podocin-related SRNS. Based on our findings, we rather opt for screening of the entire coding sequence in all sporadic and AR cases of juvenile SRNS unless epidemiological data in a particular ethnic group suggest otherwise.

The diagnosis of *WT1-associated nephropathy* is of particular clinical relevance because of its involvement in sex determination and the associated risk of Wilms' tumor and/or gonadoblastoma. Remarkable phenotypic heterogeneity has been reported for *WT1* mutations. Splice site mutations typical for Frasier syndrome may be found in

patients with disorders of sexual differentation or isolated diffuse mesangial sclerosis,[24] and patients with typical Denys–Drash syndrome mutations may present with isolated FSGS[25] or Wilms' tumor without nephrotic syndrome.[26] In this cohort with adolescent-onset SRNS, *de novo WT1* mutations were found in 4% of the sporadic and 1 of the 10 AD cases. Consistent with their late-onset nephropathy, all biopsied patients displayed FSGS rather than diffuse mesangial sclerosis, the histological diagnosis observed with early-onset *WT1*-related disease. Apart from the uniform histopathological appearance, our series illustrates the variable genotype–phenotype associations in *WT1* nephropathy: three patients presented with classical Frasier syndrome, that is, disorder of sexual differentiation with 46,XY karyotype (previously referred to as male pseudohermaphroditism), FSGS, and intron 9 splice site mutations; one karyotypic and phenotypic male with an exonic mutation showed incomplete male development upon detailed examination; three normal females with exonic mutations had a history of Wilms' tumor at infant age, and one normal female with an intronic mutation and AD family history presented with isolated SRNS. Our results are consistent with the notion that germline *WT1* mutations have a greater effect on sex determination and genital development in males than females,[7,15] resulting in a predominance of the female phenotype among mutation carriers. On the other hand, as shown here and previously,[15] *WT1* nephropathy may occasionally also occur in phenotypic males, arguing against limiting *WT1* screening to females[3,4] and in favor of karyotyping of all patients diagnosed with a *WT1* mutation.

Mutations in INF2 have recently been established as the most common cause of AD nephrotic syndrome. Among a total of 325 AD FSGS families studied to date, 38 (12%) families with pathogenic sequence variants in *INF2* have been identified.[10,22,23,27–29] Consistent with these findings, we identified 2 such patients among 10 cases of AD FSGS.

Conversely to familial FSGS, only 3 *INF2* mutations have been previously reported in 396 sporadic idiopathic FSGS cases ($<1\%$).[22,23,28] This study adds another 40 sporadic adolescent-onset cases without pathogenic mutations. Previous studies selectively screened the *hot spot* region (exons 2–5) where all mutations known to date have been detected. This region is coding the diaphanous inhibitory domain, a self-regulatory component of the protein in control of the formin homology 2 domain responsible for nucleating new actin filaments.[13] We chose to screen the entire *INF2* coding sequence, including the second autoregulatory element, the diaphanous autoinhibitory domain at the C-terminus, which is required to interact with diaphanous inhibitory domain for proper autoinhibition. No mutations were identified in either the diaphanous inhibitory domain or the diaphanous autoinhibitory domain. We detected three novel variants outside the proposed hot spots that, according to both *in silico* and experimental findings, are however unlikely to be functionally relevant. c.1736-6C>T, a putative splice- site mutation, proved benign by cDNA analysis. p.(Arg877Gln), a missense mutation resulting in substitution of a single amino acid at a nonconservative residue, was found to be a rare polymorphism by population screening. The third variant, p.(Ile685Val), was absent from the control subjects and is not catalogued in the 1000 Genomes database (www.1000Genomes.org); however, *in silico* structural analysis predicted the amino-acid substitution to be tolerable.

In summary, the 11% overall prevalence of disease-causing abnormalities in the most commonly analyzed genes in this adolescent cohort is closer to figures found in adult populations than those observed in infants. However, the low mutation detection rate even in familial cases (13% for AR and 30% for AD) points toward a large fraction of as yet undiscovered genetic forms. The advent of NG exome sequencing is expected to complete our knowledge of the genetic causes of SRNS in the near future. Even sooner, enriched targeted next generation sequencing of complete panels of disease-associated genes should increase the efficacy of detecting genetic abnormalities. Such a panel would include *MYO1E* and *PTPRO*, the two recently identified SRNS genes with AR transmission and reported cases with juvenile disease onset,[30,31] as well as genes associated with an increased disease risk such as *APOL1*. Although conventional screening of this large cohort for *MYO1E* and *PTPRO* would have exceeded current resources, next generation sequencing will remove these current limitations.

Even using conventional screening methodology and selective screening according to the algorithm applied in this study, a disease-causing genetic abnormality can be expected to be found in one out of nine adolescents with SRNS. Given the lacking efficacy (PodoNet registry results in preparation) and the significant side-effect profiles of second-line immunosuppressive protocols in SRNS, we believe that adolescent patients should undergo genetic screening as soon as steroid resistance is established. At the current state of knowledge and as long as affordable comprehensive next generation sequencing is not available, screening should include testing of the entire coding sequence of *NPHS2* and exons 8 and 9 of *WT1* in all sporadic patients. Neither prescreening for p.R229Q nor limiting *WT1* testing to females or diffuse mesangial sclerosis appears justified. Mutational analysis of *INF2* is cost effective only in AD cases and can probably be limited to exons 2–5.

MATERIALS AND METHODS

Study population

A total of 297 adolescents consecutively enrolled in the PodoNet Registry were considered for the study as they manifested first signs of kidney disease between age 10 to 20 years (mean 13.0 ± 2.5, median 12.6 years). Of these, 66 patients were eventually excluded because of lack of available DNA for testing. In four patients a genetic diagnosis had been established based on overt syndromic features (Schimke osteodystrophy, Pierson syndrome, *WT1*-associated SRNS with ambiguous genitalia, and a mitochondrial disorder). The remaining 227 nonsyndromic SRNS patients underwent genetic evaluation (Figure 1). Countries of origin included

Turkey ($n=90$), Italy ($n=48$), Germany ($n=22$), Poland ($n=22$), Serbia ($n=6$), Syria ($n=6$), Chile ($n=6$), and others ($n=27$). Of these, 48 cases from 41 families (21%) had a family history of SRNS or proteinuria, suggestive of AR inheritance in 38 (17%) patients and of AD inheritance in 10 (4%) patients.

Mutational screening

DNA was extracted from peripheral blood following the standard phenol–chlorophorm protocol. DNA samples of 350 healthy anonymous volunteers representing four different populations (Italian, German, Polish, and Turkish) were used as controls. All coding exons and adjacent intronic junctions of *NPHS2* and *INF2* genes and exons 8 and 9 of *WT1* were analyzed by direct sequencing using ABI3130 Genetic Analyser (Applied Biosystems, Foster City, CA). Mutation nomenclature is based on Human Genome Variation Society recommendations; nucleotide and residue positions are given in compliance with the reference sequences published in the human GRCh37 assembly.

We first analyzed the *NPHS2* gene in the entire cohort, with the exception of AD cases. Next, *WT1* was examined in all sporadic patients negative for pathogenic *NPHS2* mutations. Finally, we randomly selected 40 sporadic cases for sequencing of all exons of the *INF2* gene. Concurrently, AD cases were screened for *INF2*, *TRCP6*, *ACTN4*, and *WT1* mutations (Figure 1).

In silico analyses of the effect on protein structure and function

Selected bioinformatics tools were used to assess the effect of sequence variants on the structure and function of the receptor. Two indirect *in silico* predictors, PolyPhen2 (ref. 32) and SIFT,[33] were used to evaluate possibly damaging effects of single amino-acid substitutions in the INF2 protein. In addition, multiple-alignment analysis of the orthologs from different species retrieved from OMA (Orthologous MAtrix) database[34] was performed using ClustalW algorithm[35] in order to identify conservative amino-acid residues (Supplementary Material S2 online). Besides, the Protein Data Bank was searched for the structure of a protein with the most similar sequence (www.pdb.org).

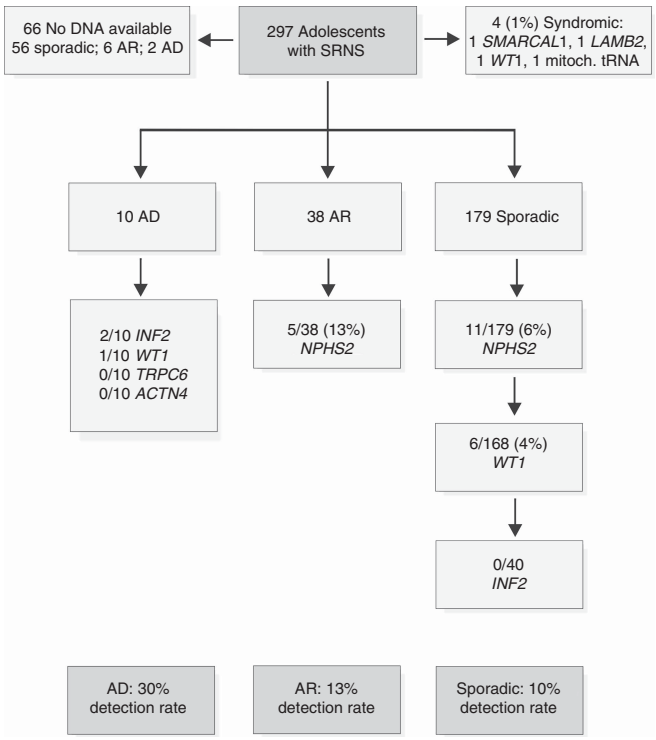

Figure 1 | Diagnostic evaluation of the PodoNet patient cohort with onset of steroid-resistant nephrotic syndrome (SRNS) in the second decade of life. AD, autosomal dominant; AR, autosomal recessive; mitoch., mitochondrial.

Verification of splicing signals

Potential effects of identified novel variants on the splicing process were explored both *in silico* (using Human Splicing Finder[36] for evaluation of exon/intron boundaries and ESEFinder[37] for detection of putative exonic splicing enhancers/silencers) and by reverse-transcriptase PCR studies. Intracellular RNA was isolated from blood samples of the index patients, their parents, and two anonymous controls using PreAnalytix RNA collection and stabilization kit (PreAnalytix, Hombrechtikon, Switzerland); later, PAXgene Blood RNA kit (Qiagen, Hilden, Germany) was used for nucleic acid purification.

DISCLOSURE

All the authors declared no competing interests.

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SUPPLEMENTARY MATERIAL

Figure S1. Sequence alignments of 25 *INF2* orthologs from different species illustrating the degree of conservation of the altered amino acid residue.

Figure S2. The crystal structure of the FH2 domain from mDia mouse protein, the homolog of *INF2* (13). Localization of the two novel variants is color-coded: yellow p.I685V; red p.R877Q; two sides (A and its 180° reverse—B) of the structure are shown.

Figure S3. Evaluation of the effect of the detected *INF2* novel sequence variants on mRNA splicing.

Supplementary Material 1. PodoNet Collaborators (www.podonet.org).

Supplementary Material 2. Evaluation of the putative pathogenic character of the detected novel *VF2* sequence variants.

Supplementary material is linked to the online version of the paper at http://www.nature.com/ki

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