Human Molecular Genetics, 2002, Vol. 11, No. 4 379–388

Genotype/phenotype correlations of *NPHS1* and *NPHS2* mutations in nephrotic syndrome advocate a functional inter-relationship in glomerular filtration

Ania Koziell*, Victor Grech[1], Sagair Hussain, Gary Lee, Ulla Lenkkeri[2], Karl Tryggvason[3] and Peter Scambler

Molecular Medicine Unit, Institute of Child Health, 30 Guilford Street, London WC1N 1EH, UK, [1]Paediatric Department, St Luke's Hospital, Guardamangia, Malta, [2]Biocenter and Department of Biochemistry, University of Oulu, Linnanmaa, 90570 Oulu, Finland and [3]Division of Matrix Biology, Department of Medical Biochemistry and Biophysics, Karolinska Institute, 17177, Stockholm, Sweden

Received September 25, 2001; Revised and Accepted December 12, 2001

Mutations of the novel renal glomerular genes *NPHS1* and *NPHS2* encoding nephrin and podocin cause two types of severe nephrotic syndrome presenting in early life, Finnish type congenital nephrotic syndrome (CNF) and a form of autosomal recessive familial focal segmental glomerulosclerosis (SRN1), respectively. To investigate the mechanisms by which mutations might cause glomerular protein leak, we analysed *NPHS1/NPHS2* genotype/phenotype relationships in 41 non-Finnish CNF patients, four patients with congenital (onset 0 to 3 months) focal segmental glomerulosclerosis and five patients with possible SRN1 (onset 6 months to 2 years). We clarify the range of *NPHS1* mutations in CNF, detecting mutation 'hot-spots' within the *NPHS1* coding sequence. In addition, we describe a novel discordant CNF phenotype characterized by variable clinical severity, apparently influenced by gender. Moreover, we provide evidence that CNF may be genetically heterogeneous by detection of *NPHS2* mutations in some CNF patients in whom *NPHS1* mutations were not found. We confirm an overlap in the *NPHS1*/*NPHS2* mutation spectrum with the characterization of a unique di-genic inheritance of *NPHS1* and *NPHS2* mutations, which results in a 'tri-allelic' hit and appears to modify the phenotype from CNF to one of congenital focal segmental glomerulosclerosis (FSGS). This may result from an epistatic gene interaction, and provides a rare example of multiple allelic hits being able to modify an autosomal recessive disease phenotype in humans. Our findings provide the first evidence for a functional inter-relationship between *NPHS1* and *NPHS2* in human nephrotic disease, thus underscoring their critical role in the regulation of glomerular filtration.

INTRODUCTION

Disruption of the normal permselective qualities of the renal glomerulus leads to loss of essential plasma proteins into the urine, which manifests in a clinical setting as nephrotic syndrome. Although the protein leak may be caused by a number of pathogenic mechanisms, the unifying feature is dysfunction of the glomerular filtration barrier (GFB), the principal structure within the glomerulus mediating protein filtration. Which GFB structure ultimately maintains glomerular permselectivity remains controversial, but mounting evidence indicates that the primary cellular target for injury is the podocyte with disruption of the actin cytoskeleton playing a key role (1). Characteristic alterations in cell structure are seen, culminating in detachment from the glomerular basement membrane and apical displacement of slit diaphragms, modified adherens junctions located between interdigitating podocyte foot processes. Eventually, adhesion of cells to the denuded glomerular basement membrane results in irreversible scarring, obliteration of the urinary space and renal failure, the most severe consequences of nephrotic disease (2).

The histological clues are supported by the recent identification of a number of genes likely to be important regulators of podocyte cell function. *NPHS1*, a 26 kb gene located on chromosome 19q13.1, was positionally cloned through linkage disequilibrium analysis of Finnish patients with a rare condition known as Finnish type congenital nephrotic syndrome (CNF) (3). Patients present with massive proteinuria, often starting *in utero* and progress to end stage renal failure by 2–3 years (4). Characteristic renal histopathological changes are immature glomeruli, mesangial cell hypercellularity and glomerular foot process fusion together with pseudocystic dilatation of the proximal tubules (5,6), and may mirror defective podocyte development (7). A conclusive link between the *NPHS1* gene and CNF was established with the detection of two discreet *NPHS1* mutations: Fin major (nt121delCT) and Fin minor (R1109X) in >90% of Finnish patients (3,8). Outside Finland, CNF constitutes the commonest type of congenital nephrotic syndrome, but the exact incidence

*To whom correspondence should be addressed. Tel: +44 207 242 9789; Fax: +44 207 404 6191; Email: a.koziell@ich.ucl.ac.uk

is unknown. Most cases emulate the classically severe clinical phenotype seen in Finland, and a variety of NPHS1 mutations distinct from Fin major and Fin minor have been detected (9,10). However, an unexplored area remains the milder disease phenotypes also seen, which include occasional remission of proteinuria despite characteristic CNF renal histology (11). Moreover, congenital nephrotic syndrome itself represents a heterogeneous group of conditions (12), each characterized by a different renal histology such as diffuse mesangial sclerosis, sometimes associated with the Denys–Drash syndrome and mutations of the WT1 gene (13), and focal segmental glomerulosclerosis (FSGS). All are thought to represent distinct entities despite the similarities in clinical presentation to CNF (14,15).

NPHS1's protein product nephrin is a single pass transmembrane protein consisting of eight extracellular class C2 immunoglobulin-like modules, a fibronectin type III-like motif and a cytosolic C-terminal tail 157 amino acid residues in length. It is an immunoglobulin superfamily member, and thus likely to have diverse mechanisms of action within podocytes such as cell surface recognition, involvement in immune response and cellular signalling. A number of observations support nephrin's critical role in maintaining glomerular permselectivity, for instance maximal expression is seen in podocyte foot processes in the region of the slit diaphragms (16) and nephrin knockout mice develop proteinuria *in utero* and die within the first 24 h (17). Furthermore, a direct interaction between nephrin and CD2-associated protein, CD2AP, a novel SH3-containing protein first characterized in murine T-cells (18) has been reported, and CD2AP knockout mice develop nephrotic syndrome and die of renal failure at 6 weeks of age rather than immuno-insufficiency (19). Although the physiological relevance of this interaction to human nephrotic disease still requires confirmation, CD2AP may anchor nephrin to the cytoskeleton and mediate nephrin–matrix interactions. CD2AP and nephrin co-localize in both mouse and human kidney (20), supporting an interactive role in maintaining the slit diaphragm and podocyte subcellular architecture. There is also increasing evidence that nephrin plays a wider role in the pathogenesis of nephrotic syndrome beyond infancy, both from experimental models of proteinuria (21–23) and studies of nephrin expression in patients, where redistribution of nephrin on podocytes has been observed in patients with primary acquired nephrotic syndrome (24).

Positional cloning also recently identified another glomerular gene associated with human nephrotic disease presenting in early life. *NPHS2* is located on chromosome 1q25–1q31 and encodes podocin, a putative 42 kDa transmembrane protein with sequence homology to the band-7 stomatin family (25). Sequence analysis suggests that it consists of a single, short transmembrane domain and cytosolic N- and C-terminal domains. The C-terminal tail contains an SPFH domain (Pfam entry: Band_7, accession no: PF1145), predicted to lie close to the membrane-associated region (http://smart.embl-heidelberg.de/). Analogous to the *NPHS1* gene and nephrin, expression of *NPHS2* in the kidney is confined to the podocyte. *NPHS2* gene mutations were originally detected in a very specific type of autosomal recessive steroid resistant nephrotic syndrome, SRN1, which presents between 3 months and 5 years of age, and FSGS renal histology (25). In contrast to CNF, this is characterized by lack of pseudo-cystic dilatation of the tubules, focal and segmental sclerosis of the glomeruli, variable mesangial cell proliferation and occasinal presence of immuno-reactants. More recently, the *NPHS2* gene has been excluded as candidate for familial steroid-sensitive nephrotic syndrome (26), but there are two reports of *NPHS2* mutations in apparently sporadic childhood FSGS, occurring in 2/9 (27) and 9/40 (28) patients, as well as one report of a homozygous *NPHS2* missense mutation occurring FSGS with an onset in adult life (29). So far, little is known about podocin's role within the podocyte, although evidence from *Caenorhabditis elegans* suggests that this group of proteins homo-oligomerize and form core components of membrane-associated proteolytic complexes (reviewed in 30). In addition, recent data suggests that podocin is able to bind nephrin either directly or indirectly, and may augment nephrin-induced stimulation of mitogen-activated protein kinases (31).

We examined genotype/phenotype correlations for the *NPHS1* and *NPHS2* genes encoding the proteins nephrin and podocin through mutational analysis of non-Finnish CNF patients, including cases of atypically mild disease, congenital FSGS and a group with SRN1-like disease. We expand the CNF clinical spectrum to include mild nephrotic syndrome, and importantly, redefine the mutation phenotype for *NPHS2* by identifying mutations as a subsidiary cause of CNF. In addition, we present data indicating co-existence of mutations in both *NPHS1* and *NPHS2* appears to modify CNF to a phenotype of congenital FSGS. This is the first described case of multiple hits in unrelated genes apparently altering an autosomal recessive renal disease phenotype in humans. Our findings support a pivotal role for *NPHS1* and *NPHS2* in regulating glomerular filtration and provide further evidence for a functional inter-relationship between these two genes within the podocyte.

RESULTS

NPHS1 analysis

CNF (n = 37). Twenty-three different mutations were detected throughout the *NPHS1* gene in 29 of 37 CNF cases analysed. Nine were novel; the remainder had been previously reported (3,8–10). All occurred in a homozygous or compound heterozygous manner and affected exons 4, 6, 7, 9, 10, 11, 14, 17, 18, 23, 24, 26, 27, intronic splicing regions and the promotor (summarized in Tables 1 and 2 and Fig. 1A–C). Isolated heterozygous mutations were not found in association with a CNF phenotype. Thirteen patients (11 families) originated from Malta, and partial haplotype analysis using highly informative markers was suggestive of a founder effect (data not shown), an observation supported by detection of the same homozygous exon 27 R1160X nonsense mutation in all Maltese cases. R1160X was also detected in CNF cases of Asian origin, but associated with a different allele. Analysis of Maltese cases 86, 87, 88 and 89 (Table 2) was not possible, although these were predicted to have homozygous R1160X mutations on the basis of a clinical history, family history and the detection of a carrier status in both parents. Heterozygous R1160X changes were present in all parents, the phenotypically normal sister of case 81, and in 1 of 44 Maltese control chromosomes and exclusively associated with a normal phenotype. An nt2335-1 (G→A) exon 18 splicing mutation was the commonest mutation

Table 1. *NPHS1* mutations detected (excluding R1160X) in classically severe CNF (17/29 patients)

Patient	Origins	Exon	Nucleotide change	Effect on coding	Effect on protein	Mutation status
Frameshift mutations						
50	Middle East	10[a]	nt1291 (insA)	Frameshift	Truncation	Hom
4	England	24	nt3250 (insG)	Frameshift	Truncation	Comp het
5	England	6	nt661 (delAG)	Frameshift	Truncation	Comp het
7	Pakistan	6	nt603 (delCACCC	Tyr205, Pro206	Del T,P,R	Hom
			CGG (ins TT)	Arg207→Ile205	Ins I	
Non-frameshift mutations and insertions						
6	Middle East	4	nt514 (del ACC)	del Thr 172	Del Thr	Hom
Nonsense mutations						
8	Turkey	26	nt3325 (C→T)	R1109X	Truncation (Fin minor)	Hom
Splicing mutations						
73	England	14[a]	nt1905 (C→T)	Exon 14 alternative splice donor site?	Aberrant splicing	Comp het
25	England	18	nt2335-1 (G→A)	Exon 18-1(G→A)	Aberrant splicing	Comp het
32	England	18	nt2335-1 (G→A)	Exon 18-1(G→A)	Aberrant splicing	Comp het
47	England	18	nt2335-1 (G→A)	Exon 18-1(G→A)	Aberrant splicing	Comp het
65	England	18	nt2335-1 (G→A)	Exon 18-1(G→A)	Aberrant splicing	Hom
2	England	23[a]	nt2335-1 (A→T)	Exon 23 donor	Aberrant splicing	Comp het
4	England	27	nt3482 (G→T)	Exon 27 donor	Aberrant splicing	Comp het
5	England	27	nt3482 (G→T)	Exon 27 donor	Aberrant splicing	Comp het
Missense mutations						
9	England/India	7[a]	nt791 (C→G)	P264R	Arg268→Pro	Comp het
47	England	7	nt808 (G→T)	G270C	Gly270→Cys	Comp het
10	India	9	nt1099 (C→T)	R367C	Arg367→Cys	Hom
9	England/India	11[a]	nt1337 (T→A)	I446N	Ile446→Asn	Comp het
11	India	11	nt1379 (G→A)	R460Q	Arg460→Glu	Hom
25	England	14	nt1868 (G→T)	C623F	Cys623→Phe	Comp het
20	India	14[a]	nt1927 (T→C)	L643P	Leu643→Pro	Hom
2	England	17	nt2227 (C→T)	R743C	Arg743→Cys	Comp het
62	India	18	nt2404 (C→T)	R802W	Arg802→Trp	Hom
Promoter						
32	England	Promoter	nt340 (G→C)			Comp het
73	England	Promoter	nt340 (G→C)			Comp het

[a]Novel mutations; other mutations have been described previously.
Hom, homozygous mutation; comp het, compound heterozygous mutation; del, deletion; ins, insertion.

in patients of English origin (Fig. 1A). We also document the first occurrence of Fin minor (R1109X) outside Finland, in a Turkish patient with no demonstrable Finnish ancestry. All *NPHS1* mutations resulted in a consistently severe CNF phenotype, regardless of location or predicted effect on the protein except for the homozygous R1160X mutation (Fig. 1B), which was associated with mild CNF disease in ~50% of cases, the majority female (Table 2). *NPHS1* mutations were not detected in eight CNF cases, five with severe and three with atypically mild CNF.

Congenital FSGS (n = 4). NPHS1 mutations were detected in all cases (Table 3). Case 39 had a homozygous nt2335-1(G→A) exon 18 splicing mutation, also identified in CNF (case 65, Table 1, and also reported in compound heterozygotes, reference 8, also this series). Cases 23 and 24 (siblings) had heterozygous N188I missense mutations, detected in one maternal allele but absent in 122 Asian, 100 Caucasian and 40 Maltese control chromosomes. The fourth, case 49, had a heterozygous intronic mutation nt1930+11(c→a), predicted to activate a cryptic intronic splice site. This was detected in one maternal allele, but absent in 222 control chromosomes. These changes were not detected in association with existing homozygous or compound heterozygous *NPHS1* mutations.

SRN1-like early onset FSGS (n = 5). No *NPHS1* mutations were detected in exons 1–29, adjacent introns or the promotor.

Table 2. R1160X NPHS1 mutation in CNF [nt 3478(C→T)]

Patient	Ethnic origin	Sex	Family history	Features at presentation		Clinical status and age at last follow-up	Mutation status
				Albumin[a]	Histology		
Classically severe CNF (n = 6)							
80	India	M	No	6	CNF	Died aged 6 months, diagnosis confirmed on postmortem	Hom
81	Malta	M	No	11	CNF	Renal transplant aged 18 months, 12 years alive and well	Hom
82	Malta	F	Yes: 83	13	CNF	Dialysis aged 4 years, failed transplant	Hom
83	Malta	M	Yes: 82	8	CNF	Died post transplant aged 6 years	Hom
84	Malta	M	No	11	–	Severe nephrotic syndrome at 15 months	Hom
85	India	M	No	15	CNF	Died aged 1 month, diagnosis confirmed on postmortem	Hom
Patients with a clinical diagnosis of classically severe CNF, mutations status not determined, but both parents heterozygous for R1160X (n = 4)							
86	Malta	M	Yes: 92	–	CNF	Died aged 3 months	–
87	Malta	M	Yes: 88	–	CNF	Died aged 5 years	–
88	Malta	M	Yes: 87	–	–	Died aged 2 months	–
89	Malta	M	Yes: 94	–	CNF	Died aged 21 months	–
Atypically mild CNF (n=6)							
26	Bangladesh	F	Yes	4	CNF	Aged 7 years; mild proteinuria, alb, 35; creat[b] 40, Nt.	Hom
90	Malta	F	No	12	CNF	Aged 11 years; remission, alb, 34; creat, Nt.	Hom
91	Malta	F	No	15	CNF	Aged 5 years; mild proteinuria; alb, 29; creat 35, Nt.	Hom
92	Malta	F	Yes: 86	–	–	Aged 19 years; mild proteinurea; alb, 34; creat, 50, Nt.	Hom
93	Malta	F	No	13	–	Aged 5 years, remission; alb, 39; creat, 42, Nt.	Hom
94	Malta	M	Yes: 89	13	–	Aged 8 years, mild proteinuria; alb, 29; creat, 40, Nt.	Hom

Nt, normotensive.
[a]Plasma albumin concentration in mg/dl.
[b]Plasma creatinine.

This included a patient previously described as having a heterozygous R138Q *NPHS2* mutation (family 4; 21).

NPHS2 analysis

CNF and no NPHS1 mutation (n = 8: 5 severe, 3 atypically mild CNF). A novel homozygous frameshift nt(465/6) ins T in exon 4, and a homozygous R138Q missense mutation in exon 3 previously described in association with SRN1 (25) were detected in two cases of severe CNF (Table 4). No mutations were found in atypically mild CNF.

Congenital FSGS (n = 4). NPHS2 mutations were detected in all four cases of congenital FSGS in conjunction with *NPHS1* mutations, described in Table 2. Case 39 had a heterozygous R229Q missense mutation absent in 120 control chromosomes, but previously detected as a homozygous event in association with adult onset FSGS (29). Cases 23 and 24 had a novel homozygous nt436delA frameshift in exon 3, and case 49 had the homozygous R138Q mutation previously described in connection with SRN1.

CNF with pre-determined NPHS1 mutations (n = 29). To clarify the extent of the *NPHS1/NPHS2* mutation phenotype overlap, CNF cases with pre-determined homozygous and compound heterozygous *NPHS1* mutations (described in Tables 1 and 2) were also screened for *NPHS2* mutations. Only two, cases 25 and 32 had heterozygous R229Q *NPHS2* missense mutations detected in association with *NPHS1* compound heterozygous mutations. Of note, *NPHS2* mutations were not identified in case 65 (CNF, Table 1), with the same homozygous nt2335-1(G→A) *NPHS1* splicing mutation detected in case 49 (congenital FSGS, Table 3). This may represent a further overlap in the *NPHS1/NPHS2* mutation spectrum. Alternatively, cases 25 and 32 were born at 35 weeks gestation and underwent renal biopsy in the immediate post-natal period rather than at 3 months. Renal histology was non-specific, and features of FSGS may not have yet developed. *NPHS2* mutations were not detected in cases of severe CNF due to homozygous R1160X mutations, virtually excluding concurrent mutations in *NPHS2* as a cause for the discordant phenotype observed.

SRN1-like early onset FSGS (n = 5). Despite the phenotypic similarity with SRN1, only one mutation was detected, a heterozygous R138Q missense mutation in a case of familial early onset FSGS, previously reported as family 4 (25) with no mutation detected in the other allele.

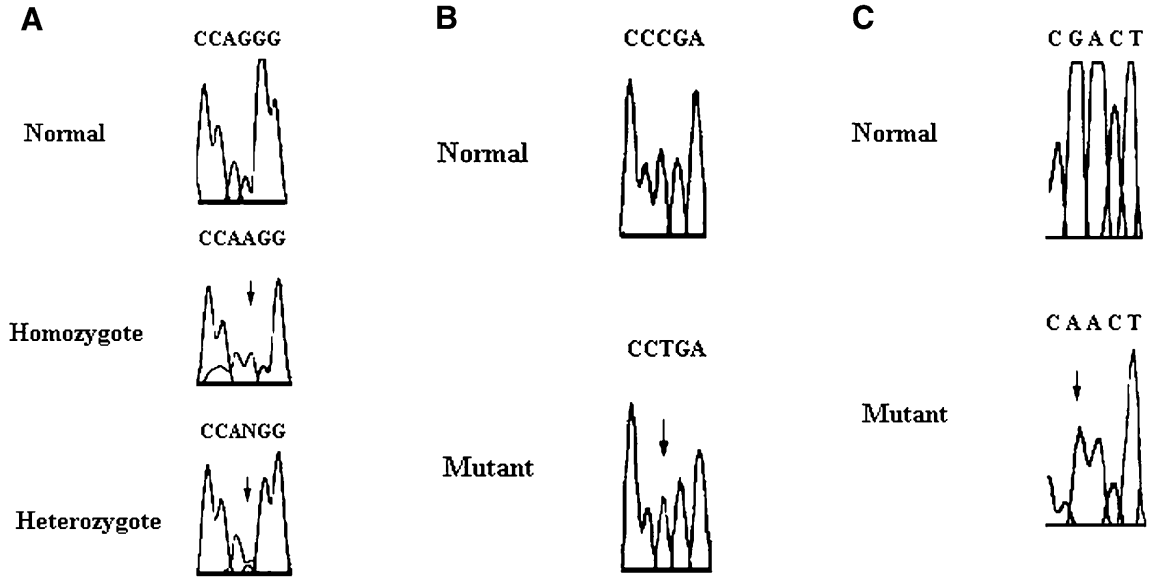

Figure 1. (**A**) Exon 18 *NPHS1* splicing mutation (nt2335-1G→A) found in association with CNF and congenital FSGS. (**B**) R1160X, the exon 27 nonsense mutation associated with a discordant CNF phenotype. (**C**) NPHS2 missense mutation R138Q associated with CNF and congenital FSGS.

Table 3. *NPHS1* and *NPHS2* mutation phenotype in congenital FSGS ($n = 4$)

Patient	Origin	Family history	Exon	Nucleotide change	NPHS1 mutation					NPHS2 mutation		
					Effect on coding sequence	Effect on protein	Mutation status	Exon	Nucleotide change	Effect on coding sequence	Effect on protein	Mutation status
39	England	No	18	2335-1 G→A	Exon 18-1 G→A	Aberrant splicing	Hom	5	623G→A	R229Q	Arg279Glu	Het
23,24	India	Yes	5	563 A→T	N188I[a]	Asn188Ile	Het	3	(436)delA[a]	Frameshift	Truncation	Hom
49	England	No	14	1930+11 (gccccgg→ gcccagg)	nt1930+11 (c→a)[a]	Cryptic splice-site activation	Het	3	413 G→A	R138Q	Arg138Glu	Hom

Table 4. *NPHS2* mutations phenotype in classically severe CNF

Patient	Origin	Exon	Nucleotide change	Effect on coding sequence	Effect on protein	Mutation status
54	England	3	413 G→A	R138Q	Arg138Glu	Hom
15	Turkey	4	nt(465/6)insT[a]	Frameshift	Truncation	Hom

[a] Novel mutation.

DISCUSSION

This study confirms the pivotal roles the *NPHS1* and *NPHS2* genes play in renal glomerular filtration and more importantly, provides further direct evidence of a potential functional relationship between them within the podocyte. A number of key conclusions can be made. Firstly, our results demonstrate that with the exception of the R1160X nonsense mutation, *NPHS1* mutations cause a severe CNF phenotype in non-Finnish patients, regardless of location or predicted effect on the nephrin protein. Moreover, *NPHS1* mutations are distributed throughout the gene (Fig. 2), emphasizing a functional requirement for both extracellular and intracellular domains. Compilation of our results with those in the literature (3,8–10), results in a total of 59 unique *NPHS1* mutations detected in CNF and congenital FSGS to date, of which two lie within the promotor region. Figure 2 illustrates the distribution of the 57 mutations within the coding region. These cluster in immunoglobulin domains two, four and seven, which gives a focus for diagnostic tests. To date, missense mutations are all located within the extracellular domain. They account for just >50% of all mutations detected, and ~66% of the mutations found within immunoglobulin domain 'hot spots' (Fig. 2), suggesting that

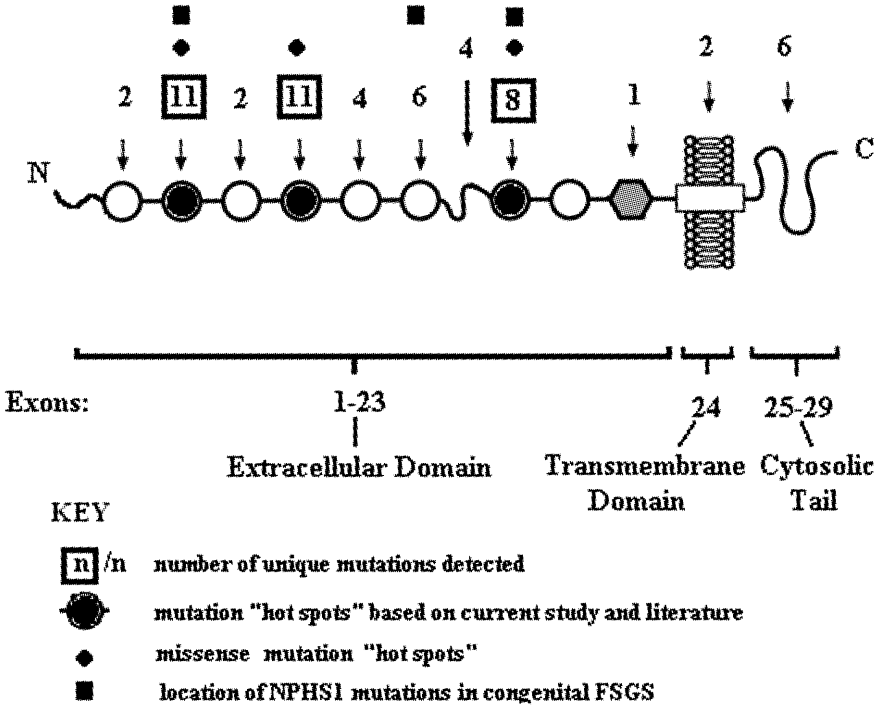

Figure 2. Distribution of unique *NPHS1* mutations detected within the coding sequence ($n = 57$) to date showing mutation 'hot-spots' and location of mutations in congenital FSGS.

these represent functionally important areas. In the absence of a suitable system able to confirm the pathogenicity of missense mutations *in vivo*, these must remain speculative. However, expression of nephrin is abnormal in podocyte cell lines cultured from some of the CNF patients with missense mutations in this study (Moin Saleem, unpublished data), and all putative missense mutations detected result in non-conservative amino acid substitutions. Eighty-one percent are predicted to be deleterious by SIFT analysis (http://www.blocks.fhcrc.org/~pauline/SIFT.html) which is based on their location in highly conserved regions of the nephrin protein. Many of the putative missense mutations target charged residues such as arginine, and hydrophobic amino acids such as leucine and isoleucine, and are likely to produce significant alterations in protein conformation. Others target cysteine residues, introducing the potential for disruption or novel formation of di-sulphide bridges. Furthermore, there is *in vitro* evidence to suggest that nephrin is a highly flexible protein, readily able to change conformation (32), and that ~75% of *NPHS1* missense mutations may result in misfolding of the nephrin protein, with the mutants becoming trapped in the endoplasmic reticulum and failing to express at the plasma membrane (33). This is similar to the effect of Fin major and Fin minor mutations in Finnish CNF patients, which create defective, unstable nonsense proteins failing to express in podocytes (8). An unexplored area remains location of ligand binding to the extracellular domains, which again could become disrupted by the introduction of a point mutation. Of note, the promotor mutation identified within this study was not detected in normal controls, and preliminary analysis suggests that this mutation lies within a novel transcription factor binding site, altering protein binding on gel shift assay (unpublished data).

Fin major and Fin minor, the prototypic mutations originally detected in Finnish patients, are rare in non-Scandinavian populations. The wide variety of different mutations detected in non-Finns are likely to be *de novo* events, especially when the ethnic diversity of the test populations is taken into account. Some populations may exhibit a founder effect, such as that detected within our series in Malta for R1160X, which facilitates the planning of diagnostic tests. Intriguingly, one Turkish CNF patient originating from Anatolia had a homozygous Fin minor mutation (R1109X) only detected in a small subset of Finnish patients. The apparent ethnic diversity of these populations implies an independent event rather migratory influence, and Fin minor has not been detected in other Turkish patients. However, the possibility of a common ancestor exists, as there is a significant contribution of Central Asian genes to the genetic makeup of both Finns and Turks from Anatolia (34), with significant population admixture between Hungarians, Slavs, Finns, Turks and Iranians (35). R1160X, a specific exon 27 nonsense mutation resulted in an unexpectedly mild CNF phenotype in ~50% of cases, and this appeared to be influenced by gender as the majority of the mildly affected cases were female. Although anecdotal reports have documented mild congenital nephrotic syndrome phenotypes in the past, we provide the first conclusive link that these form part of the CNF clinical spectrum. The discordant phenotype associated with R1160X does not appear to be population specific as cases originated from Malta, Bangladesh and India and were associated with different alleles on partial haplotype analysis. Furthermore, there were no differences in clinical

presentation, as all cases were normotensive, and initially, had normal renal function. In addition, there are no histological clues as mild cases also demonstrated typical CNF histology with microcystic dilatation and typical foot process fusion on electron microscopy. Mild disease has been thought to result from a response to anti-proteinuric drugs (8,36), and recent data suggest a link between anti-proteinuric drugs and rescue of down-regulated *NPHS1* mRNA and diminished glomerular nephrin expression in progressive renal injury in passive Heyman Nephritis (37). However, the use of anti-proteinuric agents is commonplace in the management of CNF in humans (38,39), slowing the rate of clinical deterioration, but rarely correlating with a favourable long-term outcome. Of note, two of the mild CNF cases analysed (91 and 92), did not receive anti-proteinuric drugs, and these agents were stopped in the remainder once it was apparent that the clinical status was improving. The majority of severely affected individuals were male and those mildly affected, female (Table 2). This was true both inter- and intra-family; for example patients 86 (male, severely affected) and 92 (female, mildly affected) were siblings, and patient 26 who has mild disease whereas her four brothers died in infancy. Interestingly, the original case reported with the R1160X mutation was also female with mild disease (9,36). The apparent influence on phenotype by gender in non-X-linked conditions is rare, but has been described with a classic example being autosomal recessive limb-girdle muscular dystrophy (40), where various as yet unsuccessful strategies have been used to identify the modifiers and/or epigenetic phenomena responsible. At a molecular level, the R1160X mutation results in truncation of the nephrin protein downstream of the last amino acid residue in exon 27. In contrast to Fin minor (exon 26) (9), glomerular nephrin expression in the glomeruli of patients carrying this mutation, regardless of severity in clinical phenotype (Fig. 3), suggesting that the nephrin protein remains normally distributed within the cell. Of note, other exon 27 mutations, even when situated in the immediate vicinity of R1160X, result in a severe CNF phenotype, but there are very few cases reported as yet.

Emerging data on nephrin's potential binding partners such as CD2AP (18,41) and podocin (31) underline the functional importance of the distal C-terminal cytosolic tail, and the key role it may play in protein–protein interactions. In view of a possible functional link between *NPHS1* and *NPHS2*, we tested whether the phenotypic variability associated with R1160X resulted from co-inheritance of a mutant allele within the *NPHS2* gene. However, *NPHS2* gene mutations were not detected, implying that *NPHS2* gene mutations are not modulators of the severity of the CNF phenotype, at least in these individuals. CNF cases which lacked *NPHS1* mutations were screened for *NPHS2* mutations. Renal histology was consistent with CNF, with no clinical or histological indicators suggestive of SRN1. Two of five typically severe CNF patients lacking *NPHS1* mutations had homozygous *NPHS2* mutations (Table 4), a novel frameshift mutation in exon 4, and a missense mutation R138Q, located in a mutation 'hot spot' for SRN1 (25). These findings support the genetic heterogeneity of CNF, which is further endorsed by the lack of mutations in the remaining three patients with typically severe disease, and emphasise the importance of screening for both *NPHS1* and NPHS2 mutations in CNF, especially when no *NPHS1* mutation is apparent. The role of other glomerular genes with clearly demonstrable links to nephrotic disease such as *NPHS1*, encoding a novel slit diaphragm protein homologous to nephrin (42) remains unexplored.

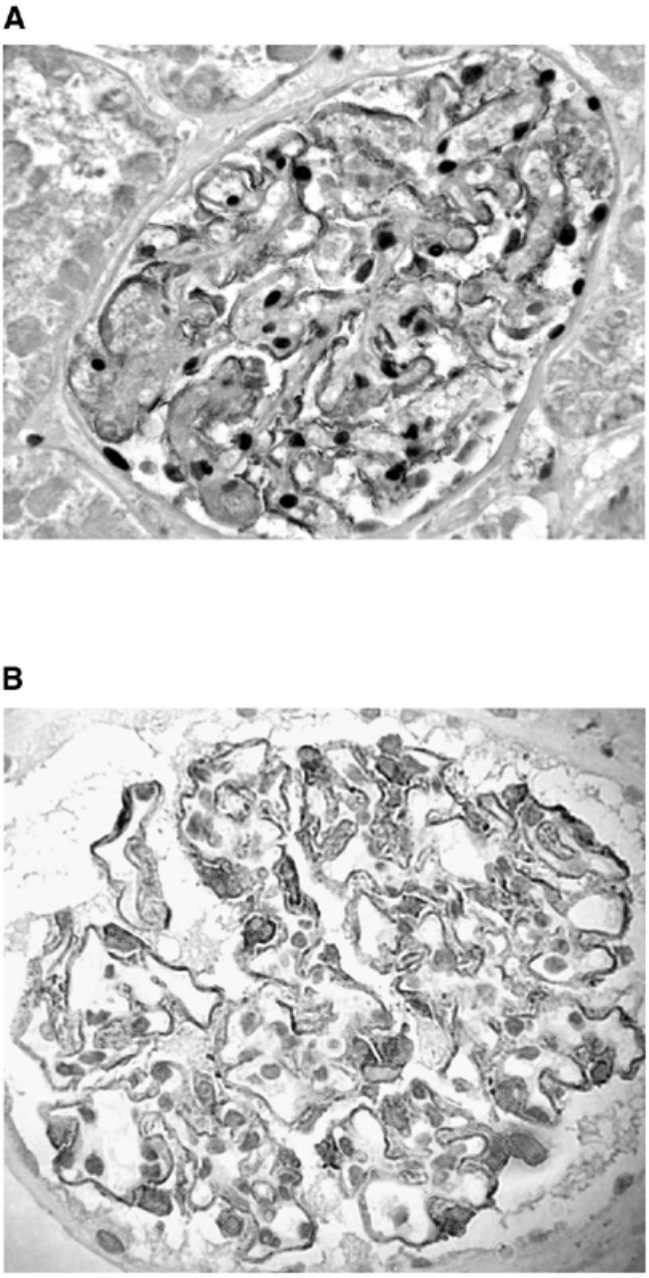

Figure 3. Nephrin protein expression associated with the R1160X mutation (**A**) Severe CNF phenotype. (**B**) Normal control.

The ability of *NPHS1* and *NPHS2* mutations to both result in CNF provided evidence for a functional link between the two genes. This was examined further by mutational analysis of patients with congenital FSGS, a congenital nephrotic syndrome traditionally distinct from CNF based on differences in renal histology (Table 5). Cases lacked pseudocystic dilatation of the proximal tubules and developed distinctive focal and segmental sclerotic lesions within the glomeruli, which differed from the global sclerosis sometimes seen in the

later stages of CNF. Co-existence of *NPHS1* and *NPHS2* mutations was detected in all congenital FSGS patients tested (Table 3), providing corroborative evidence of a functional relationship between these two genes. It appears that in order to produce a congenital FSGS phenotype, a di-genic inheritance of *NPHS1* and *NPHS2* mutations resulting in a tri-allelic hit required, i.e. mutation hits occurred as a homozygous event in one gene, with the third mutation acting as a modifier in the other. Parents who were compound heterozygote carriers of the respective *NPHS1* and *NPHS2* mutations were phenotypically normal. All changes were absent in normal control and were not detected in conjunction with a third *NPHS1*/*NPHS2* mutation in the remaining allele. The *NPHS1* N188I missense mutation potentially disrupted an *N*-glycosylation site, a mechanism shown in transfected cells to play a crucial role in the correct localization of nephrin to the plasma membrane (43). The effects of cryptic splice site activation are well described (44), and the C→A mutation introducing a putative cryptic splice site was located in the exon 14 splice donor site, a site already predicted to participate in alternative splicing (http://genome.cbs.dtu.dk/services/NetGene2/). Both *NPHS2* missense mutations lay within the SPFH functional domain. Di-genic inheritance resulting in three disease alleles occurs in other autosomal recessive conditions, but generally the phenotype does not change or improves (45–47). In addition, trans-heterozygous mutations can affect the alleles of two different genes to produce an autosomal dominant phenotype, such as in polycystic kidney disease (48,49) and Hirschsprung's disease (50). The importance of an additional disease allele in congenital FSGS is underscored by the finding that straightforward bi-allelic hit in either *NPHS1* or *NPHS2* appears to result in CNF, suggesting that this extra event may act as a modifier of disease expression, and could represent a form of genetic epistasis. Moreover, in view of the overlap in mutation phenotype between CNF and congenital FSGS, it may be that these disorders should be regarded more as part of a spectrum, with the resulting disease phenotype partially determined by the genetic background of the patient. A similar phenomenon is seen with mutations of the Wilms' tumour gene, *WT1* in Frasier and Denys–Drash syndromes (51,52), where the recent generation of specific mouse mutants has greatly advanced our understanding of the underlying molecular mechanisms (53). However, apart from the molecular implications, these findings emphasize the importance of screening cases of congenital nephrotic syndrome with a diagnosis compatible with CNF or FSGS for mutations in both *NPHS1* and *NPHS2* genes. The lack of *NPHS2* mutations in the SRN1-like group was surprising, but confirms the genetic diversity of FSGS despite the uniformities in clinical presentation and renal histology. Only one heterozygous R138Q NPHS2 mutation was detected, in a previously reported case of SRN1 (25), providing further supporting evidence that the tri-allelic inheritance is a phenomenon specific to congenital FSGS.

In conclusion, genotype/phenotype correlation of NPHS1/nephrin mutations indicates that the majority result in a severe congenital nephrotic syndrome, and that intact extracellular and cytosolic domains are required. Furthermore, immunoglobulin domains 2, 4 and 7 are likely to be particularly important for gene function and form a target for diagnostic tests. Moreover, a specific nonsense mutation within the cytosolic tail is associated with a variably severe clinical phenotype, apparently influenced by gender. This remains unexplained but might result from the co-inheritance of a disease allele in another gene, or compensation by a genetic or other determinant, such as through functional redundancy. We ascertain that a diagnosis of CNF can result from both *NPHS1* and *NPHS2* mutations, and that a molecular diagnosis of congenital nephrotic syndrome should incorporate mutational analysis of both genes. We identify a specific di-genic inheritance, resulting in three variant alleles associated with an apparent modification of the disease phenotype from CNF to congenital FSGS. Our data provide further evidence of a functional inter-relationship between nephrin and podocin and underscores the critical role these genes play in regulating glomerular protein filtration and in the pathogenesis of proteinuria. These findings, together with the overlap in mutation phenotype with other forms of FSGS, clearly substantiate a wider functional role for nephrin and podocin reaching outside congenital nephrotic disease. Our data provides the basis for a hypothesis that nephrin and podocin may form part of a multimeric complex activating or residing within intracellular pathways essential for podocyte function and slit diaphragm integrity, supported by recent co-immunoprecipitation data (31). We would predict that *NPHS1* and *NPHS2* are important mediators of the typical podocyte dysfunction seen universally in nephrotic disease. In addition, these findings can be added to a growing body of data (54), which demonstrates that inheritance of different alleles at independent genetic loci may contribute to disease phenotype.

MATERIALS AND METHODS

Patient selection and control material

Fifty patients were studied in total. Forty-one had CNF, four congenital FSGS and five an early onset FSGS compatible with SRN1. A distinction between congenital FSGS and SRN1 was made on the basis that congenital FSGS presented within the first 2 months of life, rather than between 3 months and 5 years, and was not always clearly familial. The study had ethical approval awarded by the Institute of Child Health Research Ethics committee (number 99MM05) and informed consent was obtained from individuals. Diagnosis of congenital nephrotic syndrome was based on a typical clinical history of prematurity, placental weight >25% of infant birth weight, and onset of proteinuria and nephrotic syndrome between birth and 2–3 months of age. Cases were normotensive at presentation, with normal renal function. Some had a positive family history, but the majority were sporadic. Diagnosis of an early onset nephrotic syndrome was made if presentation was between 6 months and 2 years of age. Since all five cases studied had FSGS renal histology, they were classified as possible SRN1. Diagnosis was confirmed by renal biopsy or postmortem in 44 of the 50 cases using histological criteria listed in Table 5. Renal histology was reviewed predominately by one key pathologist, and that thought to represent congenital FSGS was independently examined at two centres. Within the CNF group, 28 of 37 cases screened had classically severe nephrotic disease, the remainder atypically mild disease. The two groups appeared clinically indistinguishable for the first year of life, and there were no consistent differences in medical management to account for a difference in outcome, as anti-proteinuric agents did not prevent disease progression in this

Table 5. Renal histological criteria used to distinguish between CNF and FSGS

CNF
Early features
Glomeruli normal ± mesangial hypercellularity
Proximal tubular dilatation
Absence of immunreactants
Late features
Enlarged sclerotic glomeruli with marked microcystic dilation of the tubules
Tubular atrophy and interstitial fibrosis
FSGS
Presence of areas of both focal and segmental glomerular sclerosis
Focal and segmental tuft collapse
± segmental hyalinosis, ± glomerular deposits of IgM/C3

series. Congenital FSGS and SRN1-like cases all had severe disease and progressed to chronic renal failure by 5 years. Fifty European and North American, 61 Asian and 22 Maltese normal controls were also analysed.

DNA extraction

Genomic DNA was extracted by standard phenol/chloroform methodology or using a QIAmp Tissue extraction Kit (Qiagen).

Mutational screening

NPHS1 exons 1–29, adjacent introns and the promotor region, and *NPHS2* exons 1–8 with adjacent introns were analysed. DNA (30–60 ng) was used for PCR in a total volume of 25 µl containing DNA, 1× NH_4 buffer (Bioline), 0.2 mM nucleotides (Pharmacia), 100–200 ng primers [as previously published for *NPHS1* (9) and *NPHS2* (25)] and 0.5 U DNA polymerase (Bioline). Betane (0.2 M) was used as a co-solvent for the amplification of GC-rich regions. Thirty to thirty-five cycles of PCR amplification were performed on a Robocycler (Stratagene) with annealing temperatures ranging between 50 and 60°C, depending on primer T_m. PCR products were visualized on a 1.5–2% agarose gel against a 1 kb ladder (Gibco BRL). Ten to twenty microlitres of product was then purified from a 1.5% NuSieve® GTG® agarose gel (FMC products) using a QIAquick Gel extraction kit (Qiagen). Two to four microlitres of purified product was cycle sequenced using ThermoSequenase dye terminators (Amersham Life Science). Sequences were analysed on an ABI 377 automated sequencer (Perkin Elmer), and mutations confirmed by restriction enzyme digest, parental analysis and absence in normal controls.

NOTE ADDED IN PROOF

Recently, further evidence for a nephrin–podocin complex was provided by Schwartz *et al*. (55).

ACKNOWLEDGEMENTS

We would like to acknowledge the Renal units which provided patients for this study, and Dr Diane Rampling for histological analysis. The work was funded by The Wellcome Trust, a Charlotte Eyke and Elizabeth Wherry award from the British Medical Association, the Sir Jules Thorn Trust and the National Kidney Research Fund.

REFERENCES

1. Smoyer,W.E. and Mundel,P. (1998) Regulation of podocyte structure during the development of nephrotic syndrome. *J. Mol. Med.*, **76**, 172–183.
2. Mundel,P. and Kriz,W. (1995) Structure and function of podocytes: an update. *Anat. Embryol.*, **192**, 385–397.
3. Kestila,M., Lenkkeri,U., Mannikko,M., Lamerdin,J., McCready,P., Putaala,H., Ruotsalainen,V., Morita,T., Nissinen,M., Herva,R. *et al.* (1998) Positionally cloned gene for a novel glomerular protein nephrin is mutated in congenital nephrotic syndrome. *Mol. Cell*, **1**, 575–582.
4. Norio,R. (1966) Heredity in the congenital nephrotic syndrome. A genetic study of 57 Finnish families with a review of reported cases. *Ann. Paediatr.*, **27**, 1–94.
5. Huttunen,N.P., Rapola,J., Vilska,J. and Hallman,N. (1980) Renal pathology in congenital nephrotic syndrome of Finnish type: a quantitative light microscopic study on 50 patients. *Int. J. Pediatr. Nephrol.*, **1**, 10–16.
6. Autio-Harmainen,H. and Rapola,J. (1981) Renal pathology of fetuses with congenital nephrotic syndrome of the Finnish type. A qualitative and quantitative light microscopic study. *Nephron*, **29**, 158–163.
7. Haltia,A., Solin,M.L., Holmberg,C., Reivinen,J., Miettinen,A. and Holthofer,H. (1998) Morphologic changes suggesting abnormal renal differentiation in congenital nephrotic syndrome. *Pediatr. Res.*, **43**, 410–414.
8. Patrakka,J., Kestila,M., Wartiovaara,J., Ruotsalainen,V., Tissari,P., Lenkkeri,U., Mannikko,M., Visapaa,I., Holmberg,C. and Rapola,J. (2000) Congenital nephrotic syndrome (NPHS1): features resulting from different mutations in Finnish patients. *Kidney Int.*, **58**, 972–980.
9. Lenkkeri,U., Mannikko,M., McCready,P., Lamerdin,J., Gribouval,O., Niaudet,P.M., Antignac,C.K., Kashtan,C.E., Homberg,C., Olsen,A. *et al.* (1999) Structure of the gene for congenital nephrotic syndrome of the finnish type (NPHS1) and characterization of mutations. *Am. J. Hum. Genet.*, **64**, 51–61.
10. Beltcheva,O., Martin,P., Lenkkeri,U. and Tryggvason,K. (2001) Mutation spectrum in the nephrin gene (NPHS1) in congenital nephrotic syndrome. *Hum. Mutat.*, **17**, 368–373.
11. Haws,R.M., Weinberg,A.G. and Baum,M. (1992) Spontaneous remission of congenital nephrotic syndrome: a case report and review of the literature. *Pediatr. Nephrol.*, **6**, 82–84.
12. Martul,E.V., Cuesta,M.G. and Churg,I. (1987) Histopathologic variability of the congenital nephrotic syndrome. *Clin. Nephrol.*, **28**, 161–168.
13. Pelletier,J., Bruening,W., Kashtan,C.E., Mauer,S.M., Manivel,J.C., Striegel,J.E., Houghton,D.C., Junien,C., Habib,R., Fouser,L. *et al.* (1991) Germline mutations in the Wilms's tumor suppressor gene are associated with abnormal urogenital development in Denys-Drash syndrome. *Cell*, **67**, 437–447.
14. Sibley,R.K., Mahan,J., Mauer,S.M. and Vernier,R.L. (1985) A clinicopathologic study of forty-eight infants with nephrotic syndrome. *Kidney Int.*, **27**, 544–552.
15. Habib,R. (1993) Nephrotic syndrome in the 1st year of life. *Pediatr. Nephrol.*, **7**, 347–353.
16. Ruotsalainen,V., Ljungberg,P., Wartiovaara,J., Lenkkeri,U., Kestila,M., Jalanko,H., Holmberg,C. and Tryggvason,K. (1999) Nephrin is specifically located at the slit diaphragm of glomerular podocytes. *Proc. Natl Acad. Sci. USA*, **96**, 7962–7967.
17. Putaala,H., Soininen,R., Kilpelainen,P., Wartiovaara,J. and Tryggvason,K. (2001) The murine nephrin gene is specifically expressed in kidney, brain and pancreas: inactivation of the gene leads to massive proteinuria and neonatal death. *Hum. Mol. Genet.*, **10**, 1–8.
18. Shih,N.Y., Li,J., Karpitskii,V., Nguyen,A., Dustin,M.L., Kanagawa,O., Miner,J.H. and Shaw,A.S. (1999) Congenital nephrotic syndrome in mice lacking CD2-associated protein. *Science*, **286**, 312–315.
19. Dustin,M.L., Olszowy,M.W., Holdorf,A.D., Li,J., Bromley,S., Desai,N., Widder,P., Rosenberger,F., van der Merwe,P.A., Allen,P.M. and Shaw,A.S. (1998) A novel adaptor protein orchestrates receptor patterning and cytoskeletal polarity in T-cell contacts. *Cell*, **94**, 667–677.
20. Li,C., Ruotsalainen,V., Tryggvason,K., Shaw,A.S. and Miner,J.H. (2000) CD2AP is expressed with nephrin in developing podocytes and is found widely in mature kidney and elsewhere. *Am. J. Renal Physiol.*, **279**, F785–F792.

21. Topham,P.S., Kawachi,H., Haydar,S.A., Chugh,S., Addona,T.A., Charron,K.B. and Holzman,L.B. (1999) Nephritogenic mAb 5-1-6 is directed at the extracellular domain of rat nephrin. *J. Clin. Invest.*, **104**, 1559–1566.
22. Kawachi,H., Koike,H., Kurihara,H., Yaoita,E., Orikasa,M., Shia,M.A., Sakai,T., Yamamoto,T., Salant,D.J. and Shimizu,F. (2000) Cloning of rat nephrin: expression in developing glomeruli and in proteinuric states. *Kidney Int.*, **57**, 1949–1961.
23. Luimula,P., Aaltonen,P., Ahola,H., Palmen,T. and Holthofer,H. (2000) Alternatively spliced nephrin in experimental glomerular disease of the rat. *Pediatr. Res.*, **48**, 759–762.
24. Doublier,S., Ruotsalainen,V., Salvidio,G., Lupia,E., Biancone,L., Conaldi,P.G., Reponen,P., Tryggvason,K. and Camussi,G. (2001) Nephrin redistribution on podocytes is a potential mechanism for proteinuria in patients with primary acquired nephrotic syndrome. *Am. J. Pathol.*, **158**, 1723–1731.
25. Boute,N., Gribouval,O., Roselli,S., Benessy,F., Lee,H., Fuchshuber,A., Dahan,K., Gubler,M.C., Niaudet,P. and Antignac,C. (2000) NPHS2, encoding the glomerular protein podocin, is mutated in autosomal recessive steroid-resistant nephrotic syndrome. *Nat. Genet.*, **24**, 349–354.
26. Fuchshuber,A., Gribouval,O., Ronner,V., Kroiss,S., Karle,S., Brandis,M. and Hildebrandt,F. (2001) Clinical and genetic evaluation of familial steroid-responsive nephrotic syndrome in childhood. *J. Am. Soc. Nephrol.*, **12**, 374–378.
27. Fuchshuber,A., Karle,S., Uetz,B., Kroiss,S., Putz,M., Hildebrandt,F. and members of the APN study group (2001) Mutational analysis in patients with steroid resistant idiopathic nephrotic syndrome (srINS). Abstract FC8.6, 12th congress of the International Paediatric Nephrology Association, Seattle, WA, USA.
28. Caridi, G., Bertelli,R., Carrea, A., Di Duca,M., Catarsi,P., Artero,M., Carraro,M., Zennaro,C., Candiano,G., Musante,L. *et al.* (2001) Prevalence, genetics, and clinical features of patients carrying podocin mutations in steroid-resistant nonfamilial focal segmental glomerulosclerosis. *J. Am. Soc. Nephrol.*, **12**, 2742–2746.
29. Tsukaguchi,H., Abreu,P., Pereira,A. and Pollak,M. (2000) Missense mutations in podocin in a family with adult onset FSGS. ASN abstract A2179: paper presented at American Society Nephrology annual meeting, Toronto, Canada.
30. Tavernarakis,N., Driscoll,M. and Kyrpides,N.C. (1999) The SPFH domain: implicated in regulating targeted protein turnover in stomatins and other membrane-associated proteins. *Trends Biochem. Sci.*, **24**, 425–427.
31. Huber,T.B, Kottingen,M., Schilling,B., Walz,G. and Benzing,T., (2001) Interaction with podocin facilitates nephrin signaling. *J. Biol. Chem.*, **276**, 41543–41546.
32. Zhang,J., Ofevrstedt,L., Khoshnoodi,J., Skoglund,U. and Tryggvason,K. (2001) Molecular structure of recombinant nephrin by electron tomography. Abstract A2924, presented at the ASN/ISN World Congress of Nephrology, San Francisco, CA, USA.
33. Liu,L., Done,S., Khoshnoodi,J., Bertollero,A., Wartiovaara,J., Bergerren,P. and Tryggvason,K. (2001) Defective nephrin trafficking caused by missense mutations in the NPHS1 gene: Insight into the mechanisms of congenital nephrotic syndrome. *Hum. Mol. Genet.*, **10**, 2637–2644.
34. Di Benedetto,G., Erguven,A., Stenico,M., Castrfi,L., Bertorelle,G., Togan,I. and Barbujani,G. (2001) DNA diversity and population admixture in Anatolia. *Am. J. Phys. Anthropol.*, **15**, 144–156.
35. Guglielmino,C.R., De Silvestri,A. and Beres,J. (2000) Probable ancestors of Hungarian ethnic groups: an admixture analysis. *Ann. Hum. Genet.*, **64**, 145–156.
36. Guez,S., Giani,M., Melzi,M.L., Antignac,C. and Assael,B.M. (1998) Adequate clinical control of congenital nephrotic syndrome by enalapril. *Pediatr. Nephrol.*, **12**, 130–132.
37. Benigni,A., Tomasoni,S., Gagliardini,E., Zoja,C., Grunkemeyer,J.A., Kalluri,R. and Remuzzi,G. (2001) Blocking angiotensin ii synthesis/activity preserves glomerular nephrin in rats with severe nephrosis. *J. Am. Soc. Nephrol.*, **12**, 941–948.
38. Heaton,P.A., Smales,O. and Wong,W. (1999) Congenital nephrotic syndrome responsive to captopril and indomethacin. *Arch. Dis. Child*, **81**, 174–175.
39. Hamed,R.M.A and Shormaf,M. (2001) Congenital nephrotic syndrome: a clinico-pathological study of thirty children. *J. Nephrol.*, **14**, 104–109.
40. Zatz,M., Vainzof,M. and Passos-Bueno,M.R. (2000) Limb-girdle muscular dystrophy: one gene with different phenotypes, one phenotype with different genes. *Curr. Opin. Neurol.*, **5**, 511–517.
41. Shih,N.Y., Li,J., Cetran,R., Mundel,P., Miner,J.H. and Shaw,A.S. (2001) CD2AP localizes to the slit diaphragm and binds to nephrin via a novel C-terminal domain. *Am. J. Pathol.,*, **159**, 2303–2308.
42. Donoviel,D.B., Freed,D.D., Vogel,H., Potter,D.G., Hawkins,E., Barrish,J.P., Mathur,B.N., Turner,C.A., Geske,R., Montgomery,C.A. *et al.* (2001) Proteinuria and perinatal lethality in mice lacking NEPH1, a novel protein with homology to NEPHRIN. *Mol. Cell Biol.*, **21**, 4829–4836.
43. Yan,K., Khoshnoodi,J., Ruotsalainen,V. and Tryggvason,K. (2001) N-linked glycosylation is critical for plasma membrane localisation of nephrin. Abstract A0326 presented at the ASN/ISN World Congress of Nephrology, San Francisco, CA.
44. Teraoka,S.N., Telatar,M., Becker-Catania,S., Liang,T., Onengut,S., Tolun,A., Chessa,L., Sanal,O., Bernatowska,E., Gatti,R.A. and Concannon,P. (1999) Splicing defects in the ataxia-telangiectasia gene, ATM: underlying mutations and consequences. *Am. J. Hum. Genet.*, **64**, 1617–1631.
45. Altay,C., Oner,C., Oner,R., Mesci,L., Balkan,H., Tuzmen,S., Basak,A.N., Gumruk,F. and Gurgey,A. (1997) Genotype-phenotype analysis in HbS-beta-thalassemia. *Hum. Hered.*, **47**, 161–164.
46. Giordano,P.C., Harteveld,C.L., Haak,H.L., Batelaan,D., van Delft,P., Plug,R.J., Emonts,M., Zanardini,R. and Bernini,L.F. (1998) A case of non-beta-globin gene linked beta thalassaemia in a Dutch family with two additional alpha-gene defects: the common -alpha3.7 deletion and the rare IVS1-116 (A→G) acceptor splice site mutation. *Br. J. Haematol.*, **103**, 370–376.
47. Kerkhoffs,J.L., Harteveld,C.L., Wijermans,P., van Delft,P., Haak,H.L., Bernini,L.F. and Giordano,P.C. (2000) Very mild pathology in a case of Hb S/beta0-thalassemia in combination with a homozygosity for the alpha-thalassemia 3.7 kb deletion. *Hemoglobin*, **3**, 259–263.
48. Watnick,T., He,N., Wang,K., Liang,Y., Parfrey,P., Hefferton,D., St George-Hyslop,P., Germino,G. and Pei,Y. (2000) Mutations of PKD1 in ADPKD2 cysts suggest a pathogenic effect of trans-heterozygous mutations. *Nat. Genet.*, **25**, 143–144.
49. Pei,Y., Paterson,A.D., Wang,K.R., He,N., Hefferton,D., Watnick,T., Germino,G.G., Parfrey,P., Somlo,S. and St George-Hyslop,P. (2001) Bilineal disease and trans-heterozygotes in autosomal dominant polycystic kidney disease. *Am. J. Hum. Genet.*, **68**, 355–363.
50. Doray,B., Salomon,R., Amiel,J., Pelet,A., Touraine,R., Billaud,M., Attie,T., Bachy,B., Munnich,A. and Lyonnet,S. (1998) Mutation of the RET ligand, neurturin, supports multigenic inheritance in Hirschsprung disease. *Hum. Mol. Genet.*, **7**, 1449–1452.
51. Denamur,E., Bocquet,N., Mougenot,B., Da Silva,F., Martinat,L., Loirat,C., Elion,J., Bensman,A. and Ronco,P.M. (1999) Mother-to-child transmitted WT1 splice-site mutation is responsible for distinct glomerular diseases. *J. Am. Soc. Nephrol.*, **10**, 2219–2223.
52. Kohsaka,T., Tagawa,M., Takekoshi,Y., Yanagisawa,H., Tadokoro,K. and Yamada,M. (1999) Exon 9 mutations in the WT1 gene, without influencing KTS splice isoforms, are also responsible for Frasier syndrome. *Hum. Mutat.*, **14**, 466–470.
53. Hammes,A., Jian-Kan,G., Lutsch,G., Leheste,J., Landrock,D., Ziegler,U., Gubler M-C. and Schedl, A .(2001) Two splice variants of the Wilms' tumor 1 gene have distinct functions during sex determination and nephron formation. *Cell*, **160**, 319–329.
54. Katsanis,N., Ansley,S.J., Badano,J.L., Eichers,E.R., Lewis,R.A., Hoskins,B.E, Scambler,P.J., Davidson,W.S., Beales,P.L., Lupski,J.R. *et al.* (2001) *Science*, **293**, 2256–2259.
55. Katsanis,N. , Simons,M., Reiser,J., Saleem,M.A., Faul,C., Kriz,W., Shaw,A.S., Holzman,L.B. and Mundel,P. (2001) Podocin, a raft-associated component of the glomerular slit diaphragm, interacts with CDZAP and nephrin. *J. Clin Invest.*, **108**, 1621–1629.