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Cis and *trans* regulatory elements in *NPHS2* promoter: Implications in proteinuria and progression of renal diseases

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Podocin (NPHS2) expression in podocytes is associated with variable degrees of proteinuria and progression to renal failure in different glomerular diseases that suggests different expression profiles in NPHS2 promoter. Three functional polymorphisms in NPHS2 promoter (−51T, −116T, and −535 ins$CTTTTTT_3$) were found determining strong downregulation (−73, −59, and −82%, respectively) of the reporter gene expression when transfected in podocytes. Electrophoretic mobility shift assay experiments showed that all wild-type variants (−51G, −116C, and −535 ins$CTTTTTT_2$) formed specific DNA–protein complexes with podocyte nuclear extracts that were abolished by the presence of the rare forms (−51T, −116T, and −535 ins$CTTTTTT_3$). In the case of −51G, upstream stimulatory factor-1 (USF1) was identified as the specific *trans element* in accord to binding inhibition experiments and USF1 RNAi silencing. Haplotype analysis of 204 normal controls and 545 patients with renal diseases (308 immunoglobulin (Ig)A nephropathy and 237 focal segmental glomerulosclerosis) evidenced that −116/−51 and −535/P2OL formed two blocks in strong linkage disequilibrium in both normal and pathological cohorts. The high NPHS2 promoter profile −116C/−51G haplotype was more frequent in patients with IgA nephropathy (*P*-value = 0.005) and was associated with a better clinical outcome in terms of proteinuria and creatinine levels. Overall our study describes functional variants of NPHS2 promoter and characterizes *trans-acting* elements that modulate podocin expression in the kidney. High producer NPHS2 promoter haplotypes seem protective in patients with chronic glomerular diseases.

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Glomerular diseases, such as focal segmental glomerulosclerosis (FSGS) and IgA nephropathy (IgA), affect a bulk of patients evolving to end-stage renal failure (ESRF). Proteinuria is the common clinical feature and represents a key motif for disease progression.[1,2] Definition of the mechanisms involved in proteinuria, their prediction and prevention remain, together with the genetic contribution, a central theme in the current research.[3,4] Glomerular slit-diaphragm and the specialized proteins nephrin, podocin, and CD2AP, which form the ultrafiltration unit, are potential culprits of proteinuric insults, and podocin has a central role in it.[5–8] Associations of podocin defects with proteinuria and ESRF are increasingly reported in animal models and in humans.[9] Accordingly, targeted knockout of NPHS2 (the gene coding for podocin) leads to proteinuria and renal failure in mice[10] and inherited mutations of NPHS2 sequence are the major cause of nephrotic syndrome in children who invariably evolve to renal failure.[11–13]

Like several other glomerular proteins, podocin expression in proteinuric renal diseases appears to be a dynamic process that results in marked decrease of protein expression.[14,15] It is still unknown whether podocin mRNA is up or down-regulated and results of the literature lead to opposite conclusions. Koop *et al.*[14] reported a significant increase in podocin mRNA in minimal change nephropathy and FSGS, proposing a compensatory reaction of the damaged podocytes. Results by Schmid *et al.*[16] pointed to a differentiation between FSGS and minimal change disease, the former being

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characterized by downregulation of podocin mRNA glomerular expression. In spite of these inconsistencies, the real data are that podocin expression, at the protein level, is markedly downregulated and abnormally distributed in both conditions. According to structural models, this should interfere with slit-diaphragm architecture and function[7,17] and eventually influence proteinuria. It seems likely that restoration of normal podocin composition should modify this aspect and, depending on reparative capacities of the renal barrier, also play a role in the disease evolution. Podocin promoter has been located in the 2.5 kb of the 5′ terminus of NPHS2 gene (GenBank accession number AF487463) by demonstrating direct podocyte-specific expression in transgenic mice.[18,19] We recently demonstrated that the NPHS2 promoter fragment from –628 to ATG is able to specifically direct podocin expression in different cell lines including human glomerular epithelial cells.[20] We carried out a sequencing study of the promoter region from –628 to ATG in a large cohort of patients affected by glomerular diseases, looking for variants that could affect podocin expression.

RESULTS

Polymorphic variants in NPHS2 promoter

We evaluated 749 DNA from patients with primary nephrotic syndrome (237), IgA nephropathy (308) and controls (204). Criteria for enrolment are described below and clinical characteristics of the study group are shown in Table 1. All patients were of Italian origin; IgA patients represent the Italian branch afferent to the Consortium on IgA nephropathy.[21] According to regional enrolment, normal control DNA were obtained from Italian people enrolled in the same regions as patients. Sequencing of all DNA demonstrated 10 variants of *NPHS2* with a minor allele frequency higher than 1% in at least one group: seven were located in exons or flanking coding regions, three were in the promoter region up to –628 from ATG (Table 2). Rare promoter variants with an allele frequency lower than 1% had been described separately and were not considered in the present paper.[20] Variants at 5′ untranslated region underwent functional studies in order to demonstrate implication in podocin expression.

The haplotype frequencies were estimated from the sample of unrelated cases and controls by use of the

Table 1 | General and clinical features of normal controls and two different cohorts of patients with renal disease (nephrotic syndrome, IgA nephropathy) both characterized by glomerular damage and proteinuria

Number	Controls (*N*=204)	Nephrotic syndrome (*N*=237)	IgA (*N*=308)
Gender (♂)	120 (58%)	139 (59%)	241 (78%)
Mean age at onset (years)	38 (18–46)	17 (1–62)	21 (4–76)
Histology			
IgA grades 1–5			308 (100%) G1–2 (32%) G3–4 (54%) G5 (14%)
Nephrotic syndrome			
MCN		25 (21; 1–59)	
IgM		54 (9; 1–50)	
FSGS		138 (22; 1–62)	
Clinical presentation			
		Nephrotic syndrome 237 (100%)	Microematuria 241 (100%) Macroematuria 196 (81%) Proteinuria <1 g 122 (52%) proteinuria >1 g 119 (49%)
Therapy			
Steroid		232 (98%)	
Levam		17 (7%)	
Clo/Cyclo		83 (35%)	
CsA		103 (43%)	
Response to drugs			
Responders		16 (16; 2–21)	
Frequent relapse		22 (18; 1–61)	
Steroid dependent		43 (10; 2–51)	
Steroid resistant		150 (19; 1–39)	
ESRF		79 (33%)	53 (17%)

Csa, cyclosporin A; Clo/cyclo, chlorambucil/cyclophosphamide; ESRF, end-stage renal failure; FSGS, focal segmental glomerulosclerosis; IgM, IgM deposit; levam, levamisole; MCN, minimal change nephropathy. Age at onset for all categories, histology and response to drugs for the nephrotic syndrome group are expressed as mean and range.

Table 2 | Genetic variants with a minor allele frequency >1%, identified in cases and controls

Name	Location	Minor allele frequencies			Nucleotide substitution	db SNP identifier
		Controls	Nephrotic syndrome	IgA nephropathy		
−535	Promoter	0.012	0.015	0.016	$-535CTTTTTT_2>CTTTTTT_3$	
−116	Promoter	0.343	0.318	0.283	−116C>T	rs12406197
−51	Promoter	0.250	0.244	0.201	−51G>T	
P20L	Exon 1	0.000	0.013	0.003	c.59C>T	
G34G	Exon 1	0.012	0.019	0.008	c.102G>A	rs3818588
S96S	Exon 2	0.041	0.043	0.077	c.289C>T	rs3738423
R229Q	Exon 5	0.017	0.025	0.007	c.686G>A	
IVS7+7	Intron 7	0.076	0.066	0.063	IVS7+7A>G	
A318A	Exon 8	0.359	0.371	0.357	c.954T>C	rs1410592
L346L	Exon 8	0.038	0.049	0.061	c.1038A>G	rs3818587

SNP, single nucleotide polymorphism.

maximum-likehood estimates. All 46 possible pairwise D' values among cases and controls were calculated and the significance level was evaluated by the χ^2 test and permutation tests as implemented Haploview 3.2,[22] PHASE 2.0[23] and UNPHASED 2.404[24] programs. An unique recurrent marker pair was in complete linkage disequilibrium (LD) ($D'=1.00$) in controls, nephrotic syndrome, and IgA patients, that included the two variants at −51 and −116 from 5′ untranslated region that strictly correlated with distance (Figure 1a and b). In patients with nephrotic syndrome was striking the observation of the aminoacidic variant P20L only in individuals carrying the $-535CTTTTTT_3$ allele. Parent–child transmission (Figure 2) showed that these two variants are in fact in phase. Haplotype reconstruction from population genotype data confirmed this finding showing a block of linkage disequilibrium including $-535CTTTTTT_3$ /P20L/G34G. A second block included the two synonymous nucleotide variants L236L and A318A at exons 8 (Figure 1a).

The frequency of every single haplotype in cases and in controls are reported in Table 3.

Functional implication of NPHS2 promoter variants

Functional implications of each sequence variants in NPHS2 promoter were studied following consolidated recipes based on subcloning in pGL3 reporter vector and transient transfection experiments in glomerular epithelial cells (Figure 3) and COS7 cells. A significant reduction of luciferase expression by podocytes compared to the wild-type sequence was determined in all the three variants −51G>T, −116C>T, and $-535CTTTTTT_3$, producing −73, −59, and −82% reduction, respectively ($P<0.001$) (Figure 3) and were confirmed in COS7 cells (not shown). Binding of transcriptional factors, or protein complexes, and their characterization was investigated in the regions surrounding these polymorphisms.

−51G>T. Electrophoretic mobility shift assay (EMSA) experiments using podocyte nuclear extracts and oligonucleotide −51G demonstrated the formation of a slowly migrating complex that was not formed in case of the −51T (Figure 4a, tracks 1, 2). Competition studies with unlabelled −51G at three different concentrations of 100–200–400 M

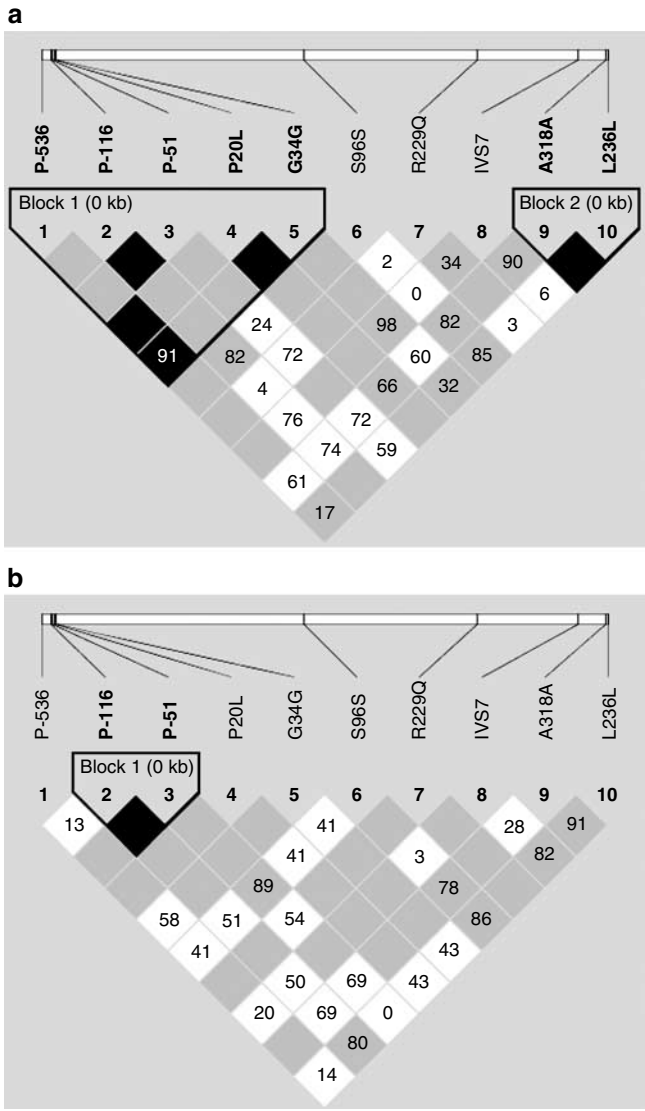

Figure 1 | Haploview showing the haplotype association of different SNPs in nephrotic and IgA patients. Pairwise LD plots between NPHS2 gene polymorphisms calculated on the cohort of (**a**) 237 nephrotic syndrome individuals and (**b**) on 308 IgA patients. Dark gray boxes correspond to pairs of polymorphisms with strong evidence of LD, light gray to uninformative pairs, white to pairs of polymorphisms with strong evidence of recombination. The numbers inside the boxes represent the D' values.

excess and lack of competition of − 51T confirmed specificity of the complex (Figure 4a, tracks 5, 6, 7, and 8). Consensus analysis for homology of −51 variant region with regulatory sequences revealed homology for two transcriptional factors, that is, USF1 and hypoxia-inducible factor 1 A. Preincubation with anti-USF1 (but not antibodies directed to another unrelated transcriptional factor such as cfos) antibodies abolished the formation of the retarded complex (Figure 4a, tracks 3 and 4), indicating that this is the transcriptional factor that binds this *cis* element. Further experiments were performed to confirm this conclusion. In a first set, degenerate (−51G: 5′-TGCGCTCC**G**GTGCCCCTAGC-3′) (the bold indicates the base that makes the difference between degenerate and typical USF E-box) and typical sequences for USF E-box (5′-TGCGCTC**AC**GTGCCCC TAGC-3′) were run in parallel and showed comigration at the same molecular weight (Figure 4b, tracks 1 and 3) whereas hypoxia-inducible factor 1 A did not form oligo-protein complexes (not shown). In the case of typical E-box, it was evident the formation of a marked protein–DNA adduct (Figure 4b, track 3) that overwhelmed by several factors the DNA–protein complex formed by degenerate sequences (compare tracks 1 and 3 in Figure 4b). The presence of anti-USF1 antibodies abolished the formation of the complex (Figure 4b, track 4) and produced a super-shift that is represented by a band with higher molecular weight and an intensity that is less than 10% of the original adduct. This suggests that only a minor part of the complex forms polymers and justify the observed lack of super-shift formation with degenerate sequences (Figure 4b, track 5). Our data support the concept that USF1 forms an adduct with a degenerate consensus sequence in NPHS2 promoter and regulates the expression of podocin. To confirm this

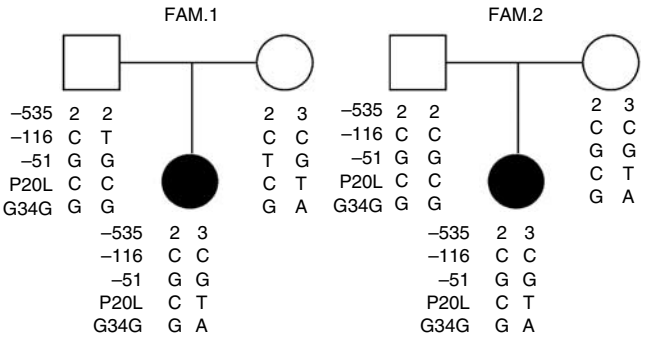

Figure 2 | Haplotypes reconstruction in two small families (out of six) in which the index case presented the P20L variant. Squares represent males, circles represent females. Filled symbols, affected individuals; open symbols, unaffected individuals.

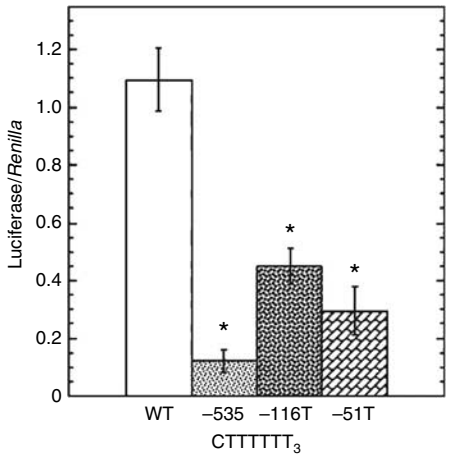

Figure 3 | Luciferase expression in podocytes transfected with constructs of the three single nucleotide polymorphisms variants −535CTTTTTT$_3$, −116C>T, and −51G>T compared to the wild-type sequences (−535CTTTTTT$_2$, −51G, −116C). In all cases, the reductions in the expression levels (−73, −59, and −82%, respectively) were highly significant ($P<0.001$) owing to the reproducibility of expression experiments.

Table 3 | Haplotypes frequencies observed in controls and cases that include nephrotic syndrome (NS) and IgA nephropathy (IgA)

Haplotype	Controls (%)	NS (%)	*P*-value	Corr. *P*-value	IgA (%)	*P*-value	Corr. *P*-value
−116/−51							
C/G	41	44	NS	NS	52	0.0006	0.005
C/T	25	24	NS	NS	20	NS	NS
T/G	34	32	NS vs controls	NS	28	0.041 vs controls	NS
−535/P20L/G34G							
−535CTTTTTT$_3$/T/A	0	1.3	0.023	0.035	0.3	NS	NS
−535CTTTTTT$_3$/C/A	1	0.2	NS	NS	0.3	NS	NS
−535CTTTTTT$_2$/C/A	0.2	0.4	NS	NS	0.2	NS	NS
−535CTTTTTT$_3$/C/G	0.2	0	NS vs controls	NS	1.2	NS vs controls	NS
A318A/L346L							
T/A	64	63	NS	NS	64	NS	NS
C/A	32	32	NS	NS	30	NS	NS
C/G	4	5	NS vs controls	NS	6	NS vs controls	NS

NS, not significant.
P-values for the case-control comparisons are reported as nominal *P*-values and corrected for multiple comparisons by permutation test (10 000 replicates).

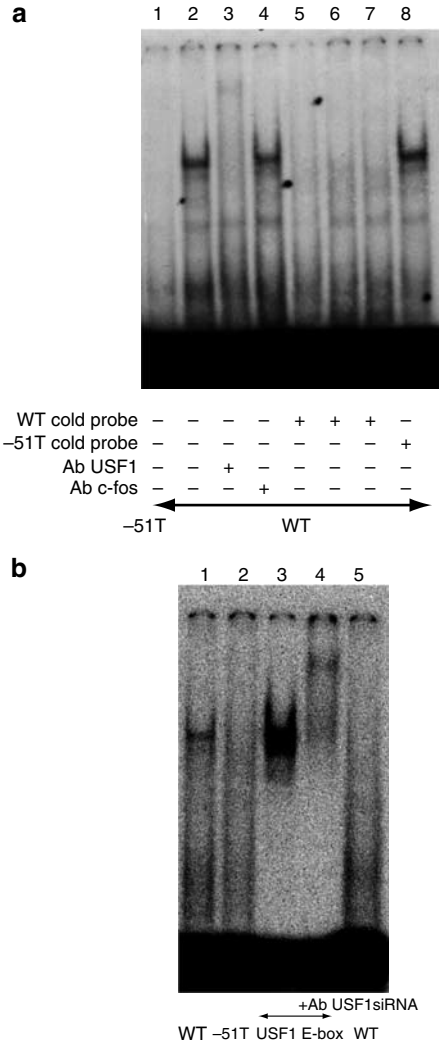

Figure 4 | (a) EMSA experiments using podocyte nuclear extracts and oligonucleotide −51G demonstrating the formation of a slowly migrating complex that was not formed in case of the less frequent variant −51T (tracks 1 and 2). Competition studies with unlabelled −51G at three different concentrations of 100–200–400 fold molar excess and lack of competition of −51T, confirmed specificity of the complex (tracks 5, 6, 7, and 8). Preincubation with anti-USF1 (but not anti-cfos) antibodies abolished the formation of the retarded complex (tracks 3 and 4) indicating that this is indeed the transcriptional factor that binds this *cis* element. (**b)** EMSA with canonical sequences for USF E-box, showing comigration at the same molecular weight of the degenerate form (tracks 1 and 3). It is evident the formation of a marked protein–DNA adduct (track 3) that overwhelms by several factors the DNA–protein complex formed by degenerate sequences (compare tracks 1 and 3). The presence of anti-USF1 antibodies abolished the formation of the complex (track 4) and produced a super-shift that is represented by a minor band with higher molecular weight and an intensity that is less than 10% of the original adduct, track 5 shows cells silenced for USF1.

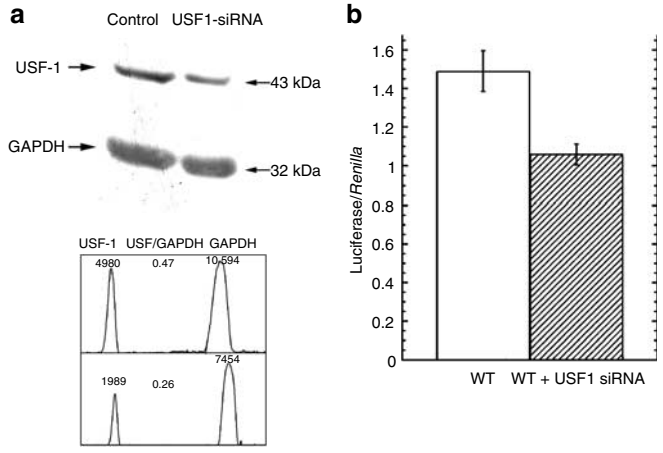

Figure 5 | Characterization of USF1 as the transcriptional factor binding –51G. USF1 RNA silencing produced (**a**) a −50% expression of USF1 at Western blot and (**b**) induced in parallel 30% reduction of luciferase expression.

point, podocytes were silenced for USF1 RNA expression and transfected with wild-type constructs containing the −51G sequence. As shown in Figure 4a and b, silencing produced a −50% expression of USF1 at Western blot (Figure 5a) and induced in parallel 30% reduction of luciferase expression (Figure 5b), a finding that conclusively supports USF1 functional implication.

−116C>T. Formation of DNA–protein complex was observed by incubation with nuclear extracts of wild-type −116C (5′-CAGCCCCCACCCGACGGTCTTTAGGGTCCC-3′) that is associated with high expression profile in the luciferase assay (Figure 6, track 2), whereas the presence of a T in the same position abolished the formation of the retarded complex (Figure 6, track 1). Also in this case, competition with unlabelled oligonucleotide 100, 200, and 400 M excess abolished the adduct formation, indicating specificity of the binding (Figure 6, tracks 3, 4 and 5). Searching databases for transcription factor binding sites failed to identify transcription factors docking to −116C.

−535CTTTTTT$_3$. Incubation of the wild-type −535CTT TTTT$_2$ with nuclear extracts did not induce the formation of any adduct. The presence of a −535CTTTTTT$_3$ at this site produced the formation of a marked protein–DNA slow migrating complex that is evident in Figure 7, track 2. As the presence of the more frequent variant is associated with a high luciferase profile whereas the rare variant observed in association with P20L in FSGS patients abolished luciferase expression by 80%, we concluded that the transcriptional factor that binds this site plays an inhibitory effect. Also in this case, analysis of consensus sequences failed to show putative transcriptional factors docking this site.

Haplotype association with nephrotic syndrome

Overall, 237 patients with primary nephrotic syndrome were analyzed. As specified in Materials and Methods section, both children and adults were included. Patients under 16 years were included if presenting sensitivity to steroids and in this case a renal biopsy was not required; other patients underwent a renal biopsy to exclude other conditions. Over 16 years, available histology was required for enrolment. As indicated in Table 1, age was the same in groups with

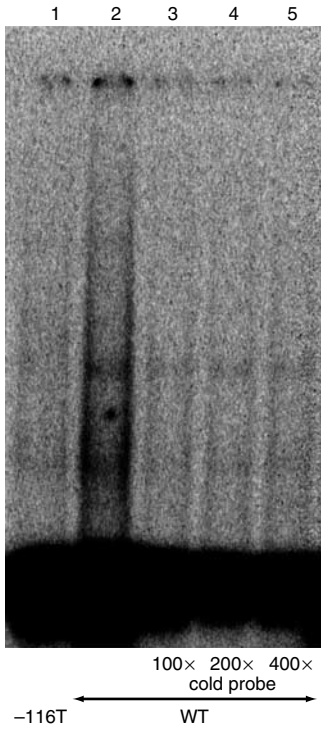

Figure 6 | EMSA experiments using podocyte nuclear extracts and oligonucleotide −116C, demonstrating the formation of a slowly migrating complex (arrow) that was not formed in case of the less frequent variant −116T (tracks 1 and 2). Competition studies with unlabelled −116C at three different concentrations of 100–200–400 fold molar excess and lack of competition of −116T confirmed specificity of the complex (tracks 3, 4 and 5).

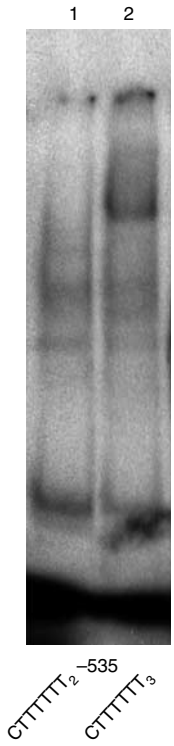

Figure 7 | EMSA experiments using podocyte nuclear extracts and oligonucleotide −535CTTTTTT$_3$ demonstrating the formation of a slowly migrating complex (track 2) that was not formed in case of the less frequent variant −535CTTTTTT$_2$ (track 1).

different histology and clinical response to drugs indicating that age is not a variable influencing these parameters. All patients with primary nephrotic syndrome were first checked for NPHS2 coding region mutations and those resulting positive were excluded from the study. Also three patients with mutations in the promoter were excluded. Details on haplotypes frequencies in patients with nephrotic syndrome are reported in Table 3. Haplotypes combinations of −116/−51 (C/G, C/T, T/G) did not present any variation in nephrotic patients and were not associated with any of the relevant clinical features, including age at onset, drugs sensitivity, histology, and progression to ESRF. The haplotype including the variants −535CTTTTTT$_3$, P20L, and G34G was found only in patients with renal disease, mostly in nephrotic patients and less frequently in patients with IgA. Haplotype comparisons using the PHASE 2.1 program[23] showed that cases are more similar to other cases than to controls, rejecting the null hypothesis at a *P*-value of 0.038 (10 000 permutations). Similarly, permutation test using Haploview 3.2[22] and UNPHASED[24] programs demonstrated a statistically significant association ($P<0.035$). This difference is due to the absence of P20L in our control population (Table 2). This finding suggests that P20L occurs on an ancestral haplotypes and produces a phenotype characterized by proteinuria when present, even as heterozygous trait.

Haplotype association with IgA

Haplotype combination frequencies and association with clinical features of IgA patients are summarized in Tables 3–5. The most relevant finding is pertinent to a discordance of frequencies of −116/−51 haplotypes combination. Patients and controls showed a statistically significant difference in haplotypes similarity (*P*-value 0.003, 10 000 permutations) according to which C/G is more frequent in IgA patients (corrected *P*-value = 0.005, Table 3) and is also associated with main clinical features such as lower proteinuria and serum creatinine level (CL) during the follow-up (Tables 4 and 5). In the former case, the association was evaluated in 197 patients with proteinuria >0.2 g/24 h at onset who had a follow-up of at least 2 years; in those who were followed for more than 5 years (136 patients) the presence of −116C/−51G haplotypes represented the unique factor inversely correlated to high proteinuria at both univariate analysis odds ratio (OR: 0.28, CL: 0.08–0.99, $P=0.026$) and multivariate analysis (OR: 0.24, CL: 0.07–0.89, $P=0.015$). A parallel association, but in a shorter follow-up, was observed between −116C/−51G and CL that were less than 1.2 mg% in those who were followed for <5 years (OR: 0.34, CL: 0.13–0.89, $P=0.029$) suggesting that proteinuria and evolution to ESRF are linked to the NPHS2 promoter background in the frame of a multifactorial pathogenesis.

Table 4 | Association between presence of −116C/−51G haplotype in NPHS2 promoter and a high blood creatinine level at the end of the follow-up period of 5 years in patients with IgA nephrites

		Univariate analysis			Multivariate analysis[a]		
−116C/−51G	Number of patients	OR	95% CI	P	OR	95% CI	P
Whole period				0.116			0.142
No	69	1 (ref.)	—		1 (ref.)	—	
Yes	184	0.64	*0.37; 1.1*		0.63	*0.34; 1.2*	
⩽5 years				**0.029**			**0.052**
No	29	1 (ref.)	—		1 (ref.)	—	
Yes	51	**0.34**	***0.13; 0.89***		**0.36**	***0.12; 1.0***	
>5 years				0.814			0.654
No	40	1 (ref.)	—		1 (ref.)	—	
Yes	133	0.92	*0.45; 1.9*		0.83	*0.38; 1.9*	

CI, confidence interval; OR, odds ratio; ref., reference.
Bold indicates statistical different OD, italic shows the 95% CI range.
[a]Adjusted for age at the onset and gender.

Table 5 | Association between presence of haplotype −116C/−51G in NPHS2 promoter and a high proteinuria level measured at the end of the follow-up period, in patients with IgA nephropathy and proteinuria at the onset >0.2 g

		Univariate analysis			Multivariate analysis[a]		
−116C/−51G	Number of patients	OR	95% CI	P	OR	95% CI	P
Whole period				0.024			0.015
No	54	1 (ref.)	—		1 (ref.)	—	
Yes	143	0.37	*0.15; 0.94*		0.34	*0.13; 0.87*	
⩽5 years				0.578			0.441
No	23	1 (ref.)	—		1 (ref.)	—	
Yes	38	0.66	*0.15; 2.9*		0.54	*0.11; 2.7*	
>5 years				**0.026**			**0.015**
No	31	1 (ref.)	—		1 (ref.)	—	
Yes	105	**0.28**	***0.08; 0.99***		**0.24**	***0.07; 0.89***	

CI, confidence interval; OR, odds ratio; ref., reference.
Bold indicates statistical different OD, italic shows the 95% CI range.
[a]Adjusted for age at the onset and gender.

DISCUSSION

Podocin has a central role in maintaining the slit-diaphragm homeostasis, connecting the main slit protein nephrin to the cell body, allowing flexibility to the whole complex. According to this concept, podocin knockout is linked to proteinuria and renal failure in mice[10] and the same occurs in patients with homozygous/compound-heterozygous mutations of the NPHS2 gene.[11–13] Functional integrity of the slit diaphragm not only requires normal podocin but also sufficient amount of protein. During proteinuria, podocin and nephrin are lost from the podocyte[14–16,25,26] body and should be restored by re-synthesis. We recently demonstrated that the region spanning from –628 base pairs to the ATG of the NPHS2 gene is able to specifically direct podocin expression in several cell lines.[20] Here we show the presence of three new variants, with a minor allele frequency >1%, suitable for case–control association studies. This region is organized, together with variants in the NPHS2 gene, in haplotype blocks, one of which (−116/−51) is in strict linkage disequilibrium in cases and controls. A second haplotype (−535CTTTTTT$_3$/P20L/G34G) appears to be represented only in patients, mainly the subgroup affected by nephrotic syndrome. All the above sequences were first evaluated on functional basis utilizing specific expression assays and gel-retardations experiments and were found critical in driving cell expression of the reporter gene. Any variant at each site determines a drastic downregulation of reporter expression up to 80% indicating the possibility of high- and low-profile NPHS2 promoter haplotypes.

A significant part of our work was devoted to the characterization of putative *trans*-acting elements, to their specificity for the binding sites and specific regulatory proteins. We furnished conclusive information on the binding at −51G of USF1, that was identified on the basis of inhibition of the DNA–protein adduct by specific antibodies and of reduction of expression in podocytes with reduced USF1 expression by the RNA interference. Therefore, USF1 has to be considered as a main regulatory factor of podocin expression in human glomeruli. Analysis of USF1 regulation should be extended to renal diseases characterized

by glomerular damage and proteinuria. As for the other two *cis* elements located at −535 and −116, the molecular approach led to the demonstration of clear DNA–protein binding, but the identity of the *trans* factors remained unknown. For the −535 it was found a clear binding with the rare allele characterized by insCTTTTTT that was abolished in the case of the more frequent allele with $-535CTTTTTT_2$. As the former allele is also associated with low promoter activity, we could conclude for a protein with inhibitory activity on podocin expression. An opposite conclusion was reached for −116, that forms a clear DNA–protein adduct in presence of a C that is abolished by the presence of a T at this site that characterizes the low producer variant. In both cases, lack of specific consensus sequences for any described transcriptional factors blocked any evolution on characterization of *trans*-acting elements and experiments using antibodies for the most common transcription factors were, consequently, unsuccessful. Association studies between clinical characteristics and outcomes, and haplotypes linked with different podocin expression profiles were carried out in one cohort of patients with IgA and one with nephrotic syndrome. In the former case, a clear association was observed between the −116C/−51G haplotype and reduction of proteinuria over years, that results in an improved preservation of renal function. The alleles we analyzed are likely to be old and the effect of the haplotype is neutral in the absence of renal disease. This is expected according to the fact that these are common variants that act as genetic modifiers on a multifactorial background. The observation of a higher frequency of the haplotype −116C/−51G in patients with IgA fits in an evolutional theory of natural selection: we can in fact hypothesize that this haplotype was subjected to a positive selective pressure as protective in a population with high prevalence of glomerular diseases. This point seems particularly interesting as prospects a new interpretative key of the mechanism leading to progression of IgA that have been historically linked to proteinuria.[27–30] Preservation of an intact slit diaphragm structure appears determinant in this setting and we support the basic application of NPHS2 promoter haplotypes study in trials on long-term outcome in IgA patients that utilize drugs that may modify the slit-diaphragm composition such as angiotensin-converting enzyme inhibithors.

As for FSGS, we could show the association of P20L with $-535CTTTTTT_3$ that was uniquely observed in such patients and in two cases with IgA. P20L is a mutation affecting exon 1 of NPHS2, not associated with functional anomalies, that has been found almost exclusively in patients with FSGS. For this reason, it was considered as a rare variant of unknown significance. The uniqueness of the association of P20L with the low producer $-535CTTTTTT_3$ variant in a single haplotype suggests strict implication in proteinuria, even if the functional effect of a heterozygous alteration in a recessive trait appears unusual and can only be explained on the basis of haplo-insufficiency. The concomitance of mild functional defect conferred by the amino-acid change (P20L) and the very low-expression profile conferred by the promoter variant ($-535CTTTTTT_3$) appears necessary for the phenotype in these patients. Apart this main finding, we could not demonstrate any association of NPHS2 promoter haplotypes with different forms of primary nephrotic syndrome. In particular, no difference related to the age and/or between FSGS vs other histology variants were found that were expected on the basis of data on podocin expression differences. Probably, other works should focus on glomerular podocin expression in different clinical and pathologic variants before any conclusion on a key aspect of primary nephrotic syndrome.

In conclusion, our study describes three functional polymorphisms in NPHS2 promoter, two of which appear to be important in determining proteinuria and long-term outcome in patients with glomerular diseases. In one case, USF1 was characterized as the *trans* element that binds specifically −51G and markedly modify the podocin profile. Future studies should consider NPHS2 functional haplotypes and *trans* element glomerular expression in different renal pathologies.

MATERIALS AND METHODS

Patient cohorts

We screened 545 patients with glomerular disease (nephrotic syndrome and IgA) and 204 healthy blood donors controls. For all we had an informed consent for molecular studies on their DNA. For individuals below age 18 years the consent was given by one parent.

Nephrotic syndrome. The patients cohort was represented by 237 cases (Table 1). Consolidated therapeutic schemes were adopted.[31,32] Renal histology was available in 217 cases: 138 had a diagnosis of FSGS based on the histological evidence of at least one segmental area of glomerulosclerosis, 54 presented mesangial IgM deposition, and 25 minimal change nephropathy.[33] Most presented strict resistance to steroids and were then treated with drug associations, including cyclosporine. Evolution to ESRF was documented in 79 patients (33.3%). These patients represent the cohort without NPHS2 mutation of an originally group of 267 patients with nephrotic syndrome who had been checked for mutation of NPHS2 coding region. Twenty-seven patients with causative mutations in the coding region and three with rare variants in the promoter were excluded from the study.

IgA nephropathy. We recruited 308 patients with biopsy-proven IgA (Table 1).

NPHS2 DNA sequence

Molecular analysis of podocin coding region was performed by direct sequencing as already described.[12,34,35]

NPHS2 promoter sequence

NPHS2 promoter from −628 to ATG was sequenced as above using the following polymerase chain reaction primers: forward: 5′-GA AAGTTGGGGATGAGGCGA-3′; reverse: 5′-CAATCAAAGCTTCCT CAGAGCTGCCGGGCGGCT-3′.

NPHS2 promoter constructs-luciferase assay

NPHS2 promoter polymerase chain reaction products were subcloned into the luciferase pGL3 reporter vector (Promega Inc.,

Madison, WI, USA). All clones, bearing a specific mutation (pPL1–pPL4) and the wild-type clone (pPL5), were sequenced prior transfection. Luciferase activity was determined in a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA, USA) according to the Promega luciferase assay (Promega Inc., Madison, WI, USA) and results were normalized to the cotransfected *Renilla* luminescence. Results are given as arbitrary units.[36]

Cell culture

COS7 (monkey renal fibroblasts) were obtained from American Type Culture Collection (Rockville, MD, USA) and were grown under humidified atmosphere of 5% CO_2 at 97°C in Dulbecco's modified Eagle's medium supplemented with 10% calf serum, 1% L-glutamine, 100 μg/ml streptomycin, 100 U/ml penicillin. An immortalized glomerular epithelial cell line was cultured in Dulbecco's modified Eagle's medium containing 25 mM glucose, 10% inactivated fetal calf serum, 100 U/ml penicillin, and 100 mg/ml streptomycin at 37°C.[25]

Transfection experiments

Transfection experiments were performed with standard podocytes, podocytes silenced for USF1 and COS7, using 4 μg plasmids following the polyethilenimine (25 kDa polyethyleneimine, Sigma, St Louis, MO, USA) procedure. Thirty microliters of a 10 μM polyethyleneimine solution were added to 10 μg DNA and incubated at 37°C for 3 h in 5% CO_2. Cells were incubated for 48 h before being processed with the luciferase assay.

Nuclear extracts and cellular fractionation

Podocytes were scraped with a rubber policeman, pelleted at 600 *g* for 10 min and resuspended in 200 μl/100 mm plate of 20 mM *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid] (pH 7.9), 1 mM ethylenediaminetetraacetic acid, 1 mM dithiotreitol, 0.5 mM phenylmethylsulfonyl flouride, 1 μg/ml of leupeptin, pepstatin, and aprotinin, 1 mM sodium vanadate, 10 mM sodium fluoride, and kept at 4°C for 15 min. Cell suspension was added with 1/4 volume of 1% NP40 to obtain a final concentration of 0.2% NP40, incubated at 4°C for 15 min and centrifuged at 600 *g* for 15 min. The supernatant (S1) was saved for further processing, whereas the pellet (P1), was resuspended in 1 volume of low salt buffer (LSB) (20 mM *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid] (pH 7.9), 1.5 mM $MgCl_2$, 0.2 mM ethylenediaminetetraacetic acid, 0.5 mM dithiotreitol, 0.5 mM phenylmethylsulfonyl flouride, 80 mM NaCl, 25% glycerol). After extensive mixing, 1 volume of high salt buffer (HSB) (as in BLS, containing 0.9 M NaCl) was added and incubated at 4°C for 30 min. P1 was centrifuged at 16 000 *g* for 30 min. The supernatant fraction (S2) was collected and used as nuclear extract for gel retardation assay.

Transient siRNA-mediated silencing of USF1

Transient transfection of small interfering RNA (siRNA) for targeting endogenous gene silencing was performed using polyethyleneimine 25 kDa and 200 nM siRNA duplex per well. The following Qiagen siRNA oligonucleotides (Hs_USF_1_HP siRNA, catalog number SI00094815) were used: sense: r(GAUUAGAGGUC GUCAUCAA) dTdT; antisense: r(UUGAUGACGACCUCUAAUC) dCdG, corresponding to base pairs between 1092 and 1112 downstream the start codon sequence GAUUAGAGGUCGUCAU CAA. Cells were assayed for 48 h after transfection.

Statistics

Haplotype reconstruction and case–control permutation tests were performed using Haploview 3.2,[22] PHASE 2.0,[23] and UNPHASED 2.404[24] programs. The Haplotypes inference was carried out through Haploview 3.2, confirmed using PHASE 2.0, analyzing cases alone, controls alone, and the whole data set. The pair-wise linkage disequilibrium statistic (D') was calculated using Haploview 3.2 with the solid spine of LD algorithm.[37] A triangular matrix of D' was used to demonstrate LD patterns in cases and controls. Case–control association study was first conducted using PHASE program, testing the null hypothesis that cases and controls haplotypes are random samples from a single set of haplotype frequencies, vs the alternative hypothesis that cases are more similar to each other than to controls. In this scenario the test gives a global *P*-value that reflects the similarity of the haplotypes between cases and controls. This test has power even when every haplotype in the data set is unique. For this analysis, 100 iterations and 10 000 permutations were run. The results have been confirmed with an independent analysis using the UNPHASED program, running 10 000 permutations as well. The haplotypes that gave significant global *P*-values were then tested for association by χ^2-test and the significance values corrected through permutation test as implemented in the Haploview 3.2 software.

The association between the presence of NPHS2 promoter haplotypes and creatinine and proteinuria levels, in patients with a length of follow-up $\leqslant 5$ years, was assessed in terms of OR by a logistic regression model, adjusting for the confounder effects of gender and age at the onset. OR values >1 indicated a positive association, whereas values <1 indicated a protective effect of the haplotype. Thersholds for normality were 1.2 mg/dl for CL and 0.2 g/day for proteinuria.

Statistical significance of OR ($\alpha = 0.05$) was assessed via likelihood ratio test and the corresponding 95% confidence intervals obtained by the Wald method, which assumes a log-normal distribution for the OR estimates. Logistic LP models were fitted using STATA software, release 7.0 (STATA Corp. LP, TX, USA, 2001).

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