Original Article

Bigenic heterozygosity and the development of steroid-resistant focal segmental glomerulosclerosis

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Abstract

Background. Focal segmental glomerulosclerosis (FSGS) is a major cause of steroid-resistant nephrotic syndrome in childhood with a central role for the podocytes in the pathogenesis. Mutated proteins expressed in podocytes cause proteinuria. The role of combined gene defects in the development of FSGS is less clear.

Methods. We analysed seven podocyte genes known to cause proteinuria and FSGS in a group of 19 non-familial childhood-onset steroid-resistant FSGS patients. These genes include NPHS1, NPHS2, ACTN4, CD2AP, WT-1, TRPC6 and PLCE1. We also screened for the mitochondrial A3243G DNA transition associated with the MELAS syndrome (mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes), and occasionally FSGS.

Results. No mutations were found in the ACTN4 and TRPC6 genes, and no mitochondrial A3243G DNA transition was found in our group of patients. Two patients showed mutations in the CD2AP gene, one combined with an NPHS2 mutation. A tri-allelic hit was found in a patient carrying compound heterozygous NPHS2 mutations and a heterozygous NPHS1 mutation. In another patient a de novo WT-1 mutation was found combined with a heterozygous NPHS1 mutation, and finally two patients showed three heterozygous PLCE1 mutations.

Conclusions. In our rather small group of 19 steroid-resistant FSGS patients, we found 11 mutations in podocyte genes in 6 patients. In four of them the found mutations could explain the pathology. Our data suggest that combined gene defects in podocyte genes may play a role in the development of FSGS.

Keywords: bigenic; CD2AP; focal segmental glomerulosclerosis in podocin

Introduction

Focal segmental glomerulosclerosis (FSGS) is a major cause of steroid-resistant nephrotic syndrome in childhood, accounting for about 75% of patients and frequently leading to end-stage renal disease. The aetiology of primary FSGS remains unknown in most cases. The recurrence of the disease after renal transplantation in approximately one-third of the patients suggests the presence of a ‘circulating FSGS factor’, which until now has not been identified [1].

Molecular studies in humans and mouse models revealed a central role of glomerular podocyte damage in the development of FSGS [2]. A growing number of proteins, expressed by podocytes, contributing to the structure of the slit diaphragm (nephrin, podocin and CD2AP) and/or function of the cytoskeleton (alpha-actinin 4) of the podocyte, cause proteinuria when mutated [3–6]. Mutations in the WT1 transcription factor [7] and in TRPC6 [8], a calcium-permeable cation channel, also lead to abnormal podocyte function and proteinuria. More recently, mutations in PLCE1, encoding a phospolipase involved in the initiation of a cascade of cellular processes resulting in cell growth, cell differentiation and gene expression, were found in familial FSGS patients [9]. The mitochondrial A3243G transition is mainly associated with the MELAS syndrome (mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes) [10], but is also found in FSGS patients possibly with or without maternally inherited diabetes and/or sensorineural hearing loss [11,12].

It might be suggested that the degree of podocyte dysfunction determines whether the patient would develop congenital nephrotic syndrome or nephrotic syndrome due to FSGS later in life. Studies in mice demonstrated that bigenic heterozygosity in podocyte genes (CD2AP± with Fyn± or Synpo± mice) led to the development of proteinuria and FSGS-like renal damage while isolated haploinsufficiency did not cause renal disease [13]. Whether combined defects in podocyte genes play a role in the development of human FSGS is less clear. In this study, we have therefore investigated the occurrence of mutations of seven podocyte genes
in a group of non-familial childhood-onset steroid-resistant FSGS patients.

**Subjects and methods**

**Patients**

Nineteen patients, aged 10–162 (average 72) months, 13 males, with steroid-resistant nephrotic syndrome due to biopsy-proven FSGS were analysed. Steroid resistance was defined as persistent proteinuria after 6 weeks of prednisone treatment (60 mg/m²/day). The relevant clinical data of the patients are provided in Table 1.

**Molecular genetic studies**

DNA was isolated from peripheral blood leukocytes and urine sediments were collected from non-transplanted patients to isolate DNA using a commercially available DNA isolation kit (Puregene™ DNA isolation kit, Gentra systems, MN, USA).

Amplification of the **NPHS1** (GenBank accession number AF190637-AF035835), **NPHS2** (AJ279246-AJ279253), **CD2AP** (AF164377/NT_007592), **WT-1** (AH003034), **ACTN4** (NM_004924.2), **TRPC6** (NP_004612) and **PLCE1** (NM_016341/NT_030059) exons was performed by PCR. Primer data are available on request. The 5′-UTR region of **NPHS1** was also analysed.

The PCR products were analysed by DNA sequencing (Dye Terminator Cycle Sequencing, PE Applied Biosystems, Foster City, CA, USA). The genomic DNA from 150 healthy ethnically matched control individuals was used to confirm novel mutations.

Screening for the mtDNA A3243G transition (MELAS mutation) was performed by PCR using the following primers: 5′-CAACTTAGTTATTTACATTC-3′ and 5′-ATTAGAATGGGTACAATGAGGA-3′, leading to a PCR product of 162 bp. PCR products were subsequently digested overnight with Apal restriction enzyme. The presence of the A3243G mutation results in an additional restriction site on position 104 bp of the PCR product. The restriction fragments were analysed on a 1% agarose gel containing ethidium bromide to visualize the DNA. Due to the heteroplasmic state of the mitochondrial mutation and the differences in threshold values of tissues, mutation analysis was performed using DNA from peripheral blood leukocytes and from urine sediment.

**Results**

All patients presented with steroid-resistant FSGS. Treatment regimen after a renal biopsy and response to this treatment are listed in Table 1.

Not a single patient had a mutation in the **ACTN4** or **TRPC6** gene and no MELAS mutation in the mtDNA was found. Four patients (21%) had mutations in the **WT1**, **NPHS1**, **NPHS2** and/or **CD2AP** genes that could explain the pathology (see Table 2 and Figures 1 and 2). In addition, two patients showed three heterozygous **PLCE1** mutations (see Table 2). The two missense mutations in patient 6 originated from the paternal allele. Short clinical summaries and the results of DNA analysis of the four patients in which the disease-causing mutations have been established are provided below.
### Table 2. Mutations detected in non-familial steroid-resistant FSGS patients

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Patient (P) numbers 1–6 correspond with the numbers in Table 1.


### Patient 1

A white male, the only child of two healthy parents, presented at the age of 3.5 years with an upper airway infection and oedema. Physical examination revealed severe peripheral oedema and ascites. Blood pressure was 120/83 mmHg. Urinalysis showed proteinuria 6 g/l and microscopic haematuria. Serum creatinine was 79 µmol/l, albumin –26 g/l and cholesterol 10.8 mmol/l. Complement factors C3 and C4 were normal. As 6 weeks of prednisone treatment 60 mg/m²/day gave no improvement, a renal biopsy was performed. Microscopic examination of renal tissue revealed FSGS. Patient 1 was treated with cyclosporin, a low dose of prednisone and enalapril without clinical response. End-stage renal disease developed at the age of 8 years, when haemodialysis was initiated. Renal transplantation with a post-mortal graft was performed a half year later. Native kidneys remained in situ. Because of delayed graft function, the renal biopsy was performed 2 weeks after transplantation and demonstrated profound acute tubular necrosis and podocyte foot effacement. Recurrence of FSGS in the renal graft was suggested and treated with plasma exchange with only slight effect on renal function and proteinuria. At present he is 10 years old, and has persisting nephrotic syndrome and pre-terminal renal failure.

Together with the earlier reported *NPSH2* (A208T) mutation of maternal origin [14], a heterozygous mutation of *CD2AP* (1488 G>A, M496I) (Figure 1a), not described before, of paternal origin was found. The amino acid substitution in podocin replaces the aliphatic amino acid alanine by threonine, an amino acid with a sulphur-containing side chain. Using a standard phosphorylation prediction site (Netphos 2.0), the presence of threonine in the mutated podocin protein may introduce an additional phosphorylation site for the protein kinase CKI and may therefore alter the secondary structure and possibly its oligomerization. The mutation is situated at the C-terminus responsible for interactions with nephrin and *CD2AP* [15] that may be affected by the mutation as well. The *CD2AP* (M496I) substitution results in the replacement of the sulphur-containing amino acid methionine in the aliphatic amino acid isoleucine. The mutation is located between the proline-rich region and the coiled-coil domain at the C-terminus. Both parents had a normal serum creatinine and no proteinuria.

### Patient 2

A white female presented at the age of 3 years with mild periorbital and pretibial oedema and slightly elevated blood pressure (120/70 mmHg). Urinalysis revealed proteinuria 4–8 g/l and microscopic haematuria. Serum creatinine was in the normal range (21 µmol/l). During the 6-week treatment with prednisone (60 mg/m²/day) the oedema became more pronounced. A biopsy was performed. Light microscopy showed collapse of some capillary loops with moderate sclerosis. Immunofluorescence demonstrated mild staining for IgM, C1q and C3 in the mesangium. Electron microscopy revealed fusion of the podocyte foot processes. Cyclophosphamide treatment gave no improvement. Her renal function gradually decreased. She received a post-mortal graft at the age of 5.5 years. The nephrotic syndrome did not yet recur.

Patient 2 presented a tri-allelic hit (Figure 1b and c) [16]. The heterozygous *NPSH1* mutation (791 C>G, P264R) has been described before [16]. The amino acid substitution is situated in the extracellular domain of nephrin, in the third immunoglobulin motif. The cyclic amino acid proline is replaced by the basic amino acid arginine. Two heterozygous *NPSH2* mutations were also found in this patient. The
first mutation (413G>A, R138Q), paternally inherited, is already known [4]. The amino acid substitution situated at the C-terminus involves the replacement of the basic amino acid arginine to the acidic amino acid glutamine. The second one (948 del T) has not been described before and results in a truncated protein and a premature stop codon 31 amino acids downstream. This mutation is situated in exon 8 and is responsible for an aberrant C-terminus of podocin. The mother had the NPSH1 mutation and the NPSH2 deletion. Two brothers of the patient were also screened for the presence of the NPSH1/NPSH2 mutations (Figure 2b). Both brothers were found to have the paternal R138Q NPSH2 mutation and one also showed the maternal P264R NPSH1 substitution. Both brothers, as well as both parents, had normal serum creatinine and no proteinuria.

Patient 3
Patient 3, a white female, presented at the age of 8.5 years with general malaise and abdominal pain. Physical examination was unremarkable; there was no peripheral oedema. Blood pressure was 120/80 mmHg. Urinalysis revealed 14.5 g/l protein and 25–50 erythrocytes per high power field. Serum creatinine was 51 µmol/l, albumin –23 g/l and cholesterol –8.6 mmol/l. Complement factors C3 and C4 were normal. Hepatitis B, C and HIV serology were negative. Renal ultrasound was normal. She did not respond to the initial treatment with prednisone 60 mg/m²/day during 6 weeks. Light microscopic examination of renal tissue revealed FSGS in 4 glomeruli and slight mesangial hypercellularity in the other 10 glomeruli. Patient 3 was treated with cyclosporin A and low-dose prednisone with an initially partial response: decrease of proteinuria to 1–2 g/l and increase of serum albumin to 34–37 g/l. The treatment with enalapril had to be discontinued because of the development of urticaria. Four years later, she developed overt nephrotic syndrome despite the continuation of cyclosporin treatment and the addition of prednisone and angiotensin II receptor blocker. Renal function deteriorated rapidly with the development of end-stage renal disease at the age of 14.5 years. After the initiation of haemodialysis, bilateral nephrectomy was performed because of the persistent nephrotic syndrome and severe hypertension. At the age of 16 years she received a post-mortal renal graft. Two and a half years later she developed proteinuria (0.9 g/l) due to acute rejection without any signs of the recurrence of FSGS on renal biopsy. Treatment by methyl prednisolone was successful. The patient is currently 19 years old and has a well-functioning renal graft.

In addition to an earlier reported de novo WT-1 mutation [14], she has a heterozygous mutation of NPSH1 (1126C>G, L376V), already previously reported [3]. The
heterozygous *NPHS1* mutation was of maternal origin. The mother had no symptoms.

**Patient 4**

This patient has a homozygous *CD2AP* mutation (1834 C>A, R612X) and is the subject of a separate report [17].

**Discussion**

This is the first report on DNA analysis of seven podocyte genes in a series of childhood-onset non-familial biopsy-proven FSGS patients. Although this series of patients is rather small, it revealed interesting data suggesting that the combined haploinsufficiency in two podocyte genes might be responsible for the development of FSGS in humans.

Patient 1 had a combination of a previously reported maternal *NPHS2* mutation [14] and a novel paternal *CD2AP* mutation. Both parents carrying one of these mutations exhibited no renal disease. This finding is extremely interesting in light of the recent studies in mouse models of FSGS, demonstrating that heterozygous *CD2AP* mutations might sensitize podocytes to mutations of other genes (synaptopodin and Fyn proto-oncogene) while isolated heterozygous mutations in these genes did not result in the development of FSGS [13]. Our data suggest that in patients with FSGS and heterozygous *NPHS2* mutations, the additional analysis of the *CD2AP* gene is warranted.

A tri-allelic digenic inheritance observed in our patient 2 has been previously reported in five patients in the series of Koziell *et al.* [16]. Both *NPHS2* mutations are situated in the nephrin-binding domain (amino acid 125–385) [15]. Most probably the compound heterozygous state of the *NPHS2* mutations is causative for the development of FSGS. This is strengthened by the fact that one of the patient's siblings carries the heterozygous R138Q *NPHS2* and P264R *NPHS1* mutations and did not show any proteinuria. Furthermore, the *NPHS1* mutation is not situated in the podocin interacting region of amino acid 1167–1256 [18].

In patient 3 the heterozygous *de novo* intron 9 splice-site mutation of *WT1* is probably responsible for the renal phenotype. This mutation is previously found in patients with the Frasier syndrome (nephrotic syndrome with FSGS, male pseudo-hermaphroditism and a higher risk of developing gonadaloblastoma). *WT1* intron 9 splice-site mutations are responsible for a disturbed DNA binding capacity of this transcription factor [19]. The role of the additional maternal inherited *NPHS1* gene mutation is unclear. The *NPHS1* mutation (L376V) is first described in a patient with congenital nephrotic syndrome of the Finnish type (large placenta, proteinuria at birth with nephrotic syndrome appearing during the first weeks of life). The patient was homozygous for one mutation (P368S) and had the L376V mutation in only one allele [3].

In one patient (number 5) a single *PLCE1* mutation was found (E603X). In the familial cases of nephrotic syndrome, two patients of one out of six families investigated were found to have FSGS [9]. The biopsies of the other families revealed diffuse mesangial sclerosis (DMS). The histological differentiation between childhood-onset FSGS and infantile DMS is however not a sinecure. The single heterozygous mutation is most probably not causative for developing FSGS since both parents, and therefore also one of the carriers of this mutation, show no renal abnormalities. In a second patient (number 6) we observed two novel missense mutations in the *PLCE1* gene (T1164M and S1177F). Both mutations originated from the paternal allele and could therefore not explain the clinical pathology. Although *PLCE1* knockout mice did not show any nephrosis-like phenotype [9, 20], knocking down the zebrafish ortholog of *PLCE1* showed foot process effacement and disorganization of the slit diaphragms. Nothing can be said about any possible effect on the glomerular filtration barrier when the zebrafish ortholog of *PLCE1* was knocked down only partially. It is not unlikely that, as in patient 1, future studies might reveal another gene to be involved in the pathogenesis of FSGS in patient 5 and maybe 6.

The meaning of a heterozygous mutation in developing a renal phenotype is not clear [21]. A single mutation in a recessive disorder is unable to induce a pathologic effect. The second mutation may have gone unnoticed or that another gene(s) may produce an additive effect. This additive effect could be the mutation in *CD2AP* in our first patient. The observed mutations in patient 1 are highly interesting in this respect. Podocin is a protein of 383 amino acids with a membrane domain and two cytoplasmic ends at the C- and N-terminus [4]. Podocin oligomers associate in a lipid raft at the podocyte membrane and clusters nephrin. *CD2AP* is an adaptor protein that functions as an integral member of the slit-diaphragm complex by interacting with podocin and nephrin and anchoring them to the actin cytoskeleton. Podocin interacts through its C-terminal end (amino acid 125–385) with *CD2AP* and nephrin [15]. The heterozygous *NPHS2* mutation in our patient was located inside the podocin interaction region and may impair the interaction with *CD2AP*. Two patients with FSGS and a mutation affecting the splice acceptor of exon 7 of *CD2AP* in one allele, described by Kim *et al.* [6], resulted in a truncated protein. The paternally inherited *CD2AP* mutation in our patient is located between the proline-rich region and the coiled-coil domain of the *CD2AP* protein. It is probable that the mutation in our patient affects the function of *CD2AP* to a lesser degree than in the patients described by Kim *et al.* [6]. The *CD2AP* mutation alone is not responsible for the development of FSGS. The father with the heterozygous mutation of *CD2AP* had a normal serum creatinine and no proteinuria.

New genetic defects explaining FSGS will be revealed in the near future. For instance, missense mutations in *LAMB2* encoding laminin β2 may cause FSGS without eye abnormalities [22]. Analyses of *LAMB2* were not performed in our group since patients with this disorder rarely lack eye abnormalities. The knowledge of a genetic defect is important and aggressive treatment of the nephrotic syndrome can be avoided and the prognosis of the renal transplant can be predicted although the finding of a genetic defect does not exclude the recurrence of nephrotic syndrome in the renal graft [23, 24, 25].

Our data demonstrate that combined genetic defects in podocyte genes may play a role in the development of FSGS in humans. Altered interactions between several podocyte...
proteins can make podocytes vulnerable for the ‘second hit’ factors and result in genetic susceptibility of a subset of patients. Further studies of the numerous podocyte genes coming from human and animal studies will provide new insights into the pathophysiology of FSGS in humans.

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Conflict of interest statement. None declared.

References


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