RESEARCH NOTE

Novel and known nephrin gene (*NPHS1*) mutations in two Greek cases with congenital nephrotic syndrome including a complex genotype

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[Fylaktou I., Megremis S., Mitsioni A., Kitsiou-Tzeli S., Kosma K., Bitsori M., Stefanidis C. J., Kanavakis E. and Traeger Synodinos J. 2013 Novel and known nephrin gene (*NPHS1*) mutations in two Greek cases with congenital nephrotic syndrome including a complex genotype. *J. Genet.* **92**, 577–581]

Introduction

About 60% of infantile nephrotic syndrome (INS) cases are caused by mutations in four genes: *NPHS1*, *NPHS2*, *LAMB2* and *WT1*. We report two cases with clinical findings compatible with congenital nephrotic syndrome (CNS). Both probands and their parents were screened for nucleotide changes in the coding regions of the *NPHS1* and *NPHS2* genes along with the two 'hot spot' exons of the *WT1* gene by direct DNA resequencing analysis and/or mutation scanning analysis. Both cases were found to have mutations in the *NPHS1* gene. The first patient was homozygous for c.1096A>C (p.S366R) mutation in exon 9. The second patient had a complex genotype involving three mutations, the c.1096A>C (p.S366R), a novel 2 bp deletion c.649_650delGT and the previously reported c.791C>G (p.P264R); the last two have been inherited in *cis* from the proband's asymptomatic father (c.649_650delGT and c.791C>G (p.P264R)) who additionally had a novel non-pathological substitution c.1619C>A (p.A540E) in *trans*.

CNS (OMIM #256300) is a rare autosomal recessive disease manifesting in the first year of life. The incidence of the disease has a frequency of 1:8200 births in Finland, but considerably lower in other countries (Hammed 2003).

Studies have shown that mutations underlying CNS occur in the following four genes: *NPHS1*, *NPHS2*, *LAMB2* and *WT1* (Hinkes *et al.* 2007). The *NPHS1* gene is most commonly affected and as illustrated by a recent study which found that about a 55% of CNS patients have disease causing mutations in the *NPHS1* gene (Hinkes *et al.* 2007). About 100 *NPHS1* mutations have been described to date, distributed throughout the gene, with no apparent mutation 'hot spot' regions (Beltcheva *et al.* 2001; Gigante *et al.* 2002; Koziell *et al.* 2002).

Here, we report the molecular investigation of *NPHS1*, *WT1* and *NPHS2* genes in two infants referred to the Medical Genetics Laboratory, Athens University, with a clinical diagnosis compatible with CNS.

Materials and methods

Patients

Blood samples of the probands and their parents were sent to our laboratory for molecular investigation to characterize the underlying cause of CNS.

Patient 1 was the first female child of two nonconsanguineous healthy parents. Caesarian section was performed at 32 weeks, premature due to rupture of the membranes, oligamnio and coloured amniotic fluids. At three months of age, the girl was admitted to the hospital with fever and edema in the eyelids, the abdomen, the lumbar region, labia major and the lower extremities. Laboratory results showed proteinuria, haematuria and anaemia.

The abdominal ultrasound showed increased echogeneity of the kidney parenchyma with elimination of the corticomedullary border. The volume of the kidneys was increased, right kidney 6.8 × 4.4 × 4.1 cm^3 and left kidney 7.4 × 4.3 × 3.7 cm^3. Yet, the kidney vessels

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Keywords. congenital nephrotic syndrome; genetic disease; kidney; nephrin; nephrotic syndrome; *NPHS1*.

presented no thrombosis. Kidney biopsy showed lesions of focal segmental glomerular sclerosis. Unfortunately, images of the histological findings were unavailable.

At that point, treatment including cyclosporine was tried unsuccessfully. Subsequently, treatment was continued with calcium carbonate, enalapril, alopurinol, alpha calcidol, sodium polystyrene sulphate, sodium bicarbonate, atorvastatin, and epoetin beta. Occasionally, the patient was given human albumin and had several blood transfusions.

Despite the treating physicians' suggestion for kidney dissection and peritoneal dialysis which would ultimately lead to kidney transplant, the parents declined and chose to take no further medical action. The girl passed away at the age of 2.5 years due to end stage renal failure.

Patient 2 was also the first child (male) of two non-consanguineous healthy parents. The proband had no family history of nephrotic syndrome. The baby was delivered in the 38th week of pregnancy by Caesarian section due to coloured amniotic fluid. Problems in nutrition were present from the day of his birth including vomiting. Two weeks after birth, he presented with edema of his eyelids. Forty days after birth, he showed an incidence of seizures due to disturbance of electrolytes. He was admitted to the local hospital and transferred to the intensive care unit for 12 days. As he presented with a massive proteinuria, he was treated with frequent human albumin infusions. During his initial evaluation, there was evidence of CMV infection by means of positive serology and urine culture for CMV. Every effort to reduce the proteinuria led to retrogression of the edema. At the age of 4.5 months, he was transferred to the Department of Pediatric Nephrology, 'P. and A. Kyriakou' Children's Hospital, to stabilize his clinical situation, to treat the infection and to proceed to nephrectomy. During hospitalization, the proband was treated with antibiotics to control the infection. In his 46th day of hospitalization, a left nephrectomy was performed and the child's condition improved (albumin, 3.3 g/dL; urea, 110 mg/dL; creatinine, 0.6 mg/dL). In addition, thyroid replacement therapy was administered. The biopsy showed that the kidney was 125 g with dimensions: 10.5 × 6 × 4 cm. Depositions of IgM elements in the glomerulus was evident. No distortion was observed in podocytes and in the basal membranes of the capillary. These lesions were typical of mesangioproliferative glomerulonephritis with diffuse IgM deposits. Moderate interstitial nephritis and areas of acute tubular necrosis were also present. Unfortunately, there are no available images of the histological findings. Since then a progressive reduction of his renal function was noticed with progressive increase of his serum creatinine values. He was on chronic kidney disease conservative management (human recurrent erythropoietin, oral iron, thyroid replacement therapy and phosphate binders). At the age of 2.9 years, chronic peritoneal dialysis was initiated and after two months, a right nephrectomy was performed. At the age of 5.1 years, he underwent a successful cadaveric transplantation. During his last follow up at the age of 6.4 years, he had normal renal graft function.

DNA extraction and exon amplification

Genomic DNA was extracted from peripheral blood lymphocytes, using the QIAamp DNA Blood Mini Kit (Qiagen GmBh, Hilden, Germany).

All 29 exons of the *NPHS1* gene, all 8 exons of *NPHS2*, and exons 8 and 9 of the *WT1* gene, also including approximately 100 bp of flanking intronic sequences around all exons, were subjected to DNA analysis. PCR primers for amplification were designed based on public gene sequence databases (NCBI: http://www.ncbi.nlim.nih.gov, ENSEMBL: http://www.ensembl.org) regarding exon–intron boundaries. The primers used for the *NPHS1* gene exons and the exon 8 of the *WT1* gene are listed in table 1 and those used for *NPHS2* and exon 9 of the *WT1* gene as previously published (Megremis *et al.* 2009, 2011). Primers were designed with Primer3 (Rozen and Skaletsky 1998). The PCR protocol used for the amplification of the specific fragments was based on the manufacturer's recommendations, HotStarTaq® Master Mix kit (Qiagen, Hilden, Germany). The PCR programme consisted of an initial denaturation step at 95°C for 15 min followed by 30–40 cycles (depending on the fragment being amplified) of the following steps: 95°C for 1 min, 55–60°C (depending on the T_m of the primers) for 1 min 72°C for 1 min and a final extension at 72°C for 8 min.

The SURVEYOR Mutation Detection Kit (Transgenomic, Omaha, USA), which cleaves heteroduplexes at their mismatched bases, was used to screen 10 of 29 exons of *NPHS1*, including exons 2, 3, 9, 6, 12, 13, 15, 16, 25 and 26. The method was based on Vogiatzakis *et al.* (2007) with minor modifications, including a reduced volume of the substrate (6 μL vs a total volume of 20 μL), as well as the analysis of digestion products by electrophoresis in a 10% PAGE gel run at 180 V in 1× TBE for 3 h rather than an agarose gel. Positive control samples previously characterized by direct sequencing analysis were analysed in parallel. Samples giving an indication of cleavage were subject to direct sequencing analysis.

Sequencing analysis

The coding regions of *NPHS2* and *WT1* (exons 8 and 9) were analysed by direct sequencing, as well as the remaining exons of the *NPHS1* gene and any exons found to be positive with the SURVEYOR endonuclease assay. Sequencing analysis was performed with the 7-Deaza-dGTP Cytm5/Cy5.5 Dye Primer Cycle Sequencing Kit (GE HealthCare Little Chalfont, Buckinghamshire, UK), run on the OpenGene Visgen automated sequencer (Visgen, Ontario, Canada) and analysed with the Unix Based Gene Objects 3.1 software (www.visgen.com/products/geneobjects.html).

In silico analysis tools

The consequence of any novel nonsynonomous nucleotide variations that were identified within exons were evaluated

Table 1. Primers and conditions of the *NPHS1* gene and exon 8 of the *WT1* gene.

exon	Primers (all 5′–3′)	PCR size (bp)	Annealing T/cycles
NPHS1			
1	F: AGGGAGGAGAGTAACGGAAAGAGA R: AAGGAAAGGGCAGAGGGTTTGTCT	280	58°C/30
2/3	F: TGCTGACTGAAGGTGAGTGGGA R: CCAGCACTGAGAAGGACTTGAAGA	673	55°C/35
4	F: AGCCTCTCCTCTCCCAGACT R: TCCCACACTGGCCTCAGCATCT	382	55°C/35
5	F: GGCATTCAGGCAGTCCAGAAAGT R: TCCCAGATGTTCATACCTAGCCCA	365	59°C/40
6	F: AATCTCTCCCATGGAACCCTGGAT R: ATATCAGTCACTCAGTGGGCCTGGA	436	55°C/35
7/8	F: TTCAGGCACTCAGAGAAACATGGG R: ACTGGAGACAGATGCTGAGAT	601	58°C/32
9	F: TGCCTCAGAGAACCATCTGTGTGT R: TTGGACCCATGGTCCTCAAGGAGAAA	424	59°C/40
10/11	F: ACTCAGGCCTCTAGCACGATGGATA R: TGTGTCTTTCCTGATTCCCTGCCA	620	58°C/30
12/13	F: AAAGGCTTTGTTTGGGCTGGC R: AGAGGCTGGAGAGGCACTA	725	55°C/32
14	F: TGTCCTGGGACAAGGAAGGG R: GACTGCAGTGACCTATGATTGCGT	489	55°C/35
15/16	F: ACCTGTGCCTGATCTCCAATCTGT R: AGACTCCACAATGGGCAAGGTT	496	55°C/35
17	F: TGGAGACAACCTCACCAACCTTGA R: TATTCATTCTGGGAGCATGCCCTG		59°C/32
18/19	F: TGATTGTGGCTAGGAGAATGAGGC R: TTCCTCCACCCATTCGTCTTCCTT	634	58°C/30
20	F: GGATGAATGGATAGATAGGCAGACGG R: CCATCCTCACACATACACAGAACTTCC	387	58°C/30
21/22	F: CAGAGCAGTGTTCACCATGACCTT R: GGTTGGTCTCAAGTTCATCTGCCT	719	58°C/35
23	F: GCAGTCATATACCAGTTCCCATGA R: TGAGCTTGGCCAGAACTAAGTCGT	291	58°C/40
24	F: ACAGAGACCTCTGCCCATCTGTTT R: TTCAGTATGCAGCAACCACAGGGT	328	58°C/30
25/26	F: AACCCTGTGGTTGCTGCATACTGA R: ACAAAGCCCTTTCCATCCTCTGAC	425	58°C/30
27/28	F: ACAATCAGGGCACCGACG R: ATCATGCCCAGCCGACTGTCTTTA	573	58°C/30
29	F: CGTGCCCAGCCTGATATTGTTGAA R: ACCAGCTGAACCATCTCTGTCACT	518	58°C/30
WT1			
8	F: TACCCTAACAAGCTCCAGCGAAGT R: AATCAACCCTAGCCCAAGGGAACA	276	59°C/35

with the *in silico* analysis tools sorting intolerant from tolerant (SIFT) prediction (Kumar *et al.* 2009) (http://blocks.fhcrc.org/sift/SIFT.html) and polyphen 2 (Adzhubei *et al.* 2010) (http://genetics.bwh.harvard.edu/pph).

Results

No mutations were found in *NPHS2* and *WT1* genes in either proband, but both were characterized with mutations in the *NPHS1* gene. Patient 1 was homozygous for the c.1096A>C (p.S366R) in exon 9. Patient 2 was found to have a complex genotype with three mutations: a known nucleotide change in exon 9, c.1096A>C (p.S366R), a novel 2 bp deletion in exon 6, c.649_650delGT (p.Cys217fsX) (figure 1) and a nucleotide change in exon 7, c.791C>G, leading to the missense mutation p.P264R. Analysis of the mother of patient 2 found a carrier state for p.S366H. The father of patient 2, in addition to mutations in exon 6 and exon 7, was found to carry a novel amino acid change in exon 12 c.1619C>A (p.A540E), leading to a substitution of alanine to glutamine which was not transmitted to the neonate. To identify the nature of the change and its impact on the function of the protein we used two *in silico* analysis tools. The SIFT results found a score of 0.91, consistent with a nonpathological change while the polyphen 2 analysis

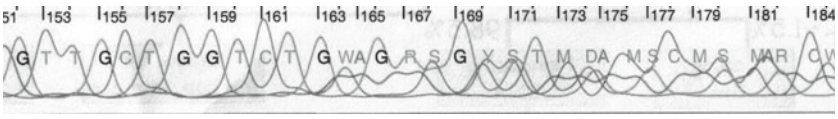

Figure 1. The frameshift mutation in exon 6. The frameshift starts at position 164.

characterized the mutation as benign with a score of 0.025 (sensitivity, 0.96; specificity, 0.80). These results indicate that the substitution is tolerated and has no effect on the protein's structure.

Discussion

CNS is a rare disease manifest during the first year of life. Children born with CNS show a variety of symptoms including proteinuria, hypoalbuminaemia and edema that often leads to end stage renal disease. CNS is mainly caused by mutations in *NPHS1* gene and rarely by mutations in *NPHS2* gene (Sonmez *et al.* 2008).

Recently, different studies have shown that mutations in the nephrin gene can also lead to other forms of nephrotic syndrome, indicating a wider spectrum of nephrin mutations influence the normal biology of the podocyte (Philippe *et al.* 2008). In fact, mutations of the nephrin gene have also been implicated to underlie the development of childhood onset steroid-resistant nephrotic syndrome (Philippe *et al.* 2008; Santin *et al.* 2009).

In this study, we examined two patients with clinical findings consistent with congenital nephrotic syndrome. The molecular analysis revealed nucleotide changes in the *NPHS1* gene. Patient 1 was characterized to be homozygous for the mutation c.1096A>C, first described by Lenkkeri *et al.* (1999).

Patient 2 revealed a more complex genotype, as a result of the existence of three mutations. The c.1096A>C (p.S366R) mutation, was inherited from the mother. The novel dinucleotide deletion c.649_650delGT (p.Cys217fsX) in exon 6 and the known missense mutation c.791C>G (p.P264R) in exon 7, were inherited from his father and are implicated to be in *cis*. The c.649_650delGT (p.Cys217fsX) causes a frameshift resulting in a premature termination of translation at nucleotide 649, hence the creation of a truncated protein of 217 amino acids likely precluding the read through of mRNA to the missense mutation in exon 7.

The novel substitution c.1619C>A (p.A540E), found in the father's sample is considered as nonpathological, taking in consideration the *in silico* analysis tools as well as the absence of clinical findings in the father. The substitution is in *trans* with the other two mutations based on the segregation of the family.

Recent studies have proposed a classification of mutations taking in consideration the severity of NS. As reported, there are three distinct mechanisms by which mutations in nephrin can lead to dysfunction of the SD (Shono *et al.* 2009). The p.S366R as well as the frameshift mutation (p.Cys217fsX) belong to class I and class II mutations, respectively (severe mutations), that agree with the phenotype observed. It is also reported that the S366R mutant is accumulated in the ER and shows complete lack in the plasma membrane (Liu *et al.* 2001). Frameshift mutations are considered to lead to total absence of functional protein (Shono *et al.* 2009). Patients 1 and 2 have the same missense mutation (S366R), but due to the fact that patient 2 has also a frameshift mutation we can assume that he will exhibit a more severe phenotype since the one allele will not produce a functional protein.

In conclusion, the molecular analysis of both children shows that *NPHS1* mutations are the major cause of congenital nephrotic syndrome verifying that *NPHS1* gene is probably the most important component of the slit diaphragm (McCarthy and Saleem 2011). The screening of mutations for the *NPHS1, NPHS2* and *WT1* genes supports definitive diagnosis, which may be useful for clinical management of such patients, as well as family counselling and family planning.

Acknowledgements

We would like to thank the Greek Society of Families with Kidney Disease 'ELPIDA' whose donations have partly funded this project and also the Athens University Research Institute for the Study of Genetic and Malignant Disorders in Childhood, Greece.

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Received 27 March 2013, in revised form 28 June 2013; accepted 3 July 2013
Published on the Web: 27 November 2013