Original Article

Mutational Analysis of the *NPHS2* Gene in Czech Patients with Idiopathic Nephrotic Syndrome

(nephrotic syndrome / *NPHS2* gene / focal segmental glomerulosclerosis / mutation / polymorphism)

J. REITEROVÁ[1,2], H. ŠAFRÁNKOVÁ[1], L. OBEIDOVÁ[2], J. ŠTEKROVÁ[1,2], D. MAIXNEROVÁ[1], M. MERTA[2,3], V. TESAŘ[1]

[1]Department of Nephrology, First Faculty of Medicine, [2]Institute of Biology and Human Genetics, Charles University in Prague and General University Hospital in Prague, Prague, Czech Republic
[3]Department of Metabolic Care and Gerontology, University Hospital in Hradec Králové, Charles University in Prague, Hradec Králové, Czech Republic

Abstract. Focal segmental glomerulosclerosis and minimal change disease represent frequent histological patterns of renal injury in patients with nephrotic syndrome. Few cases carrying *NPHS2* gene variants have been described to date. Mutational analysis of the *NPHS2* gene was performed in 50 Czech adult patients with histologically proved FSGS/MCD. The common p.P20L and p.R229Q polymorphisms of the *NPHS2* gene were tested in 169 patients with IgA nephropathy and in 300 individuals of the control group. No mutation in the *NPHS2* gene in patients with adult onset was identified. One homozygous mutation p.V290M in a patient with onset in early childhood was found. One new heterozygous variant in the non-conservative area of the *NPHS2* gene, p.G97S, was identified in a patient with childhood-onset FSGS. In one adult patient, there were two polymorphisms, p.P20L and p.R229Q, in trans-heterozygous state, which could contribute to steroid-resistant nephrotic syndrome. The most common polymorphism p.R229Q was identified in 12 % of FSGS/MCD patients, in 11.8 % of IGAN patients and in 10% of controls. The heterozygosity of p.R229Q polymorphism was similar in the IGAN group, with non-significantly higher prevalence in IGAN patients with progressive form of the disease (15.9 % versus 9.4 %). The prevalence of p.P20L polymorphism was not significantly different among the groups (6 % in FSGS patients, 1.8 % in IGAN patients, 1 % in the control group). To conclude, *NPHS2* mutations are rare in patients with adult onset of FSGS/MCD. The R229Q polymorphism is frequent in the Czech population and probably could have some influence on IGAN.

Introduction

Nephrotic syndrome (NS) is characterized by proteinuria, hypoalbuminaemia, oedema, and dyslipidaemia. Clinically, NS has been divided into two categories based on the response to steroid therapy: steroid-sensitive NS (SSNS) and steroid-resistant NS (SRNS). Approximately 10 % of children and 50 % of adults with idiopathic NS have SRNS, fail to respond to immunosuppressive treatment and progress to end-stage renal disease (ESRD) within seven years (Korbet, 2002). In these cases, renal histology typically shows focal segmental glomerulosclerosis (FSGS) or minimal change disease (MCD). The incidence of FSGS has increased two- to four-fold in the past two decades, although the reasons for this increase are unclear. To date, mutations in seven genes (*NPHS1, NPHS2, CD2AP, PLCE1, ACTN4, TRPC6,* and *INF2*) expressed by glomerular podocytes have been identified in patients with NS (Table 1). Mutations in three new genes (encoding membrane-associated class I myosin, actin regulatory protein, and protein tyrosine phosphatase receptor type O) were identified in individual cases with FSGS in 2011 (Akilesh et al., 2011; Mele et al., 2011; Ozaltin et al., 2011).

The *NPHS2* gene, encoding podocin, was identified as the causative gene in early-onset autosomal-recessive SRNS (Boute et al., 2000). Podocin is a 383-amino acid lipid-raft-associated protein localized at the slit diaphragm, where it is required for the structural organiza-

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Corresponding Author: Jana Reiterová, Department of Nephrology, First Faculty of Medicine, Charles University in Prague and General University Hospital in Prague, U Nemocnice 2, 128 08 Prague 2, Czech Republic, Phone: (+420) 224 966 790; Fax: (+420) 224 923 268; e-mail: jreiterova@seznam.cz

Abbreviations: ESRD – end-stage renal disease, FSGS – focal segmental glomerulosclerosis, HRM – high-resolution melting, IgA – immunoglobulin A, IGAN – IgA nephropathy, MCD – minimal change disease, NS – nephrotic syndrome, PCR – polymerase chain reaction, SRNS – steroid-resistant nephrotic syndrome, SSNS – steroid-sensitive nephrotic syndrome.

Table 1. The genes involved in genetic forms of idiopathic nephrotic syndrome

Gene	Location	Protein	Heredity	Usual onset
NPHS1	19q13.1	nephrin	autosomal recessive	Prenatally or in early childhood
NPHS2	1q25-31	podocin	autosomal recessive	Early childhood
ACTN4	19q13	α4-actinin	autosomal dominant	Adolescence
TRPC6	11q21-22	TRPC6	autosomal dominant	Adolescence
CD2AP	6p12	CD2AP	autosomal dominant	Childhood
PLCE1	10q23-24	fosfolipase Cε	autosomal recessive	Early childhood
IFN2	14q32	formin	autosomal dominant	Adolescence

tion and regulation of the glomerular filtration barrier. Its interaction with nephrin, CD2AP and TRPC6 manage mechanosensation signalling, podocyte survival, cell polarity, and cytoskeletal organization. Although recessive *NPHS2* mutations were initially reported to cause familial SRNS in children with ESRD, occurring between three months and five years of age, recent studies have shown that they are associated with a broader clinical spectrum. Most patients with two *NPHS2* pathogenic mutations develop NS before the age of six years, present mostly with FSGS, do not respond to immunosuppressive therapy, reach ESRD before the first decade of life, and have a minimal risk for recurrence of FSGS after kidney transplantation (Caridi et al., 2001; Karle et al., 2002; Hinkes et al., 2008). *NPHS2* mutations were not found in four large cohorts of adult-onset FSGS (McKenzie et al., 2007; Caridi et al., 2003; Aucella et al., 2005; Monteiro et al., 2006). On the other hand, *NPHS*2 variants in 23 % of late-onset familial cases and in 2 % of sporadic ones were described in Japan (Tsukaguchi et al., 2002). Recently, a French group identified *NPHS2* changes in 14 % of cases presenting with SRNS after 18 years of age (Machuca et al., 2009). To sum up, 15 sporadic and 11 families with adult-onset FSGS carrying *NPHS2* gene variants have been reported.

The polymorphism R229Q is one of the most commonly reported podocin sequence variations. The arginine residue at protein position 229 is highly conserved across species, and arginine-to-glutamine substitution R229Q has been reported to alter functional properties of podocin *in vitro* and possibly *in vivo* (Zhang et al., 2004). It has been found repeatedly with slightly increased frequency in SRNS compared to healthy controls (Weber et al., 2004; Franceschini et al., 2006). On the other hand, subsequent studies reported similar frequencies of this polymorphism in SRNS and in normal subjects (5.13 and 3.75 %, respectively) (Ruf et al., 2004). Further, the influence of the R229Q functional variant on microalbuminuria was investigated. First, the R229Q functional variant was associated with microalbuminuria in the general population (Pereira et al., 2004). In the second study, the R229Q variant did not appear to alter the risk of proteinuria in general population and in diabetic population (Tonna et al., 2008).

To further define the clinical relevance of *NPHS2* mutations, we undertook a comprehensive mutation screening study in Czech adult patients. In addition, we tested the significance of the most common R229Q polymorphism of the *NPHS2* gene in patients not only with FSGS, but also in patients with IgA nephropathy (IGAN) and in a large cohort of Czech healthy individuals.

Material and Methods

Patients

This study was performed in 50 patients with histologically proved FSGS/MCD (20 males, 30 females, mean age at the time of diagnosis 42.2 ± 16 years). The diagnosis of FSGS/MCD was established on the basis of histological examination of a specimen of renal tissue gained by renal biopsy. Renal biopsies were performed in the years 2004–2008 at the Department of Nephrology of the First Faculty of Medicine, Charles University in Prague and General University Hospital in Prague. Written informed consent was obtained from all included subjects. Mean age at the time of onset of NS was 33.2 ± 18.6 years. There were 10 patients with first manifestation of NS already during childhood (3–17 years). Six % of cases were familial forms of FSGS.

Based on the therapy response, there were 36 steroid-resistant patients (SRNS) and 14 steroid-sensitive patients (SSNS). Patients defined as SRNS did not respond to prednisone (dose 1 mg/kg) during six months of therapy. Three hundred Czech individuals (150 males, 150 females) formed the control group with mean age 64.5 ± 17.5 years. The control group was randomly selected from blood donors. All individuals had normal urine sediment and no proteinuria.

In addition, the significance of the most common R229Q and P20L polymorphisms of the *NPHS2* gene was tested in 169 patients with IGAN, which is the most common primary chronic glomerulonephritis. The diagnosis was established based on histological examination of a specimen of renal tissue gained by renal biopsy. The mean age of the IGAN group was 45.2 ± 14.8 years at the time of diagnosis. The follow-up of IGAN patients was at least four years. The patients were divided into a stable group (63 patients) defined by renal function within normal ranges, and a progressive group (106 patients) with ESRD during four years of the study or pa-

tients whose serum creatinine at least doubled during this follow-up.

Methods

Isolation of genomic DNA was accomplished by a salting-out procedure from peripheral-blood lymphocytes. All eight exons of the *NPHS2* gene were amplified by polymerase chain reaction (PCR) using exon-flanking primers in MyCycler 1.065 (Bio-Rad Laboratories, Hercules, CA), as described elsewhere (Boute et al., 2000). Mutation analysis was performed by direct sequencing of all exons in ABI Prism™ 3130 Genetic Analyzer (Applied Biosystems, Carlsbad, CA).

P.P20L (in exon 1) and p.R229Q (in exon 5) polymorphisms were screened by the high-resolution melting method (HRM) in 169 IGAN patients and in 300 patients of the control group. PCR of exon 1 and exon 5 were performed by using Light Cycler 480 (Roche Diagnostic, GmbH, Mannheim, Germany) in a total volume of 10 µl of the mixture containing 1 µl genomic DNA, 1 × Light Cycle 480 High-resolution melting master mix (Roche Diagnostic) and 5 pmol of each primer. PCR amplicons were analysed with Light Cycle 480 Gene Scanning Software (Roche Diagnostic). This software analyses the high-resolution melting curve data to identify changes in the shape of the curve that indicate both homozygous and heterozygous allelic variants in a sample. The Gene Scanning Software generates a difference plot by subtracting the curves from a reference curve and automatically groups samples with similar melting curves. All suspected samples from these two substitutions were analysed by direct sequencing in ABI Prism™ 3130 Genetic Analyzer (Applied Biosystems).

The χ^2-test was used to compare the distribution of genotypes of polymorphisms between the FSGS, IGAN and control groups.

Results

Homozygous *NPHS2* pathogenic mutation C.G868A (P.V290M) that causes conservative amino acid substitution in the carboxy-terminal cytoplasmic tail of podocin was identified in one young woman only (Fig. 1). Severe nephrotic syndrome manifested itself already at three years of age of this patient. Steroids and other immunosuppressive drugs (cyclophosphamide, mycophenolate and cyclosporin) reduced proteinuria only transiently. The 24-year-old woman now suffers from advanced renal insufficiency and renal transplantation is considered.

The C.G291A (P.G97S) substitution was identified on one allele in another patient with SRNS. This substitution of hydrophobic amino acid glycine to hydrophilic amino acid serine was not present in 300 controls. On the other hand, this substitution is not located in the conservative area of the gene and the same substitution is present in another mammal (wild boar) (Table 2). P.G97S is probably not a causal mutation in the *NPHS2*

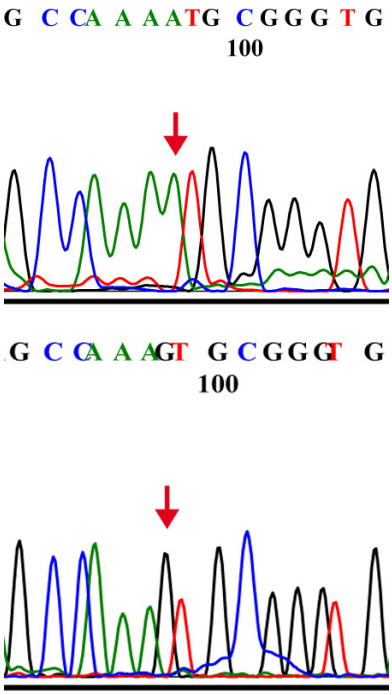

Fig. 1. Homozygous *NPHS2* pathogenic mutation c.G868A (p.V290M)

gene. No other mutation was found on the second allele of this patient. There could be a change in introns leading to abnormal splicing or mutation in other genes responsible for genetic forms of the nephrotic syndrome. Nephrotic syndrome was already diagnosed at the age of nine years of this patient. Renal biopsy showed FSGS, and he was treated with steroids, cyclosporin, chlorambucil, mycophenolate with transient therapeutic response and with repeated infectious complications. He had renal failure at the age of 25 years and now is on haemodialysis.

P.P20L and p.R229Q polymorphisms in trans-heterozygous state were identified in a patient with manifestation of NS at the age of 33 years. There was no therapeutic response to cyclophosphamide, and after 10 years of therapy the patient suffers from advanced renal insuf-

Table 2. The graphic alignment of p.G97S substitution in the NPHS2 gene

	↓ **p.G97S**
1. Homo sapiens	KSS**G**LGACEWLL
2. Oroctolagus cuniculus	KSS**G**LGACEWLL
3. Sus scrofa	KSS**S**LGACEWLL
4. Mus musculus	KPS**G**LGACEWLL
5. Monodelphis domestica	KSS**H**LGMCEWLL
6. Gallus gallus	KSP**G**LNICEWLL

Sequence alignment of protein sequences was performed with distant species: 1. *Homo sapiens* podocin /ref. NP_055444.1/; 2. *Oroctolagus cuniculus* /ref.XP_002715053.1/; 3. *Sus scrofa* /ref. XP_003130389.1/; 4. *Mus musculus* /ref.NP_269723.1/; 5. *Monodelphis domestica* /ref.XP_001374197.1/; 6. *Gallus gallus* /ref.XP_422265.2/. The position of putative amino acid is in boldface.

ficiency. She has two children who inherited only the R229Q allele and have no proteinuria.

The two above-mentioned polymorphisms, p.P20L and p.R229Q, were also identified in other patients. The p.P20L polymorphism in exon 1 was found in three FSGS patients (6 %). P.P20L was present only in steroid-resistant patients. P.P20L was also identified in three IGAN patients with progressive form of the disease (1.8 %) and in three patients of the control group (1 %). The distribution of this polymorphism was not significantly different between FSGS patients, IGAN patients and the control group.

The most common polymorphism p.R229Q in heterozygous state was identified in six FSGS patients (12 %), in four steroid-resistant patients and in two steroid-sensitive patients. It was also found in 30 patients of the control group (10 %), and in one patient the polymorphism was in homozygous state. The heterozygosity of R229Q in the IGAN group was similar, 11.8 %. The distribution of R229Q heterozygosity was slightly higher in a progressive IGAN subgroup (15.9 %) than in stable IGAN patients (9.4 %).

Discussion

Recessive *NPHS2* mutations are rare in adult patients with idiopathic nephrotic syndrome. We did not identify any mutation in the *NPHS2* gene in patients with adult onset of FSGS. Our results are consistent with the finding of an Italian study in which only three putative heterozygous *NPHS2* mutations (on one allele) were found in a cohort of 64 adult patients with SRNS (Caridi et al., 2003). A Canadian group found only one compound heterozygous mutation of the *NPHS2* gene in 87 adult patients with FSGS (He et al., 2007). On the contrary, four mutations (all heterozygous mutations in combination with p.R229Q polymorphism) in 47 patients with adult onset FSGS were found in a recent Spanish study (Santín et al., 2011). We identified one homozygous mutation p.V290M in a patient with onset of FSGS in early childhood. This mutation has already been described in compound heterozygous state (in combination with another missense mutation) in a sporadic case of SRNS with childhood onset of the disease (Karle et al., 2002).

One heterozygous variant of unknown significance in the non-conservative area of the *NPHS2* gene, p.G97S, was identified in a patient with childhood-onset FSGS. We could speculate that this variant could be implicated in the pathogenesis of SRNS because it was not found in 300 individuals of the control group. On the other hand, the clinical course and drug response was comparable in FSGS patients with a single *NPHS2* mutation as in FSGS patients without mutation (Caridi et at., 2009). No pathogenic *NPHS2* mutation was identified in our three familial cases of FSGS with adult onset of the disease.

In one adult patient, there were two polymorphisms, p.P20L and p.R229Q, in trans-heterozygous state, which could contribute to SRNS in this adult patient. The incidence of the p.P20L variant is very low (0–1 %) (Weber et al., 2004; McKenzie et al., 2007). The p.P20L variant is a non-conservative amino acid exchange, and was predicted to be deleterious. DiDuca et al. (2006) found that p.P20L is a part of a haplotype with a variant in the *NPHS2* promoter that is associated with marked down-regulation of podocin expression. On the contrary, Santín et al. (2011) assessed the p.P20L variant in heterozygous state in patients with SRNS and in controls with the same frequency. We identified p.P20L in heterozygous state in 6 % of FSGS patients, 1.8 % IGAN patients and in 1 % of control individuals, which was not significantly different.

The most common polymorphism, p.R229Q, was identified in 12 % of FSGS patients and in 10 % cases of the control group (in one individual of the control group in homozygous state). There is uneven p.R229Q allele distribution throughout different populations. This polymorphism is more frequent among South Americans, Europeans and European Americans (4–7 %) than among Africans, African Americans, and Asians (0–1.5 %), suggesting that this variant emerged in Europe, although it is not possible to discern its specific geographic origin (Dusel et al., 2005; Yu et al., 2005; Machuca et al., 2009). The highest frequency 7.3 % was described in Chileans and Argentineans (Machuca et al., 2009). In European population, the highest occurrence of p.R229Q polymorphism, 4.5 %, was described in France, 3.2 % in Italian population and 3.1 % in Spanish population (Weber et al., 2004; Aucella et al., 2005; Santín et al., 2011). The frequency of the p.R229Q polymorphism was not studied in Slaves.

The high frequency of R229Q in the Czech population suggests an old mutation. One individual from the control group was homozygous for the R229Q polymorphism. In accordance with our findings, another report already mentioned that the R229Q homozygosity probably leads to mild or no phenotype (Tsukaguchi et al., 2002). The heterozygosity of p.R229Q polymorphism was similar in the IGAN group, with non-significantly higher prevalence in IGAN patients with progressive form of the disease.

To conclude, *NPHS2* mutations are rare in patients with adult onset of FSGS/MCD. The R229Q polymorphism is frequent in the Czech population. The possible influence of the p.R299Q polymorphism on IGAN should be further studied.

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