Short Communication

TRPC6 Gene Variants in Czech Adult Patients with Focal Segmental Glomerulosclerosis and Minimal Change Disease

(focal segmental glomerulosclerosis / nephrotic syndrome / TRPC6)

L. OBEIDOVÁ[1], J. REITEROVÁ[1,2], P. LNĚNIČKA[1], J. ŠTEKROVÁ[1], H. ŠAFRÁNKOVÁ[2], M. KOHOUTOVÁ[1], V. TESAŘ[2]

[1]Institute of Biology and Medical Genetics, First Faculty of Medicine, Charles University in Prague and General University Hospital in Prague, Czech Republic
[2]Department of Nephrology, First Faculty of Medicine, Charles University in Prague and General University Hospital in Prague, Czech Republic

Abstract. Blood filtration and formation of primary urine in the kidney glomerulus is provided by a specialized membrane called slit diaphragm located between well-branched pedicels of podocytes. Actually, the slit diaphragm is a protein supercomplex, whose disruption can cause failure of renal filtration, and patients usually manifest nephrotic syndrome. Recently, familial forms of nephrotic syndrome have been described which arise from malfunction of mutated proteins making up the slit diaphragm. In 2005 it was found that one of the proteins present in this complex was non-selective cation channel TRPC6. The aim of this work was to screen mutations and polymorphisms of the *TRPC6* gene in a group of 64 Czech patients with nephrotic syndrome and subsequently, on the basis of these data, evaluate the role of mutations in the *TRPC6* gene in Czech population. The analysis was performed by the PCR method followed by direct sequencing and high-resolution melting method. We have not identified any mutations in our group of patients. Two additional single nucleotide polymorphisms – p.P15S and p.A404V – were detected along with nucleotide changes that did not result in amino acid changes and with a few intronic changes. P.P15S heterozygotes were more frequent in patients with steroid-resistant FSGS than in steroid-sensitive patients (29 % versus 12.1 %). To conclude, we did not find any probable disease-causing mutation in the *TRPC6* gene in the cohort of 64 Czech patients. The p.P15S polymorphism might have some influence on the therapeutic response of FSGS patients.

Received November 18, 2011. Accepted February 17, 2012.

This project was supported by the grant project from the Ministry of Health of the Czech Republic NS/9779-4 and research project of the Ministry of Education, Youth and Sports of the Czech Republic MSM 0021620806.

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) 224923268; e-mail: jreiterova@seznam.cz

Abbreviations: ESRD – end-stage renal disease, FSGS – focal segmental glomerulosclerosis, GFR – glomerular filtration rate, GN – glomerulonephritis, GPCR – G protein-coupled receptor, HRM – high resolution melting, MCD – minimal change disease, NS – nephrotic syndrome, PCR – polymerase chain reaction, SRNS – steroid-resistant nephrotic syndrome, SSNS – steroid-sensitive nephrotic syndrome, TRPC6 – transient receptor potential cation channel, subfamily C, member 6.

Introduction

Nephrotic syndrome (NS) is characterized by proteinuria, hypoalbuminaemia, oedema, and dyslipidaemia. Approximately 10 % of children and 50 % of adults with idiopathic NS are steroid-resistant NS (SRNS), failing to respond to immunosuppressive treatment and progressing 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 by 2- to 4-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 SRNS.

The *TRPC6* gene is located on the long arm of chromosome 11 (11q22.1) and codes for the short transient receptor potential channel (TRPC6) (Reiser et al., 2005; Winn et al., 2005). TRPC6 belongs to the family of transient receptor potential channel proteins (TRP), a group of voltage-independent cation-permeable channels that are expressed in many tissues (Clapham et al., 2001). TRP channels have been implicated in diverse biological functions such as cell growth, ion homeostasis, mechanosensation dependent on calcium entry into

cells. The TRPC subfamily (TRPC1–7) is a group of calcium-permeable cation channels that are important for the increase in intracellular calcium concentration after direct stimulation via G protein-coupled receptors (GPCR) (Montell, 2005). The special feature of TRPC6 is the presence of ankyrin-binding repeats in the N terminus. TRPC6 is an important component of the glomerular slit diaphragm that co-localizes with CD2AP, nephrin and podocin in podocytes (Mukerji et al., 2007).

So far, twelve different *TRPC6* gene mutations have been identified in cases with familial FSGS and four in sporadic cases with a late onset of kidney disease (between 14 and 57 years, with a few exceptions – onset at 1, 2, 4, 8, 7 and 9 years of age) and a variable rate of progression to ESRD (Winn et al., 2005; Heeringa et al., 2009; Santín et al., 2009; Zhu et al., 2009; Büscher et al., 2010; Gigante et al., 2011; Mir et al., 2012). Affected individuals typically presented with high-grade proteinuria in their third or fourth decade of life and 60 % progressed to ESRD. The average time between initial presentation and the development of ESRD was 10 years.

All identified mutations were missense mutations, apart from two truncated mutations (p.K874X, p.89fsX8). Eight of these missense mutations (p.P112Q, p.N125S, p.H218L, p.R895C, p.R895L, p.E897K, p.Q889K and p.M132T) were gain-of-function mutations that resulted in increased Ca^{2+} current amplitudes. The rest of missense mutations are likely pathogenic variants according to genetic studies based on the bio-physical and biochemical differences between the wild-type and mutant amino acid residues and on the evolutionary conservation in orthologues and homologues. Mutations are distributed throughout the N and C terminal cytosolic domains. No mutations have been described in transmembrane domains. Most mutations are accumulated in the ankyrin repeats, which correlates with their important functional role.

To further define the clinical relevance of the *TRPC6* gene mutations, we undertook a comprehensive mutation screening study in 64 patients with histologically proven FSGS/MCD.

Material and Methods

This study was performed on 64 patients with histologically proven FSGS/MCD (30 males, 34 females, mean age at the time of diagnosis 41.8 ± 18.5 years). The diagnosis of chronic GN was established on the basis of histological examination of a specimen of renal tissue gained by renal biopsy. Renal biopsy with the histological finding of FSGS/MCD (44 samples evaluated as FSGS and 20 as MCD) was performed in the years 2004–2008 at the Department of Nephrology of General University Hospital in Prague. Written informed consent was obtained from all included subjects and the study was performed with the approval of the Ethics Committee of the General University Hospital in Prague. Family history for proteinuria was positive in three patients.

At the time of diagnosis (which is equal to the time of the renal biopsy) and at the end of the follow-up period, clinical data on arterial hypertension, serum creatinine (S-creatinine), GFR (glomerular filtration rate), proteinuria and serum albumin (S-albumin) were collected. Estimated GFR was assessed based on the modification of diet in renal disease formula (MDRD formula). The mean follow-up period was 36.7 ± 22.8 months. Arterial hypertension (defined as systolic blood pressure higher than 140 mmHg and diastolic blood pressure higher than 90 mmHg and/or use of antihypertensive drugs) was present in 69.4 % of patients. Clinical characteristics of the patients are summarized in Table 1.

Patients were divided into two groups on the basis of therapy response. As steroid-resistant were defined patients who did not respond to prednisone (dose 1 mg/kg) during six months of therapy. Thirty-one patients were steroid-resistant, 33 patients were steroid-sensitive.

Three hundred Czech individuals formed the control group with mean age 64.5 ± 17.5 years. The control group was randomly selected from individuals who are blood donors. All individuals had normal urine sediment and no proteinuria.

Isolation of genomic DNA was accomplished by a salting-out procedure from peripheral-blood lymphocytes. The coding regions and intron-exon boundaries of the *TRPC6* gene were screened for mutations using polymerase chain reaction (PCR) with following direct sequencing and/or with high-resolution melting analysis (HRM). Primers for all 13 exons of the *TRPC6* gene were designed using Primer-Blast program through the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) (Table 2). HRM analysis was performed in 10 ml volumes using LightCycler 480 (Roche Diagnostics, GmbH, Mannheim, Germany). The reaction mixture included LightCycler 480 HRM Master kit (consisting of 2x conc. Master mix, 25 mM $MgCl_2$ and PCR H_2O), 0.05 mM of each primer and 10 mg/l of genomic DNA. Dimethyl sulphoxide was added in some cases to improve specificity of the reaction. The PCR phase of HRM analysis consisted of polymerase activation (95 °C/2 min), followed by the amplification step which differed in the number of cycles and annealing temperatures among the exons (50–60 cycles, annealing

Table 1. Clinical characteristics of patients with FSGS

	At the time of diagnosis	At the end of follow-up period
Serum creatinine (μmol/l)	128.4 ± 131.9	126.9 ± 108.5
GFR (ml/s/1.73 m^2)	1.5 ± 0.95	1.41 ± 0.85
Proteinuria (g/24 h)	5.7 ± 4.7	1.77 ± 2.85
Serum albumin (g/l)	27.4 ± 9.9	39.6 ± 6.5

Table 2. Primers used for all 13 exons and intron-exon boundaries of the TRPC6 gene

Exon	Primer: Forward (5'-3') / Reverse (5'-3')	Range [bp]
1	F: TGGAACTGCCCACTCGGCTC R: ACTCCTGCGAGCGCACAACC	298
2A	F: GGCAAGTCATTTGGCACACT R: CCAACTGTAGGGCATTCTGG	316
2B	F: CAGTGGTGCGGAAGATGTTA R: TGGAGTCACATCATGGGAGA	332
2C	F: TTTGCTGAAGGCAAGAGGTT R: AGCCGTCATGACTGGATCTT	336
2D	F: TGACTCGTTTAGCCACTCCA R: GGTAGCGATCACAACTTTTGC	328
3	F: TCTGAAGCATAGTAAAACGTGGT R: CCCTTTATCCTTATTTAGCACCAA	313
4	F: TCGTTTATGCTGAACCTTTCTT R: ACCCAACTGTGATTCCCTGA	326
5	F: GGAGATCATTGGAATGTGCAG R: CCAACTGCTAAGACTGCAAACA	365
6	F: CAGCTAAGGCTGAATGCGAT R: TGAGAATTGTGCAGTAACCGA	377
7	F: CGCAGAAAAAGAAGTTACCTAAA R: CCCATGGACTTACATAAACGC	420
8	F: TTTGCAGACACTAAACAAACTCA R: AGCAGTCCATGCTTTCATCC	383
9	F: TGCATTTCCTTGCTGAACTG R: AAAGGGATGTGGCATAGTGG	316
10	F: AGCACTTGCAAAGGGAAGAA R: AGATAAGCCCGATCATGTGC	271
11	F: GACAACCTCTAACAAACAGCCA R: AAGAATCACATAGTTCAAGAACCTAAA	369
12	F: TCGCCTGCATCCTGGCTGAATC R: AGCTCTCCAGGCACTCTGCG	305
13	F: GTTTTTCCGCATTGCGTATT R: GCCCATTGGCACTTAAGAAA	292

T 55–65 °C) and terminated by final elongation (72 °C/7 min). After PCR phase the samples were heated to 95 °C and quickly cooled to 40 °C for duplex formation. Then the samples were heated to 60 °C and proper HRM analysis began by raising the temperature 0.02 °C/s up to 98 °C. Data were analysed with the Light Cycler Gene Scanning Software. Suspect samples were sequenced using an automatic fluorescent genetic analyser ABI Prism™ 3130 Genetic Analyzer (Applied Biosystems, Carlsbad, CA) in accordance with the manufacturer's instructions. The Hardy-Weinberg equilibrium was tested in the control group by the Pearson χ^2-test. A P value greater than 0.05 was assumed to be at Hardy-Weinberg equilibrium in a population (P = 0.61 for p.P15S control group and P = 0.10 for p.A404V control group).

Results and Discussion

In this study we performed mutation screening of the *TRPC6* gene in a group of 64 patients with histologically proven FSGS or MCD to investigate the role of these mutations in Czech adult population.

No *TRPC6* gene mutation was found. Two additional single nucleotide polymorphisms (SNPs) with an amino acid change, p.P15S, c.43C>T (rs3802829) and p.A404V, c.1211C>T (rs36111323), were identified. To investigate the prevalence and possible effect of these polymorphic changes on individuals we performed mutation analysis by the high-resolution melting method in a group of controls counting 300 samples. We also evaluated the distribution of these polymorphisms among the groups of patients with SRNS and SSNS, respectively (Table 3). The distribution of polymorphism p.A404V was almost equal among the groups of patients with SRNS (22.9 %), SSNS (29.4 %) and control group (23 %). On the other hand, the results show more frequent prevalence of heterozygotes of the p.P15S polymorphism in patients with SRNS than in SSNS (29 % versus 12.1 %). Since FSGS caused by mutations in the *TRPC6* gene is characteristic by its incomplete penetrance and wide clinical spectrum, several proteins involved in the glomerular filtration barrier might need to be abnormal to give rise to a severe phenotype. Thus, this change might apparently have some influence on FSGS along with another change. Therefore, we intend to continue screening the p.P15S change in a larger cohort of patients to determine its possible influence on FSGS manifestation.

Other variants that did not result in an amino acid change were detected in a significant portion of our patients: 1. p.T296T (1 patient), 2. p.N561N (36 patients), 3. p.Y705Y (1 patient), 4. p.T714T (1 patient), 5. p.F843F (16 patients) and 6. p.Q904Q (48 patients). We can speculate that they may contribute to the phenotype of FSGS in the patients. In addition, a few intronic changes were found which could also have influence on the proper function of the protein (Table 4).

Although we did not find any possible disease-causing mutation of the *TRPC6* gene in our cohort of Czech adult patients, our results are not in contradiction with other studies. Most studies of the *TRPC6* gene were done mainly in patients with familial FSGS (e.g. 71 families by Reiser et al., 2005; 21 families by Heeringa et al., 2009; 31 families by Zhu et al., 2009). So far, only four mutations of the *TRPC6* gene have been discovered

Table 3. Prevalence of heterozygotes of p.P15S and p.A404V polymorphisms in different groups of FSGS patients and in the control group

	Steroid-resistant FSGS patients	Steroid-sensitive FSGS patients	Control group
p.P15S heterozygotes (C/T)	29.0 %	12.1 %	16.5 %
p.A404V heterozygotes (C/T)	22.9 %	29.4 %	23.0 %

Table 4. Summary of all exonic and intronic changes found in our group of patients

Change		Position
c.43C>T	p. Pro15Ser	exon 1
c.171-20A>G	probably no splice defect	intron 1
c.888G>A	p. Thr296Thr	exon 2
c.1211C>T	p. Ala404Val	exon 4
c.1683T>C	p. Asn561Asn	exon 6
c.2115C>T	p.Tyr705Tyr	exon 8
c.2142G>T	p.Thr714Thr	exon 8
2485-138C>T	probably no splice defect	intron 8
c.2529C>T	p.Phe843Phe	exon 11
c.2645-23_-25delCTT	probably no splice defect	intron 11
c.2712G>A	p. Gln904Gln	exon 13

in sporadic cases of FSGS (Santín et al., 2009; Gigante et al., 2011; Mir et al., 2012). There were only three patients with familial history of proteinuria in our group of patients.

In our cohort of 64 patients from the Czech Republic we did not find any disease-causing mutation of the *TRPC6* gene. The HRM method as a rapid sensitive analytical method was optimized for *TRPC6* mutation screening. Nevertheless, the c.43C>T polymorphism could be investigated in a larger cohort of patients to establish its possible influence on the prognosis of FSGS.

Acknowledgement

The authors declare that they have no competing financial interests.

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