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Mutational screening of inverted formin 2 in adult-onset focal segmental glomerulosclerosis or minimal change patients from the Czech Republic

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Abstract

Background: Mutations in *INF2* are frequently responsible for focal segmental glomerulosclerosis (FSGS), which is a common cause of end stage renal disease (ESRD); additionally, they are also connected with Charcot-Marie-Tooth neuropathy. *INF2* encodes for inverted formin 2. This protein participates in regulation of the dynamics of the actin cytoskeleton, involving not only the polymerisation, but also the depolymerisation of filaments. The present study is the first mutational analysis of *INF2* done in the Czech Republic.

Methods: Mutational analysis of *INF2* was performed on 109 patients (mean age at onset 41.44 ± 18.91 years) with FSGS or minimal change disease (MCD); and also in 6 patients without renal biopsy who had already developed chronic kidney disease (CKD)/ESRD at the time of diagnosis. We used high resolution melting method (HRM), with subsequent Sanger sequencing, in suspect samples from HRM analysis. The HRM method is an effective method for the screening of large cohorts of patients.

Results: Two pathogenic mutations (p.Arg214His and p.Arg218Gln) were detected in *INF2*. The first (p.Arg214His) was identified in the FSGS patient with a positive family history. The second mutation (p.Arg218Gln) was found in two brothers with ESRD of unknown etiology. The most frequent sequence change was the substitution p.P35P, the incidence of which corresponded with the frequencies available in the ExAC Browser and gnomAD Browser databases. This analysis also detected different exonic and intronic changes that probably did not influence the phenotype of the included patients.

Conclusions: The *INF2* mutational screening is useful in familial FSGS cases as well as in patients with an unknown cause for their ESRD, but with a positive family history. *INF2* seems to be not only the cause of FSGS, but also of ESRD of unknown etiology. Our study has confirmed that the HRM analysis is a very useful method for the identification of single nucleotide substitutions.

Keywords: Focal segmental glomerulosclerosis, Minimal change disease, End stage renal disease, High resolution melting method, *INF2*

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Background

Podocytes are highly specialized cells with a unique and highly differentiated cytoarchitecture. Their main functions include: support of the glomerular capillaries, synthesis of glomerular basement membrane (GBM), and regulation of permselectivity. Any damage to the podocytes is usually characterized by nephrotic syndrome and (if persistent) it may progress into end stage renal disease (ESRD). Minimal change disease (MCD) and focal segmental glomerulosclerosis (FSGS) are common causes of nephrotic syndrome.

The major pathological feature of both MCD and FSGS is increased permeability of the glomerular capillary filter. Under light microscopy the glomeruli of patients with MCD have a normal size and cellularity and show no evidence of segmental sclerosis; whereas electron microscopy demonstrates the same podocyte foot processes effacement as in glomeruli affected by FSGS. Whereas the outcome of MCD in terms of long-term preservation of renal function is good; by comparison, FSGS often leads to ESRD. We still have only limited insight into the pathogenesis of both MCD and FSGS. Moreover, both the etiology and histology of FSGS as well as of MCD may be very variable, including: viral infections, toxic agents, adaptive structural-functional responses, and mutations in the genes encoding proteins specific for podocytes (or highly expressed in them) [1–3], such as: *ACTN4* [4], *INF2* [5], and *TRPC6* [6].

Inverted formin 2, encoded by *INF2*, is a member of the formin family of proteins, which regulates the dynamics of the actin cytoskeleton. It not only accelerates actin polymerisation, but also filament depolymerisation. INF2 is expressed in many tissues such as: kidney, liver, heart, skeletal muscles, and placenta. In renal podocytes, it plays a key role in the function and structure of the actin cytoskeleton. The main regions of INF2 are: the N-terminal diaphanous inhibitory domain (DID), the formin homology FH1 and FH2 domains, and the terminal diaphanous autoregulatory domain (DAD) [7, 8]. Most of the reported mutations are localized in the DID domain, which probably functions as a regulatory domain of the polymerisation and depolymerisation of the actin filaments. The previous experiments demonstrated that substitutions in INF2 caused an abnormal distribution of the slit diaphragm proteins nephrin and podocin, and dysregulation of the podocyte cytoskeleton; suggesting its involvement in the pathogenesis of the autosomal dominant form of FSGS [5, 9]. Mutations in *INF2* are not only connected with FSGS, but also with Charcot-Marie-Tooth neuropathy [10].

In this study, we present a mutational analysis of *INF2* (whose mutations are frequent causes of FSGS in adults) in patients with FSGS or MCD as well as in a group of patients with a positive family history of ESRD of unknown etiology, using a high resolution melting method (HRM) and Sanger sequencing.

Methods

Patients and study design

Our study, focused upon *INF2*, was carried out in 109 patients with biopsy-proven FSGS or MCD (see Table 1 for detailed information). The origins of FSGS or MCD of the involved individuals were both familial (inherited) in 19 cases, and idiopathic (sporadic). The study also included a group of 6 patients with a positive family history for ESRD in combination with developed advanced chronic kidney disease (CKD)/ESRD at the time of diagnosis. There were no renal biopsies performed in this group of patients thought to have burnt out diagnostic features because of advanced disease. Healthy controls from anonymous volunteers were used to test for variants in healthy individuals.

Ethical approval

The study was performed with the approval of the Ethics Committee of the General University Hospital in Prague.

Table 1 Clinical data of 109 patients with FSGS/MCD included in the analysis of *INF2*

Characteristic	Group	Number/Value
Diagnosis	FSGS	77 (70.6%)
	MCD	32 (29.4%)
Sex	Male	51 (46.8%)
	Female	58 (53.2%)
Family history	Positive	19 (17.4%)
	Negative	90 (82.6%)
NS at the time of diagnosis	Yes	78 (71.6%)
	No	31 (28.4%)
Effect of corticosteroid therapy	Sensitive	34 (31.2%)
	Resistant	41 (37.6%)
	No therapy	34 (31.2%)
Mean age at the time of diagnosis	Years	41.44 ± 18.91
Mean proteinuria at the time of diagnosis	All: Grams/24 h	5.67 ± 4.64
	FSGS: Grams/24 h	4.99 ± 4.14
	MCD: Grams/24 h	7.42 ± 5.32
Mean serum albumin level at the time of diagnosis	All: Grams/Litre	28.05 ± 9.48
	FSGS: Grams/Litre	30.36 ± 9.1
	MCD: Grams/Litre	22.15 ± 7.67
Average serum creatinine level at the time of diagnosis	All: μmol/Litre	109.52 ± 61.26
	FSGS: μmol/Litre	112.05 ± 52.21
	MCD: μmol/Litre	103.31 ± 78.91

Data are presented by mean ± SD or number (percentage)
FSGS Focal segmental glomerulosclerosis, *MCD* Minimal change disease, *NS* Nephrotic syndrome (defined as presence of proteinuria ≥3 g/24 h plus serum albumin level ≤ 30 g/L)

The blood of patients, their relatives, and the anonymized controls was obtained after written informed consent was given by all in accordance with the protocol approved by the institutional review board at the General University Hospital.

Mutational analysis

Genomic DNA was isolated from the peripheral blood lymphocytes using standard procedures. The complete coding region and intron-exon boundaries of *INF2* were amplified, and were subsequently analyzed using the HRM method and LightCycler 480, as well as HRM Master Mix (both by Roche Diagnostics). The data obtained from the HRM analysis were analyzed using Light Cycler Gene Scanning Software (Roche Diagnostics). The suspect samples from the HRM analysis were screened for substitutions by Sanger sequencing performed on an ABI Prism™ 3130 Genetic Analyser using BigDye® Terminator v1.1 Cycle Sequencing Kit (both by Applied Biosystems). All primer sequences and annealing temperatures are available upon request. Targeted Sanger sequencing was also performed in the parents and relatives of probands with causal mutations or novel substitutions in order to determine their carryover and position on homologous chromosomes.

The HRM method is a fast, simple, and cheap method for the analysis of large cohorts of patients. Especially for SNP scanning, this is a highly sensitive and specific method, with some limitations (e.g., GC-rich fragments, or detection of homozygous variants). Sanger sequencing is the technique commonly used in molecular laboratories. On the one hand, it is a simple method in terms of sample preparation, and an effective way to analyze single genes, or to confirm variations identified by next generation sequencing. Then again, the procedure is an expensive procedure with detection limitations (e.g., mosaicism).

Sequence analysis and assessment of sequence changes

Sequence chromatograms were analyzed using a BioEdit Sequence Alignment Editor (http://www.mbio.ncsu.edu/BioEdit). All sequenced samples were compared to the reference coding sequence NM_022489.3 as well as the reference genome sequence NG_027684.1, respectively. Substitutions found in this analysis were checked against the Human Gene Mutation Database (http://www.hgmd.cf.ac.uk/ac/index.php) and/or the Ensembl [11] and NCBI dbSNP database (https://www.ncbi.nlm.nih.gov/projects/SNP/). In the case of novel missense variants, their pathogenicity was assessed computationally using Mutation Taster [12], PolyPhen-2 [13], PROVEAN [14], and SIFT [15]. The possible splice effect of intronic changes was evaluated using Human Splicing Finder [16] and Net Gene2 (http://www.cbs.dtu.dk/services/NetGene2/).

Results

INF2 mutational analysis was performed in a total of the 115 patients with FSGS (77 patients) /MCD (32 patients), or the characteristics of a separate group including a positive family history for ESRD in combination with developed advanced CKD/ESRD at the time of diagnosis. The described mutations and both the exonic and intronic polymorphisms had been identified. See Table 2 for the exonic variants identified.

Two mutations in *INF2* (p.Arg218Gln and p.Arg214His) already known to be responsible for the familial form of FSGS [5] were detected in two different families with a positive family history for FSGS, or ESRD of unknown etiology. Both of the mutations found are localized in exon 4, which is involved in the highly conserved DID region, and that is situated at the N-terminus of the INF2 protein.

The most frequent substitution in *INF2* (p.P35P) located in exon 2, had been found in all patients, mostly in the homozygous state (allele frequency = 97.4%). Exon 21 of *INF2* seemed to be a very variable region because of the high number of changes identified. However, these substitutions probably did not influence the phenotype of the patients included in our study.

Family no. 133 (*INF2*, p.Arg218Gln)

This family (see Fig. 1a), with a positive family history of ESRD of unknown etiology, and with mild proteinuria (affecting two brothers, their father, aunt, and cousin), had already been identified as one described by the causal missense mutation p.Arg218Gln (c.653 G > A) in *INF2*, with a damaging effect on podocyte function [5]. The substitution was first detected in the two brothers who had already developed ESRD. Afterwards, the same mutation was found in their father and cousin. The ages of the brothers at ESRD were 27 and 31 years, respectively; while their father developed ESRD at age 57. For that reason, in the case of these two patients, we presume the co-action of more factors, such as other substitutions in *INF2* or some influence from the environment. The younger affected brother as well as his healthy mother also had the SNP p.Pro528Ser (c.1582 C > T) in *INF2*. Additionally, we found other different intronic and exonic same-sense substitutions; however, there is not any hypothesis of their effects on the phenotype.

Family no. 50 (*INF2*, p.Arg214His)

Also identified was the known mutation p.Arg214His (c.641 G > A), with a proven damaging effect [5], in a 51 year old woman with FSGS (from a renal biopsy) and with a positive family history (her father had ESRD at age 33, and an uncle at age 30) (see Fig. 1b). Her two daughters were healthy. At her age, this patient has only mild chronic renal insufficiency with moderate proteinuria (1-3 g/24 h), although she suffers from diabetes

Table 2 All *INF2* exonic variants found in our two cohorts of patients including allele frequencies from ExAC Browser and gnomAD Browser

Exone	cDNA level	Protein level	Allele frequency % (*n* = 115)	ExAC Browser Af	gnomAD Browser Af	Reference or rs number
2	c.42 G > A	p.Leu14Leu	0.9	0.03997	0.01817	rs62638758
2	c.105 C > T	p.Pro35Pro	97.4	0.9783	0.9783	rs4983530
4	c.579 C > T	p.Tyr193Tyr	0.4			Novel
4	**c.641 G > A**	**p.Arg214His**	**0.4**	**NF**	**NF**	**[5]**
4	**c.653 G > A**	**p.Arg218Gln**	**0.9**	**NF**	**NF**	**[5]**
7	c.879G > A	p.Ser293Ser	0.9	0.007779	0.007394	rs184709736
7	c.885G > A	p.Leu295Leu	0.4	0.00009769	0.00009.272	rs370680236
8	c.1472C > T	p.Pro491Leu	0.4			Novel
8	c.1499C > T	p.Pro500Leu	0.4	0.0009303	0.0003025	rs561201601
8	c.1582C > T	p.Pro528Ser	0.9	0.007560	0.005266	rs181694819
18	c.2640 T > C	p.Asp880Asp	60.0	0.8608	0.8619	rs10133301
21	c.3066 T > C	p.Asp1022Asp	68.3	0.8060	0.7871	rs4983535
21	c.3108 T > C	p.Leu1036Leu	0.4	0.007002	0.004700	rs186075307
21	c.3163C > T	p.Pro1055Ser	0.4			Novel
21	c.3169C > T	p.Pro1057Ser	2.2			Novel
21	c.3170C > T	p.Pro1057Leu	1.3			Novel
21	c.3177C > T	p.Pro1059Pro	0.9			Novel
21	c.3179C > T	p.Thr1060Ile	3.5			Novel
21	c.3180C > T	p.Thr1060Thr	1.7			Novel
21	c.3181C > A	p.Leu1061Met	0.9			Novel
21	c.3181C > T	p.Leu1061Leu	1.3			Novel
21	c.3207A > C	p.Pro1069Pro	40.4	0.6103	0.6053	rs1128840
21	c.3207A > G	p.Pro1069Pro	40.9	0.2469	0.2524	rs1128840
21	c.3286C > T	p.Pro1096Ser	7.8	0.07527	0.06494	rs34251364

Pathogenic mutations are showed in bold. Allele frequency was counted for the current study
Af Allele frequency
NF Not found in browser

mellitus, hypertension, and obesity (BMI = 35.58). The biopsy sample showed a perihilar variant of FSGS, with a mild increase of the mesangial matrix (see Fig. 2). This type of injury represents a common part of the so-called secondary FSGS, which is mediated by adaptive structural-functional responses, and frequently is associated with obesity of all of grades with a BMI ≥ 30.0. Even though the renal biopsy looked like secondary FSGS, a mutational analysis was done because of the positive family history of ESRD of unknown etiology.

Discussion

Several genes highly expressed in podocytes (e.g., *ACTN4, INF2,* and *TRPC6*) had previously been reported to be a cause of familial and sporadic autosomal dominant forms of FSGS. Herein, we present the molecular genetic analysis of *INF2* (using the HRM method and Sanger sequencing) performed on a large cohort of patients with FSGS/MCD (FSGS = 77 patients, MCD = 32 patients), as well as in a group of patients (6) characterized by a positive family history for ESRD in combination with advanced CKD/ESRD at the time of diagnosis. These types of analyses can lead to the identification of new causal mutations; therefore, they are very important in clinical practice for the diagnosis of patients.

The INF2 protein, belonging to the formin family, plays a key role in the influence of the actin cytoskeleton dependent processes in podocytes because of its capability to accelerate both the polymerisation and depolymerisation of actin. The protein consists of three main parts including: the DID domain, two FH domains, and the DAD domain. Previous studies have demonstrated the abnormal distribution of the detected mutations; all of them having been identified in exons encoding the DID domain, suggesting its crucial role for the protein function. The DID domain appears to be important for actin cytoskeleton regulation [7, 8].

All identified substitutions in the exons were determined to be disease causing candidates if they: (a) segregated with the disease (in the case that other relatives

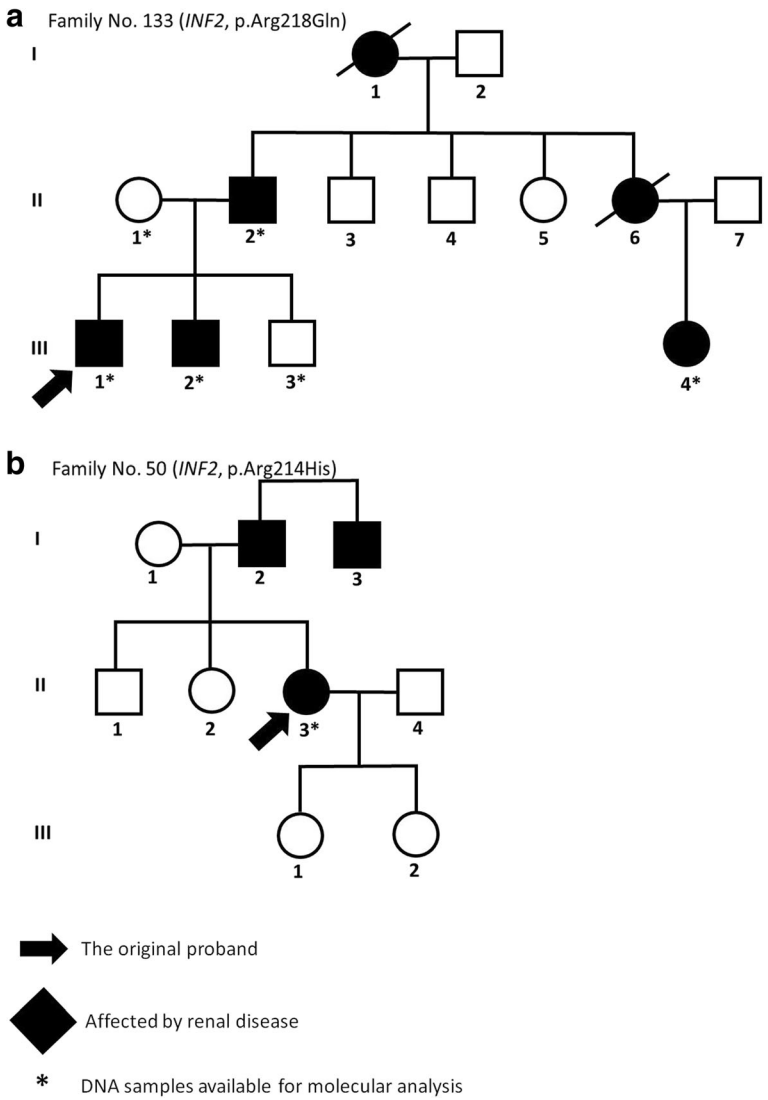

Fig. 1 Pedigree diagrams of selected families from our cohorts of patients. The figure shows the pedigrees of the *INF2* mutated families; black arrows indicate original probands; black filled individuals were affected by renal disease. All affected individuals whose DNA was available for analysis carried the mutation in *INF2*

were also affected, and we had samples of their DNA); (b) were predicted to be damaging by the Mutational Taster program [12], PolyPhen-2 [13], PROVEAN [14], and SIFT [15]; (c) were not carried by both healthy family members (whose DNA samples we had) as well as the healthy controls; and (d) were not present in control chromosomes in the db SNP and 1000 Genomes project.

We identified the mutation p.Arg218Gln in *INF2* with a damaging effect on the podocyte function in two brothers from a family with the positive history for ESRD. This mutation was first described by Brown et al. in a large family with the familial form of FSGS [5]. Both brothers quickly progressed to ESRD, aged 27 and 31, respectively.

The other detected casual mutation in *INF2* was p.Arg214His, also first identified by Brown et al. [5]. The mutation was found in a woman with a perihilar variant of FSGS and simultaneously suffering from diabetes mellitus. Although the family history of the proband was positive for renal diseases (father and uncle), her two healthy daughters had refused the opportunity for genetic testing. The incidences of this variant in our analysis were 0.9% (the whole cohort of patients), 1.3% (only the cohort of FSGS patients), and 5.6% (the cohort of FSGS patients with a positive family history), respectively. Both identified casual mutations are localized in the DID domain of INF2, which is necessary for normal protein function because it is involved in actin cytoskeleton regulation.

The mutational analysis of *INF2* also identified other different exonic changes and intronic substitutions that

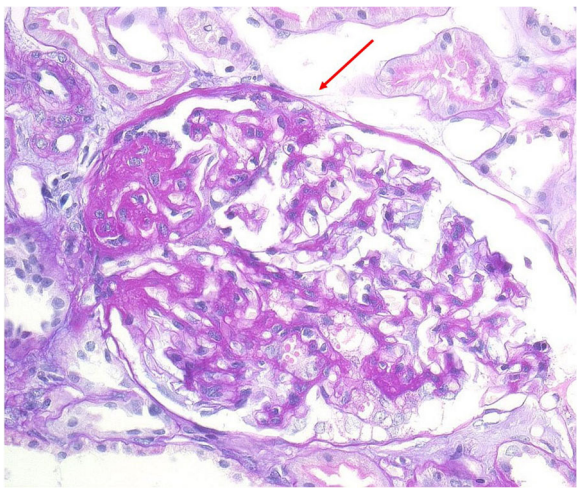

Fig. 2 Kidney biopsy findings in a proband from family No. 50. Enlarged glomerulus with perihilar segmental sclerotic lesion. Simultaneously, there is showed a small sclerotic lesion (arrow) with a cell bridging between the Bowman capsule and the GBM (PAS, high power field, objective 40×)

seemed to have no effect on the phenotype of the patients included in this study.

The most frequent substitution in *INF2* was p.P35P within exon 2, and with an allele frequency of more than 97%. This incidence corresponds with the results available in the ExAC Browser (http://exac.broadinstitute.org/) and gnomAD Browser (http://gnomad.broadinstitute.org/) databases, confirming the sensitivity of HRM. Other highly frequent changes in *INF2* were p.D1022D and p.D880D, with allele frequencies of 68% and 60%, respectively.

The HRM analysis was a very effective method for mutational screening in such a large cohort of patients. It proved to be of sufficient sensitivity in the detection of single nucleotide substitutions.

According to previous studies focused on *INF2*, it appears that this gene is responsible for a high percentage of patients with familial FSGS [5, 17, 18]. Even though the proportion of mutations in sporadic FSGS is significantly lower [9, 18], we included both familiar and idiopathic patients with FSGS. Although the incidence of pathogenic mutations was lower than our assumptions at the beginning of the study, we established the method for the molecular analysis of *INF2* and analyzed a large cohort of patients with FSGS/MCD; confirming that MCD cases are less likely to harbor deleterious genetic variants in those genes implicated in FSGS [4–6, 19]. This was the first molecular genetic analysis focused on *INF2* in the Czech Republic. A mutational analysis of *INF2* should be performed in all patients with a positive family history of FSGS or unknown ESRD with an autosomal dominant inheritance. Proteinuria is frequently mild or moderate, the patients are asymptomatic, and advanced renal insufficiency is already present, so a renal biopsy is not indicated in many cases.

Conclusions

In conclusion, pathogenic mutations of *INF2* are not a frequent cause of FSGS nor of MCD in the Czech Republic. The *INF2* mutations must be taken into account; not only in patients with FSGS and a positive family history, but also in patients with ESRD of unknown etiology and a positive family history of ESRD in other generations. The HRM method seems to be a reliable and cheaper method than is direct sequencing for an analysis of the concrete gene in large cohorts of patients.

Abbreviations

ACTN4: Alpha-actinin 4; BMI: Body mass index; CKD: Chronic kidney disease; DAD: Diaphanous autoregulatory domain; DID: Diaphanoury domain; ESRD: End stage renal disease; FH: Formin homology domain; FSGS: Focal segmental glomerulosclerosis; GBM: Glomerular basement membrane; HRM: High resolution melting; INF2: Inverted formin 2; MCD: Minimal change disease; PAS: Periodic acid-Schiff stain; R: Spectrin repeat; SNP: Single nucleotide polymorphism; TRPC6: Transient receptor potential cation channel, subfamily C, member 6.

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Availability of data and materials

Data supporting the findings are included in the article. Any further information is available from the corresponding author upon reasonable request.

Authors' contributions

MS carried out the molecular genetic analysis and the drafting of the manuscript. JS conceived and designed the study, and coordinated the analyses. EH made the histopathologic analyses. VH recruited the participants and collected clinical data. VT participated in the design of the study. JR recruited the participants, collected the clinical data, and helped to draft the manuscript. All of the authors have read and approved the final manuscript.

Ethics approval and consent to participate

The study was performed in accordance with the Helsinki Declaration, and with the approval of the Ethics Committee of the General University Hospital in Prague, Ref. No. 1443/11 S-IV. The blood of patients, their relatives, as well as anonymized controls was obtained after their written informed consent had been obtained.

Consent for publication

The written consent for publication of case presentations was obtained from each relevant participant.

Competing interests

The authors declare that they have no competing interests.

Publisher's Note

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Original Article

Mutational Analysis of *ACTN4*, Encoding α-Actinin 4, in Patients with Focal Segmental Glomerulosclerosis Using HRM Method

(focal segmental glomerulosclerosis / minimal change disease / α-actinin 4 / mutational analysis / / high-resolution melting method)

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Abstract. α-Actinin 4, encoded by *ACTN4*, is an F-actin crosslinking protein which belongs to the spectrin gene superfamily. It has a head-to-tail homodimer structure with three main domains. Mutations in *ACTN4* are associated with idiopathic nephrotic syndrome (NS). However, until today only a few mutations have been described in this gene. We used genomic DNA of 48 patients with focal segmental glomerulosclerosis (FSGS) and minimal change disease (MCD) to screen for *ACTN4* mutations by high-resolution melting analysis (HRM). Suspect samples were sequenced and compared with healthy controls. To investigate the prevalence and possible effect of some substitutions found in FSGS/MCD patients we also looked for these changes in patients with IgA nephropathy (IgAN) and membranous glomerulonephritis (MGN). We found 20 exonic and intronic substitutions in the group of 48 Czech patients. The substitution 2242A>G (p.Asn748Asp) is a candidate mutation which was identified in one patient but not in any of the 200 healthy controls. Exon 19 seems to be a variable region due to the amount of revealed polymorphisms. In this region we also found three unreported substitutions in IgAN patients, c.2351C>T (p.Ala784Val), c.2378G>A (p.Cys793Tyr) and c.2393G>A (p.Gly798Asp). These substitutions were not found in any tested healthy controls. To conclude, the *ACTN4* mutations are not a frequent cause of FSGS/MCD in Czech adult patients. One new *ACTN4* mutation has been identified.

Introduction

α-Actinin 4, encoded by the *ACTN4* gene, is a member of the spectrin gene superfamily that crosslinks F-actin filaments (Maruyama and Ebashi, 1965; Davison et al., 1989). It is a non-muscle isoform expressed in many human tissues (Löwik et al., 2009). Its structure is a head-to-tail antiparallel homodimer with three main parts. The N-terminal domain is composed of two calponin homologous domains (CH1, CH2) with actin-binding sites (ABS), followed by four spectrin repeats (R1-R4). The C-terminal domain consists of two EF-hand domains that can bind Ca^{2+} ions (Baron et al., 1987; Imamura et al., 1988; Leinweber et al., 1999). The protein is located in the cytoplasm and also in the cell nucleus (Kumeta et al., 2010). α-Actinin 4 can interact with phosphatidylinositol 3-kinase, vinculin, β_1 integrins, synaptopodin, membrane-associated guanylate kinase (MAGI-1), zonula occludens 1 (ZO-1), plasminogen activator inhibitor type 1 (PAI-1) and brain-expressed RING finger protein (BERB) (Otey et al., 1990; McGregor et al., 1994; Shibasaki et al., 1994; El-Husseini et al., 2000; Patrie et al., 2002; Magdolen et al., 2004; Asanuma et al., 2005; Chen et al., 2006). It is affected by Ca^{2+} ions and phosphorylation (Weins et al., 2007; Shao et al., 2010). It participates in cell adhesion and locates along stress fibres, where it interacts with

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Abbreviations: ABS – actin-binding sites, BERB – brain-expressed RING finger protein, CH – calponin homologous domain, CLP-36 – PDZ-domain and LIM-domain protein, FSGS – focal segmental glomerulosclerosis, HRM – high-resolution melting, IgAN – immunoglobulin A nephropathy, MAGI-1 – membrane-associated guanylate kinase 1, MCD – minimal change disease, MGN – membranous glomerulonephritis, NS – nephrotic syndrome, OR – odds ratio, PAI-1 – plasminogen activator inhibitor type 1, R – spectrin repeats, ZO-1 – zonula occludens 1.

the PDZ-domain and LIM-domain protein (CLP-36) (Vallenius et al., 2000; Michaud et al., 2006). α-Actinin 4 was studied in connection with some types of cancer, including breast and lung cancer, pancreatic ductal carcinoma, ovarian cancer and glioma tumours (Honda et al., 1998; Echchakir et al., 2001; Kikuchi et al., 2008; Sen et al., 2009; Yamamoto et al., 2009).

Mutations in *ACTN4* are associated with idiopathic nephrotic syndrome, which is identified as focal segmental glomerulosclerosis (FSGS) and minimal change disease (MCD) (Reiterová and Šafránková, 2010). Nephrotic syndrome is defined by nephrotic proteinuria, hyperlipidaemia, hypoproteinaemia and oedemas (Ryšavá et al., 2005). Until today only a few mutations have been described in this gene (Kaplan et al., 2000; Weins et al., 2005; Choi et al., 2008; Dai et al., 2009, 2010). This is the first study in the Czech Republic focused on Czech patients with FSGS and MCD analysing the *ACTN4* gene.

Material and Methods

The study was performed in 48 Czech patients with biopsy-proven FSGS and MCD (17 males and 24 females, mean age 42.3 ± 16.5, mean age at the time of diagnosis 35.9 ± 18.2 years, these data were not available in seven patients). Thirty-one patients were steroid-resistant and four patients were steroid-sensitive. Four patients were treated with ACEi and AT1 blockers. These data were not available in nine patients. As steroid-resistant were defined patients who did not respond to prednisone (dose 1 mg/kg) during six months of therapy. Two hundred unrelated healthy males and females without history of renal disease or abnormal urinary findings were included as controls. The control group was randomly selected from individuals who are blood donors. To investigate the prevalence and possible effect of some substitutions found in FSGS/MCD patients we also looked for these changes in patients with immunoglobulin A nephropathy (IgAN) and membranous glomerulonephritis (MGN). The group of IgAN patients included 155 members (100 males and 55 females, mean age 46.7 ± 14.5). The group of MGN patients included 56 members (34 males and 22 females, mean age 60 ± 13.8). The study was performed with the approval of the Ethics Committee of the General University Hospital in Prague. The blood of patients and healthy controls was obtained after informed consent was given in accordance with a protocol approved by the institutional review board at the General University Hospital in Prague.

Genomic DNA was isolated from peripheral blood lymphocytes using QIAGEN spin columns in a QIAcube device (Qiagen, GmbH, Hilden, Germany). The coding region and intron-exon boundaries of the *ACTN4* gene were screened for mutations by high-resolution melting analysis (HRM) using LightCycler 480 (Roche Diagnostics, Mannheim, Germany). All primers were designed using Primer-Blast program through the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). PCR was performed in 10 ml volumes in LightCycler 480. The amplification mixture included the High Resolution Melting Master kit (Roche Diagnostics) (consisting of 2x conc. Master mix, 25 mM $MgCl_2$ and PCR H_2O), 0.01 mM each of two primers and 10 mg/l or 20 mg/l genomic DNA. Dimethyl sulphoxide was added to some exons to increase specificity. The temperature was initiated with a 2 min hold at 96 °C for activation of the polymerase, followed by amplification steps and terminated by final elongation. After PCR the samples were heated to 95 °C and cooled to 40 °C, which caused duplex formation. After that the samples were heated to 60 °C and then the temperature was let to raise by 20 °C/s to 98 °C. Data were analysed with Light Cycler Gene Scanning Software (Roche Diagnostics). Suspect samples were sequenced in both directions using an automatic fluorescent genetic analyser ABI Prism™ 3130 Genetic Analyzer (Applied Biosystems, Carlsbad, CA) in accordance with the manufacturer's instructions. Graphic views of nucleotide sequences were performed using BioEdit Sequence Alingment Editor (http://www.mbio.ncsu.edu/BioEdit).

The entire statistical computation was performed using freeware program PLINK (Purcell et al., 2007). First, all studied markers were analysed for Hardy-Weinberg equilibrium ($\alpha = 0.01$). Then, an association test was performed; P values less than 0.01 were considered statistically significant. The odds ratio (OR) was computed to the markers that were polymorphic in our study. In our case, OR represents the degree of association of the studied polymorphism with the disease condition. For example, SNP marker OR represents the odds of having disease given the presence of a particular SNP allele divided by the odds of having disease given the presence of another SNP allele. The higher is the OR value, the stronger is the association of minor allele with the disease phenotype. For markers that showed statistically significant association with the disease phenotype, lower and upper boundaries of the 95% confidence interval and standard error of the mean were computed (Purcell et al., 2007).

Results

We found 20 exonic and intronic substitutions in our group of patients with FSGS and MCD. In exon 19 we found additional substitutions in the groups of patients with IgAN and MGN and in healthy controls. All results are summarized in Fig. 1 and Table 1. All studied markers were in Hardy-Weinberg equilibrium, both the cases and control groups ($P > 0.01$). Association test revealed that one marker was significantly associated with the disease phenotype, the substitution c.2360C>T (p.Pro787Leu) for IgAN patients. OR for this marker was 2.021, which indicates mild association of the T allele presence with the disease phenotype. Lower and upper limits of the 95% confidence interval for c.2360C>T (p.Pro787Leu) OR was 1.246 to 3.279.

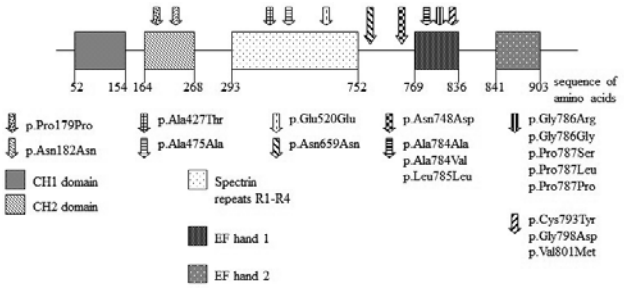

Fig. 1. The scheme of found exonic substitutions in the protein α-actinin 4

Substitutions

p.Ala427Thr (c.1279G>A)

This substitution was identified by Weins et al. (2005) as tolerated change. It is a change of a non-polar neutral amino acid for a polar neutral amino acid. We found it in a 42-year-old woman with FSGS and negative family history, but it was not detected in any of the 200 healthy controls. The age of onset of nephrotic syndrome in this woman was 39 years.

Table 1. Substitutions found in the studied patients with FSGS and MCD, IgAN, MGN and healthy controls. Novel exonic changes in boldface type (ND = not done)

Exon/intron	Nucleotide change: c.NM_004924.3	Amino acid change	Number of patients with FSGS/MCD (48)	Number of patients with IgAN (155)	Number of patients with MGN (55)	Healthy controls (200)
IVS3	c.397+27T>A		1	ND	ND	ND
5	c.537G>A	p.Pro179Pro	4	ND	ND	ND
5	c.546C>T	p.Pro182Asn	12	ND	ND	ND
IVS6	c.573-52G>C		1	ND	ND	ND
IVS8	c.819+50G>A		1	ND	ND	ND
IVS8	c.819+26G>T		1	ND	ND	ND
IVS9	c.912+65C>A		1	ND	ND	ND
11	c.1279G>A	p.Ala427Thr	1	ND	ND	0
12	c.1425C>T	p.Ala475Ala	1	ND	ND	ND
IVS13	c.1551+49C>T		6	ND	ND	ND
14	**c.1560G>A**	**p.Glu520Glu**	**1**	ND	ND	ND
IVS15	c.1875+22G>A		1	ND	ND	ND
IVS15	c.1875+23G>A		2	ND	ND	ND
IVS15	c.1875+28G>A		6	ND	ND	ND
IVS15	c.1875+29G>A		1	ND	ND	ND
16	c.1977T>C	p.Asn659Asn	6	ND	ND	ND
18	**c.2242A>G**	**p.Asn748Asp**	**1**	**0**	**0**	**0**
19	**c.2351C>T**	**p.Ala784Val**	**0**	**2**	**0**	**0**
19	c.2352G>A	p.Ala784Ala	1	13	9	16
19	c.2353C>T	p.Leu785Leu	0	12	1	10
19	c.2355G>A	p.Leu785Leu	0	9	2	3
19	c.2356G>A	p.Gly786Arg	0	15	5	13
19	c.2358G>A	p.Gly786Gly	0	4	1	3
19	c.2359C>T	p.Pro787Ser	0	8	1	13
19	c.2360C>T	p.Pro787Leu	3	41	6	29
19	c.2361C>T	p.Pro787Pro	1	18	2	6
19	c.2378G>A	p.Cys793Tyr	0	1	0	0
19	**c.2393G>A**	**p.Gly798Asp**	**0**	**1**	**0**	**0**
19	c.2401G>A	p.Val801Met	0	1	1	0
IVS19	c.2418+8A>G		0	1	0	0
IVS19	c.2418+13G>A		0	0	1	1
IVS19	c.2418+14G>A		0	0	0	3
IVS19	c.2418+15G>A		0	0	0	1
IVS19	c.2418+16C>T		0	2	0	7
IVS19	c.2418+17C>T		0	2	0	9

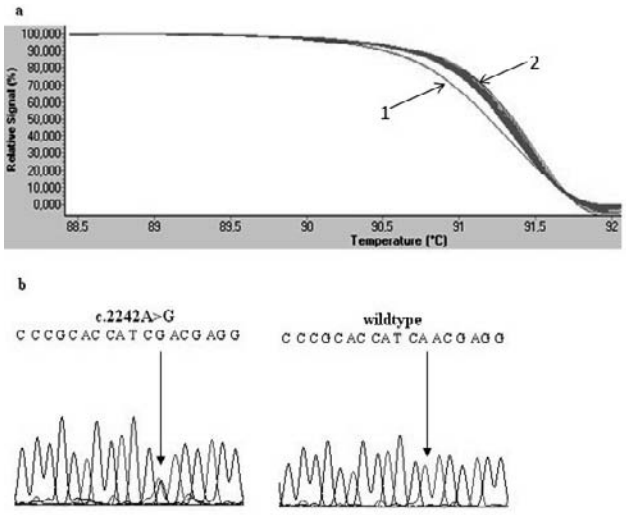

Fig. 2. **a)** The melting curve analysis of nucleotide change c.2242A>G (p.Asn748Asp) in the group of patients. Number 1 shows a patient with substitution, number 2 are patients without substitution. **b)** Sequencing pattern of part of exon 18; right – patient with substitution (c.2242A>G, p. Asn748Asp), left – patient without substitution (wild-type)

p.Asn748Asp (c.2242A>G)

We identified this substitution in one patient. It was a 59-year-old woman with FSGS and positive family history. Her age at diagnosis of the nephrotic syndrome was 54 years. This substitution was found neither in 200 healthy controls nor in 155 patients with IgAN and 56 patients with MGN (see Fig. 2). We found the patient's daughter, who also suffered from FSGS. Today, this woman is after renal transplantation and her DNA is not available. We also compared the protein sequence in different species using BioEdit Sequence Alignment Editor. The substituted amino acid is conservative in the compared vertebrates (see Fig. 3). This substitution represents a change of a polar neutral amino acid for an acidic amino acid. This also supports the probable causal significance.

p.Pro787Leu (c.2360C>T)

The substitution p.Pro787Leu was identified in three patients with FSGS and MCD and in 29 of 200 healthy controls. Both amino acids are non-polar and neutral. The other exonic substitutions in this region were p.Ala784Ala (c.2352G>A) and p.Pro787Pro (c.2361C>T) in the group of patients with FSGS and MCD, and even p.Pro787Ser (c.2359C>T) in 13 healthy controls, representing a change of a non-polar neutral amino acid for a polar neutral amino acid. We also looked for these changes in patients with IgAN and MGN. We found all these substitutions and seven additional changes in these patients. The additional changes were p.Ala784Val, p.Leu785Leu, p.Gly786Arg, p.Gly786Gly, p.Cys793Tyr, p.Gly798Asp and p.Val801Met. All substitutions in this region are summarized in Table 1.

p.Ala784Val (c.2351C>T)

We identified this substitution in two patients who suffered from IgAN. The first patient was a 67-year-old man with negative family history. He had mild proteinuria of 0.32 g per 24 h. The second case was a 25-year-old woman. She also had a negative history and her proteinuria was 0.18 g per 24 h. This substitution was found neither in the 200 healthy controls nor in patients with FSGS/MCD and MGN. We did not find this substitution in any databases or publications. Both amino acids are non-polar and neutral.

p.Cys793Tyr (c.2378G>A)

This substitution was identified in a 35-year-old woman with negative family history who had suffered from IgAN since her childhood. There was not only IgAN in renal biopsy, but also thin membrane nephropathy in the electron microscope finding. She did not respond to immunosuppressive therapy. Her proteinuria was 2.4 g per 24 h. Both amino acids are polar and neutral. We did not find this substitution in any databases or

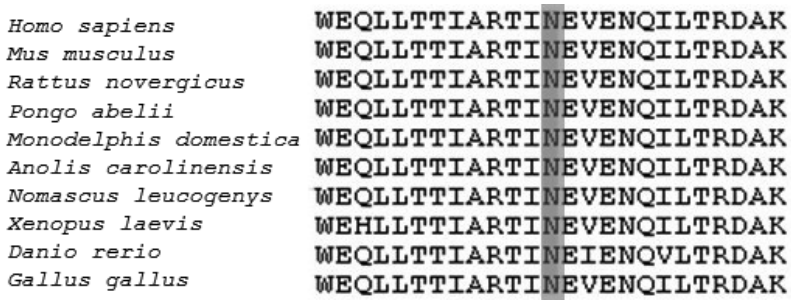

Fig. 3. Protein sequence comparison of p.Asn748Asp in different species. The substitution is marked grey. *Homo sapiens*: ref|NP_004915.2| α-actinin 4, *Mus musculus*: ref|NP_068695.1| α-actinin 4, *Rattus novergicus*: ref|NP_113863.2| α-actinin 4, *Pongo abelii*: ref|NP_001127286.1| α-actinin 4, *Monodelphis domestica*: ref|XP_001362530.1| predicted α-actinin 4 isoform 1, *Anolis carolinensis*: ref|XP_003228576.1| predicted α-actinin 4-like isoform 2, *Nomascus leucogenys*: ref|XP_003252699.1| predicted α-actinin 4-like isoform 2, *Xenopus laevis*: ref|NP_001087030.1| α-actinin 4, *Danio rerio*: ref|NP_955880.1| α-actinin 4, *Gallus gallus*: ref|NP_990457.1| α-actinin 4.

publications. We also compared the protein sequence in different species using BioEdit Sequence Alignment Editor. The substituted amino acid is conservative in the compared vertebrates.

p.Gly798Asp (c.2393G>A)

The substitution p.Gly798Asp (c.2393G>A) was found in a 59-year-old woman with IgAN but not in the 200 healthy controls or patients with FSGS/MCD and MGN. It is a change of a non-polar neutral amino acid for an acidic amino acid. We did not find this substitution in any databases or publications. The proteinuria of the woman was 5 g per 24 h. We also compared the protein sequence in different species using BioEdit Sequence Alignment Editor. The substituted amino acid is conservative in the compared vertebrates.

Discussion

We screened DNA from 48 unrelated individuals with FSGS and MCD for mutations in the *ACTN4* gene using the high-resolution melting method. We identified one candidate mutation in exon 18, which is probably the causal mutation. This substitution p.Asn748Asp (c.2242A>G) was found in a 59-year-old woman with positive family history, but not in 200 healthy controls.

Exon 19 seems to be a variable region. We observed three substitutions in the group of patients with FSGS and MGN. We also found seven additional changes in patients with IgAN and MGN and in healthy controls. The substitutions p.Ala784Val, p.Gly798Asp and p.Cys793Tyr were found in patients with IgAN but not in any of the 200 healthy controls. We can speculate that this could affect the clinical course of the disease. Cysteine and tyrosine are both polar and neutral amino acids. However, cysteine contains sulphur atoms in the form of sulphhydryl groups, allowing it to form disulphide bonds. This fact could affect formation of other protein structures. In exon 19 we also detected the substitution c.2360C>T (p.Pro787Leu). In patients with IgAN it significantly associated with the disease phenotype. OR for this marker was 2.021, which indicates mild association of the T allele presence with the disease phenotype. The number of IgAN patients was limited, and it is therefore difficult to verify the association of this polymorphism in the variable area of the gene with the disease.

The substitution p.Ala427Thr (c.1279G>A) in exon 11 was found in one patient. This substitution was described as tolerated change by Weins et al. (2005), but not found in our group of 200 healthy controls. We speculate that it could have some significance for the clinical course.

It is also interesting that we detected two identical substitutions in six patients with FSGS/MCD, c.1551+49C>T and c.1977T>C (p.Asn659Asn). We presume that these two substitutions could be in linkage. As the size of the screened groups was limited, it would be advisable to extend these groups of patients in order to exclude possible false positive results.

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The authors declare that they have no competing financial interests.

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Ověření použitelnosti poměru expresí genů *NPHS2* a *SYNPO* při diagnostice fokální segmentální glomerulosklerózy a minimálních změn glomerulů

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SOUHRN

Cíl: Cílem této studie bylo ověřit použitelnost poměru expresí genů *NPHS2* a *SYNPO* při diagnostice fokální segmentální glomerulosklerózy (FSGS) a minimálních změn glomerulů (MCD) u českých pacientů a zároveň identifikovat nové markery, které by mohly vést k jednoznačnému rozlišení mezi oběma diagnózami.
Typ studie: Pilotní studie
Materiál a metody: Do studie bylo zařazeno 24 vzorků od pacientů s FSGS a MCD. Exprese genů byla stanovena metodou relativní kvantitativní real-time PCR provedené na kartách TaqMan® Array Micro Fluidic Cards s následnou normalizací dat k referenčnímu genu *GAPDH*.
Výsledky a závěry: Nebyly prokázány žádné statisticky významné rozdíly v expresi studovaných genů mezi pacienty s FSGS a MCD. Zároveň se nepodařilo potvrdit použitelnost poměru expresí genů *NPHS2* a *SYNPO* pro rozlišení FSGS a MCD u českých pacientů.
Klíčová slova: FSGS, MCD, genové exprese, kvantitativní real-time PCR.

SUMMARY

Šafaříková M., Pazourková E., Hořínek A., Reiterová J., Maixnerová D., Honsová E., Zadražil J., Štekrová J., Kohoutová M., Tesař V.: The verification of the applicability of *NPHS2*/*SYNPO* ratio for diagnosis of FSGS and MCD

Objective: The aim of this study was the verification of the applicability of *NPHS2*/*SYNPO* ratio for diagnosis of focal segmental glomerulosclerosis (FSGS) and minimal change disease (MCD) in Czech patients and identification of new markers for distinguishing between MCD and FSGS.
Material and methods: Our study was performed on 24 samples from patients with FSGS and MCD. Gene expressions were determined using relative quantitative real-time PCR and TaqMan® Array Micro Fluidic Cards and normalized to the reference gene *GAPDH*.
Results and conclusion: Our results did not find any significant difference in the podocin/synaptopodin ratio and in any other studied genes between FSGS and MCD.
Keywords: FSGS, MCD, gene expressions, quantitative real-time PCR.

Úvod

Fokální segmentální glomeruloskleróza (FSGS) a minimální změny glomerulů (MCD) patří mezi časté příčiny nefrotického syndromu, který je charakterizován nefrotickou proteinurií, hypoproteinémií, hyperlipidémií a otoky [1]. MCD se podílí na vývoji nefrotického syndromu převážně u dětí a mladistvých. U dospělých má nejvyšší podíl výskytu FSGS, zatímco prevalence MCD výrazně klesá [2]. FSGS velmi špatně odpovídá na léčbu kortikosteroidy a často vede k renálnímu selhání. Prognóza u MCD je lepší a tito pacienti zpravidla dobře reagují na léčbu [3]. Etiologie FSGS a MCD je obvykle neznámá. Nicméně obě onemocnění jsou dávána do souvislosti např. s virovými infekcemi, léky a drogami [2,4]. U FSGS existují i familiární formy, které vznikají v důsledku mutací v genech kódujících podocytární proteiny, např. *ACTN4*, *INF2* a *NPHS2* [5]. Podocyty jsou vysoce specializované renální buňky, které spolu s fenestrovaným endotelem kapilár a glomerulární bazální membránou tvoří filtrační bariéru. Svými dlouhými výběžky, tzv. pedicely, pokrývají celý povrch kapilár. Prostor mezi nimi vyplňuje nejdůležitější složka filtrační bariéry, tenká proteinová membrána zvaná slit diaphragm. Mezi hlavní funkce podocytů patří syntéza složek glomerulární bazální membrány a regulace filtrace v ledvinách [6].

Patogeneze FSGS a MCD je stejná. Dochází k poškození podocytů s následným splynutím jejich výběžků, které zapříčiňuje zvýšenou propustnost renální filtrační bariéry. U pacientů s MCD se histologický nález ve světelném mikroskopu jeví normálně a patologické procesy je možné prokázat pouze pomocí elektronové mikroskopie. U FSGS jsou tyto změny patrné již při rutinním histologickém vyšetření. V raných fázích FSGS nalézáme splynutí pedicel převážně v juxtamedulárních glomerulech.

S progresí onemocnění se poškození následně rozšiřuje i do ostatních glomerulů. V případě, že je pro histologické vyšetření pacienta v rané fázi FSGS k dispozici pouze malý vzorek biopsie, který neobsahuje juxtamedulární glomeruly, může dojít k chybné diagnóze [7,8].

Vzhledem k tomu, že potřeba správné diagnózy je klíčová k predikci progrese onemocnění a volbě vhodné léčby, jsou hledány vhodné markery pro jednoznačné rozlišení FSGS a MCD. Jedním z těchto markerů se zdá být poměr expresí genů *NPHS2* a *SYNPO* [9].

Cílem této studie bylo ověřit použitelnost poměru expresí genů *NPHS2* a *SYNPO* při diagnostice FSGS a MCD u českých pacientů (Tabulka 1) a zároveň identifikovat nové markery, které by mohly vést k jednoznačnému rozlišení mezi oběma diagnózami. V rámci studie byla zkoumána exprese 28 vytipovaných genů (Tabulka 2), které jsou spojovány s různými nefropatiemi nebo imunitním systémem člověka.

Table 1. The characteristics of analyzed diagnostic groups.

Diagnosis	N	Age at the time of biopsy (years±SD)	Gender (f/m)
MCD	7	44.7 ± 17.4	3/4
FSGS	17	46.1 ± 10.3	10/7

N=number of samples

Materiál a metody

Do studie bylo zařazeno 24 pacientů s histologicky potvrzenou diagnózou primární FSGS nebo MCD. Základní charakteristika souboru pacientů je uvedena v Tabulce 1. Vzorky renálních biopsií pocházely ze Všeobecné fakultní nemocnice v Praze, Fakultní nemocnice v Hradci Králové, Fakultní nemocnice u Svaté Anny v Brně a Fakultní nemocnice v Olomouci. Všichni pacienti podepsali před renální biopsií informovaný souhlas s molekulárně-genetickým vyšetřením.

Větší část renální biopsie byla použita pro rutinní histologické vyšetření. Tkáň určená pro analýzu genových expresí byla ihned po odběru vložena do stabilizač-

Table 2. The alphabetical order of studied genes with results of the statistical analysis. All values were rounded.

Gene	P value (FSGS vs MCD)	Expressions in FSGS patients			Expressions in MCD patients		
		Med.	Min.	Max.	Med.	Min.	Max.
ACE	0.45	37.73	2.42	518.82	40.05	26.15	239.94
AGTR1	0.85	1.78	0.05	9.63	2.36	0.87	4.88
AGTR2	0.11	0.75	0	6.10	2.31	0.31	2.24
APOL1	0.90	27.36	10.85	111.19	34.70	10.48	390.12
CCL5	0.95	9.43	1.86	20.00	10.27	1.14	183.39
CCR3	0.95	0.64	0.04	10.18	0.43	0.12	8.88
CCR5	0.75	5.07	1.18	23.37	3.70	0.75	28.07
CD2AP	0.31	367.80	161.99	1629.02	455.65	274.14	813.94
CD80	0.95	0.24	0.08	0.84	0.33	0.07	1.73
CXCR3	1.00	0.50	0.09	1.64	0.30	0.10	1.86
IFNG	0.25	0.17	0	0.53	0.03	0	0.96
IL10	0.70	0.27	0.04	1.11	0.46	0.10	3.08
IL12A	0.35	0.41	0.22	1.85	0.33	0.14	2.41
IL18	0.43	9.60	0.88	22.32	12.54	0.28	23.10
IL2RA	0.95	0.78	0.21	6.38	1.42	0.23	15.55
INF2	0.75	0.07	0.02	0.20	0.08	0.02	0.37
KIRREL2	0.37	1.07	0.13	3.60	1.14	0.36	10.81
MMP14	1.00	49.14	12.24	269.99	40.27	10.54	500
NPHS1	0.25	53.47	0.52	647.97	77.86	19.38	587.64
NPHS2	0.23	28.66	0	163.39	53.73	6.97	249.77
PLA2R1	0.31	32.60	4.89	274.35	35.95	14.28	40.34
PLCE1	0.31	12.31	3.59	63.60	14.05	3.30	105.77
PODXL	0.41	239.48	16.40	2138.20	290.78	106.95	2283.11
SYNPO	0.23	30.27	5.87	274.92	45.09	10.45	341.04
TGFB1	1.00	14.00	5.16	47.15	17.06	5.92	167.01
TNFSF12	0.44	1.38	0.66	6.51	1.25	0.64	29.26
TRPC6	0.51	1.38	0.57	4.20	0.89	0.54	10.11
VEGFA	0.41	173.50	29.02	1521.49	197.92	120.57	1783.86

Med.= median, Min.= minimum, Max.= maximum

ního pufru RNA later (Ambion) a do izolace RNA byla uchovávána při teplotě -20°C. Pro izolaci celkové RNA byl použit GenElute™ Mammalian Total RNA Miniprep Kit (Sigma-Aldrich). Koncentrace získané RNA byla změřena nanofotometrem (Implen) na základě optické denzity OD260/280. Pro následnou syntézu cDNA byl použit SuperScript® VILO™ Synthesis Kit (Invitrogen). Produkt byl uskladněn při -20°C. Exprese genů byla stanovena metodou relativní kvantitativní real-time PCR v celkovém objemu 100 µl (přístroj ABI Prism® 7900HT Sequence Detection System, Applied Biosystems) provedené na kartách TaqMan® Array Micro Fluidic Cards (Applied Biosystems). Získaná data byla analyzována v programu ExpressionSuite Software v1.0.3 (Life Technologies, Inc.) a normalizována k referenčnímu genu *GAPDH*. Porovnání expresí genů bylo provedeno vzhledem k negaussovskému rozdělení hodnot neparametrickým Mann-Whitneyho testem s Bonferroniho korekcí a hladinou významnosti $p<0,05$. Pro všechny statistické výpočty byl použit statistický program STATISTICA 10 (StatSoft CR s.r.o.).

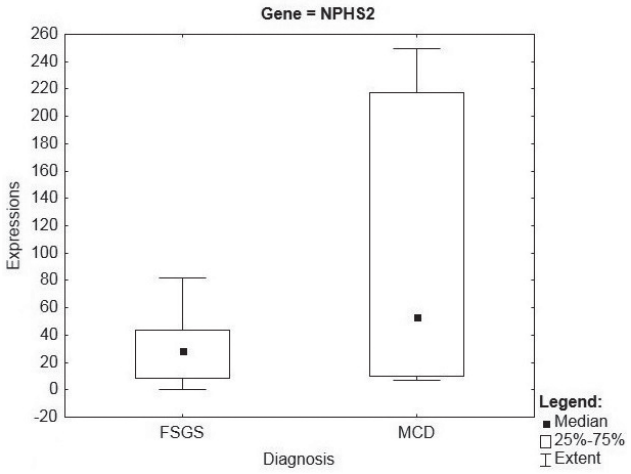

Fig. 1. The variable gene expressions of the *NPHS2* gene in FSGS and MCD patients. Relative gene expressions were calculated using normalization against the reference gene (*GAPDH*).

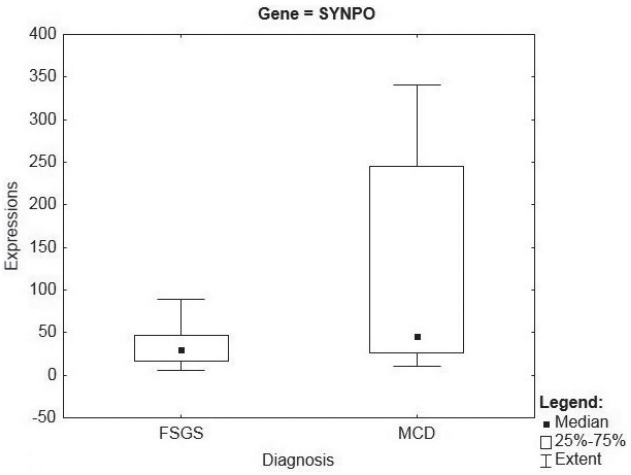

Fig. 2. The variable gene expressions of the *SYNPO* gene in FSGS and MCD patients. Relative gene expressions were calculated using normalization against the reference gene (*GAPDH*).

Výsledky a diskuse

Naše výsledky neprokázaly žádné statisticky významné rozdíly v expresi studovaných genů mezi pacienty s FSGS a MCD, což se dá vysvětlit vysokou variabilitou naměřených hodnot v obou skupinách pacientů. Detailně v Tabulce 2. Zároveň se nám nepodařilo potvrdit použitelnost poměru expresí genů *NPHS2* a *SYNPO* pro rozlišení FSGS a MCD [9]. Poměr expresí těchto genů vykazoval v našich vzorcích opět vysokou variabilitu. Podrobně zobrazeno na Obr. 1 a 2.

FSGS, zvláště její primární forma, může být velmi heterogenní nejen v etiologii, ale i v histologickém nálezu, pro jehož klasifikaci se standardně používá Kolumbijský systém [4]. Do naší skupiny pacientů s primární FSGS byly zařazeny vzorky s různými histologickými nálezy v rámci Kolumbijské klasifikace, což pravděpodobně způsobilo variabilitu jejich expresí. Pro další analýzy bude nutné získat více vzorků a roztřídit je do jednotlivých kategorií podle Kolumbijské klasifikace. Názory na klasifikaci MCD jsou rozdílné, a to ovlivňuje variabilitu klinického i histologického obrazu těchto pacientů. První přístup popisuje MCD jako samostatné onemocnění. Podle druhého přístupu jsou minimální změny glomerulů mírnější variantou FSGS [2]. Pacienti s MCD zařazení do studie neměli identické histologické nálezy, což pravděpodobně vedlo k variabilitě expresí analyzovaných genů. Na druhou stranu, všichni pacienti zařazení do studie byli kavkazského původu, takže je možné vyloučit variabilitu pocházející z etnického původu.

Bennett a kol. analyzovali exprese u vzorků od pacientů s FSGS a kontrol, které pocházely ze zdravé části ledvin po nefrektomii pacientů s Wilmsovým tumorem. Jejich výsledky prokázaly zvýšenou i sníženou expresi u mnohých genů, např. *NPHS2* a *VEGFA*, Naše studie se sice zabývala rozdíly mezi FSGS a MCD, avšak žádný z výše jmenovaných genů nevykazoval abnormální hladinu exprese při srovnání mezi oběma skupinami pacientů. Dle našeho názoru má studie Bennetta a kol. dva zásadní nedostatky. Za prvé, studie zahrnovala pouze čtyři dospělé pacienty s FSGS a tři kontrolní vzorky, které pocházely od dětí. Za druhé, srovnání nádorové tkáně s pacienty s FSGS mohlo vést ke zkreslení výsledků [10].

Velký přínos problematice rozlišení FSGS a MCD přinesli Hodgin a kol. Jejich studie zahrnovala geny účastnící se mnohých buněčných procesů, např. vývoje a diferenciace, migrace, adheze a organizace cytoskeletu. Zároveň použili vzorky pacientů s FSGS, jejichž histologické nálezy byly řádně roztříděné do skupin podle Kolumbijské klasifikace. Hodgin a kol. prokázali, že při srovnání pacientů s FSGS oproti pacientům s MCD a kontrolám se mnoho genů exprimuje rozdílně, např. *NPHS2, PODXL, PLCE1, NPHS1, SYNPO, PLA2R1*, a *INF2*. Naše analýzy nepotvrdily rozdílné hladiny exprese v genech, které byly zahrnuty v obou studiích. To mohlo být zapříčiněno i tím, že byly použity různě uchované vzorky. Hodgin a kol. analyzovali tkáň z parafínového bločku

fixovanou formalínem a naše studie byla provedena na vzorcích uchovávaných ve stabilizačním pufru při -20°C [11].

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