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.

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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.

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

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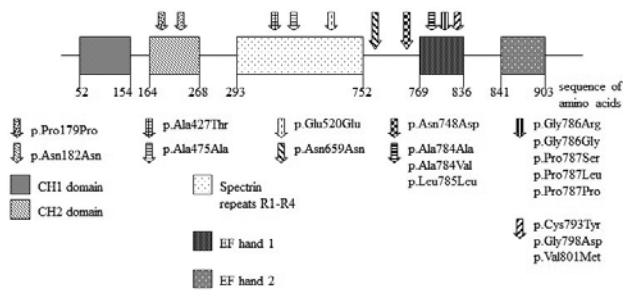
Fig. 1. The scheme of found exonic substitutions in the protein α-actinin 4

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)

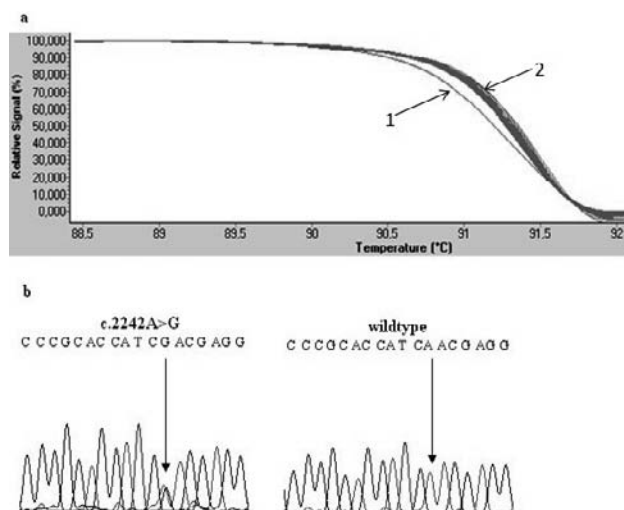| Exon/intron	Nucleotide change: c.NM_004924.3	Amino acid change	Number of patients with FSGS/MCD (48)	Number of patients with IgAN (155)	Number of patients with MGN (55)	Healthy controls (200)
IVS3	c.397+27T>A		1	ND	ND	ND
5	c.537G>A	p.Pro179Pro	4	ND	ND	ND
5	c.546C>T	p.Pro182Asn	12	ND	ND	ND
IVS6	c.573-52G>C		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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