

New TRPC6 gain-of-function mutation in a non-consanguineous Dutch family with late-onset focal segmental glomerulosclerosis

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Keywords: focal segmental glomerulosclerosis, podocyte, proteinuria, TRPC6

ABSTRACT

Background. Focal segmental glomerulosclerosis (FSGS) is a leading cause of steroid-resistant nephrotic syndrome. Hereditary FSGS is frequently caused by mutations in important structural podocyte proteins, including the slit diaphragm-associated transient receptor potential channel C6 (TRPC6).

Methods. In five patients with biopsy-proven autosomal-dominant FSGS from five different Dutch families, all 13 exons of *TRPC6* were sequenced. Upon identification of a novel *TRPC6* sequence variant, the resultant amino acid change was introduced in the wild-type TRPC6 protein and functionally tested using patch-clamp analyses and cell-surface biotinylation experiments.

Results. None of the previously described *TRPC6* mutations were found in our cohort. In one family, we identified a novel

c.524G>A sequence variant resulting in a p.Arg175Gln (R175Q) substitution in the TRPC6 protein. This sequence variant was absent in 449 control subjects and from public SNP databases. The mutation was located in the third ankyrin repeat domain (ANK3) in the cytoplasmic N-tail of TRPC6, important for protein–protein interaction and regulation of ion channel activity. Patch-clamp analyses of the mutant channel indeed showed an increased TRPC6 channel-mediated current. However, cell-surface expression of the mutant channel was not increased.

Conclusions. We identified a novel TRPC6 p.Arg175Gln gain-of-function mutation that shows increased TRPC6-mediated current, which is not due to altered cell-surface expression. This is the first mutation identified in ANK3 of the TRPC6 N-tail and is most likely responsible for the late-onset autosomal dominant FSGS in this family.

INTRODUCTION

Focal segmental glomerulosclerosis (FSGS) is one of the leading causes of steroid-resistant nephrotic syndrome, responsible for up to 40% of idiopathic nephrotic syndromes, and can rapidly progress to end-stage renal disease (ESRD) [1, 2]. FSGS is a histological diagnosis characterized by sclerosis in some but not all glomeruli (focal), while the lesions demonstrate a segmental glomerular distribution [3, 4]. FSGS can be classified as primary idiopathic FSGS or secondary FSGS. Several causes for secondary FSGS have been recognized including viral infections, certain drugs and glomerular hyperfiltration secondary to a reduced number of nephrons or due to comorbidities like diabetes mellitus, hypertension or obesity. A further category is comprised of the hereditary forms of FSGS or congenital nephrotic syndromes, including mutations in the genes encoding nephrin (NPHS1), podocin (NPHS2), Wilms' tumour 1 (WT1), alpha-actinin 4 (ACTN4), CD2-associated protein (CD2AP), phospholipase C epsilon-1 (PLCE1), laminin β 2 (LAMB2), myosin heavy chain 9 (MYH9), inverted formin 2 (INF2), mitochondrial proteins and the cation channel transient receptor potential channel C6 (TRPC6) [1, 5].

The majority of the aforementioned proteins are expressed by the podocyte in the glomerulus. They are often crucial to the integrity of the glomerular slit diaphragm that connects the podocyte foot processes. This complex of interconnected proteins provides both physical linkage and a signalling platform that regulates podocyte behaviour and architecture, and thereby affects glomerular permeability. Damage to the glomerular capillary filter, in particular at the level of the podocyte and the slit diaphragm, is of crucial importance in the pathophysiology of FSGS, as well as other forms of proteinuric disease [6].

Previously, TRPC6 was identified as a slit diaphragm-associated protein in podocytes [7]. Gain-of-function mutations in TRPC6 were shown to cause autosomal-dominant FSGS [7–10]. In the kidney, TRPC6 is expressed in both the podocyte and the distal nephron, but podocyte-specific overexpression of either wild-type (WT) or mutant TRPC6 in mice was recently shown to lead to albuminuria and histological findings similar to human FSGS [10, 11]. In addition, glomerular TRPC6 expression is increased in acquired human proteinuric diseases, including non-familial FSGS and membranous glomerulopathy (MGP), as well as in animal models for human FSGS and MGP [9, 12]. TRPC6 is a receptor-operated non-selective cation channel, which allows Ca^{2+} to flow into the cell in response to activation by diacylglycerol secondary to a phospholipase C-mediated signal coming from the stimulation of specific cell-surface receptors [13]. TRPC6 is, therefore, thought to function as a part of the signalling complex at or near the slit diaphragm, which functionally connects nephrin, podocin, CD2AP, PLCE1 and TRPC6 with the podocyte actin cytoskeleton [7, 14]. We, and others, have previously demonstrated that angiotensin II (AngII) activates specific TRPC6-mediated intracellular pathways in the podocyte, which might also lead to functional slit diaphragm

alterations and, thereby, cytoskeletal changes [12, 15–17]. Furthermore, the anti-proteinuric angiotensin receptor blockers (ARB), angiotensin-converting enzyme inhibitors (ACEi) and calcineurin inhibitors were shown to inhibit these TRPC6-mediated cascades [12, 16]. Thus, TRPC6 appears to be involved in the pathogenesis of both hereditary and acquired FSGS, and possibly other proteinuric diseases.

In the present study, we screened patients from five families with presumed hereditary biopsy-proven FSGS known in our centre, in which mutations in *ACTN4* and *INF2* were excluded, for variants in the gene encoding TRPC6. In doing so, we identified a novel, previously unpublished *TRPC6* variant that is likely the cause of the late-onset autosomal dominant FSGS in one of these families.

MATERIALS AND METHODS

Patients and TRPC6 mutational analysis

We identified patients with biopsy-proven FSGS from families with suspected autosomal-dominant inheritance. In affected individuals of which blood samples and informed consent were present, we performed *TRPC6* mutational analysis. Genomic DNA was purified from blood samples by isolation of DNA from leucocytes by standard column-based methods. The isolated DNA was used as a template to sequence the 13 exons, including intron–exon boundaries, of the *TRPC6* gene using sense and anti-sense primers as depicted in Table 1. The published reference sequence for human *TRPC6* (NM_004621.5) was used as the WT *TRPC6* sequence for means of comparison. The identified *TRPC6* sequence substitution was checked in public SNP databases including the Exome Variant database (>10 000 alleles) (NHLBI Exome Sequencing Project; <http://evs.gs.washington.edu/EVS/>) and was also analysed in 292 healthy Caucasian controls and 157 patients with MGP from the same geographical area.

DNA constructs

An expression plasmid containing human influenza haemagglutinin-derived (HA)-tagged human TRPC6 was constructed using the bicistronic pCI-Neo/IRES-GFP vector (pCI-Neo/HA-hTRPC6 WT IRES-GFP). By site-directed mutagenesis, plasmids including TRPC6 mutations were constructed resulting in pCI-Neo/HA-hTRPC6-R175Q-IRES-GFP and pCI-Neo/HA-hTRPC6-R895C-IRES-GFP, which were verified by sequence analysis.

Electrophysiology

Human embryonic kidney (HEK293) cells seeded in 12-well plates were maintained at 37°C in Dulbecco's modified Eagle's medium (Bio Whittaker Europe, Vervier, Belgium) supplemented with 10% (v/v) fetal calf serum (PAA, Linz, Austria), 2% (v/v) L-glutamine, 10 $\mu\text{L}/\text{mL}$ (v/v) essential amino acids and 0.01 mg/mL (w/v) ciproxin at 37°C in a humidity controlled incubator with 5% (v/v) CO_2 atmosphere. Cells were transiently transfected with 1 μg of the respective constructs using Lipofectamine²⁰⁰⁰ (Invitrogen-Life Technologies, Breda, the Netherlands). After 24 h, transfected cells were

Table 1. Primers used to sequence the 13 exons of TRPC6

Exon	F/R primer	Primer sequence 5' → 3'
1	F primer	GTCTGCCCAGGTCCAGTTC
1	R primer	GTACACACGCGGGTTCAG
2_01	F primer	GATGAATGGCAAGTCATTGG
2_01	R primer	AACCTCTTGCCTTCAGCAAA
2_02	F primer	GCCAATGAGCATCTGGAAAT
2_02	R primer	AGCCGTCATGACTGGATCTT
2_03	F primer	GATTGAACGGCCTCATGATT
2_03	R primer	GGTAGCGATCACAACCTTTTGC
3	F primer	AACGTGGTATTCTCCATTATTGC
3	R primer	AGCACCAACAAGAACCAAAA
4	F primer	GCCATTTGTTTGTGCCTGT
4	R primer	TGGAGATAAGATTTTCCCCACT
5	F primer	GGAGATCATTGGAATGTGCAG
5	R primer	AATGAACCCAAGGCAACTGT
6	F primer	TTGGGACCAAATTTTGAAGG
6	R primer	GAGAATTGTGCAGTAACCGAACT
7	F primer	GGAGACTTCCATTTCGAAAACC
7	R primer	CCAAAACATTATCCCATGGAC
8	F primer	TCACTAATTTGCAGACACTAAACAA
8	R primer	CGAAGAGCAGTCCATGCTT
9	F primer	CGATCACTGGGGTCTGAGAG
9	R primer	AAAGGGATGTGGCATAGTGG
10	F primer	AGGGAAGAACCCCGTAAGAA
10	R primer	GCTTCTGAACATCTGTCCCTTT
11	F primer	TTGGCAGCCACAAAGTCTAA
11	R primer	AAGAATCACATAGTTCAAGAACCTAAA
12	F primer	GGCTCACTACAGGGAGGAAG
12	R primer	GCTCTCCAGGCACTCTGC
13	F primer	TTTCCTCCTGTCCCACAGTC
13	R primer	GGCTCCAGATGATAGGATGG

F, forward; R, reverse.

plated at low density on 18-mm glass coverslips coated with fibronectin (Roche Diagnostics, Almere, the Netherlands). A stock solution of 100 mM 1-oleoyl-2-acetyl-*sn*-glycerol (OAG; Sigma-Aldrich) dissolved in dimethyl sulfoxide was diluted to a final concentration of 100 μ M in standard extracellular solution, which was used as an agonist for TRPC6 activation. Whole-cell configuration of the patch-clamp technique was used for the functional characterization of the ion channels. An EPC-9 patch-clamp amplifier controlled by the Pulse

software 8.8 (HEKA Elektronik, Germany) was used in the whole-cell configuration. Borosilicate patch pipettes had a resistance of 2–3 M Ω . Series resistance (R_s) change was <10 M Ω . All experiments were done at room temperature. A stimulation protocol consisting of repetitive voltage ramps ranging from –100 to +100 mV was applied for 450 ms each 2 s from a 0-mV holding potential. After 40 s, 100 μ M of OAG was applied for \approx 300 s. The extracellular bath solution consisted of (in mM): 135 NaCl, 5 CsCl, 2 CaCl₂, 1 MgCl₂, 10

4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 10 glucose (adjusted to pH 7.4 with CsOH). The intracellular solution was (in mM): 145 caesium methanesulfonate, 2 MgCl₂, 0.3 CaCl₂, 10 ethylene glycol tetraacetic acid and 10 HEPES (adjusted to pH 7.25 with CsOH). Analysis and display of patch-clamp data was performed with Igor Pro software version 6.0 (WaveMetrics, Lake Oswego, OR).

Cell-surface biotinylation assays

HEK293 cells were seeded in 6-well plates and transiently transfected with 1.2 µg DNA of the respective DNA constructs using Lipofectamine²⁰⁰⁰ reagent (Invitrogen-Life Technologies, Breda, the Netherlands) according to the manufacturer's protocol. After 3 days, cells were incubated with 500 µL EZ-link[®] Sulfo-NHS-LC-LC-Biotin (500 µg/mL; Thermo Scientific) during 30 min at 4°C. Thereafter, cells were lysed, centrifuged and exposed overnight at 4°C to NeutrAvidin beads (NeutrAvidin[™] Agarose Resins; Thermo Scientific). The biotinylated fraction was then eluted in Laemmli sample buffer, resolved by electrophoresis on NuPAGE[®] 10% Bis-Tris Gels and blotted to nitrocellulose.

Statistical analysis

Data are expressed as the mean ± standard error of the mean, with *n* representing the number of tested cells. Statistical analysis was performed applying one-way analysis of variance. P-values <0.05 were considered significant.

RESULTS

TRPC6 sequence analyses in families with proposed autosomal-dominant FSGS

From our centre, we selected patients with biopsy-proven FSGS and a family history that could be compatible with an autosomal-dominant mode of inheritance, and in which mutations in *ACTN4* and *INF2* had been excluded. In this way, we identified five patients in which a possible hereditary form of FSGS was suspected. Sanger sequence analysis of all 13 exons of the gene encoding TRPC6 excluded the presence of known TRPC6 gain-of-function mutations in these patients. However, one of the patients demonstrated a formerly unknown heterozygous mis-sense TRPC6 variant, in which a G was substituted for an A in exon 2 of the TRPC6 gene (c.524G>A) (Figure 1A). This currently 33-year-old male patient presented with proteinuria at the age of 27. His kidney biopsy showed a limited number of globally sclerosed glomeruli, along with several glomeruli demonstrating segmental glomerulosclerosis of the perihilar variant. Electron microscopy showed complete podocyte effacement. Patient is currently treated with both an angiotensin converting enzyme inhibitor (ACEi) and an angiotensin receptor blocker (ARB), and his latest estimated glomerular filtration rate is ≈95 mL/min with a proteinuria <1 g/24 h. The c.524G>A nucleotide substitution leads to a predicted arginine-to-glutamine change at amino acid 175 in the TRPC6 protein (p.Arg175Gln or R175Q). This arginine at position 175 is highly conserved throughout

evolution and is present in TRPC6 protein homologues of multiple species (Figure 1B).

Characterization of the index patient's pedigree

After identification of this TRPC6 gene variant in the index patient, we set out to characterize the family, of which the non-consanguineous pedigree is depicted in Figure 2. In addition to the index patient (IV.6), there are two other family members with biopsy-proven FSGS (III.3 and III.4). Subject III.3 is a currently 67-year-old male with ESRD, who presented with proteinuria at the age of 40, was treated with haemodialysis since the age of 50 and eventually underwent renal transplantation. Subject III.4 is the 57-year-old mother of the index patient with ESRD at the age of 53, who is currently being treated with peritoneal dialysis. Interestingly, subjects I.1 and II.1 were reported to have died from ESRD at a relatively young age, with no further data available regarding (probable) diagnosis. Upon screening of the siblings of the index patient, one currently 37-year-old sister (IV.5) has mild proteinuria, detected after pregnancy, and has a normal renal function. Renal biopsy was not performed in this subject. The other sibling, a currently 39-year-old sister (IV.4), has no proteinuria and a normal renal function. Subject V.1 is the 3-year-old son of the index patient, who was not tested, but appears clinically healthy.

TRPC6 c.524G>A variant analysis in the pedigree of the index patient and healthy controls

Sequence analysis revealed the c.524G>A (G524A) sequence variant in several family members in the pedigree (Figure 2). In short, subjects III.3, III.4 and IV.6, all known to have biopsy-proven late-onset FSGS, tested positive for this TRPC6 variant. In addition, the proteinuric sibling (IV.5) also showed the same nucleotide substitution. Interestingly, the clinically unremarkable sibling IV.4 also harbours the TRPC6 variant that appears to segregate with FSGS and/or proteinuria in this family. In subject III.1, a healthy currently 67-year-old sibling of the index patient's mother, the c.524G>A variant, was not detected. As expected, subject III.5, father of the index patient who does not display signs of renal disease, also tested negative for the c.524G>A variant. No mutation analysis was done in the other family members either because of unavailability of genetic material or the absence of informed consent. To exclude that the identified c.524G>A sequence is a common TRPC6 variant or SNP, we analysed 292 healthy Caucasian controls and 157 patients with MGP from the same geographic area for the c.524G>A sequence variant, which all tested negative. The substitution was also absent from public SNP databases including the ESP database (>10 000 alleles). Therefore, the c.524G>A nucleotide change found in the genomic DNA of the subjects in this pedigree and the resultant p.Arg175Gln amino acid substitution appear to represent a true and novel TRPC6 mutation. The presence of mutated channel proteins could be responsible for the late-onset autosomal dominant FSGS in this family.

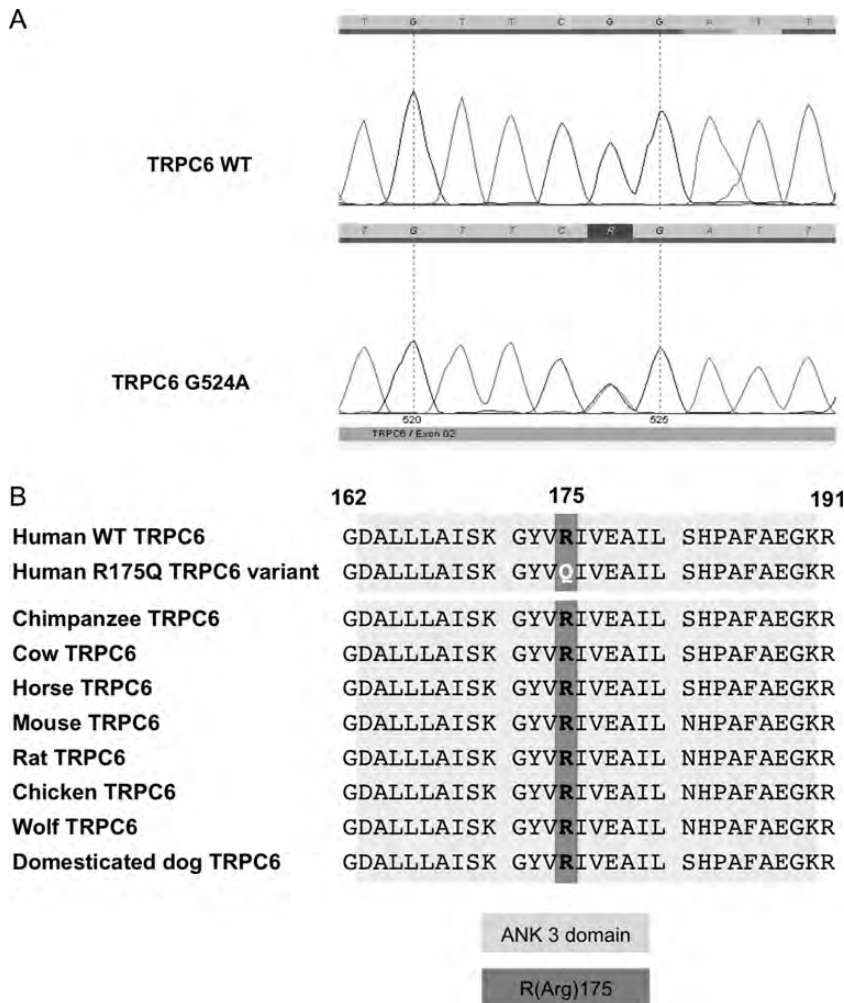


FIGURE 1: Novel *TRPC6* c.524G>A sequence variant gives rise to p.Arg175Gln amino acid substitution. (A) Sequence chromatograms showing a *TRPC6* c.524G>A (G524A) sequence variant was demonstrated in patients in a family with hereditary, biopsy-proven FSGS. (B) This sequence variant gives rise to a *TRPC6* arginine to glutamine substitution at amino acid position 175 (p.Arg175Gln or R175Q) in the third ANK3 domain of the N-terminal tail of the *TRPC6* channel (as represented in Figure 5). For comparison, the *TRPC6* WT amino acid sequence of ANK3 is shown, which is highly conserved in a large number of animal species. See online Supplementary material for a colour version of this figure.

Functional electrophysiological characterization of mutant and wild-type *TRPC6* channels

To characterize the functional consequences of the p.Arg175Gln substitution, HEK293 cells were transiently transfected with expression vectors, which encode WT *TRPC6*, p.Arg175Gln (R175Q) or the previously characterized p.Arg895Cys (R895C) *TRPC6* gain-of-function mutant [7]. The p.Arg895Cys mutant was included in our experimental set-up as a positive control, while empty vector was used as a negative control to control for the possibility of endogenous-mediated responses. The exposure of transiently transfected HEK293 cells to 100 μ M OAG resulted in a sustained activation of WT *TRPC6*-mediated currents (Figure 3A), consisting of an outwardly rectifying current with Cs^+ as a charge carrier. Measured currents were significantly larger in the p.Arg895Cys and, importantly, p.Arg175Gln mutants (Figure 3A and B). The *TRPC6* current amplitude after OAG treatment was in agreement with previous studies [18]. Specifically, the

current density at +80 mV was 31 ± 4 pA/pF for WT *TRPC6*, while values of 54 ± 6 and 67 ± 9 pA/pF were collected for the p.Arg895Cys and p.Arg175Gln mutants, respectively (Figure 3C).

Cell-surface expression of mutant and wild-type *TRPC6* channels

To determine whether the observed gain-of-function phenotype results from enhanced plasma membrane expression of the p.Arg175Gln mutant channel, we performed cell-surface biotinylation experiments. Figure 4A demonstrates that the cell-surface expression, depicted by the biotinylated protein, of the p.Arg175Gln (R175Q) mutant was not increased when compared with the *TRPC6* WT channel. Neither was the cell-surface expression of the *TRPC6* p.Arg895Cys (R895C) mutant increased. The ratio of cell-surface to total expression was even significantly reduced in the p.Arg895Cys (R895C) mutant when compared with the WT *TRPC6* (Figure 4B).

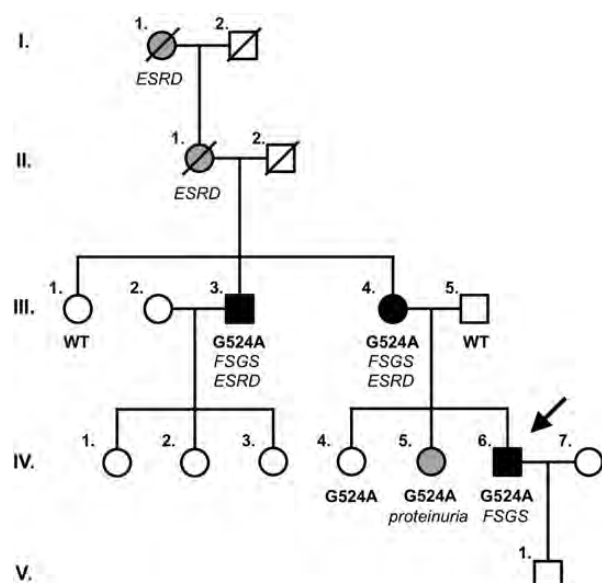


FIGURE 2: Pedigree in which the *TRPC6* c.524G>A sequence variant (G524A) co-segregates with late-onset autosomal dominant FSGS. The *TRPC6* c.524G>A sequence variant (G524A) was found in three family members with biopsy-proven FSGS, one family member with proteinuria and one currently asymptomatic family member. Two additional family members died of ESRD, but no genetic material was available for testing. Solid symbols depict biopsy-proven FSGS and grey symbols depict probable FSGS without histological confirmation. Arrow indicates the index patient.

DISCUSSION

In this study, we identified a new *TRPC6* variant that co-segregated with the cases of autosomal dominant, late-onset, biopsy-proven FSGS in a Dutch non-consanguineous family. The detected nucleotide substitution was found neither in a large cohort of healthy controls, nor in a large cohort of patients with MGP. The c.524G>A nucleotide substitution leads to a predicted change from a positively charged arginine to a neutral glutamine at amino acid position 175 (p.Arg175Gln) in the *TRPC6* channel protein. When the mutant *TRPC6* channel was functionally tested by patch-clamp analysis, it showed a clear gain-of-function phenotype when compared with the WT *TRPC6* channel. The p.Arg175Gln *TRPC6* mutant did not show an increased cell-surface expression. This demonstrated that the measured increased channel currents are due to an intrinsically enhanced channel conductance secondary to the introduced amino acid substitution. Thus, although causality of this mutation cannot be conclusively proven based on the above data, it is highly likely that this new gain-of-function *TRPC6* mutation is the disease-causing mutation responsible for the late-onset autosomal-dominant FSGS in this family.

The novel p.Arg175Gln *TRPC6* mutation identified in this study is the first mutation located in the ankyrin repeat domain 3 (ANK3; predicted AA 163–189 by UniProtKB; Figure 5). Importantly, there are no sequence conflicts or putative alternative splicing sites near to this location in *TRPC6*.

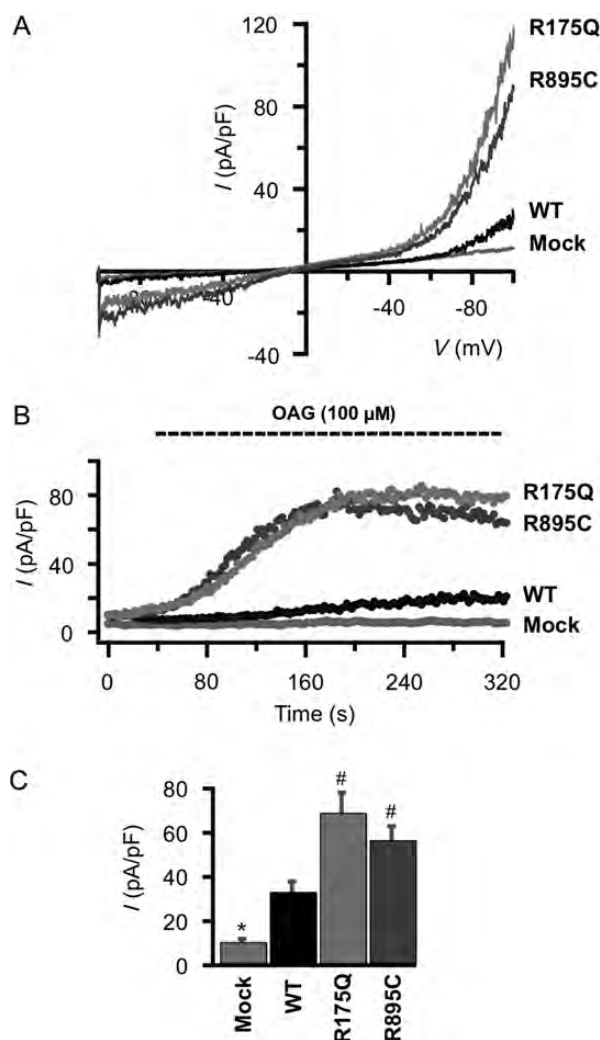


FIGURE 3: Functional characterization of the novel *TRPC6* p.Arg175Gln (R175Q) substitution showing a gain-of-function phenotype. (A) HEK293 cells were either mock-transfected or transfected with WT *TRPC6*, the novel *TRPC6* p.Arg175Gln (R175Q) mutation or the previously identified *TRPC6* p.Arg895Cys (R895C) mutation, and subsequently characterized by patch-clamp analysis. Representative current–voltage relationships (*I*–*V*) are depicted. (B) The exposure of cells to 100 μ M OAG resulted in a slow time developing and sustained activation of *TRPC6*-mediated currents. (C) Current densities at +80 mV were compared for mock-, WT-, p.Arg175Gln- or p.Arg895Cys-transfected cells. **P* < 0.05 compared with WT, #*P* < 0.05 compared with WT and mock.

The cytoplasmic N-terminal tail of *TRPC6* harbours four ankyrin domains, which are conserved domains of ≈ 33 amino acids, generally suggested to function as protein–protein interaction domains [13]. Of the previously identified *TRPC6* mutations, three were located in ANK1, two in ANK2, one in ANK4, and two additional cytoplasmic N-terminal mutations are located outside of the predicted ANK domains [7, 8, 19–22]. Six other *TRPC6* mutations were located to the C-terminal cytoplasmic tail [7, 19, 20, 23]. No mutations have been identified in the transmembrane, extracellular or pore-forming regions of the channel. This could suggest that the tails are more susceptible to acquire mutations, or that sequence

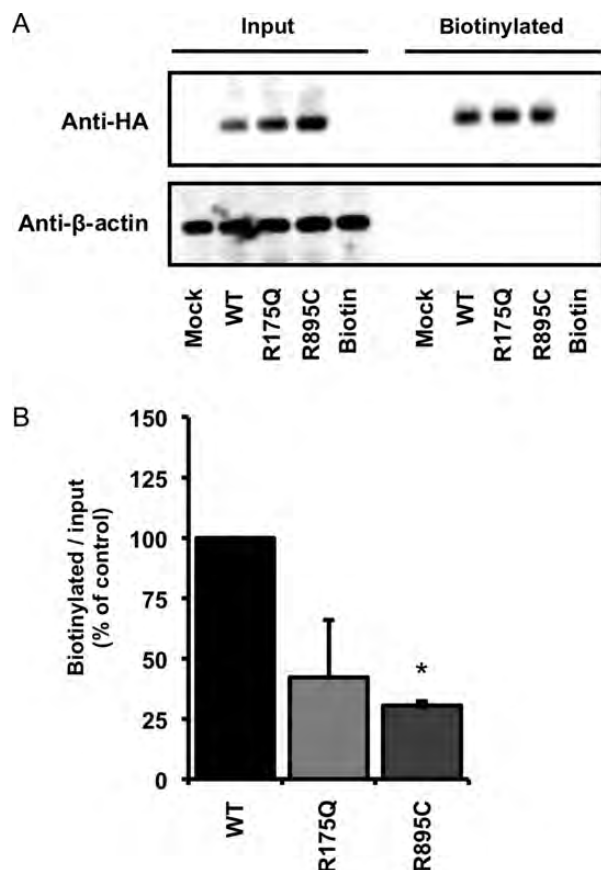


FIGURE 4: Cell-surface expression does not explain the gain-of-function phenotype of the TRPC6 p.Arg175Gln (R175Q) and p.Arg895Cys (R895C) mutants. (A) Cell-surface biotinylation experiments were performed on HEK293 cells that were either mock-transfected or transfected with HA-tagged WT TRPC6, the novel TRPC6 p.Arg175Gln mutation or the previously identified TRPC6 p.Arg895Cys mutation. β -actin was used as loading control. (B) Immunopositive bands were quantified, and the ratio of biotinylated protein versus input was determined as a measure of cell-surface expression relative to total expression for WT TRPC6, the p.Arg175Gln and p.Arg895Cys variants. * $P < 0.05$ compared with WT.

variations in the other regions are not compatible with life or do not lead to a clinical (renal) phenotype. Surprisingly, patients with FSGS due to *TRPC6* mutations do not show any other pathological phenotype apart from FSGS, while TRPC6 is expressed in many other tissues, e.g. cardiac myocytes, pulmonary and non-pulmonary vascular smooth muscle cells, endothelial cells and neurons [13]. TRPC6 knockout mice, however, do not show a clear renal phenotype apart from reduced susceptibility to AngII-induced albuminuria [17, 24]. To our knowledge, apart from mice in which WT or mutant TRPC6 was overexpressed in a podocyte-specific manner, there are no reports on TRPC6-overexpressing or knock-in transgenic models mimicking the human FSGS phenotype [10]. Such animal models would certainly contribute towards solving these unclarified issues.

Our electrophysiological characterization of the p.Arg175Gln mutation showed that the channel displays a gain-of-function phenotype, in terms of whole-cell channel

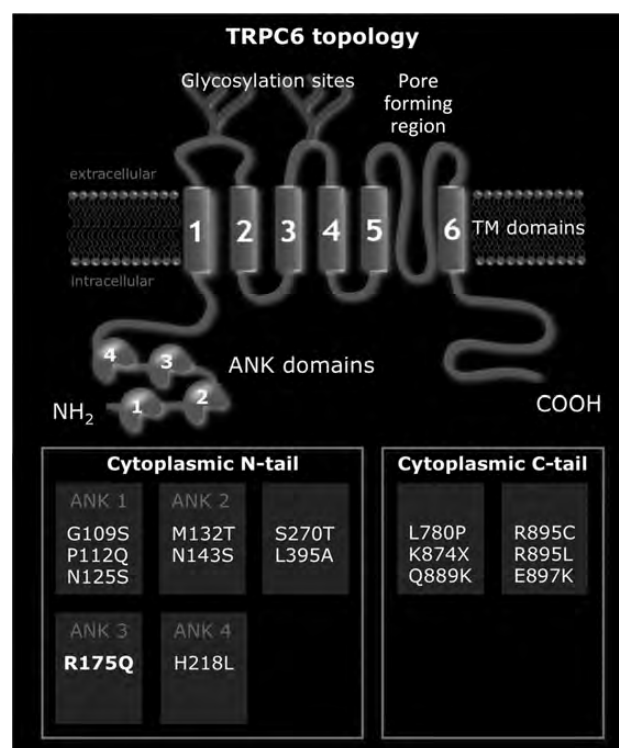


FIGURE 5: Channel topology of the TRPC6 cation channel, including all known TRPC6 mutations associated with hereditary FSGS. The TRPC6 channel is a six-transmembrane (TM) spanning protein with two glycosylation sites on the extracellular loops connecting TM segments 1 and 2, as well as TM segments 3 and 4, respectively. The pore-forming region is located between TM segments 5 and 6, and the protein contains two large intracellular N- and C-terminal tails, which harbour all TRPC6 mutations associated with hereditary FSGS known to date. The N-terminal tail contains four ankyrin repeat (ANK) domains that harbour seven of the known TRPC6 mutations, including the novel p.Arg175Gln (R175Q) mutation in ANK3. Two additional mutations are located outside of the ANK domains in the N-terminal tail, whereas the other five known mutations are located distally in the C-terminal tail. See online Supplementary material for a colour version of this figure.

currents, that is comparable with the previously described p.Arg895Cys TRPC6 mutation, which is not caused by an enhanced cell-surface expression of the mutated channel proteins. In contrast, it was previously shown that another N-terminal tail mutation (p.Pro112Gln, P112Q) did display an enhanced cell-surface expression [7, 8, 16]. Nine of the 14 TRPC6 mutations known to date showed an increased channel activity as determined by electrophysiology and/or intracellular Ca^{2+} measurements. For two mutations, no effect could be shown at initial characterization, while the remaining three mutations were not functionally tested [7, 8, 19–23]. However, *in silico* analysis of these mutations suggested that these too can display a gain-of-function phenotype [19]. Therefore, an increased TRPC6 channel activity is likely involved in the pathogenesis of the podocytopathy and resultant renal function decline in these patients. It is suggested that TRPC6 relays information from the slit diaphragm to the podocyte cytoskeleton, enabling the cell to adapt to environmental challenges

in the glomerulus [14]. Possibly, increased Ca^{2+} influx through the mutated channels impairs these signalling processes at the slit diaphragm, ultimately affecting podocyte plasticity. Indeed, TRPC6 mutations or overexpression of the WT TRPC6 has been shown to affect the podocyte cytoskeleton and plasticity *in vitro* [9]. Particularly, since the cytoplasmic tails of TRP-type channels are important in protein–protein interactions that affect both channel function itself and downstream signalling pathways, altered binding of auxiliary proteins to these domains could be involved. There are data demonstrating that TRPC6 interacts with, e.g. nephrin, podocin, PLC- γ 1 and large-conductance Ca^{2+} -activated K^+ (BK_{Ca}) channels, the latter suggested to preserve the driving force for Ca^{2+} influx through TRPC6 (reviewed in ref. [14]). Whether the latter mechanism involves the direct physical interaction or binding to the cytoplasmic tails of TRPC6 has not been shown.

Several studies suggested that (over)activation of the calcineurin/NFAT pathway is an important consequence of TRPC6 gain-of-function mutations [16]. Recently, we showed that the calcineurin/NFAT pathway is part of an AngII-induced feedforward signalling mechanism in the podocyte that can ultimately lead to increased expression of TRPC6 itself [12]. This mechanism seems relevant in both hereditary FSGS and acquired proteinuric diseases, which have been associated with increased TRPC6 expression by the podocyte. NFAT activation *per se* induces TRPC6 expression, glomerulosclerosis and albuminuria [12, 25]. Furthermore, AngII-induced Ca^{2+} influx through TRPC6 is thought to influence podocyte cytoskeletal stability by activating the Ca^{2+} -dependent RhoA signalling pathway, and/or stimulating synaptopodin degradation [26, 27]. Because AngII stimulates signalling cascades involving TRPC6 and the calcineurin/NFAT pathway, and ACEi, ARBs and calcineurin inhibitors were shown to downregulate TRPC6 expression in animal models for human FSGS, treatment with ACEi, ARBs or calcineurin inhibitors seems pathophysiologically rational in patients with TRPC6 mutations [12, 15–17]. However, it is not known whether prophylactic treatment in TRPC6 mutation carriers, before the development of proteinuria, is beneficial in inhibiting disease progression.

The family pedigree of our index patient clearly demonstrated the characteristics of a late-onset, autosomal-dominant pattern of inheritance. When the first TRPC6 mutations in hereditary FSGS were demonstrated, it indeed appeared that it was associated with presentation at adult age [7, 8]. However, thereafter several reports showed TRPC6 mutations and associated disease in children [20, 21]. In the presented family, FSGS was diagnosed after the age of 25 and in some family members considerably later, with one mildly proteinuric subject aged 37 and one currently asymptomatic 39-year-old carrier of the p.Arg175Gln mutation. It is not uncommon to identify asymptomatic TRPC6 mutation-carrying relatives in the younger generation of pedigrees, as shown before [19]. Whether the type and localization of the mutation, or the therapy initiated upon identification of initial proteinuria, alters the clinical course, or penetrance of the disease is currently unknown. Interestingly, upon podocyte-specific

overexpression of two TRPC6 variants corresponding to previously identified human TRPC6 mutations in mice, only 23–45% of adult mice showed a mild non-nephrotic range albuminuria, suggesting incomplete penetrance [10]. Thus, TRPC6 mutations may lead to relatively subtle changes in podocyte function or, as previously suggested, podocyte adaptive responses to environmental challenges. This, in turn, could ultimately enhance susceptibility to the development of FSGS at a later age. Regarding mutations in other podocyte proteins that cause proteinuric disease, often at younger age, combinations of bigenic heterozygosity in several of these proteins have been shown to enhance susceptibility to as well as penetrance of the clinical disease manifestations [28, 29]. Altogether, these data suggest that environmental factors, or compound effects of genetic background, influence penetrance of glomerular disease in patients carrying TRPC6 mutations, possibly in a multi-hit setting.

SUPPLEMENTARY DATA

Supplementary data are available online at <http://ndt.oxfordjournals.org>.

ACKNOWLEDGEMENTS

We thank Dr Jochen Reiser (University of Miami Miller School of Medicine) for kindly supplying an HA-tagged TRPC6 wild-type construct. J.M.H. was financially supported by a Dutch Kidney Foundation grant (OW08). In addition, this work was supported by a Kolff Career Stimulation Grant from the Dutch Kidney Foundation (KJPB 07.0001), a grant from the Genzyme Renal Innovations Program (GRIP) and a Ruby Diabetes Research Grant from the Dutch Diabetes Fund (2009.80.118) to T.N., as well as a European Young Investigator award (EURYI) to J.G.J.H.

CONFLICT OF INTEREST STATEMENT

None declared.

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Received for publication: 20.6.2012; Accepted in revised form: 6.11.2012