

## TRPC6 mutational analysis in a large cohort of patients with focal segmental glomerulosclerosis

Sheila Santín<sup>1</sup>, Elisabet Ars<sup>1</sup>, Sandro Rossetti<sup>2</sup>, Eduardo Salido<sup>3</sup>, Irene Silva<sup>1</sup>, Rafael García-Maset<sup>4</sup>, Isabel Giménez<sup>4</sup>, Patricia Ruíz<sup>1</sup>, Santiago Mendizábal<sup>5</sup>, José Luciano Nieto<sup>6</sup>, Antonia Peña<sup>7</sup>, Juan Antonio Camacho<sup>8</sup>, Gloria Fraga<sup>9</sup>, M<sup>a</sup> Ángeles Cobo<sup>10</sup>, Carmen Bernis<sup>11</sup>, Alberto Ortiz<sup>12</sup>, Augusto Luque de Pablos<sup>13</sup>, Ana Sánchez-Moreno<sup>14</sup>, Guillem Pintos<sup>15</sup>, Eduard Mirapeix<sup>16</sup>, Patricia Fernández-Llama<sup>4</sup>, José Ballarín<sup>4</sup> and Roser Torra<sup>4</sup> on behalf of the FSGS Study Group\*

<sup>1</sup>Molecular Biology Laboratory, Fundació Puigvert, Universitat Autònoma de Barcelona, REDinREN, Instituto de Investigación Carlos III, Barcelona, Spain, <sup>2</sup>Division of Nephrology and Hypertension, College of Medicine, Mayo Clinic, Rochester, MN, USA, <sup>3</sup>Molecular Biology Laboratory, Hospital Universitario de Canarias, Tenerife, <sup>4</sup>Nephrology Department, Fundació Puigvert, Universitat Autònoma de Barcelona, REDinREN, Instituto de Investigación Carlos III, Barcelona, <sup>5</sup>Pediatric Nephrology Department, Hospital Universitario La Fe, Valencia, <sup>6</sup>Pediatric Nephrology Department, Hospital Vall d'Hebron, Barcelona, <sup>7</sup>Pediatric Nephrology Department, Hospital Infantil La Paz, Madrid, <sup>8</sup>Pediatric Nephrology Department, Hospital Sant Joan de Déu, Barcelona, <sup>9</sup>Pediatric Nephrology Department, Hospital de la Santa Creu i Sant Pau, Barcelona, <sup>10</sup>Nephrology Department, Hospital Universitario de Canarias, Tenerife, <sup>11</sup>Nephrology Department, Hospital Universitario de La Princesa, Madrid, <sup>12</sup>Nephrology Department, Fundación Jiménez Díaz, Madrid, <sup>13</sup>Pediatric Nephrology Department, Hospital General Universitario Gregorio Marañón, Madrid, <sup>14</sup>Pediatric Nephrology Department, Hospital Infantil Universitario Virgen del Rocío, Sevilla, <sup>15</sup>Pediatric Nephrology Department, Hospital Germans Trias i Pujol, Badalona and <sup>16</sup>Nephrology Department, Hospital Clinic, Barcelona, Spain

Correspondence and offprint requests to: Roser Torra; E-mail: rtorra@fundacio-puigvert.es

\*Other investigators in the FSGS Study Group are listed below: *Hospital Universitario La Fe*- Isabel Zamora; *Hospital Vall d'Hebron*- Joan López-Hellin, Álvaro Madrid, Clara Ventura, Ramón Vilalta; *Hospital Infantil La Paz*- Laura Espinosa, Carmen García, Marta Melgosa, Mercedes Navarro; *Hospital Sant Joan de Déu*- Antonio Giménez, Jorge Vila Cots; *Fundación Jiménez Díaz*- Simona Alexandra, Carlos Caramelo<sup>†</sup>, Jesús Egido; *Hospital General Universitario Gregorio Marañón*- M Dolores Morales San José; *Hospital Infantil Universitario Virgen del Rocío*- Francisco de la Cerda; *Hospital de Barcelona*- Pere Sala, Frederic Raspall, Àngel Vila; *Hospital Torrecárdenas*- Antonio María Daza; *Hospital Niño Jesús*- Mercedes Vázquez, José Luis Écija; *Hospital Universitario Reina Sofía*- Mario Espinosa; *Hospital Infantil Miguel Servet*- M<sup>a</sup> Luisa Justa; *Hospital Príncipe d'Espanya*- Rafael Poveda; *Hospital Universitario de Getafe*- Cristina Aparicio; *Hospital Materno-Infantil Son Dureta*- Jordi Rosell; *Hospital Infantil doce de Octubre*- Rafael Muley; *Hospital de Galdakao*- Jesús Montenegro; *Hospital Universitario Marqués de Valdecilla*- Domingo González; *Hospital Materno-Infantil de Badajoz*- Emilia Hidalgo; *Hospital Universitario Virgen de las Nieves*- David Barajas de Frutos; *Hospital Son Llàtzer*- Esther Trillo; *Hospital Universitario Virgen de la Arrixaca*- Salvador Gracia; *Hospital de Cruces*- Francisco Javier Gainza de los Ríos.

### Abstract

**Background.** Mutations in the *TRPC6* gene have been reported in six families with adult-onset (17–57 years) autosomal dominant focal segmental glomerulosclerosis (FSGS). Electrophysiology studies confirmed augmented calcium influx only in three of these six *TRPC6* mutations. To date, the role of *TRPC6* in childhood and adulthood non-familial forms is unknown.

**Methods.** *TRPC6* mutation analysis was performed by direct sequencing in 130 Spanish patients from 115 unrelated families with FSGS. An *in silico* scoring matrix was developed to evaluate the pathogenicity of amino acid substitutions, by using the bio-physical and bio-chemical differences between wild-type and mutant amino acid, the evolutionary conservation of the amino acid residue in orthologues, homologues and defined domains, with the addition of contextual information.

**Results.** Three new missense substitutions were identified in two clinically non-familial cases and in one familial case. The analysis by means of this scoring system allowed us to classify these variants as likely pathogenic mutations. One of them was detected in a female patient with unusual clinical features: mesangial proliferative FSGS in childhood (7 years) and partial response to immunosuppressive therapy (CsA + MMF). Asymptomatic carriers of this likely mutation were found within her family.

**Conclusions.** We describe for the first time *TRPC6* mutations in children and adults with non-familial FSGS. It seems that *TRPC6* is a gene with a very variable penetrance that may contribute to glomerular diseases in a multi-hit setting.

**Keywords:** focal segmental glomerulosclerosis; *in silico* scoring system; missense substitutions; mutation analysis; *TRPC6* gene

## Introduction

Focal segmental glomerulosclerosis (FSGS) is an important cause of end-stage renal disease (ESRD) in both children, where it accounts for 7–20% of cases, and adults, where it accounts for up to 35% of cases [1,2]. FSGS is a pathological entity defined by segmental sclerotic lesions involving only a subpopulation of glomeruli. Typical manifestations include proteinuria, hypertension, nephrotic syndrome (NS), renal insufficiency and eventual kidney failure. Steroid therapy is the main treatment; however, the response rate is estimated to be 30–50% at 10 years, with the persistence of proteinuria and the majority still reaching ESRD [3,4].

Conventionally, FSGS has been defined as primary (idiopathic), secondary or familial. It has been estimated that up to 18% of FSGS cases are familial [5]. Two recent studies by Winn *et al.* and Reiser *et al.* have discovered a new causative gene for familial FSGS [6,7]. This gene, *TRPC6*, is a member of the transient receptor potential channel (TRPC) subfamily, which has been reported to be a non-selective cation channel [8]. Taking the results of both studies together, six families with six different *TRPC6* gene mutations were identified. These mutations lead to a late onset kidney disease (between 17 and 57 years of life) and a variable rate of progression to ESRD.

Among these six *TRPC6* mutants, only three (P112Q, R895C, E897K) [6,7] were gain-of-function mutations that resulted in increased  $Ca^{2+}$  current amplitudes. The remaining three mutations (N143S, S270T and K874X) did not modify calcium influx during the functional assay, although genetic data supported their role as pathogenic variants [7]. This discrepancy between functional and genetic data underlines the fact that a functional analysis may not always provide definitive proof to discern between mutations and polymorphisms for amino acid substitutions in the *TRPC6* gene. Recently, an *in silico* approach has been developed for the evaluation of amino acid substitutions in human disease genes [9–14]. This approach takes into consideration the bio-physical and biochemical differences between the wild-type and the mutant amino acid residue (Grantham Difference, GD) [15] and the evolutionary conservation in orthologues and homologues (Grantham Variation, GV) [9,12]. These factors were used as *in silico* predictors in a scoring matrix for the evaluation of missense substitutions in the *TRPC6* gene.

To date, *TRPC6* mutations have only been described in familial FSGS with adult onset. Our purpose was to evaluate the prevalence of *TRPC6* mutations also in non-familial forms of FSGS in both adults and children.

## Subjects and methods

### Patients

A total of 130 Spanish patients belonging to 115 unrelated families were included after informed consent was obtained. Of these 115 families, 10 showed evidence of disease in two members of a single generation and 2 showed evidence of disease in multiple generations.

We classified our population according to age at FSGS diagnosis: congenital (<1 year;  $8.4 \pm 4$  months;  $N = 10$ ), early childhood (1–5 years;  $36.9 \pm 12$  months;  $N = 25$ ), late childhood (6–12 years;  $100 \pm 27.2$  months;  $N = 16$ ), adolescent (13–18 years;  $188 \pm 13.2$  months;  $N = 9$ ) and adult (>18 years;  $33.5 \pm 12$  years;  $N = 55$ ).

For all 115 index cases, the kidney biopsy report was available and showed that 95 patients had FSGS and 20 patients had mesangial proliferation with FSGS. FSGS diagnosis was based on the histological diagnosis of primary FSGS as defined by the Columbia FSGS classification system [16].

### Mutation analysis

Genomic DNA was isolated from peripheral blood samples using the ‘salting out’ method [17]. We analyzed PCR amplicons of each of the 13 exons of *TRPC6* by direct sequencing using primers and conditions previously described [6,7]. Novel primers were designed with the Primer Express software (Applied Biosystems, Foster City, CA) for exons 2 and 11. All PCR products were sequenced using the Big Dye DNA Sequencing kit v1.1 (Applied Biosystems, Foster City, CA) and an ABI PRISM 3100-Avant genetic analyser.

These patients were previously screened for *NPHS2*, *ACTN4* and *CD2AP* mutations. When available, DNA samples of other family members were also obtained.

### Classification of amino acid substitutions

We have developed an *in silico* scoring matrix to evaluate the pathogenicity of amino acid substitutions (missense mutations) identified in the *TRPC6* gene. A similar approach has been developed and used to classify amino acid substitutions found in other human genes associated with common disease phenotypes [9–15,18]. This scoring matrix takes into consideration a number of *in silico* predictors:

- (1) The bio-physical and bio-chemical differences between the wild-type and the mutant amino acid (Grantham Difference, GD) [15].
- (2) The evolutionary conservation of the amino acid residue in a multi-sequence alignment (MSA) of orthologue and homologue *TRPC6* proteins (Grantham Variation, GV) [9]. GV was defined as the largest GD for a specific position within the MSA. The MSA was generated using Clustal-W software. Sequences were obtained from NCBI or ENSEMBL (Figure 2).
- (3) The distance between the mutant amino acid and the range of variation present at their position in MSA (Grantham Deviation, GDev) [12].
- (4) Each variant was evaluated for affecting splicing using the algorithm Neural Network Splice Site Prediction ([http://www.fruitfly.org/seq\\_tools/splice.html](http://www.fruitfly.org/seq_tools/splice.html)).
- (5) We used the UniProtKB/Swiss-Prot program (<http://www.expasy.org/sprot/>) and the published literature [19] to identify the defined domains [four ankyrin repeats (ANK-1, ANK-2, ANK-3, ANK-4), six transmembrane domains, TRP II domain and TRP domain].
- (6) Variants were also evaluated using ‘sort intolerant from tolerant’ (SIFT) (<http://blocks.fhcrc.org/sift/SIFT.html>) and ‘Polymorphism Phenotype’ (Polyphen) ([www.bork.embl-heidelberg.de/PolyPhen](http://www.bork.embl-heidelberg.de/PolyPhen)) programmes. Based on specific cut-off values, SIFT and Polyphen assign a score and class the amino acid substitution as tolerated/not tolerated or benign/probably/possibly damaging.
- (7) Contextual information included population data such as previous description of the variant in databases (as derived from the HGMD and SNPDB) and analysis of a control population of 400 normal chromosomes (matched by race, by ethnicity and geographically with the study cohort).

We assigned points for each of these factors, the sum of which resulted in an overall variant score (VS). In detail, the GD and the largest GV value observed in the MSA were classed into six groups: 0–5, 6–35, 36–80, 81–130, 131–179 and >180 (as previously suggested by Rossetti *et al.* [13]) and built into a scoring matrix. Each GD–GV match in the matrix was assigned a score, from –2 to +8. A similar matrix was constructed for GDev–GD; when GDev was very similar to GD, +2 points were given, but when GDev dropped precipitously as further diverged sequences were

included in the analysis,  $-2$  points were given. Additional points were added for the following items: if the residue was located within a defined and conserved domain (Ank-1, Ank-2 and TRP II) (+1 to +4); if the substitution matched a residue in the MSA ( $-4$ ); if the change was not present in the control population (+2), if present ( $-2$ ); if the change was not described in an SNP database (+1), if described ( $-1$ ); if the change was predicted by SIFT programme to be non-tolerated (+2) or tolerated ( $-2$ ); if the change was predicted to be probably damaging/possibly damaging/benign (+2/+1/ $-2$ ) by the Polyphen programme; and if the change was not predicted to affect splicing (0). The overall VS were classified into four groups, specifically  $VS \leq -5$  (neutral polymorphism), VS between  $-4$  and 4 (indeterminate), VS between 5 and 10 [mutation group (MG) = C, likely pathogenic], and VS higher than 11 (MG = B, highly likely pathogenic).

The scoring matrix was tested using previously described and classified TRPC6 amino acid substitutions, as positive controls (pathogenic variants, positive training set) or negative controls (polymorphisms, negative training set). The values assigned to each specific factor are inspired by the scoring matrix developed for the *PKD1/2* genes [13], with some minor modifications. The trained scoring matrix was then used to evaluate the actual amino acid substitutions found in our study cohort.

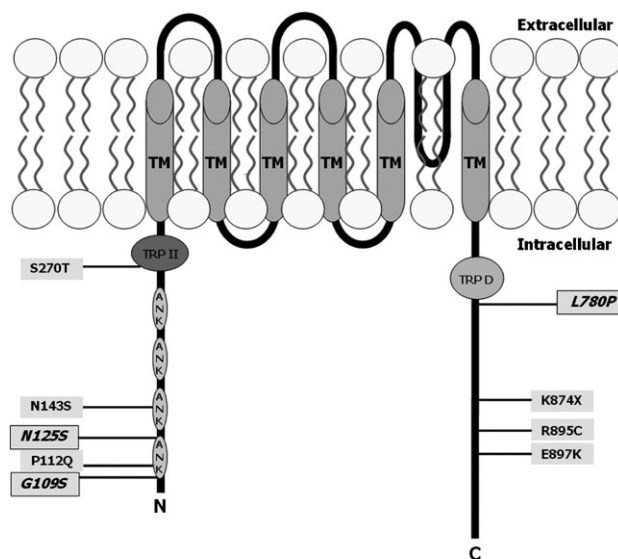
## Results

We have analysed 115 FSGS unrelated cases, 90% with apparently unaffected relatives and 10% familial cases. Three novel heterozygous missense variants in three FSGS patients were identified, two of whom presented with NS in adulthood and one in late childhood.

### CASE 1

The proband (a 50-year-old female) developed proteinuria during her first pregnancy at 21 years of age. During her second pregnancy at the age of 25, she developed preeclampsia and proteinuria was again present and persisted after delivery. Treatment with prednisone had no effect, and the renal biopsy showed FSGS with mesangial deposits of IgM, C3 and C1q. After 14 years, she developed ESRD and renal transplantation was performed a year later. No recurrence of FSGS has been observed so far. A new missense variant (c.325G>A) was found in exon 2 causing a glycine-to-serine substitution (G109S) within the first ankyrin repeat of the TRPC6 protein (Figure 1). Her mother presented with a mesangioproliferative glomerulonephritis and ESRD at the age of 47 years. We could not study the DNA from the mother because she had died. We studied the DNA from two members of her family (her brother and son) and both carried the G109S substitution. At the time of the study, the spot urine protein/creatinine ratio was 42.6 mg/mmol at the age of 40 years and 4.8 mg/mmol at the age of 25 years, respectively.

To determine the effect of the G109S amino acid substitution on the protein, we used our scoring system analysis. According to Abkevich *et al.* [9], the G109S variant is moderately conservative (GD = 56) but at a highly conserved site in the MSA (GV = 0) (Figure 2), so the GD/GV matrix score is +5 and it could be classed as a deleterious/high-risk substitution. According to Tavtigian *et al.* [12], this change could be also classified as a high-risk change since GDev is the same as GD (+2). Moreover, G109 is located in a conserved domain (ankyrin repeat-1) (+1) and no abnor-



**Fig. 1.** Distribution of substitutions within the predicted TRPC6 protein. TRPC6 comprises six transmembrane domains (TM), four ankyrin repeats (ANK) and the TRP II and TRP domains. New *TRPC6* substitutions are shown in italics. The remaining mutations we show here have been published previously [6,7].

mal splicing was predicted (+0). This variant is predicted to be not tolerated by the SIFT programme (+2) and is predicted to be ‘probably damaging’ by the Polyphen programme (+2). Finally, G109S missense substitution was not detected in any public SNP database (+1) and it was not found in 400 control chromosomes (+2), giving a VS of 15. This is a high score and according to our scoring matrix it defines G109S as a highly likely pathogenic amino acid substitution (MG = B) in the *TRPC6* gene (Table 1).

### CASE 2

A 42-year-old female presented with NS at the age of 41 years. Treatment with prednisone had no effect, and the renal biopsy showed FSGS with negative immunofluorescence. She has not developed ESRD to date (1-year follow-up). A new missense change (c.374A>G) was identified in exon 2 causing an asparagine-to-serine substitution (N125S) within the first ankyrin repeat of the TRPC6 protein (Figure 1). Her parents were not available because they had died, but neither of them was clinically affected.

This substitution is a conservative change (GD < 100) and occurs in a moderately conserved site in MSA (Figure 2), giving a GD/GV matrix score of +4 (including the zebrafish sequence). When GDev was calculated, this variant could be classified as deleterious/high-risk substitution because GDev is very similar to GD (+2). Furthermore, the N125S change occurs in a conserved domain (ankyrin repeat-1) (+1), is a non-tolerated change by the SIFT programme (+2) and is predicted to be ‘probably damaging’ by the Polyphen programme (+2). Finally, we did not find it in any of the public SNP databases (+1) as well as in 400 control chromosomes (+2). All these parameters together gave a high score (VS = 14), and the missense substitution

**A**

	<b>G109S</b>	<b>N125S</b>	
Human TRPC6	RFLDAAEYGNIPVVRKMLEECHS---	LNVCVDYMGQNALQLAVANEHLEITELLKKEN	157
Patient 1	RFLDAAEYGNIPVVRKMLEECHS---	LNVCVDYMGQNALQLAVANEHLEITELLKKEN	157
Patient 2	RFLDAAEYGNIPVVRKMLEECHS---	LNVCVDYMGQNALQLAVANEHLEITELLKKEN	157
Chimpanzee	RFLDAAEYGNIPVVRKMLEECHS---	LNVCVDYMGQNALQLAVANEHLEITELLKKEN	227
Orang-utan	RFLDAAEYGNIPVVRKMLEECHS---	LNVCVDYMGQNALQLAVANEHLEITELLKKEN	157
Rhesus monkey	RFLDAAEYGNIPVVRKMLEECHS---	LNVCVDYMGQNALQLAVANEHLEITELLKKEN	102
Gray lemur	RFLDAAEYGNIPVVRKMLEECHS---	LNVCVDYMGQNALQLAVANEHLEITELLKKEN	157
Horse	RFLDAAEYGNIPVVRKMLEECHS---	LNVCVDYMGQNALQLAVANEHLEITELLKKEN	157
Cow	RFLDAAEYGNIPVVRKMLEECHS---	LNVCVDYMGQNALQLAVANEHLEITELLKKEN	156
Dog	RFLDAAEYGNIPVVRKMLEECHS---	LNVCVDYMGQNALQLAVANEHLEITELLKKEN	324
Platypus	RFLDAAEYGNIPVVRKMLEECHS---	LNVCVDYMGQNALQLAVANEHLEITELLKKEN	216
Gray opossum	RFLDAAEYGNIPVVRKMLEECHS---	LNVCVDYMGQNALQLAVANEHLEITELLKKEN	176
Rat	RFLDAAEYGNIPVVRKMLEECHS---	LNVCVDYMGQNALQLAVANEHLEITELLKKEN	156
Mouse	RFLDAAEYGNIPVVRKMLEECHS---	LNVCVDYMGQNALQLAVANEHLEITELLKKEN	156
Guinea Pig	RFLDAAEYGNIPVVRKMLEECHS---	LNVCVDYMGQNALQLAVANEHLEITELLKKEN	119
Chicken	RFLDAAEYGNIPVVRKMLEECHS---	LNVCVDYMGQNALQLAVANEHLEITELLKKEN	148
Frog	RFLDAAEYGNIPVVRKMLEECHS---	LNVCVDYMGQNALQLAVANEHLEITELLKKEN	148
Zebrafish	RFLDAAEYGNIPVVRKMLEECHS---	LNVCVDYMGQNALQLAVANEHLEITELLKKEN	140
Human TRPC7	RFLDAAEYGNIPVVRKMLEECHS---	LNVCVDYMGQNALQLAVANEHLEITELLKKEN	102
Human TRPC3	RFLDAAEYGNIPVVRKMLEECHS---	LNVCVDYMGQNALQLAVANEHLEITELLKKEN	98
Human TRPC4	AYLNAVEKGDYASVKKSLSEAEIYFKININCIDPLGRITALLIAIENENLEITELLKFN-		93
Human TRPC5	AFLNAVEKGDYATVKQALQEAETIYNVNNINCMDPLGRSALLIAIENENLEITELLKHS-		93
Human TRPC1	LFLACDKGDYVMVKKILEENSS-GDLNINCVDVLRNAVTTIENENLDILQLLLDY--		106

**B**

	<b>L780P</b>	
Human TRPC6	DDADVEWKFARAKLWFSYFEEGRTLVPVFNLVSPKSLFYLLKLLKKWISSELFQGHKKGF	795
Patient 3	DDADVEWKFARAKLWFSYFEEGRTLVPVFNLVSPKSLFYLLKLLKKWISSELFQGHKKGF	795
Chimpanzee	DDADVEWKFARAKLWFSYFEEGRTLVPVFNLVSPKSLFYLLKLLKKWISSELFQGHKKGF	865
Orang-utan	DDADVEWKFARAKLWFSYFEEGRTLVPVFNLVSPKSLFYLLKLLKKWISSELFQGHKKGF	716
Rhesus monkey	EDADVEWKFARAKLWFSYFEEGRTLVPVFNLVSPKSLFYLLKLLKKWISSELFQGHKKGF	740
Gray Lemur	DDADVEWKFARAKLWFSYFEEGRTLVPVFNLVSPKSLFYLLKLLKKWISSELFQGHKKGF	795
Horse	DDADVEWKFARAKLWFSYFEEGRTLVPVFNLVSPKSLFYLLKLLKKWISSELFQGHKKGF	796
Cow	DDADVEWKFARAKLWFSYFEEGRTLVPVFNLVSPKSLFYLLKLLKKWISSELFQGHKKGF	795
Dog	DDADVEWKFARAKLWFSYFEEGRTLVPVFNLVSPKSLFYLLKLLKKWISSELFQGHKKGF	963
Platypus	DDADVEWKFARAKLWFSYFEEGRTLVPVFNLVSPKSLFYLLKLLKKWISSELFQGHKKGF	853
Gray opossum	EDADVEWKFARAKLWFSYFEEGRTLVPVFNLVSPKSLFYLLKLLKKWISSELFQGHKKGF	814
Rat	DDADVEWKFARAKLWFSYFEEGRTLVPVFNLVSPKSLFYLLKLLKKWISSELFQGHKKGF	794
Mouse	DDADVEWKFARAKLWFSYFEEGRTLVPVFNLVSPKSLFYLLKLLKKWISSELFQGHKKGF	794
Guinea Pig	DDADVEWKFARAKLWFSYFEEGRTLVPVFNLVSPKSLFYLLKLLKKWISSELFQGHKKGF	757
Chicken	DDADVEWKFARAKLWFSYFEEGRTLVPVFNLVSPKSLFYLLKLLKKWISSELFQGHKKGF	785
Frog	DDADVEWKFARAKLWFSYFEEGRTLVPVFNLVSPKSLFYLLKLLKKWISSELFQGHKKGF	779
Zebrafish	DDADVEWKFARAKLWFSYFEEGRTLVPVFNLVSPKSLFYLLKLLKKWISSELFQGHKKGF	771
Human TRPC7	EDADVEWKFARAKLWFSYFEEGRTLVPVFNLVSPKSLFYLLKLLKKWISSELFQGHKKGF	740
Human TRPC3	DDSDVEWKFARAKLWFSYFEEGRTLVPVFNLVSPKSLFYLLKLLKKWISSELFQGHKKGF	734
Human TRPC4	DHADVEWKFARAKLWFSYFEEGRTLVPVFNLVSPKSLFYLLKLLKKWISSELFQGHKKGF	685
Human TRPC5	DHADVEWKFARAKLWFSYFEEGRTLVPVFNLVSPKSLFYLLKLLKKWISSELFQGHKKGF	692
Human TRPC1	NHEDKEWKFARAKLWFSYFEEGRTLVPVFNLVSPKSLFYLLKLLKKWISSELFQGHKKGF	676

**Fig. 2.** Sequence alignments (using CLUSTAL-W) showing evolutionary conservation of TRPC6 substitutions. (A) Positions 109 and 125 are marked in grey and ankyrin repeat-1 in italics. (B) Position 780 is marked in grey and TRP domain in italics.

could be classified as a highly likely pathogenic mutation (MG = B) (Table 1).

### CASE 3

A 9-year-old female presented at the age of 7 years with oedema, >100 mg/m<sup>2</sup>/h proteinuria and microscopic haematuria. Treatment with prednisone had no effect, but she responded to immunosuppressive drugs (CsA and MMF) with serum albumin normalization but persistence of non-nephrotic range proteinuria. The renal biopsy showed FSGS with mesangial proliferation and deposits of IgM and IgG. She did not develop ESRD so far (after 2 years of follow-up). No members of the family (parents and two twin brothers) were clinically affected.

A new *missense* variant (c.2339T>C) was found in exon 9 causing a leucine-to-proline substitution (L780P) near the TRP domain and within the C-terminus of the TRPC6 protein (Figure 1). We obtained DNA from family members; the father (40 years of age) and the two brothers (5 years old) had the same change but they had no clinical symptoms.

We scored this change using our scoring matrix and classified it as group C because L780P variant is a non-conservative change (GD = 98) but at a moderately conserved hydrophobic residue in the MSA (Figure 2), giving a GD/GV score of +3; GDev did not drop precipitously as further diverged sequences were included in the analysis (Figure 3) (+1); the SIFT programme predicts that this substitution affects function (+2) and the Polyphen programme predicts that this L780P change could be 'possibly damaging' (+1); we did not find it in any of the public

**Table 1.** Classification of amino acid substitutions

Amino acid substitutions	Exon	Previous description	GD <sup>a</sup>	GV <sup>b</sup>	GD/GV matrix score <sup>c</sup>	GDev <sup>d</sup>	Defined domain (degree of conservation) <sup>e</sup>	Splicing prediction <sup>f</sup>	Present in controls	Described in SNP database	Polyphen prediction <sup>g</sup>	SIFT predicted tolerated <sup>h</sup>	Variant score	Mutation group <sup>i</sup>
Positive and negative controls														
P15S	1	SNP database	74	260 [fi]	-2	0 [fi] (-2)	No (0)	Not predicted (0)	Yes (-2)	Yes (-1)	0 (benign) (-2)	Yes (-2)	-11	P
P112Q	2	Winn <i>et al.</i>	76	0	+5	76 (+2)	Ank-1 (C) (+1)	Not predicted (0)	Not (+2)	Not (+1)	2.15 (probably damaging) (+2)	Not (+2)	15	B
N143S	2	Reiser <i>et al.</i>	46	0	+5	46 (+2)	Ank-2 (HC) (+2)	Not predicted (0)	Not (+2)	Not (+1)	2.23 (probably damaging) (+2)	Not (+2)	16	B
S270T	2	Reiser <i>et al.</i>	58	0	+5	58 (+2)	TRP II (HC) (+2)	Not predicted (0)	Not (+2)	Not (+1)	2.05 (probably damaging) (+2)	Not (+2)	16	B
A404V	4	SNP database	64	0	+5	64 (+2)	No (0)	Not predicted (0)	Yes (-2)	Yes (-1)	0.25 (benign) (-2)	Yes (-2)	0	I
R895C	13	Reiser <i>et al.</i>	180	0	+8	180 (+2)	No (0)	Not predicted (0)	Not (+2)	Not (+1)	3.24 (probably damaging) (+2)	Not (+2)	17	B
E897K	13	Reiser <i>et al.</i>	56	0	+5	56 (+2)	No (0)	Not predicted (0)	Not (+2)	Not (+1)	2.1 (probably damaging) (+2)	Not (+2)	14	B
Novel variants identified in our cohort														
G109S	2	Novel	56	0	+5	56 (+2)	Ank-1 (C) (+1)	Not predicted (0)	Not (+2)	Not (+1)	2.15 (probably damaging) (+2)	Not (+2)	15	B
N125S	2	Novel	46	23 [fi]	+4	45 [fi] (+2)	Ank-1 (C) (+1)	Not predicted (0)	Not (+2)	Not (+1)	2.15 (probably damaging) (+2)	Not (+2)	14	B
L780P	9	Novel	98	51 [fi]	+3	68 [fi] (+1)	No (0)	Not predicted (0)	Not (+2)	Not (+1)	1.95 (possibly damaging) (+1)	Not (+2)	10	C

<sup>a</sup>GD (Grantham distance); score of chemical difference between the normal and mutated residue (high score, greater difference).

<sup>b</sup>GV (Grantham variation); score of chemical difference between 16 orthologues (ranging from orangutan to Zebrafish[fi]) and 5 homologues (0 = completely conserved).

<sup>c</sup>GD/GV matrix score; lower matrix scores corresponded to low GD and high GV (conservative change and strong variation within the MSA), while higher matrix scores corresponded to high GD and low GV (non-conservative change and strong conservation within the MSA).

<sup>d</sup>GDev (Grantham deviation); score of chemical difference between the mutated residue and the range of variation between orthologues (GD similar to GDev, higher difference).

<sup>e</sup>Domain containing residue: ANK repeats, transmembrane domains, TRP II domain, TRP domain; I, invariant; HC, highly conserved (>80%); C, conserved (80–50%); NC, not conserved (<50%); No (not defined domains).

<sup>f</sup>Not predicted by the Splice Site Prediction Neural Network.

<sup>g</sup>Polyphen assessment: ratio polyphen >2 (probably damaging), ratio polyphen >1 (possibly damaging), ratio polyphen <1 (benign).

<sup>h</sup>SIFT tolerated: not tolerated = +2; tolerated = -2.

<sup>i</sup>Mutation Group (MG) = B → Variant Score (VS) > 11; MG = C → 5 > VS < 10; MG = I → -4 > VS < 4; MG = P → VS < -5; B, highly likely pathogenic; C, likely pathogenic; I, variant with indeterminate effect; P, neutral variant or polymorphism.

L780P	AA	c	p	v	Cmax	Cmin	Pmax	Pmin	Vmax	Vmin	GV	Gdev
Human	L	0.00	4.90	111.00	0.00	0.00	4.90	4.90	111.00	111.00	-	97.78
Chimpanzee	L	0.00	4.90	111.00	0.00	0.00	4.90	4.90	111.00	111.00	0	97.78
Orang-utan	L	0.00	4.90	111.00	0.00	0.00	4.90	4.90	111.00	111.00	0	97.78
Rhesus monkey	I	0.00	5.20	111.00	0.00	0.00	5.20	4.90	111.00	111.00	4.86	95.38
Gray Lemur	L	0.00	4.90	111.00	0.00	0.00	5.20	4.90	111.00	111.00	4.86	95.38
Horse	L	0.00	4.90	111.00	0.00	0.00	5.20	4.90	111.00	111.00	4.86	95.38
Cow	L	0.00	4.90	111.00	0.00	0.00	5.20	4.90	111.00	111.00	4.86	95.38
Dog	L	0.00	4.90	111.00	0.00	0.00	5.20	4.90	111.00	111.00	4.86	95.38
Platypus	I	0.00	5.20	111.00	0.00	0.00	5.20	4.90	111.00	111.00	4.86	95.38
Gray opossum	I	0.00	5.20	111.00	0.00	0.00	5.20	4.90	111.00	111.00	21.82	95.38
Rat	F	0.00	5.20	132.00	0.00	0.00	5.20	4.90	132.00	111.00	21.82	95.38
Mouse	F	0.00	5.20	132.00	0.00	0.00	5.20	4.90	132.00	111.00	21.82	95.38
Guinea Pig	F	0.00	5.20	132.00	0.00	0.00	5.20	4.90	132.00	111.00	21.82	95.38
Chicken	L	0.00	4.90	111.00	0.00	0.00	5.20	4.90	132.00	111.00	21.82	95.38
Frog	V	0.00	5.90	84.00	0.00	0.00	5.90	4.90	132.00	84.00	51.26	67.79
Zebrafish	V	0.00	5.90	84.00	0.00	0.00	5.90	4.90	132.00	84.00	51.26	67.79
<b>Human missense</b>	<b>P</b>	<b>0.39</b>	<b>8</b>	<b>32.5</b>								

**Fig. 3.** Calculation of GV and GDev according to Tavtigian *et al.* using L780P as an example. For each amino acid aligned to human position 780, we give the amino acid sidechain composition (C), polarity (P) and volume (V). Starting with human and working sequentially to the Zebrafish sequence, we determine the minimum and maximum observed values of C, P and V; these are used to calculate GV. We then determine the difference between C, P and V for the missense variant (Pro in this case) and the range of variation observed to that point in the alignment. The differences are used to calculate GDev according to Tavtigian *et al.* [12].

SNP databases (+1) nor in 400 controls studied (+2). We obtained a VS of 10; thus, the variant could be classified as a likely pathogenic amino acid substitution (Table 1).

These three cases were also screened for *NPHS2*, *NPHS1*, *WT1*, *ACTN4* and *CD2AP* genes, and no mutations were found (unpublished data).

## Discussion

TRPC6 is a member of the transient receptor potential (TRP) superfamily of cation-selective ion channels, which mediate both store-operated and receptor-operated cation influx in many body tissues [20]. TRP channels have been implicated in diverse biological functions such as cell growth, ion homeostasis, mechanosensation and PLC-dependent calcium entry into cells. The TRPC subfamily (TRPC1–TRPC7) is a group of calcium-permeable cation channels that are important for the increase in intracellular calcium concentration after direct stimulation via G protein-coupled receptors (GPCR) [19]. A special feature of some TRPC proteins, including TRPC6, is the presence of ankyrin binding repeats in the N terminus. TRPC6 is an important component of the glomerular slit diaphragm that colocalizes with CD2AP, nephrin and podocin in cultured mouse podocytes and coimmunoprecipitates with nephrin and podocin [7]. To date, only six *TRPC6* mutations causing familial adult onset FSGS have been reported [6,7].

We searched for *TRPC6* mutations in 12 familial and 103 non-familial cases with FSGS, which represent the largest cohort of patients screened for *TRPC6* mutations published

to date. We describe one new missense substitution in a familial case and two new missense variants identified for the first time in apparently non-familial cases. Moreover, one of these missense changes was identified in a child with FSGS. Although testing of *TRPC6* missense mutations by a functional assay may seem the best approach to determine its pathogenicity, only three of the six *TRPC6* described mutations produced detectable changes in calcium current amplitude. This may be due to technical challenges in detecting minimal calcium amplitude variations in an *in vitro* cellular system. For this reason, we opted to develop an *in silico* predictive model, a scoring matrix based on a mixture of different approaches previously reported for other genes such as *BRCA1*, *BRCA2*, *PKD1* and *PKD2* [12,13], for the evaluation of amino acid substitutions at the *TRPC6* gene. In order to validate the performance of this scoring system to the *TRPC6* gene, we included in the analysis the previously reported missense changes (P112Q, N143S, S270T, R895C and E897K) and SNPs (P15S and A404V). All these missense substitutions were classified as highly likely pathogenic mutations whereas one SNP (P15S) was classed as polymorphism and the other (A404V) as a variant of indeterminate pathogenicity (Table 1). Then, we used the scoring matrix to evaluate the amino acid substitutions found in our study cohort (G109S, N125S and L780P) and they were also classified as highly likely or likely pathogenic mutations.

The three new variants we describe here are missense, two of them located in the first ankyrin repeat domain. The other published changes include five missense changes (two in ankyrin domains, one in the TRP II domain and two

in the C-terminal) and one stop codon at the end of the C-terminal. These mutations are distributed throughout the N and C terminal cytosolic domains. No mutations have been described in transmembrane domains. Moreover, four of nine changes identified to date affect the ankyrin 1 and 2 repeat domains. The accumulation of mutations in the ankyrin repeats correlates with the important functional roles ascribed to them [21].

Some families described here and in the literature [6,7] show members of a single family with the mutation and normal urine sediment while other members are on renal replacement therapy by the same age. The third case presented here is clinically sporadic; however, the mutation has been detected in asymptomatic relatives. This frankly incomplete penetrance or wide clinical spectrum could be explained by a multi-hit hypothesis; several proteins involved in the glomerular filtration barrier might need to be abnormal to give rise to a severe phenotype. The most well-known genes involved in FSGS have been screened in our patients and no mutation was detected (*NPHS2*, *NPHS1*, *WT1*, *ACTN4*, *CD2AP*), which does not rule out the multi-hit hypothesis as the number of known genes implicated in the slit diaphragm is increasing every day. In mice, FSGS can be oligogenic in aetiology. Huber *et al.* [22] showed that bigenic compound heterozygosity for mutations in Fyn, Synaptopodin and/or CD2AP can cause FSGS in mice, suggesting that a similar mechanism may also underlie the disease in humans. The extent to which genetic factors contribute to most cases of FSGS remains unknown. Moreover, the increased incidence of idiopathic FSGS suggests that both genetic and environmental factors may play an important role in the pathogenesis of this disease [23]. A report by Ghiggeri *et al.* [24] describing two pairs of identical twins with FSGS but very different clinical courses highlights the importance of non-genetic factors.

The clinical phenotype associated with the L780P variant is of particular interest because it represents the first FSGS child carrying a variant in the *TRPC6* gene described to date. The reason why a mutation in *TRPC6* gene usually takes a long time to give rise to a clinical phenotype may be explained by the fact that the abnormal protein may produce subtle changes in the cell behaviour visible after time in the presence of other renal insults [25]. A certain level of protein redundancy may also account for the late onset of this disease. The early onset case may have unknown genetic or environmental risk factors triggering a severe phenotype. An outstanding feature of this case is the unusual satisfactory response to anticalcineurin therapy. Exaggerated calcium signalling conferred by the *TRPC6* mutations may activate the calcium-dependent phosphatase calcineurin, which promotes apoptosis and disrupts the podocyte actin cytoskeleton [26]. Blocking this calcineurin activity might improve proteinuria [27]. This may also be true for other forms of FSGS.

Even though the *in silico* approach is very useful for predicting probably mutations, to date the functional assay is the definitive step to determine if a variant is a mutation. Further clinical data of patients with *TRPC6* mutations and their response to treatments may permit to better know their associated phenotypes and tailoring of initial medication choices. On the other hand, patients with *TRPC6* mutations

seem to be less likely to develop recurrent FSGS; therefore, preemptive screening of potential kidney transplant recipients may be useful, particularly when considering living donor transplantation. Also, family members should be screened for the *TRPC6* mutations to be followed up in case they have the mutation and to discard them as kidney donors.

Although being a carrier of a *TRPC6* mutation seems to be a risk factor for developing FSGS, some people with a mutation in this gene show either no signs or mild signs of the disease. Thus, the knowledge of the mutated gene carrier status may encourage the nephrologist to consider relatively innocuous interventions such as lifestyle or more aggressive management such as ACEI or ARBs.

In summary, our results plus the ones published to date suggest that *TRPC6* mutations account for ~6% of familial FSGS and ~2% of sporadic FSGS. Carrying a mutation in the *TRPC6* gene seems to be a predisposing factor towards developing FSGS. Knowing the carrier status for this gene might have significant consequences regarding the follow-up of asymptomatic carriers, study of potential kidney donors and tailoring of therapy in affected individuals.

*Acknowledgements.* This work has been supported by REDinREN (Red renal de investigación española 16/06, RETICS, Instituto de Investigación Carlos III) and a grant from the Spanish Health Ministry (FIS-05/761). The authors wish to thank the patients and their families for taking part in this study and Dr Peter Harris for reading the manuscript and providing valuable comments.

*Conflict of interest statement.* None declared.

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Received for publication: 12.1.09; Accepted in revised form: 27.4.09

Nephrol Dial Transplant (2009) 24: 3096–3102

doi: 10.1093/ndt/gfp196

Advance Access publication 25 April 2009

## Cystatin C is correlated with mortality in patients with and without acute kidney injury

Max Bell<sup>1</sup>, Fredrik Granath<sup>2</sup>, Johan Mårtensson<sup>1</sup>, Erland Löfberg<sup>3</sup>, Anders Ekblom<sup>2</sup> and Claes-Roland Martling<sup>1</sup> of KING (Karolinska Intensive care Nephrology Group)

<sup>1</sup>Department of Anaesthesiology and Intensive Care, <sup>2</sup>Department of Medicine, Clinical Epidemiology Unit and <sup>3</sup>Division of Nephrology, Department of Medicine, Karolinska University Hospital, Solna, Sweden

Correspondence and offprint requests to: Max Bell; E-mail: max.bell@karolinska.se

### Abstract

**Background.** Recent research has shown cystatin C to predict mortality and cardiovascular morbidity independent of renal function. The aim of this study was to evaluate the prognostic value of cystatin C on mortality in adult general ICU patients with acute kidney injury (AKI). We later expanded the study and included patients without signs of AKI.

**Methods.** A total of 845 ICU patients were analysed for cystatin C and classified according to the RIFLE criteria. Of these, 271 patients with either creatinine >150 µmol/l, urea >25 or anuria/oliguria entered the AKI cohort. The remaining 562 patients entered the non-AKI cohort. Both cohorts were divided into quartiles according to cystatin C at entry. In the non-AKI cohort, we split the highest cystatin C quartile into two. The relationship between the different cystatin C quartiles and mortality in patients with and with-

out AKI was estimated by hazard ratios (HR) derived from the Cox proportional hazards regression model.

**Results.** A relationship between cystatin C and mortality was found in patients with and without AKI, being stronger in patients without AKI. In AKI patients, the HR comparing cystatin C above and below the median more than doubled from the second year on compared to the first year follow-up. After exclusion of patients in the non-AKI cohort with 'potential AKI' (creatinine >100 µmol/l or urea >20 mmol/l), the correlation between cystatin C levels and risk of death was strengthened.

**Conclusions.** Cystatin C is correlated with mortality independently of renal function measured by creatinine in patients entering the general ICU.

**Keywords:** AKI; critical care; cystatin C; epidemiology; RIFLE