TRPC6 Mutational Analysis in Iranian Children With Focal Segmental Glomerulosclerosis

Alaleh Gheissari,[1,2] Rokhsareh Meamar,[3] Majid Kheirollahi,[4,5] Maedeh Rouigari,[6] Moein Dehbashi,[6,7] Leila Dehghani,[8] Amin Abedini[1]

[1]Isfahan Kidney Diseases Research Center, Isfahan University of Medical Sciences, Isfahan, Iran
[2]Department of Pediatric Nephrology, Isfahan University of Medical Sciences, Isfahan, Iran
[3]Isfahan Clinical Toxicology Research Center, Isfahan University of Medical Sciences, Isfahan, Iran
[4]Pediatric Inherited Diseases Research Center, Research Institute for Primordial Prevention of Non-communicable Disease, Isfahan, Iran
[5]Department of Genetics and Molecular Biology, School of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran
[6]Isfahan Neurosciences Research Center, Alzahra Hospital, Isfahan University of Medical Sciences, Isfahan, Iran
[7]Genetics Division, Department of Biology, Faculty of Science, University of Isfahan, Isfahan, Iran
[8]Department of Tissue Engineering and Regenerative Medicine, School of Advanced Technologies in Medicine, Shahid Beheshti University of Medical sciences, Tehran, Iran

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Introduction. Focal segmental glomerulosclerosis (FSGS) ranks among nephrotic syndromes. Research shows that FSGS is brought about by several genes including transient receptor potential cation channel subfamily c member 6 (*TRPC6*). This study aimed to investigate *TRPC6* gene in Iranian FSGS children.
Materials and Methods. Twenty-six FSGS patients were included. They were all under 16 years old. Polymerase chain reaction amplification and sequencing were performed to examine exons 2 and 13 of *TRPC6* gene.
Results. Sampling was performed when the patients had a mean age of 9.26 ± 3.19 years. Sixteen children were boys (61.5%); male-female ratio was 1.35:1. Four patients (15.4%) were diagnosed with *TRPC6* variants. Three missense nonsynonymous mutations (C121S, D130V, and G162R) and 1 synonymous mutation (I111I) were detected. All variants were novel; in silico analysis predicted D130V and G162R as pathogenic. Patients with and without mutations were not different significantly regarding age at disease onset, sex, consanguinity, hypertension, hematuria, serum creatinine and albumin, rate of progression to kidney failure, response to steroids, and resistance to cyclosporine A and cyclophosphamide.
Conclusions. This study examined exons 2 and 13 of *TRPC6* gene in Iranian FSGS children. Four novel *TRPC6* variants were detected; in silico analysis showed that 2 variants (D130V and G162R) could be pathogenic. It could be concluded that *TRPC6* may be useful for genetic screening in Iranian FSGS children.

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INTRODUCTION

Focal segmental glomerulosclerosis (FSGS) is a type of nephrotic syndrome identified by edema, proteinuria, hypoalbuminemia, and hyperlipidemia.[1,2] It is responsible for 20% of all causes of nephrotic syndrome and more than 75% of steroid-resistant nephrotic syndrome (SRNS) in children.[2,3] Focal segmental glomerulosclerosis

commonly progresses to end-stage renal disease (ESRD).[4] It can be classified as primary (idiopathic) and secondary (due to viruses, drugs, structural pathologies, ischemia, and familial or genetic causes).[2]

Several genes have been identified as genetic causes for FSGS which encodes proteins that are involved with construction of podocytes and slit diaphragms, including *NPHS1*, *NPHS2*, *NPHS3*, *CD2AP*, *Myo1E*, *ACTN4*, *INF2*, and *TRPC6* (transient receptor potential cation channel subfamily c member 6).[5] The TRPC6 protein is a member of the transient receptor potential family of cation channels which has been suggested to play a role in the pathogenesis of SRNS, especially FSGS.[6-8] Initially, it had been assumed that *TRPC6* was responsible for familial adult onset of FSGS, but recent studies have shown that this gene can result in disease in sporadic cases and also in the pediatrics.[9,10] The majority of mutations identified in *TRPC6* were missense mutations existing in exons 1, 2, 3, 4, 7, 9, 10, 12, and 13.[3,7-18] Exons 2 and 13 had the highest frequency of reported mutations.

To date, limited data is available about genetic causes of SRNS in Iranian children, and the few existing studies are limited to *NPHS1* and *NPHS2*.[19-22] The main aims of this study were to evaluate the mutations in exons 2 and 13 of the *TRPC6* gene in Iranian children with FSGS and to assess the pathogenicity of probable mutations. We also evaluated the association of *TRPC6* mutations with clinical characteristics of patients.

MATERIALS AND METHODS

Study Design and Population

This study was conducted from March 2016 to March 2017 on children with FSGS at Imam-Hossein Children's Hospital, Isfahan, Iran. This hospital is the main tertiary care center for pediatric nephrology diseases in Isfahan province. Our inclusion criteria were biopsy-proven diagnosis of FSGS, age less than 16 years, and a minimum clinical follow-up of 6 months. Our exclusion criteria were presence of any cause of secondary types of FSGS (sickle cell disease, reflux nephropathy, and renal agenesis), incomplete clinical and pathological data of patients and their families, and having mutations in other genes that cause FSGS in previous screenings. The study protocol was approved by the ethics committee of Isfahan University of Medical Sciences. This study followed the guidelines of the Declaration of Helsinki. Informed consent was obtained from all parents or caregivers of the patients in this study.

Patients and Data Collection

Overall, 26 Iranian pediatric patients were enrolled to the study according to the inclusion criteria. A venous blood sample was obtained from patients for mutational analysis of *TRPC6*. Demographic and clinical data of patients were recorded including age, sex, age at disease onset, weight, height, hypertension, hematuria, serum creatinine and albumin levels, proteinuria amount, progression to chronic kidney disease (CKD) or ESRD, history of transplantation, and response to different treatments.

Definitions

A diagnosis of FSFS was based on pathologic criteria.[23] Hypertension was defined as systolic or diastolic blood pressure above the 95th percentile for age, sex, and height. Hematuria was established in patients with more than 5 erythrocytes per high-power field. Glomerular filtration rate was estimated using the updated Schwartz equation.[24] Chronic kidney disease was defined as a glomerular filtration rate less than 60 mL/min/1.73 m^2 for 3 months or more. End-stage renal disease was defined as a glomerular filtration rate less than 15 mL/min/1.73 m^2 or need for renal replacement therapy. Prednisolone was administered at a dose of 60 mg/m^2/d or 2 mg/kg/d for 4 weeks and after that followed for more than 4 weeks by same dose every other day. Early nonresponse to steroids was defined as failure to remission after 8 weeks of therapy and patients who initially responded to steroids and after that became resistant are considered as late nonresponders. Early or late nonresponders to steroids were treated by cyclosporine A or cyclophosphamide. Complete remission was defined as no proteinuria and partial remission was considered as no edema and a proteinuria between 4 mg/m^2/h and 40 mg/m^2/h. Complete remission was considered as response to treatment and partial remission was considered as resistance to treatment.

Mutation Analysis

DNA was extracted from ethylenediaminetetra-

acetic acid anticoagulated peripheral blood by QiAamp DNA Blood Mini Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instruction. Primers for exons 2 and 13 of *TRPC6* (Table 1) were designed based on *TRPC6* sequence (NM_004621.5) using Gene Runner version 6.5.28. Polymerase chain reaction (PCR) was used to amplify exons 2 and 13 of the TRPC6 gene. Polymerase chain reaction was carried out in 100-µL volume containing 100 ng of DNA, 10 µL of 1X PCR buffer, 1.5 mM of magnesium chloride, 0.8 mM of nucleoside triphosphate, 0.5 µM of each forward and reverse primer and 2.5 U/100 µL reaction for Taq DNA polymerase (Sinaclon Co, Iran). The PCR program was started with initial denaturation at 95°C for 2 minutes, followed by 30 cycles with a strand separation step at 95°C for 30 seconds, an annealing step at 58°C for each primer pair for 30 seconds and an extension step at 72°C for 70 seconds. Finally, PCR was finished with a 5-minute extension period at 72°C. After PCR performing and prior to sequencing, the PCR products were stained with ethidium bromide and were run on 1.5% agarose gels and after that visualized on an ultraviolet transilluminator. Then, PCR products were sequenced using a cycle sequencing kit (BigDye terminator v.3.1 kit) on an automated DNA sequencing machine (Applied Biosystems 3730/DNA analyzer). All sequences were compared with *TRPC6* reference sequence (NM_004621.5) using NCBI blast and nucleotide/amino acid changes were recorded. When a variation was seen we performed PCR and sequencing again for confirming the variation.

In silico Evaluation of *TRPC6* Variants

For evaluating the effect of variants and mutations on biological protein function of *TRPC6* which were found in the study we used PROVEN (http://provean.jcvi.org/index.php), Polyphen-2 (http://genetics.bwh.harvard.edu/pp2), SIFT (Sorting Intolerant From Tolerant) (http://sift.jcvi.org/), Mutation Taster (http://www.mutationtaster.org/) and PhD-SNP (Predictor of human Deleterious Single Nucleotide Polymorphisms) (http://snps.biofold.org/phd-snp/phd-snp.html).

Statistical Analysis

Continuous data were expressed as the mean ± standard deviation and categorical variables were shown as percentage. For mean value comparison between patients with and without *TRPC6* mutations we performed the independent sample *t* test or the Mann Whitney *U* test as appropriate. Categorical variables were compared between different groups using thee chi-square test. A *P* value less than .05 was considered significant. Statistical analysis was performed using the SPSS software (Statistical Package for the Social Sciences, version 22.0, SPSS Inc, Chicago, IL, USA).

RESULTS

Patients

The mean age of patients at the time of sampling was 9.26 ± 3.19 years (range, 2 to 15 years). Sixteen patients (61.5%) were male and male-female ratio was 1.35:1. The mean age at disease onset was 5.44 ± 2.28 years (range, 1 to 10 years). Consanguinity was present in 20 patients (76.9%) and all of them were first cousin marriages. All FSGS cases were sporadic and they had no other family member with confirmed diagnosis of FSGS. Progression rate to CKD and ESRD were 15.4% and 30.8%, respectively. All patients had SRNS among whom 14 patients (53.8%) were early nonresponders and 12 patients (46.2%) were late nonresponders. Table 2 shows the demographic and clinical data of participants in details.

TRPC6 Mutation Analysis

TRPC6 variants were identified in 4 patients (15.4%). We identified 3 missense nonsynonymous mutation (*C121S*, *D130V*, *G162R*) and 1 synonymous mutation (*I111I*). All variants were heterozygous (Table 3) and were novel based on single nucleotide polymorphism database (https://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?geneId = 7225). Figure 1 shows the sequence chromatography of mutant and wild type of these mutations. According to all in silico analysis which were used, D130V

Table 1. TRPC6 primer sequences which were used in the study.

Exon	Forward (5' to 3')	Reverse (3' to 5')	Size, bp	Temperature, °C
2	TTAGTGATCGCTCCACAAG	TCACTAGACAATGACAGGTAAG	610	56.8
13	TCCTGTCCCACAGTCACTAG	CTACAGCCTTTACCCTGAAC	422	59.45

Table 2. Characteristics of Studied Patients

Variables	Value
Sex	
Male	15
Female	11
Age at disease onset, y	5.44 ± 2.28
Age at time of sampling, y	9.26 ± 3.19
Weight, kg	35.5 ± 17.63
Height, cm	127.6 ± 25.84
Parental consanguinity	20 (76.9)
Hypertension	6 (23.1)
Hematuria	2 (7.7)
Serum creatinine, mg/dL	2.2 ± 2.27
Serum albumin, g/dL	3.71 ± 1.17
Proteinuria, g/d	1.09 ± 1.47
Progression to chronic kidney disease	4 (15.4)
Progression to end-stage renal disease	8 (30.8)
Progression time to end-stage renal disease (years from disease onset)	4.22 ± 1.71
Kidney transplantation	6 (75.0)
Transplant rejection	1 (16.7)
Primary response to steroids	
Complete remission	12 (46.2)
Partial remission	8 (30.8)
No remission	6 (23.1)
Response to cyclosporine A	
Complete remission	12 (57.1)
Partial remission	6 (28.6)
No remission	3 (14.3)
Response to cyclophosphamide	
Complete remission	0
Partial remission	0
No remission	10 (100)
Early nonresponders to steroids	14 (53.8)
Late nonresponders to steroids	12 (46.2)
Resistance to cyclosporine A	9 (42.8)
Resistance to cyclophosphamide	10 (100)

and G162R could have adverse effect on biological function of *TRPC6*. *D130V* cause substituting an aspartic acid to valine and in *G162R* missense mutation glycine substitutes to arginine. Figure 2 shows evolutionary conservation of amino acids in positions 130 and 162 which shows these 2 positions are highly conserved. The other 2 variants (*I111I*, *C121S*) are predicted by Mutation taster as disease causing but other in silico analyses showed that these 2 variants probably did not have an adverse effect on *TRPC6* function. According to the protein topology of TRPC6 (http://www.uniprot.org/uniprot/Q9Y210), 2 mutations (*D130V* and *G162R*) which probably are disease causing are located in ankyrin domain 1 and 2 of the *TRPC6* structure, respectively.

Table 3. *TRPC6* Mutations and Variants in 2 Studied Exons and Their Pathogenicity Based on in Silico Analysis

				PROVEAN		Polyphen-2		SIFT		Mutation Taster	phD-SNP
Exon	Nucleotide Change	Amino Acid Change	Zygosity	Score	Prediction	Score	Prediction	Score	Prediction	Prediction	Prediction
2	c.333 C>T	p.I111I	Heterozygote	0	Neutral	-	-	1	Tolerated	Disease causing	Neutral
2	c.362 G>C	p.C121S	Heterozygote	0.11	Neutral	0.7	Possibly damaging	0.96	Tolerated	Disease causing	Neutral
2	c.389 T>A	p.D130V	Heterozygote	-7.23	Deleterious	0.99	Probably damaging	0	Damaging	Disease causing	Disease-related polymorphism
2	c. 484 G>C	p.G162R	Heterozygote	-4.74	Deleterious	0.99	Probably damaging	0.02	Damaging	Disease causing	Disease-related polymorphism

Table 4. Characteristics of Patients With *TRPC6* Variants*

Variants	Consanguinity	Age at disease onset, y	Sex	Hypertension	Hematuria	Proteinuria, g/d	Serum Albumin, g/dL	Serum Creatinine, mg/dL	Response to Cyclophosphamide	Response to Cyclosporine	Primary Response to Steroids	Progression to ESRD	Progression to CKD
I111I	Yes	1	Female	No	No	1.2	2.2	0.8	NU	NR	NR	No	Yes
C121S	Yes	4	Female	No	Yes	2.45	2.7	0.8	NU	CR	NR	No	No
D130V	Yes	5	Male	No	No	2.2	3.5	0.8	NU	CR	PR	No	No
G162R	Yes	9	Female	Yes	No	1.9	2.5	0.9	NR	CR	PR	No	No

*CKD indicates chronic kidney disease; ESRD, end-stage renal disease; CR, complete remission; PR, partial remission; NR, no remission; and NU, not used.

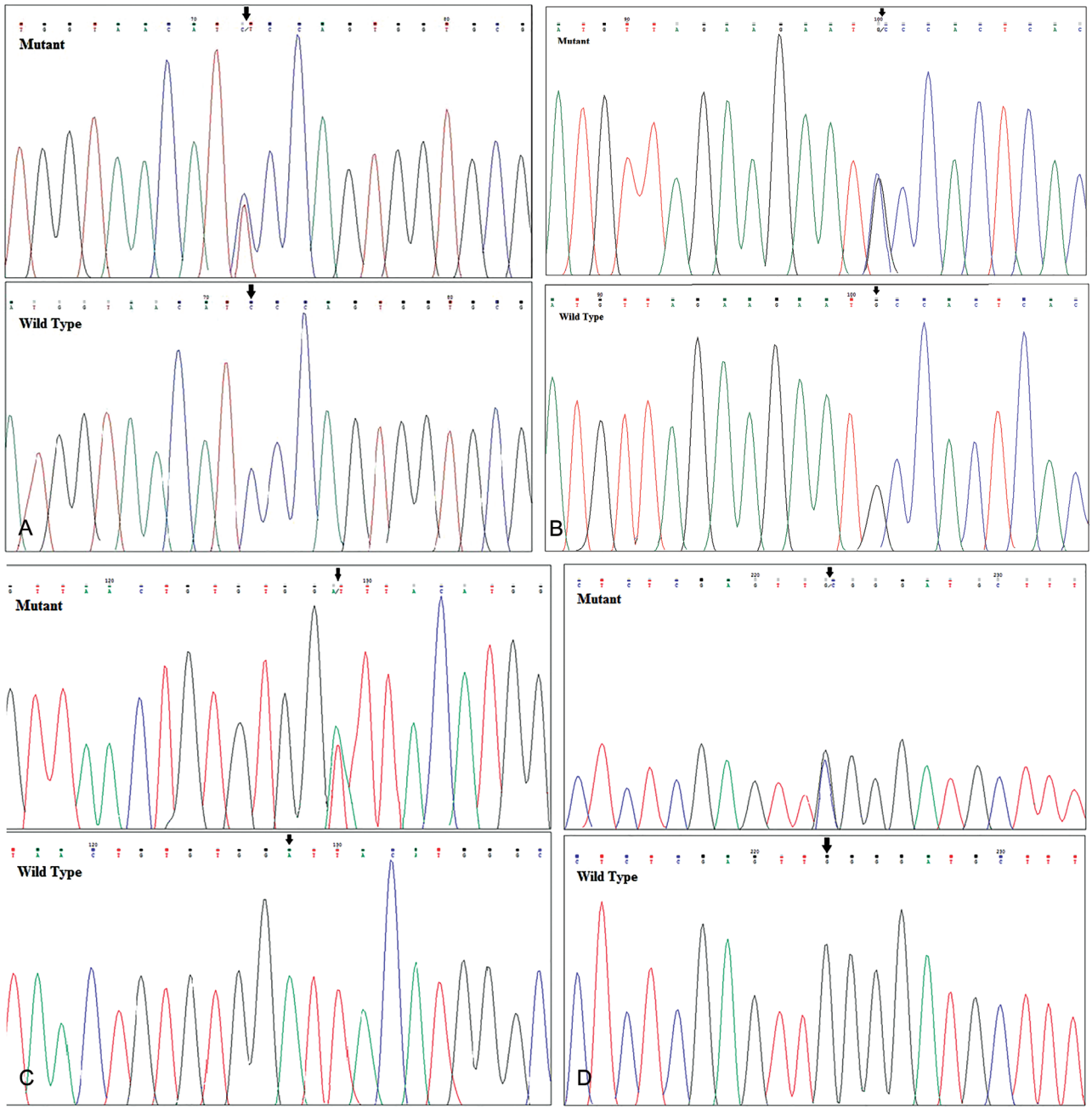

Figure 1. Sequence chromatograms of identified variants in *TRPC6* gene in children with focal segmental glomerulosclerosis. A, c.333 C > T, p.I111I; B, c.362 G > C, p.C121S; C, c.389 T > A, p.D130V; and D, c. 484 G > C, p.G162R.

Characteristics of Patients With *TRPC6* Variants

Table 4 shows the features of 4 patients with *TRPC6* variants. Three patients (75%) were female. The mean age at their disease presentation was 4.75 ± 3.3 years. All patients were born from consanguineous parents. All parents of subjects with *TRPC6* variants had normal kidney function with no proteinuria. One patient (25%) progressed to CKD 1 year after disease onset. All patients with *TRPC6* variants were early nonresponders to steroids and resistance rate to cyclosporine was 25%. When comparing the patients with and without mutations, the mean age of disease onset was lower in patients with mutations than subjects without variants but this difference was not significant (4.75 ± 3.3 versus 5.56 ± 2.12 years, $P = .50$). Eight patients (47%) of 17 patients without *TRPC6* mutations had resistance to cyclosporine

species	match	gene	aa	alignment
Human			130	E E C H S L N V N C V D Y M G Q N A L Q L A V
mutated	not conserved		130	C H S L N V N C V V Y M G Q N A L Q L A V
Ptroglodytes	all identical	ENSPTRG00000004207	129	C R S L N V N C V D Y M G Q N A L Q L A V
Fcatus	all identical	ENSFCAG00000010432	73	E C L S L N V N C V D Y M G Q N A L Q L A V
Mmusculus	all identical	ENSMUSG00000031997	129	C H S L N V N C V D Y M G Q N A L Q L A V
Ggallus	all identical	ENSGALG00000017194	72	E E C P S L N V N C V D Y M G Q N A L Q L A V
Drerio	all identical	ENSDARG00000056625	113	E E L P E L D V N C V D Y M G Q N A L Q L A V
Xtropicalis	all identical	ENSXETG00000005481	75	E E C R S L N V N C V D Y M G Q N A L Q L A V

species	match	gene	aa	alignment
Human			162	L L L K K E N L S R V G D A L L L A I S K G Y
mutated	not conserved		162	L L L K K E N L S R V R D A L L L A I S K G Y
Ptroglodytes	all identical	ENSPTRG00000004207	161	L L L K K E N L S R V G D A L L L A I S K G Y
Fcatus	all identical	ENSFCAG00000010432	105	L L L K K E N L S R V G D A L L L A I S K G Y
Mmusculus	all identical	ENSMUSG00000031997	161	L L L K K E N L S R V G D A L L L A I S K G Y
Ggallus	all identical	ENSGALG00000017194	104	L L L K K E N L S R V G D A L L L A I S K G Y
Drerio	all identical	ENSDARG00000056625	145	L L L K K D N L S R I G D A L L L A I S K G Y
Xtropicalis	all identical	ENSXETG00000005481	107	L L L K K E N L A R V G D A L L L A I S K G Y

Figure 2. Aspartic acid at position 130 and glycine at position 162 are highly conserved in evolution in *TRPC6* gene. Data was obtained from: http://www.mutationtaster.org

and this rate was 25% for patients with mutations but this was not significant (P = .60). There were no significant differences in other variables between patients with and without mutations including gender, hypertension, hematuria, serum creatinine and albumin, rate of progression to CKD and ESRD, consanguinity, response to steroids and resistance to cyclophosphamide.

DISCUSSION

In this study, we evaluated the two exons of *TRPC6* gene in Iranian children with FSGS and identified 4 novel *TRPC6* variants. To the best of our knowledge the present study is the first study which was carried out on Iranian population and also in the Middle East region that assessed the *TRPC6* gene in patients with FSGS. The previous studies have been done in European,[3,7-10,12,13,15,17,25] Chinese,[18,26,27] African-American, and Hispanic[7] populations. In this study, *TRPC6* mutations were found in patients with sporadic FSGS supporting the idea that this gene could cause FSGS in patients without family members with SRNS and autosomal dominant pattern and this issue was confirmed in some studies previously.[3,9,10,26] In our study, all *TRPC6* variants were seen in patients whom were born from consanguineous parents and this issue did not exist in previous studies. Since we did not analyze DNA samples of the patients' parents, we are not able to define the pattern of disease inheritance in this population and find out that this observation is just a coexistence or due to other underlying reasons.

In this study, we found that the mean age at disease onset was lower in patients with *TRPC6* mutation but the difference was not significant probably due to our small sample size. This observation is similar to a study conducted by Mir and colleagues on Turkish children.[3] In our study, 25% of patients with *TRPC6* variants progressed to CKD, but no patient progressed to ESRD. The rate of ESRD progression in patients with *TRPC6* mutations varies in different studies including zero,[10,26] 25%,[17,25] and 43.7%.[3] This discrepancy is most probably due to different time of follow-up. In fact Mir and colleagues[3] had longer time of follow-up and they reported the highest progression rate to ESRD in children with *TRPC6* mutations. Another issue which is comparable between patients with and without *TRPC6* is response to immunosuppressive medications. In our study, resistance to cyclosporine was 25% in patients with mutations and 47% in patients without *TRPC6* variants, but this difference was not significant.

In one study conducted on Turkish children the resistance rate to cyclosporine in children with and without *TRPC6* mutations was reported to be 62.5% and 55.5%,[3] respectively, but similar to our study this finding was not significant. In other studies on children with *TRPC6* mutations, resistance was reported zero,[10] 50%,[17] and 100%,[15] but in these studies differences with patients without mutation were not investigated. In two studies on children evaluating the differences between treatment response in patients with and without genetic causes of SRNS, it was determined that patients with genetic causes had significantly lower rate of response to cyclosporine,[15,28] but in these studies the most prevalent genes with mutations were *NPHS1*, *NPHS2,* and *WT1* while *TRPC6* mutations had low prevalence. Therefore, it seems that those studies have failed to show that patients with *TRPC6* mutations have more or less resistance to cyclosporine. This issue needs larger cohort studies for further evaluation.

In this study, we identified four novel missense mutations in *TRPC6* gene and 3 of them were nonsynonymous. These three mutations all existed on exon 2 and 2 of these novel variants were predicted pathogenic by 5 in silico analysis programs. To date, 28 different disease causing mutations have been re ported,[3,7-13,15-18,26,27,29,30] among which 25 mutations were missense nonsynonymous, [3,7-13,15-18,26,27,30] 1 of them was nonsense mutation,[7] and 2 others (89fsX8, D873fsX878) were frameshifts mutations.[15,29] Nearly, 40% of mutations occurred in exon 2 and after that exon 13 with 19% of mutations was the most prevalent. In this study, we evaluated these two exons since the possibility of finding mutations were higher and finally all variants we found were located on exon 2 which had the highest mutation rate. By matching the positions of mutations with *TRPC6* protein topology this issue can be inferred that the majority of mutations in *TRPC6* are occurred in intracellular N- and C-terminal tails.[13] In fact, the exons which encodes these two parts of *TRPC6* protein (mostly exons 2 and 13) can be considered as hot spots and used in genetic screening of patients with FSGS. N-terminal tail of *TRPC6* have 4 ankyrin domains which have role in protein-protein interaction.[13,31] In our study, *D130V* and *G162R* which were predicted pathogenic by 5 in silico programs were located in ankyrin domain 1 and 2, respectively, and this finding is comparable with previous studies which showed that the majority of mutations in N-terminal are exist an ankyrin domains.[10,13,18]

Functional analysis revealed that *TRPC6* mutations can be classified into two groups.[30] The first group of mutations cause gain of function, which can be observed in G109S, N110H, P112Q, M132T, N143S, R175Q, H218L, A404V, Q889K, R895C and E897k.[7,9,10,13,17,18,30] These mutations cause increased intracellular calcium which can affect signaling pathway from the slit diaphragm to the cytoskeleton of podocytes and decrease the resistance of glomeruli to environmental changes.[13,32] Also, intracellular calcium increase can cause apoptosis and reduction of podocytes which are a terminal barrier against protein loss in the kidney.[6] The second group of mutations cause loss of function, which happened in N125S, L395A, G757D, L780P, and R895L.[30] These mutations cause reduction in channel activity.[30] It seems that mutations which cause loss of function are more prevalent in children with FSGS than gain of function.[30] Unfortunately we did not perform functional analysis in identified mutations in our study and we are not able to classify our mutations as gain of function or loss of function.

TRPC6 could be a target for treatment of SRNS and FSGS. Schlondroff et al. showed *TRPC6* mutation can activate calcineurin-NFAT (nuclear factor of activated T cells) pathway.[17,33] On the other hand, angiotensin II through calcineurin-NFAT pathway could increase the expression of *TRPC6*. Altogether, the mentioned pathways ultimately cause glomerulosclerosis and albuminuria.[13,34,35] Given this issue patients with *TRPC6* mutations can benefit from calcineurin inhibitors such as cyclosporine and also angiotensin converting enzyme inhibitors and angiotensin II receptor blockers.[13] In our study 3 of 4 patients with mutations in *TRPC6* had complete remission by cyclosporine; but as mentioned previously, the value of response to cyclosporine in different studies are varied. Therefore, for better evaluation of calcineurin inhibitors in patients with *TRPC6* variants clinical trial studies should be performed. Another target for reducing the expression of *TRPC6* on glomeruli and finally improvement the proteinuria is activating peroxisome proliferator–activated receptor g pathway.[36] Sildenafil is a phosphodiesterase 5A inhibitor which can activate

the peroxisome proliferator–activated receptor g pathway and through this way reduce the expression of *TRPC6* and finally decrease the proteinuria.[36] This issue was shown in vitro and in vivo but the usefulness of this method should be evaluated in patients.[36]

Our study had some limitations; there was no control group in our study for evaluating the existence of identified *TRPC6* variants and confirming them as a mutation which cause disease. We did not analyze the samples of patients' parents for segregation and also evaluation for existing the mutations. In this study, we did not assess the all exons of *TRPC6* which could cause identification more variants of *TRPC6* in our population. We did not perform functional evaluation on identified mutations for assessing the effect of them on *TRPC6* protein.

CONCLUSIONS

In this study we evaluated exons 2 and 13 of *TRPC6* gene in Iranian children with FSGS. We identified four novel *TRPC6* variants among which two variants (D130V, G162R) by in silico analyzing predicted as pathogenic. *TRPC6* can be useful for genetic screening in Iranian children with FSGS.

CONFLICT OF INTEREST

None declared.

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Correspondence to:
Amin Abedini, MD
Isfahan Kidney Diseases Research Center, Isfahan University of Medical Sciences, Hezar Jerib Street, Isfahan, Postcode: 81746-73695, Iran.
Tel: +98 913 289 0262
Fax: +98 314 261 3636
E-mail: amin69.med@gmail.com

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