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ORIGINAL PAPER

Mutation analysis of *SLC3A1* and *SLC7A9* genes in patients with cystinuria

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Abstract Cystinuria is an autosomal inherited disorder of renal reabsorption of cystine, arginine, lysine, and ornithine. Increased urinary excretion of cystine results in the formation of kidney stones. Considering the few studies on the genetic basis of the cystinuria in the Middle East and the population-specific distribution of mutations in the *SLC3A1* and *SLC7A9* genes, in the present study, mutation analysis of these two genes was performed in a cohort of Iranian patients with cystinuria. Thirty unrelated cystinuria patients were analyzed for four of the most common mutations using ARMS-PCR (M467T, T216M) and RFLP-PCR (G105R, R333W) methods. For negative sample, two exons of both genes, which harbor many mutations, were subject to DNA sequencing. Eight variants were identified including missense, polymorphism, intron variant, and a novel variant. The most frequent mutations were not detected in our patients and only G105R was found. Since the molecular genetic testing results may influence the therapy and prognosis of cystinuria, this paper contributes to understanding of the molecular basis of cystinuria in the Iranian patients.

Keywords Mutation · *SLC3A1* · *SLC7A9* · Cystinuria · Iran

Introduction

Cystinuria (MIM# 220100) is an autosomal inherited disorder characterized by hyperexcretion of cystine, arginine, lysine, and ornithine into urine [1, 2]. The disease is caused by the impaired transport of these amino acids in the proximal renal tubule and gastrointestinal tract [3, 4]. Cystine

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is of low solubility in the normal urine pH (pH >7) which can lead to the formation of kidney stones [5] and can have pathological consequences [6].

The worldwide prevalence of this disease is 1/7000, but significantly differs among different populations and ranges from 1:2500 persons in Libyan Jews to 1:100,000 in Sweden [6, 7]. Iran is located in the stone belt area with the prevalence of stones reaching 2–3 % [8]. Males usually tend to have a more severe phenotype in comparison to females [5], but the incidence is rather equal between males and females [7].

So far, two genes have been identified to cause cystinuria *SLC3A1* and *SLC7A9*. *SLC3A1* (*CSNU1*, MIM#104614) is located on chromosome 2 (2p16.3) and encodes the heavy subunit rBAT of the renal $b^{0,+}$ transporter [9]. The *SLC7A9* (*CSNU3*, MIM# 604144), which encodes the light subunit $b^{0,+}$AT, is located on chromosome 19 (19q13.1) [1, 10]. The $b^{0,+}$AT protein represents the catalytic subunit of the transporter complex, whereas the rBAT is mainly involved in the trafficking and possible stabilization of the transporter in the brush border membrane [11]. In addition, the rBAT protein modulates the activity of $b^{0,+}$AT protein [7].

Two subtypes of cystinuria including type I and non-type I have been identified based on the amount of urinary cystine excreted and the pattern of inheritance. Whereas, Type I is transmitted as an autosomal recessive disorder, and heterozygotes show a normal aminoaciduria, non-type I heterozygotes exhibit moderate or high excretion of cystine and dibasic amino acids in urine. Therefore, non-type I cystinuria has an autosomal dominant inheritance with incomplete penetrance [12]. In 2002, a new classification based on the type of mutations was proposed by the International Cystinuria Consortium (ICC) which includes type A, type B, and type AB [5, 13]. Type A of cystinuria is caused by mutations in the *SLC3A1* gene (genotype AA). Whereas, type B includes *SLC7A9* mutations (genotype BB), and type AB is the result of one mutation in *SLC3A1* and one mutation in *SLC7A9* each of which is inherited from one parent [3, 7]. More than 100 mutations have been identified for the *SLC3A1* gene [14] and nearly 100 mutations for the *SLC7A9* gene [2, 12].

We sequenced exons 6 and 3 of the *SLC3A1* and *SLC7A9* genes, respectively, which harbor many mutations [15]. Also, the patients were studied for the four of the most common mutations which have been reported previously including M467T, T216M, G105R, R333W [11–13, 15].

In view of the facts that there are limited number of studies on the genetic basis of the cystinuria in the Middle East and that each population may have a specific distribution of mutations in the responsible genes for this disease, the present study was launched which presents the results of the first comprehensive mutation analysis on patients with cystinuria in Iran.

Materials and methods

Subjects

We analyzed 30 unrelated cystinuria patients including 14 women and 16 men. These patients were selected according to the type of stones (cystine stones) present in the patients who had been subjected to operation to remove kidney stones in Alzahra Hospital, Isfahan University of Medical Sciences. From 1500 patients with staghorn stones, 54 patients from 30 families had cystine stones. All the patients had a history of recurrent cystine stones and elevated levels of urine. Informed consent was obtained from all the patients. About 10 mL of peripheral blood was taken from the patients. Genomic DNA was extracted using standard procedures according to the manufacturer's instructions (Bio genet kit, Korea).

This study was approved by the Ethics Committee of the Medical University of Isfahan according to the National Health and Medical Research Council guidelines (Permission No. 391433). In this study we used three methods that included PCR–RFLP Analysis, PCR-ARMS, and PCR/Sequence Analysis.

Molecular study

PCR/sequence analysis

The exons 6 and 3 of the *SLC3A1* and *SLC7A9* genes, respectively were analyzed using polymerase chain reaction (PCR). The coding sequence of exons, intron–exon boundaries and some intron sequence (on average 50 bp on each of exons) were amplified by intron-derived primers. PCR was carried out on 25 μL containing 150 ng of genomic DNA and 2.5 μL of 10× PCR buffer, 1 U of Taq DNA polymerase, 200 μmol/L of dNTPs and 400 nmol/L of primer forward and reverse, and 0.75 μmol/L of $Mgcl_2$. The temperature profile for the 35-cycle amplification reaction was as follows: initial denaturation at 94 °C for 4 min, followed by 35 cycles at 94 °C for 20 s, annealing at 55.5 (exon 3), 62.5 °C (exon 6) for 30, extension at 72 °C for 40 s, and final extension at 72 °C for 5 min. The PCR products were sequenced using the Applied Biosystems® 3730/Genetic Analyzer and using BigDye terminator kit.

PCR–RFLP analysis

We used PCR-based restriction fragment length polymorphism (RFLP) assays for the known mutations G105R (exon 4) and R333W (exon10) in *SLC7A9* using the restriction enzymes *Apa*I and *Ncil (Bcnl),* respectively [12]. The mentioned mutations abolish restriction sites. Genomic DNA was amplified by appropriate primers and subsequently PCR products were digested with restriction

enzymes. PCR of G105R (20 s at 94 °C, 30 s at 57 °C, 30 s at 72 °C) and R333W (20 s at 94 °C, 30 s at 57 °C, 30 s at 72 °C) was carried out with a protocol consisting of initial denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 57 °C for 30 s, and extension at 72 °C for 30 s, followed by a final extension at 72 °C for 5 min. Validity of PCR–RFLP method was confirmed by direct DNA sequencing of some randomly selected PCR products.

PCR-ARMS

We detected point mutations T216M (exon 3) and M467T (exon 8) in the *SLC3A1* gene using the ARMS assay. For each mutation, three primers were used: one forward primer (common to both amplifications) and two reverse primers (mutation specific and normal). Each mutant reverse primer has two mismatches among the first seven 3′ end nucleotides. PCR was carried out for 38 cycles for M467T (20 s at 94 °C, 30 s at 55 °C, 30 s at 72 °C) and T216M (20 s at 94 °C, 30 s at 57 °C, 40 s at 72 °C), with initial denaturation at 94 °C for 4 min and final extension at 72 °C for 10 min. To confirm the PCR-ARMS results, some of the randomly selected PCR products were sent for direct sequencing.

All primers were designed with reference to the genomic sequences (Ensemble) (Table 1). All PCR products were separated on 1 or 2 % agarose gels, according to the size of the product.

Results

Two variations to the reference sequence were identified in the *SLC3A1* including the polymorphism c.1136 + 3delT and the pathogenic mutation R362C. Six other sequence variants were also detected in the *SLC7A9* gene including polymorphisms c.235 + 22T>G, p.C137C, c.478 + 10T>C, and missense mutations p.G105R, p.V142A and finally one novel variant c.177G>A (Table 2).

Table 1 Sequence of primers for amplifications

Gene	Exon	Forward primer	Reverse primer	Method
SLC3A1	3	ATGAGGTTTGAGAGAAGCAC	RN:GAAACCAAATATGTTTATCACTCG RM:GAAACCAAATATGTTTATCACTCA RS:ATCTGCCTTTTACCCCTTTG	ARMS Sequence
	8	ACCCTTTTCTTGCTCATCAG	RN:AGGGAGTGTGAAAAGAAGCA RM: AGGGAGTGTGAAAAGATGCG RS: ATAAGCTCTCAGACCACCAA	ARMS Sequence
	6	TATAGAGCGAGCTGTGGGCA	TGCCTTGGCCTCCTACAGTG	Sequence
HBB	In1	ACAATTTCTGCCAATCAGGA	AGACAGTTTTCAAGACCCTG	ARMS
SLC7A9	3	TACCGAGGGAGGGTGGC	AAGAGGGATACTGGAGGGT	Sequence
	4	CCCTTCCTCTCTGTGTTCCAG	GTCTTTTCTGACCCCTGCCC	RFLP
	10	TCTCAGTGCGTTTAACCTCCTC	GCATCTGGGTCATTTGGAAGC	RFLP

RN normal reverse primer, *RM* mutant reverse primer, *RS* reverse primer for sequencing the fragment containing point mutation

Table 2 Description of mutations identified in the *SLC3A1* and the *SLC7A9* genes and their frequencies

Gene	Ex/Int	Nucleotide change	Amino acid change	Type of mutation	Allele frequency
SLC3A1	In 6	c.1136 + 2/3delT	–	Intron variant	8 of 60
	Ex 6	c.1084C/T	R362C	Missense	4 of 60
	Ex 8	c.1400T/C	M467T	Missense	0 of 60
	Ex 3	c.647C/T	T216M	Missense	0 of 60
SLC7A9	Ex 3	c.177G/A	–	Polymorphism/mutation?	1 of 60
	In 3	235 + 22T/G	–	Intron variant	9 of 60
	Ex 4	c.425T/C	V142A	Missense	–
	Ex 4	c.498G/A	G105R	Missense	2 of 60
	In 4	c.478 + 10T/C		Intron variant	–
	Ex 4	c.411T/C	C137C	Polymorphism	–
	Ex 10	c.1182C/T	R333W	Missense	0 of 60

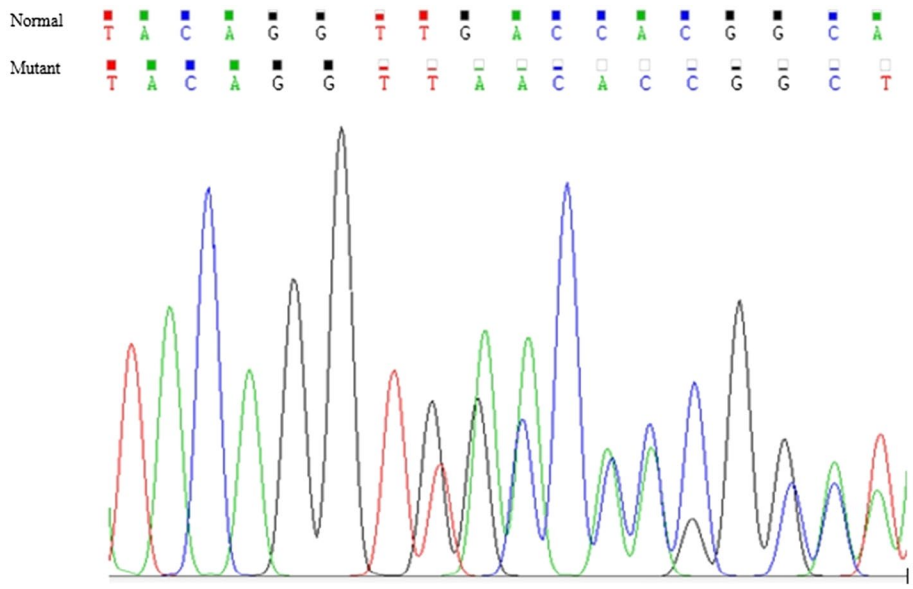

Fig. 1 Electrophoretogram of a heterozygous patient for intron variant c.1136 + 2/3delT in *SLC3A1* gene

SLC3A1 mutations

Two patients were found to be heterozygous for the polymorphism c.1136 + 2/3delT (C4 and C27). In addition, three patients (C2, C7, and C15) were homozygous for this polymorphism (Fig. 1). Also the mutation R362C was identified in two patients in homozygosity (C9 and C16).

SLC7A9 mutations

A novel nucleotide substitution c.177G>A in exon 3, which is a silent substitution, was found in heterozygosity in one patient (C7). Among previously reported mutations in the *SLC7A9* gene, we found p.V142A and p.G105R in our patients (Fig. 2). The amino acid alteration p.V142A (c.425T>C) was found in two patients (C4 and C20). One patient was heterozygous and another one was homozygous for this mutation. In our study, G105R was detected in two heterozygotes (C6, C20) (Fig. 2). We found three polymorphisms including p.C137C, c.478 + 10T>C, and c.235 + 22T>G in our patients which were previously reported in the *SLC7A9* gene. The polymorphisms c.478 + 10T>C and p.C137C were identified in two patients. One patient (C20) was homozygous and another (C4) was heterozygous. Moreover, we detected the third polymorphism c.235 + 22T>G in seven patients, five of whom were heterozygous and two were homozygous for this intron variant. M467T, T216M, and R333W mutations were not detected in our patients (Fig. 3), but G105R was found. Altogether, we identified 33 genetic changes in 30 affected patients including four polymorphisms, three missense mutations, and a novel variant. In total, twelve out

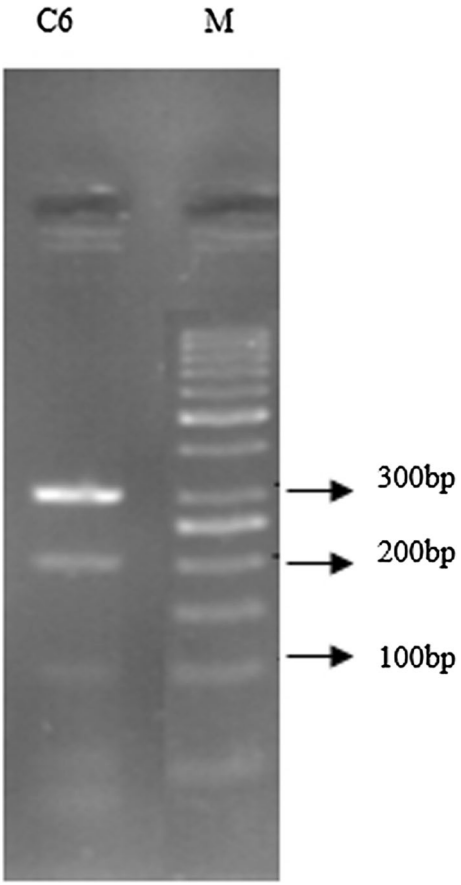

Fig. 2 RFLP analysis for G105R mutation in a heterozygous patient (*M* marker 50 bp). The PCR product was incubated at 37 °C overnight. The enzyme-digested products were electrophoresed in 2 % agarose gel. In the absence of G105R cleaves the PCR product into two fragments of 100 and 200 bp

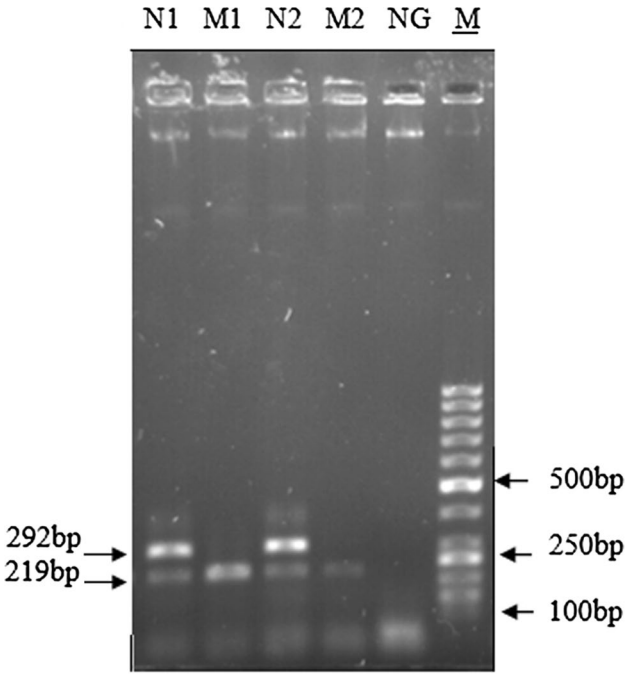

Fig. 3 Gel electrophoresis of ARMS products two wild-type individuals for M467T. (*N* normal primer, *M* mutated primer, *NG* negative control, *M* marker 50 bp, *lanes 1* and *2* are related to C1 and C2 patients). Internal control size: 219 bp, main band size: 292 bp

of thirty patients had one or more changes. One patient (C7) was heterozygous for c.177G>A and homozygous for c.1136 + 3delT changes. Other patient (C4) was heterozygous for c.235 + 22T>G, p.C137C, c.478 + 10T>C, V142A (*SLC7A9*), and c.1136 + 3delT (*SLC3A1*) changes. Another patient (C6) was heterozygous for c.235 + 22T>G and G105R (*SLC7A9*). One patient (C20) was heterozygous for G105R and homozygous for c.235 + 22T>G, p.C137C, c.478 + 10T>C, V142A (*SLC7A9*). A patient (C15) was homozygous for c.1136 + 3delT (*SLC3A1*) and heterozygous for c.235 + 22T>G (*SLC7A9*). The remaining seven patients had one mutation in the *SLC3A1* gene or in the *SLC7A9* gene. Two patients (C9, C16) were homozygous for R362C (*SLC3A1*). One patient (C2) had one allele of c.1136 + 3delT and another (C27) was heterozygous for this mutation. A patient (C12) had two alleles of c.235 + 22T>G and two patients were heterozygous (C8, C24) for c.235 + 22T>G. No mutation was detected in the remaining 18 patient in these exons.

Discussion

Cystinuria is an inherited disorder due to defective renal reabsorption of cystine and dibasic amino acids. In our study, 30 unrelated cystinuric patients were selected. We found cystinuria in 54 patients from 1500 patients with staghorn calculi (3.6 %). Kheradpir et al. studied 148 Iranian children with staghorn calculi and observed cystinuria in six patients (4 %) [16]. In addition to the sequencing of exon 3 (*SLC7A9*) and exon 6 (*SLC3A1*), we also studied the most common mutations including M467T, T216M (*SLC3A1*) and G105R, R333W (*SLC7A9*) in our cystinuric patients. We found eight genomic variants, two of which in *SLC3A1* and the other six in the *SLC7A9* gene, including a new variant, four polymorphisms, and three missense mutations.

A novel variant c.177 G>A with substitution of G by A at position c.177 in axon 3 (*SLC7A9)* was found which changes ACA codon to ACG codon of threonine amino acid [17]. This substitution occurs in the extracellular domain of the light subunit $b^{0,+}$AT. This residue is highly conserved among other species. Variant Effect Predictor (Ensemble) was performed to predict the functional effects of this variant which predicted it to have a neutral effect on protein. The annotation included predictions by SIFT and PolyPhen.

The missense mutation G105R has been mentioned as the most frequent mutation in the *SLC7A9* gene in cystinuric patients in different studies [10, 12, 13, 18]. Worldwide, G105R has been reported as the most frequent mutation in the *SLC7A9* gene [15]. The worldwide frequency of this mutation in white patients is 21 %. It is located just after helix IL1 loop [4]. According to the data of the ICC, there is a severe increase of cosine excretion in heterozygous carriers of the variant G105R [12]. Although G105R change affects a highly conserved amino acid residue, a small percentage of the heterozygotes carrying this mutation exhibits elevated levels of urinary cystine and dibasic amino acids [12]. The levels of G105R $b^{0,+}$AT protein produced in HeLa cells were ~10 % of wild-type $b^{0,+}$AT [12]. This mutation was reported in European cystinuria patients with ethnic origins of German [13, 18], Italian [18], Macedonia, Serbia [3], Czech [1], Greek [19], Spanish [15], former Yugoslav, and Turkish patients [11, 13] but it was not found in China [2], USA [10], and japan [20].

Polymorphism C137C was detected in our study which was previously reported in cystinuria patients from Greece [19], Portugal [21], and Sweden [22]. C137C (also reported as c.411T>C) is apparently a silent mutation. However, it has been noted that many apparently silent mutations could have significant deleterious consequences as the result of regulatory sequence disruption or defective splicing [21].

Another missense mutation in *SLC7A9* was V142A (axon 4) which is located in the EL2 domain of $b^{0,+}$AT protein [20]. This mutation has been found in normal subjects without the cystinuria phenotype and therefore, there are conflicting views on the pathogenicity of this variant [21]. V142A has been identified in Japanese [20], Portugal [21], Swedish [22], Greek [19, 23], Canada, and German

patients [13]. R362C, a known missense mutation in exon 6 of *SLC3A1*, has been reported in previous researches, for example in Italy [24] and Germany [18].

Also two known intron variants including c.235 + 22T>G in intron 3 and c.478 + 10T>C in intron 4 of *SLC7A9* were identified in our patients. The variant c.235 + 22T>G has been found in the studies on cystinuria patients in Germany [25], Portugal [21], and Greece [23, 26]. The variant c.478 + 10T>C has been reported in Greece [26] and Portugal [21]. Moreover, we found that the polymorphism c.1136 + 3delT that occurs in intron 6 of the *SLC3A1* gene has been previously detected in some populations including the Swedish [27], Chinese [2], and Italians [28]. This deletion is an intronic variant located at the splicing junction site. Computer model analysis revealed that this mutation could increase the strength of the splicing [28].

Chatzikyriakidou et al. concluded that the polymorphic background of the *SLC7A9* gene, including intronic variants 235 + 22T>G and 478 + 10T>C, probably affect the manifestation of cystinuria in carriers of *SLC7A9* mutations [26].

A study showed significant differences between patients and controls for IVS3 + 22T>G and V142A [23]. According to the literature, some SNPs may have a role in the pathogenesis of the disease especially in *SLC7A9*. These SNPs could explain the occurrence of the disease in patients that have no mutations in *SLC3Al* and *SLC7A9* and interpret the marked differences between siblings with the same mutations [23].

Exon 3 (*SLC7A9*) and exon 6 (*SLC3A1*) were sequenced. We also focused on the most common mutations reported so far including M467T, T216M, G105R, R333W in our cystinuric patients. R333W, as one of the most frequent mutations [4, 12, 15] is located in exon 10 of the *SLC7A9* gene. The worldwide frequency of this mutation is 6 % in Caucasians and Japanese patients [4]. R333W was observed in the German [11, 13, 25], Czech [1], Greek [19, 29], Japanese [20], Italian [11], and Spanish [4] populations. The residue R333W is conserved for all the human members of the heterodimeric amino acid transporter family [20]. R333W was not detected in our study.

T216M was originally reported in an Italian Gypsy patient [24]. Also, it has been reported as a frequent mutation in Greece [11, 29], Turkey [11], and Germany [13]. This mutation has been detected in patients from Yugoslavia [11, 15], Turkey [18], the Czech Republic [1], Poland [30], and Spain [15]. T216M was not found in our patients in this study.

M467T, as the most common mutation in the *SLC3A1* gene, was first described in Spanish patients [9]. In addition, M467T in *SLC3A1* has been described as the most important mutation in German [11, 13, 18], Swedish [27], Spanish-Italian [24, 28], Czech [1], Turkish [6], Spanish [28], and American patients [31]. This substitution contributed to the cystinuria phenotype in French, German [18], Italian and Turkish [6], Greek [29], and American [32] patients. In contrast, M467T was not found in this study as well as in Japanese patients [14]. Since no previous studies have been conducted on this issue in the Middle East, we cannot compare our results in this region.

In conclusion, the most frequent mutations in Europe (M467T, T216M, and R333W) were not found in the studied Iranian population. The frequent mutation G105R occurred only in two alleles. In addition, this study describes one novel variant in the *SLC7A9* gene (c.177G>A) and expands the spectrum of cystinuria genetic variations. In view of the clinical practice, the molecular genetic testing results might influence the therapy and prognosis of cystinuria [13]. This research contributes to our understanding of the molecular basis of cystinuria in Iranian patients.

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Conflict of interest The authors declare no conflict of interest.

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