ORIGINAL ARTICLE

Novel and heteroplasmic mutations in mitochondrial tRNA genes in Brugada syndrome

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Abstract

Background: *Brugada syndrome (BrS) is a rare cardiac arrhythmia characterized by sudden death associated with electrocardiogram patterns characterized by incomplete right bundle-branch block and ST-segment elevations in the anterior precordial leads. This syndrome predominantly is seen in younger males with structurally normal hearts. Mitochondrial variants particularly mt-tRNA mutations, are hot spots that lead to cardiological disorders. Previous studies have shown that mutations in mitochondrial tRNA genes play an important causal or modifying role in BrS. The present study aims to evaluate the involvement of mitochondrial tRNA genes in arrhythmogenic BrS.*

Methods: *In this study, 40 Iranian patients were investigated for the presence of the mutations in 6 mitochondrial tRNA genes (tRNA Ile, Met, Gln, Asn, Ala and Trp) by PCR-SSCP analysis.*

Results: *There were 4 mutations in tRNA genes, that for first time, were found in BrS patients and these mutations were not in controls. Three of them were heteroplasmic and located in tRNA^{Gln} (T4377A) and tRNA^{Met} (G4407A and C4456T) which were assessed as pathogenic mutations. A homoplasmic variant (5580T > C) in tRNA^{Trp} gene was located within the junction region between tRNA^{Trp} and tRNA^{Ala} genes. This mutation may disturb the processing of mt-tRNA^{Trp}.*

Conclusions: *The results of this study suggest that mutations in mitochondrial tRNA genes might lead to deficiencies in translational process of critical proteins of the respiratory chain and potentially lead to BrS in Iranian subjects.* (Cardiol J 2018; 25, 1: 113–119)

Key words: cardiac arrhythmia, Brugada syndrome, mitochondrial tRNAs, variations

Introduction

Brugada syndrome (BrS) (OMIM 601144) is inherited in an autosomal dominant manner and is characterized by a variable ST segment elevation in the right precordial electrocardiogram (ECG) leads and has a high incidence of ventricular arrhythmias and sudden death in patients with structurally normal hearts [1]. Interestingly, ST segment elevation may disappear after intravenous isoprenaline or exercise [2]. A definitive diagnosis can be made when a type of ST segment elevation is observed in more than 1 right precordial lead (V1 to V3) in the presence or absence of a sodium channel-blocking agent, and in conjunction with one of the following: documented ventricular fibrillation, polymorphic ventricular tachycardia, a family history of sudden cardiac death at less than 45 years of age, coved-type ECGs in family members, syncope, or abnormal nocturnal respiration [3, 4].

The ECG pattern of BrS may be present at imaging or may become apparent only under special conditions (such as vagal stimulation, fever or exposure to a sodium channel blocking drug) [5, 6]. The clinical diagnosis of BrS is determined when

it is associated with a previous history of syncope or sudden cardiac death [7].

This syndrome typically manifests during adulthood, with a mean age at first arrhythmic event ranging between 22 and 65 years, but also occurs in infants and children [8]. Over 90% of these cases had been in male patients. Symptoms of BrS occur mostly at night and seem to be most prevalent in Southeast Asia and Japan [9]. Screening of some families with the Brugada phenotype has revealed distinct mutations in the SCN5A gene, which encodes the pore-forming alpha-subunit of the cardiac sodium channel [10]. In patients with this syndrome, researchers found missense mutations in SCN5A and assessed the functional significance of these mutations on cardiac sodium channel characteristics [11]. Alterations seemed to be associated with an increase in inward sodium current during the action potential upstroke [12].

At the present time, it is known that an increasing number of human diseases is found to be associated with point mutations in mitochondrial tRNA genes [13]. The heart is highly dependent on oxidative energy generated in mitochondria and this suggests that defects in ion channels which are adenosine triphosphate (ATP)-sensitive, are responsible for channelopathy diseases such as heart arrhythmia [14].

Also, it is distinguished that tRNA^{Met}, tRNA^{Ile}, tRNA^{Trp} and tRNA^{Gln} genes were hot spots for cardiovascular diseases [15]. Yet, most of studies focused on nucleic genes and family-based screening in arrhythmia, and knowledge of mitochondrial tRNA genes and population-based Iranian patients with arrhythmia especially BrS, was limited. Thus, we have paid special attention to determine the relationship between six mitochondrial tRNA genes and BrS. We performed a methodical screening of mitochondrial genes in two regions, including tRNA^{Ile}, tRNA^{Met}, tRNA^{Gln}, tRNA^{Asn}, tRNA^{Ala} and tRNA^{Trp}.

Methods

Study samples

The study was performed on 40 Iranian patients diagnosed by specialists from the center of Arrhythmia in Tehran, Iran. Diagnosis of type 1 BrS was based on the presence of a prominent coved ST-segment elevation ≥ 2 mm or 0.2 mV at its peak followed by a negative T wave. Also, clinical diagnostic included an electrophysiology test to assess of ventricular arrhythmias measurement and conduction times [16]. Table 1 shows the main characteristics of the patient population.

Table 1. Characteristics of 40 patients with Brugada syndrome (BrS) included in the study.

Age range at diagnosis (mean age)	12–63 years (31.4 ± 5.6)
Men/women	25/15
Age range in men	17–63 years
Age range in women	12–59 years
Family history of BrS	8 (20%)
Family history of sudden death (≤ 45 years)	15 (37%)
Syncope	4 (10%)

Also, 45 unrelated healthy individuals (30 males and 15 females) who were matched with the patients regarding sex, age (mean age: 29.8 ± 4.5) and ethnicity and had no family history of arrhythmia were enrolled into the control group. They exhibited normal ECGs and no signs of myocardial disorders during exercise testing; these examinations were performed as part of a clinical checkup in the absence of cardiovascular symptoms. Recruitment of the patients and laboratory protocols in this study were approved by the Committees on the Ethics of Human Research of Yazd University and written informed consent was obtained from each participant.

Mutational analysis of mitochondrial genome

Genomic DNA from all participants was isolated, including controls, from peripheral blood samples, using DNA isolation kit (Qiagene Co, Tehran, Iran). By using two pairs of specific primers designed from the genomic sequence of the mitochondrial MT-tRNA genes (4211-4521 and 5461--5721), the two coding segments (320 and 279 bp, respectively) were amplified by polymerase chain reaction (PCR) (Table 2). These two segments have the entire sequence of the six transfer RNA genes (tRNA Ile, Met, Gln, Asn, Ala and Trp).

The primers were designed by primer design software (Primer Premier 5.0; Premier Bio soft Inc., Canada) and their secondary structure was examined using Gene Runner, version 3.05 (Hastings Software Inc. Hastings, NY, USA, http://www.generunner.com).

Optimized PCR conditions in a total volume of 25 μL mixture containing 1 × PCR buffer, 25 ng genomic DNA, 10 pmol/each forward and reverse primers, 1.5 mmol/L MgCl$_2$, 0.2 mmol/L in each dNTPs, and 1 U Taq DNA polymerase were performed (Cat No: YT1591, Yekta Tajhiz Azma Co,

Table 2. Designed primers and fragment length of each segment in polymerase chain reaction.

Primer sequence (5′-3′)	Nucleotide position	Tm (°C)	Size (bp)	Fragments
F- TATGATATATATGTCTCCATACCC	4211-4230	54	320	tRNA^{Ile-Gln-Met}
R- GAGCTTAGCGCTGTGTGATGAG	4531-4521			
F- CCCTTACCACGCTACTCCTA	5461-5480	54	279	tRNA^{Asn-Ala-Trp}
R- TTCAATCTACTTCTCCCGCC	5740-5721			

Tehran, Iran). The DNA amplification program included one cycle of an initial denaturation step at 94°C for 5 min followed by 35 cycles at 95°C for 35 s, 57°C for 45 s, 72°C for 50 s, and a final extension at 72°C for 10 min. The amplified products were then analyzed for size on 1.5% agarose gel.

Possible mutations were tested using single-strand conformation polymorphism (SSCP) analysis. For PCR-SSCP assay, 5 μL of PCR products were mixed with 10 μL SSCP loading solution (80% formamide/0.25% xylene cyanol FF/40% sucrose). DNA was made single stranded by heating at 94°C for 10 min, chilled immediately on ice for 2 min, and loaded onto a polyacrylamide/TBE 0.5 × gel. The gel concentrations and running conditions were as follows: 8% non-denaturing polyacrylamide gel (Sigma, Germany), 16 h, room temperature, 5 mA. After the run, the gel was removed from the apparatus and the DNA bands were visualized through silver staining and abnormal conformers got sequenced from a commercial agency (Macrogene Seoul, South Korea). The results of DNA sequencing were compared with the Cambridge consensus sequence.

Software and databases

In this study, online multiple sequence alignment was performed using Molecular Evolutionary Genetics Analysis (MEGA6) software (https://www.ncbi.nlm.nih.gov/pmc/) and Blast analysis were used to determine the homology of the sequences obtained in the study and all other sequences of other species. This program is available at the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/Blast).

The study of pathogenicity of the whole mtDNA was accomplished by the human mitochondrial genome database (Mitomap) which is briefness of polymorphisms and mutations in human mitochondrial DNA (http://www.mitomap.org/MITOMAP). Mamit-tRNA (http://mamit-trna.u-strasbg.fr) was also used for detection of the structural features of mammalian mitochondrial tRNAs and is a helpful tool in the frame of human diseases linked to point mutations

in mitochondrial tRNA genes. Finally, mitotRNAdb and RNAfold web server which are two database of mitochondrial tRNA genes (http://mttrna.bioinf.uni-leipzig.de/mtDataOutput/) and secondary structures of single stranded RNA or DNA sequences (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi), were used in order to identify the graphical representations of tRNA secondary structures.

Statistical analysis

The Fisher exact test was used to determine the association between mitochondrial tRNA mutations and the genetic risk of arrhythmia. The quantitative measures in groups were presented as mean ± standard deviation. The statistical analyses were performed using the Graph Pad Prism software (Graph Pad Software, Inc. USA) and p value less than 0.05 was considered to be statistically significant.

Results

In a total of 40 unrelated Iranian patients and 45 healthy subjects, PCR-SSCP analysis revealed aberrant conformers corresponding to 4 mutations in 3 transfer RNA genes (Table 3, Fig. 1). Results show that one heteroplasmic mutation was located in tRNA^{Gln} (T4377A) and according with MITOMAP database (Reported Mitochondrial DNA Base Substitution Diseases: rRNA/tRNA mutations) was, for first time, reported in these BrS patients. T4377A mutation was found in a 35-year-old man with a previous history of syncope and occurred at D-stem of tRNA and exactly in a position that was highly evolutionarily conserved nucleotide of the corresponding tRNA (Fig. 2). This mutation was absent in unrelated controls.

Also, two heteroplasmic mutations in tRNA^{met} were observed: G4407A in a 40-year-old man with previous syncope that was reported, for first time, in arrhythmia and C4456T in 2 patients with syncope experienced in adolescence. Previous research showed that variants in this region may lead to impairment in tRNA 3′ end metabolism and deficiency

Table 3. Characteristics of four mutations in Brugada syndrome patients by sequence analysis.

Gene	NT change	Hetero/Homo	Family history of sudden death	Carrier, age/sex
tRNA-Gln	T4377A	Hetero	Yes	45/male
tRNA-Met	G4407A	Hetero	Yes	55/male
	C4456T	Hetero	Yes	30/male
			No	20/female
tRNA-Ala	T5580C	Homo	Yes	43/male
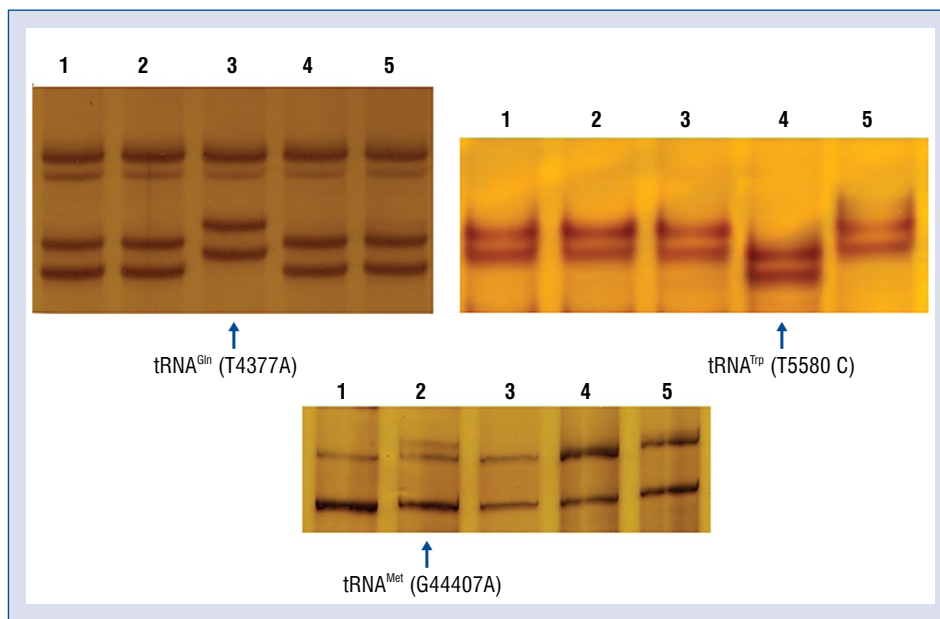
Figure 1. The polymerase chain reaction single-strand conformation polymorphism (PCR-SSCP) gel electrophoresis results show two heteroplasmic mutation in tRNA^{Gln} (T4377A) and tRNA^{Met} (G4407A) genes and a homoplasmic variant (5580T>C) in tRNA^{Trp} gene. The arrows indicate abnormal conformers on the gel.
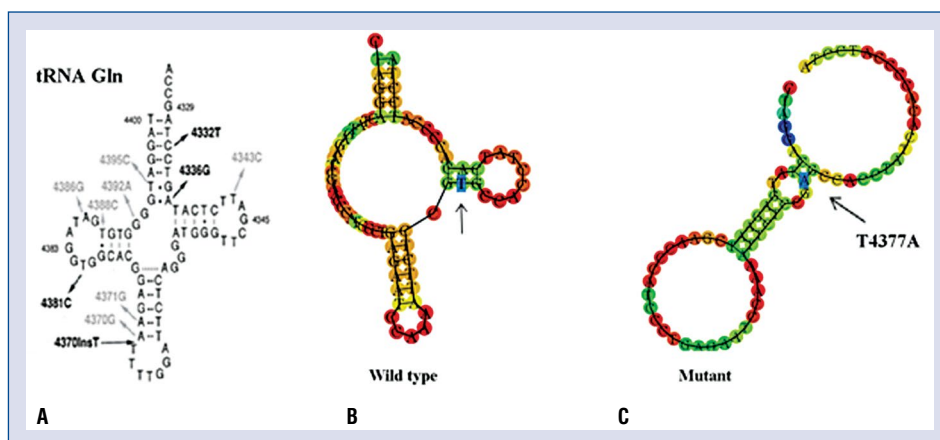
Figure 2. The location of T4377A in tRNA^{Gln}; **A.** Cloverleaf structure of human mt-tRNA^{Gln} that is a heavy tRNA and transcribed from the light DNA strand and thus has a high G content; **B, C.** Predictions of the secondary structure of mt-tRNA^{Gln} with and without the mutation.
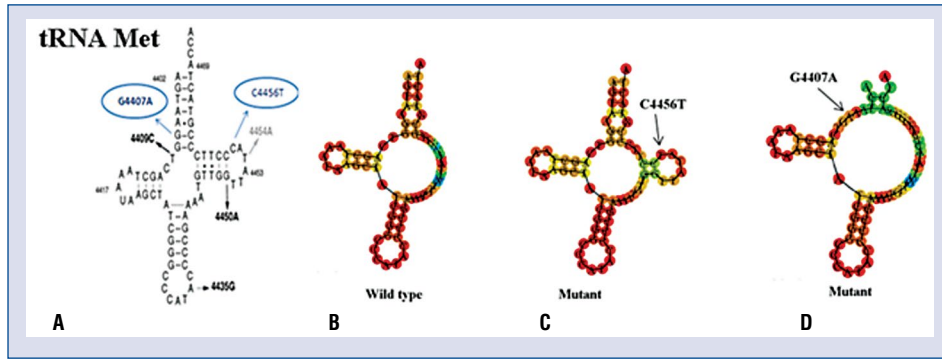
Figure 3. A schema of locations of C4456T and G4407A in tRNA[Met]; **A.** Cloverleaf structure of human mt tRNA[Met] that is a light tRNA and transcribed from the heavy DNA strand and have a low G content; **B–D.** Predictions of the secondary structure of mt-tRNA[Met] with and without the mutations.

of important subunits of the respiratory chain in hypertension diseases, because they reported that oxygen consumption rate in cells containing these mutations decreased in comparison to the average level of control cases [8, 15].

The results of Mamit-tRNA site also showed that the positions of G4407A and C4456T mutations were in acceptor stem and T-loop of tRNA[met] respectively (Fig. 3). Both mutations affected conserved nucleotides and were not found in any of the healthy controls.

To assay whether nucleotide variations affect the secondary structure of mt-tRNAs, secondary structures of normal type and mutant tRNAs were predicted using the RNA fold software. All of 3 above mutations in tRNA[Gln] and tRNA[Met] cause instability in the secondary structure of corresponding tRNAs, therefore these mutations may be involved in the pathogenesis of BrS.

The next mutation was a novel and homoplasmic T to C transition at the position 5580 mutation in a man with BrS but not in the healthy controls (Fig. 4). This variation locates at the junction of tRNA[Trp] at the heavy strand and tRNA[Ala] at the light strand. The 3' end of the normal flanking sequence of tRNA[Trp] gene is 5580 T/GTCTTT, whereas the 3' end of the mutant flanking sequence this gene is 5580 C/GTCTTT. This nucleotide change has not been previously reported in various arrhythmias but the effect of this variation on the phenotype could not be assessed.

Discussion

All 22 human mitochondrial tRNAs are encoded in the mtDNA [17]. The several diseases associated with mutations in these genes may
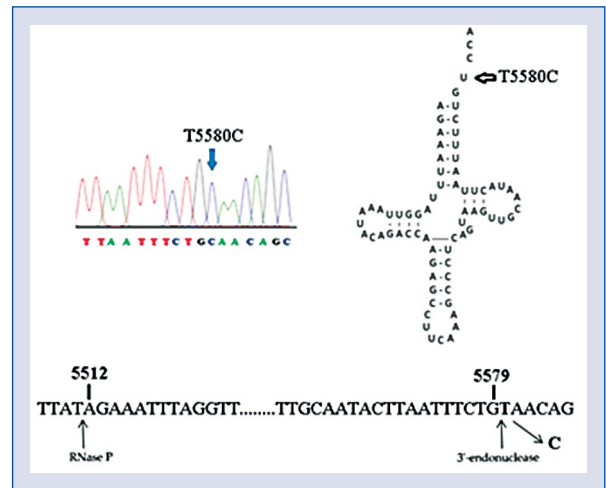

Figure 4. Identification of the T5580C mutation in 3' end immediately after last nucleotide (G) in mitochondrial tRNA[Trp] gene. Sequence chromatogram show homoplasmic T5580C mutation from an affected individual. In cloverleaf structure of tRNA[Trp], arrows indicate the location of the base change at position 3' end. Processing of the mt-tRNA[Trp] precursor has been shown by RNase P and 3'-endonuclease.

manifest from reduction in aminoacylation and tRNA stability [18], disability in the processing functional tRNA and decrease of interaction with mitochondrial ribosomes and elongation factor Tu [19]. More than 350 polymorphic and pathogenic mutations (MITOMAP) have been observed in human mitochondrial tRNA genes [20]. However, according to available research, few studies represent mutational analyses to evaluate the involvement of mtDNA in inherited arrhythmogenic diseases [21–23], and genetic studies on the mitochondrial

DNA of BrS patients are ongoing and have yet to be reported. Previous studies also showed accumulation of mitochondrial genome variations in patients affected by long QT syndrome (LQTS), an inherited channelopathy responsible for sudden cardiac death in young individuals. Moreover, ion channels are ATP sensitive and the heart is highly dependent on oxidative energy generated in the mitochondria, thus, it cannot be excluded that arrhythmia may be due to mitochondrial dysfunction. Recently, Stocchi et al. [23] performed association analysis between mtDNA alterations and 16 BrS patients. In their study, only one novel synonymous variant (C9600T) was detected in one patient. Also, they showed what appears to be an association between patients with the highest number of variants and four mt single nucleotide polymorphism (SNPs) (T4216C, A11251G, C15452A, T16126C) and the most severe BrS phenotype (p = 0.002). Because of the importance of mitochondrial genome mutations, especially tRNA genes, the present study investigates the link between mt-tRNA mutations and BrS in an Iranian population.

Protein biosynthetic systems normally, have two tRNA^{Met} classes. One is used specially for initiation, and the other recruits in polypeptide chain elongation. Mammalian mitochondria are actually unusual in that they have a single gene for tRNA^{Met}, which functions in both the initiation and elongation phase of polypeptide synthesis. Likely, it is the main reason for a high degree of conservation in this tRNA. Therefore, this tRNA^{Met} has critical significance in mitochondrial translation [24]. The D-loop in human tRNA^{Met} is slightly small and lacks the G nucleotides that in the tertiary structure facilitate interactions with the T-loop. The T-stem has two neighboring pyrimidine: pyrimidine pairs and T-loop contains only six nucleotides instead of the normal seven that suggest human mitochondrial tRNA^{Met} may have an innately weak tertiary structure [25]. In the present research, two interesting point mutations (G4407A and C4456T) occur in the tRNA^{Met} gene but methodical examinations of the structural and biochemical consequences of these mutations are missing. The novel G4407A mutation (Fig. 2A) results in a G6 to A change at the end of the acceptor stem of mtRNA^{Met}. Because this mutation leads to loss of the base pairing in acceptor stem, and RNA Fold results show that it causes changes in the structure of tRNA (Fig. 2D), therefore heteroplasmic and highly conserved G4407A mutation may cause pathogenicity through disruption and the formation of the acceptor stem

and reduces correct amino acid recognition. Also detected was a C-to-T base change at np 4456 in T-loop that causes instability in the secondary structure of tRNA (Fig. 2C). This change was revealed to be heteroplasmic in two patients, and has also been reported as a polymorphic change in MITOMAP. Previous functional analysis showed that mutant cell lines in this region of mtRNA^{Met}, had a significant reduction of oxygen consumption rate in patients with hypertension. Therefore, defecting to retain the equilibrium of oxygen consumption and production may cause cardiovascular diseases and is probably of particular importance in the pathogenesis of arrhythmia.

Also, in one patient, 4377 T>A base change was detected; this mutation had not been previously reported. This transition affected a highly conserved position in the tRNA^{Gln} gene (Fig. 3). The results of SSCP analysis also showed that this mutation was heteroplasmic and, consequently, it was considered for pathogenicity. It was determined that mismatches in this stem may lead to shortening of the D-stem and enlargement of the D-loop (Fig. 3C) [25]. The analysis of potential tertiary structures shows that only a few human mt tRNA species have the possibility of folding into a three dimensional structure through D- and T-loop interactions and that one of them is tRNA^{Gln} [24]. Since most tRNA families show high conservation within individual domains of the secondary structure, like D-stem, therefore the novel T4377A mutation can be introduced as a pathogenic alteration in BrS patients.

The homoplasmic 5580 T>C transition lies in the non-coding region immediately following the 3' end of the tRNA^{Trp}. Interestingly, the thymidine at the 5580 position of the mitochondrial genomes is highly conserved among various species. Actually, the processing of precursors in mt-tRNAs requires the accurate endonucleolytic cleavage at both 3' and 5' ends. At 3' end, excision of tRNA from primary transcript is catalyzed by 3' endonuclease, whereas at 5' end, extra nucleotides are removed by RNase P [19, 26]. Thus, it is expected that the T-to-C transition at position 5580 in the H-strand may lead to defective tRNA^{Trp} 3' end processing in the precursor transcript. It is known that the 5' and 3' end processing deficiency consequently of pathogenic mitochondrial tRNA mutations could associate with many disorders [27].

These finding would suggest that minor changes in base content or structure by these substitutions may result in impairment of translation and affect the respiratory chain which likely triggers cardiac diseases.

Conclusions

Four mitochondrial tRNA base pair substitutions were reported that could possibly have a significant role in the development of BrS. It appears that variability at several loci will not be sufficient to increase arrhythmia risk but these mutations are associated with the risk of mitochondrial dysfunction in BrS.

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Conflict of interest: None declared

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