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Association of angiotensin-converting enzyme and nitric oxide synthase genes polymorphisms with the risk of myocardial infarction in Bangladesh

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ABSTRACT

Myocardial infarction is one of the leading manifestations of illness and death worldwide. The aim of the study was to find out the associations of ACE (I/D) and NOS3 (G894T & 4b/a) genes polymorphisms with the occurrence of myocardial infarction (MI) in Bangladeshi population. The study was conducted on 100 cardiac patients experiencing MI and 150 healthy volunteers with no complications. The genotyping was done using PCR and PCR-RFLP methods and the biochemical parameters were measured using auto biochemistry analyzer. Over all, the serum Troponin I, AST and ALP levels were significantly ($p<0.01$, and 0.001, respectively) higher and the albumin level was significantly ($p<0.001$) lower among the patients. The percentage of DD genotypes of ACE gene was significantly ($p<0.05$) higher in patients. The individual with DD allele was at 3.28-fold increased risk (OR=3.28; 95% CI=1.6 to 6.7; $p < 0.01$) of experiencing MI while individual with ID genotype was at lower risk. In addition, the cigarette smokers with DD genotypes were found to have a 4.1-fold increased risk to develop cardiac disease (OR=4.1; 95% CI=1.5 to 11.2; $p<0.01$). The frequencies of NOS3 (G894T & 4a/b) genotypes in the patients and controls were almost similar. There were no significant differences among the biochemical parameters for different genotypes. Thus our recent study suggested that the ACE (I/D) gene may have strong associations with the occurrence of MI and DD genotype would be considered as a risk factor.

1. Introduction

Myocardial infarction (MI) is one of the major manifestations of coronary artery disease (CAD). CAD is considered to be one of the main causes of morbidity around the world (Gouvinhas et al., 2013). According to the World Health Organization (WHO, 2019), every year approximately 17.9 million people die of cardiovascular diseases worldwide that is 31% of all deaths and most of these occur in developing countries. CAD is a multifactorial disorder that is a consequence of an interaction between genetic background and environmental factors such as diet, smoking and physical activity (Smith et al., 1997; Prins et al., 2012). The genes conferring susceptibility to coronary artery disease are largely unknown. Atherosclerotic plaque formation, hypercholesterolemia, hypertension, and

diabetes are considered as major risk factors for CAD (Smith et al., 1997). Nitric oxide (NO), an important relaxation factor in the human body, plays a key role in the relaxation of vascular smooth muscle, inhibits adhesion of platelets and leukocytes to the endothelium, reduces vascular smooth muscle cells migration and proliferation, and limits the oxidation of atherogenic low-density lipoproteins (Schmidt and Walter, 1994). Thus intracellular NO has been considered as an inhibitor of atherosclerotic plaque formation and reduces the risk of CAD. NO is synthesized by the action of nitric oxide synthase (NOS) from L-arginine. There are at least three isoenzymes of NOS: Inducible NOS, neuronal NOS, and endothelial NOS (eNOS/NOS3) (Nathan and Xie, 1994).

The NOS3 gene is located on chromosome 7q35-36, consists of 26 exons with a total size of 21 kb, encodes for intracellular NO production (Marsden et al., 1993). This gene is expressionally and functionally regulated through multiple regulatory steps, and entails several polymorphisms, some of which have functional consequences (Hingorani et al., 1999). Study conducted by Hingorani et al. first described that exchange of guanine to thymine at nucleotide 1917 in exon 7, replaces glutamic acid to aspartic acid of NOS3 gene which has been associated with coronary spasm (Hingorani et al., 1995; Yoshimura et a., 1998), essential hypertension (Miyamoto et al., 1998) and the risk of acute myocardial infarction (AMI) (Hingorani et al., 1999). Several variable number of tandem repeats (VNTRs) such as polymorphic repeats close to the 5' end, the 27 base pair (bp) repeat in intron 4 are the most studied. The resulting rare 4-repeat allele (4a/b) has been shown to be associated with CAD among subjects belonging to European ancestry (Wang et al., 1996). This 4a/b mutation of NOS3 gene was found to be associated with MI, among subjects of Turkish descent (Cine et al., 2002). Several studies reported that significant association of the 4a/b polymorphism with CAD and MI has been found in several ethnic populations (Wang et al., 1996; Park et al., 2000), though a few groups have also reported lack of association of this SNP (Single Nucleotide Polymorphism) with CAD (Granath et al., 2001; Jaramillo et al., 2010).

The renin-angiotensin system is one of the key regulatory systems, play important role in cardiovascular physiological processes such as cardiovascular remodeling, sodium homeostasis and maintenance of vascular tone (Dzau, 1994). The angiotensin-converting enzyme (ACE) is a major component of the renin-angiotensin system, which is found in the lungs, kidneys, cardiomyocytes and other tissues. ACE converts angiotensin I to angiotensin II a potent vasoconstrictor and also inactivates bradykinin a potent vasodilator (Murphey et al., 2000; Cicoira et al., 2001). Elevated levels of angiotensin II and decreased bradykinin levels is accompanied by the inactivation of ACE can cause increased vascular resistance and high blood pressure found in CAD. The ACE gene is located on chromosome 17q23, and bears 26 exons and 25 introns (Hubert et al., 1991). Even though the human ACE gene contains a large number of polymorphic regions that can be used in the genetic analysis of populations (Reider et al., 1991). The insertion/deletion (I/D) polymorphism of ACE, present in intron 16, where 287 Alu repeat is deleted, has been extensively investigated (Howard et al., 1990). It has been reported that ACE genotypes affect the levels of ACE, which has been consider as a high risk factor for the development of MI (Leatham et al., 1994). It is considered that I allele has a sequence that silences ACE enzyme causing low activity while D allele lacking silencer sequence causes higher ACE activity (Choi et al., 2004). To the best of our knowledge there is no study presenting the possible relation of NOS3 and ACE (I/D) polymorphism with MI in Bangladesh yet. The aim of the present study was to find out the effects of NOS3 and ACE gene (I/D) polymorphisms in the risk of Myocardial Infarction in Bangladeshi population.

2. Materials and methods

The study was a case-control study conducted in 250 subjects (Table 1). The case group comprises 100 cardiac patients who have experienced MI one or more times. The studied MI patients were referred by their treating physicians, and severe chest pain and serum high troponin I level were also measured to include the patients as MI groups. The cardiac patients were recruited for this study immediately after being hospitalized with MI symptoms at Coronary Care Unit (CCU) of Sir Salimullah Medical College Hospital without any medical history of other chronic diseases. A total of 150 healthy controls with no history of cardiac or chronic diseases were recruited from different hospitals of Dhaka city where they came for regular health check-up (Table 1). There was no significant difference in baseline characteristics between control and cases (Table 1).

All participants were given an explanation of the nature of the study, and informed consent was obtained. They completed a structured questionnaire covering information on age, gender, medical, family history of chronic diseases and smoking status. Smoking status was summarized as smoker or nonsmoker (Table 1). The study was approved by the departmental ethical committee. The study was conducted in accordance with the declaration of Helsinki and its subsequent revisions (World Medical Association, 2013).

Sample collection

Approximately 5.0 mL of venous blood was drawn from each individual following all aseptic precautions

with the help of a trained person, using a disposable syringe. About 2.0 mL of drawn blood was immediately transferred into a tube containing EDTA (1.20 mg/ml) and remaining blood into a plain tube for the separation of serum and transported to the laboratory using ice-box. The blood samples of EDTA tubes were stored at –20°C until genomic DNA extraction. The serum was isolated by centrifugation and the biochemical parameters were measured by Dimension Xpand auto biochemistry analyzer.

Allele genotyping

Genotyping of ACE (I/D) gene

The ACE (I/D) genotypes were determined using the previously described PCR method (Uddin et al., 2007). The genomic DNA was extracted from peripheral leukocytes according to our previous method (Hosen et al., 2015). Then polymerase chain reaction (PCR) was performed to amplify the genomic DNA. PCR conditions and primer sequences were used according to the method of Uddin et al. (2007). PCR was carried out using Go Taq polymerase (Go Taq® Flexi, Promega Corporation, USA). Approximately 0.5 µg of genomic DNA was added to a PCR mix composed of 2.5 units Taq polymerase, 200 µmol dNTPs, 50 pmol of each primer, and PCR buffer composed of 50 mol/mL KCl, 10 mol/mL Tris-HCl (pH 8.3), and 2.5 mol/mL MgCl2 in a volume of 50 µL. The PCR products were separated by electrophoresis and visualized under UV light after ethidium bromide staining. Presence of 490bp and 190bp fragment demonstrates insertion (I) and deletion (D) variants respectively while presence of both bands indicates I/D variants of ACE gene (Fig. 1).

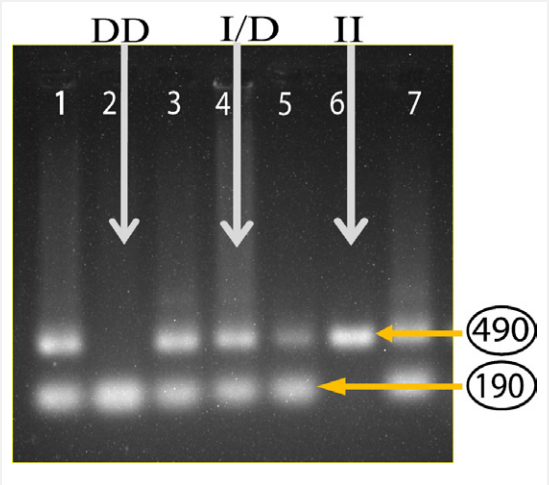

Fig. 1. Polymorphic variants of ACE (I/D) alleles. Both 490 bp and 190 bp fragments at well 1, 3, 4, 5 and 7 demonstrate insertion/deletion (ID) variants; 190 bp fragment at well 2 indicates DD variants and 490 bp fragment at well 6 depicts II variants of ACE gene.

Genotyping of NOS3 (G894T & 4a/b) gene

The genomic DNA amplification for NOS3 G894T and 4a/b genotyping were done by PCR-RFLP (Polymerase Chain Reaction-Restriction Fragment Length Polymorphism) and PCR methods respectively, using our newly designed primers and PCR was carried out similar ways as described for ACE genotyping (Hosen et al., 2015). The forward and reverse primers for G894T genotyping were 5'-TCCCTGAGGAGGGCATGAGGCT-3' and 5'-TGAGGGTCACACAGGTTCCT-3' respectively and the forward and reverse primers for 4a/b genotyping were 5'-AGGCCCTATGGTAGTGCCTT-3' and 5'-TCTCTTAGTGCTGTGGTCAC-3' respectively. About 5.0 μL PCR product (457bp) of G894T genotype was digested with Msp I restriction enzyme (Fig. 2a). The presence of 320bp and 137bp fragments demonstrate GG genotype, presence of 457bp, 320bp and 137bp bands indicate GT genotypes while only one band of 457bp depicts TT genotype (Fig. 2a). For 4a/b genotyping, the PCR product of 393bp indicates "a" genotype and the insertion of 27bp VNTR (420bp) indicates "b" genotype and presence of both 393bp and 420bp fragments demonstrate a/b genotypes (Fig. 2b).

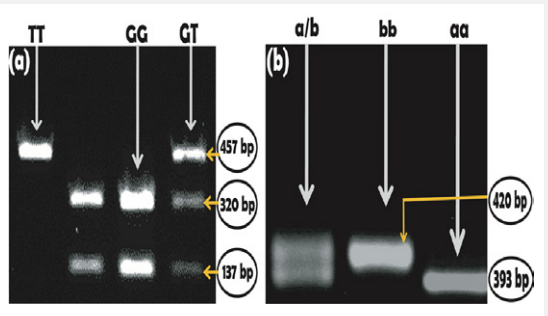

Fig. 2. Polymorphic variants of NOS3 (G894T & 4a/b) alleles. (a) After Msp I digestion, 320 bp and 137 bp fragments demonstrate GG alleles, presence of 457bp, 320 bp and 137 bp bands indicate GT alleles while only one band of 457 bp depicts TT alleles. (b) Presence of both 420 bp and 393 bp fragments indicates a/b variants whereas only 393 bp band demonstrates aa variants and only 420 bp band bb variants.

Statistical analysis

Statistical Package for Social Science (SPSS), windows version 17.0 and GraphPad Prism software were used to perform statistical analyses. The relative association between cases and controls were assessed by calculating the odds ratio (OR). ORs, as a measure of relative risk, at 95% confidence intervals (95% CI) were estimated using logistic regression models. Data were considered as statistically significant according to P values < 0.05. One way ANOVA test was used to estimate the levels of biochemical parameters for different genotypes.

Table 1. Baseline characteristics and clinical parameters.

	Healthy Control (n=150)	CAD Patients (n=100)	p value
Age	53 ± 17	55 ± 12	ns
Gender (n, %)			
Male	113 (75)	76 (76)	ns
Female	37 (25)	24 (24)	
Smoking Status			
Non-Smoker	60 (40)	41 (41)	ns
Smoker	90 (60)	59 (59)	
Family history			
Parents Affected		28 (28)	
Siblings Affected		23 (23)	
No comments		22 (22)	
No History		27 (27)	
Biochemical parameters			
Troponin I (ng/mL)	0.028 ± 0.002	22.3 ± 4.3	<0.01
Total Protein (g/dL)	6.95 ± 0.2	6.89 ± 0.15	ns
ALT (IU/L)	34.4 ± 3.2	27.6 ± 2.2	ns
AST (IU/L)	29.1 ± 2.2	55.2 ± 4.8	<0.001
ALP (IU/L)	74.5 ± 3.8	92.3 ± 3.1	<0.001
ALB (g/dL)	4.11 ± 0.1	3.3 ± 0.1	<0.001

Results are expressed as Mean ± SEM and as number (percentage). Student t-tests were performed to estimate the level of significances. p<0.05 was taken as level of significance. ns; Non-significant. ALT; Alanine aminotransferase, AST; Aspartate aminotransferase, ALP; Alkaline phosphatase, ALB; Albumin.

3. Results

In this study, we examined the association of ACE (I/D) and NOS3 (G894T and 4a/b) genes polymorphisms with the susceptibility of MI in Bangladeshi population. The genotypic distribution (p<0.01) and the allelic frequency (p<0.001) of ACE (I/D) genotypes among the study subjects were significantly different (Table 2). In contrast, the genotype proportions of different NOS3 (G894T and 4a/b) genotypes in both groups were not significantly different (Table 2). The percentages of homozygous insertion, homozygous deletion and heterozygous insertion/deletion alleles were 36.7%, 42.5% and 20.8% respectively in control subjects and 22%, 37% and 41% respectively in MI patients. On the other hand, the percentages of different variants of NOS3 gene were almost similar among the study subjects.

Table 2. Genotypic frequencies of ACE and NOS3 genes in study subjects.

Genes	Controls subjects	CAD Patients (n=100)	p value
ACE			
Genotype (I/D) frequency			
II	**55 (36.7)**	22 (22)	
ID	63 (42.5)	37 (37)	<0.01
DD	32 (20.8)	41 (41)	
NOS3			
Genotype (G894T) frequency			
GG	67 (44.7)	47 (47)	
GT	81 (54)	51 (51)	>0.05
TT	02 (1.3)	02 (02)	
Genotype (VNTR Variants; 4a/b) frequency			
bb	101 (67.3)	69 (69)	
a/b	45 (30)	27 (27)	>0.05
aa	04 (2.7)	04 (04)	

Results are expressed as number (percentage). Chi-square test was performed. p<0.05 was taken as level of significance.

Genotypic analysis of ACE (I/D) and NOS3 (G894T & 4a/b) genotypes

The risk of myocardial infarction associated with the ACE (I/D) and NOS3 (G894T & 4a/b) genotypes were estimated and presented in Table 3. In consideration of ACE (I/D) genotypes, there were four genotyping groups while the subjects with II genotypes was considered the reference group. Individual with DD genotypes was in high risk of experiencing myocardial infarction when compared (OR, 3.28; 95% CI, 1.60-6.70; p<0.01) to the control. In addition, patients having either DD or ID genotypes showed higher risk for MI compared to control group (OR=2.05; 95% CI=1.20-3.70; p< 0.05). On the other hand, association of ID genotypes with MI was not statistically significant (OR, 1.50; 95% CI, 0.75-2.80, p>0.05).

Table 3. Odds ratios of developing MI for ACE and NOS3 genotypes.

Gene	Control subjects (n=150)	CAD Patients (n=100)	P value	Odd ratio (95% CI)
ACE				
Genotypes (Insertion/Deletion)				
II	55	22	-	1 (Ref.)
ID	63	37	ns	1.5 (0.75 to 2.8)
DD	32	41	<0.01	3.28 (1.6 to 6.7)
ID+DD	95	78	<0.05	2.05 (1.2 to 3.7)
NOS3				
Genotypes (G894T)				
GG	67	47	-	1 (Ref.)
GT	81	51	ns	0.92 (.5 to 1.5)
TT	02	02	ns	1.4 (0.2 to 10.5)
Genotypes (VNTR Variants; 4a/b)				
bb	101	69	-	1 (Ref.)
a/b	45	27	ns	0.88 (.5 to 1.5)
aa	04	04	ns	1.5 (0.35 to 6.1)

Odds ratios (OR) and 95% confidence interval (95% CI), OR adjusted for ages and gender. Fisher's exact test was performed. p<0.05 was taken as level of significance.

The NOS3 G894T and 4a/b alleles are considered to be rare alleles and their relationship with study variables was analyzed and presented in Table 3. The GG and TT variants at 894 position of NOS3 did not exert any relation (OR, 1.40; 95% CI, 0.20-10.50, p >0.05) with myocardial infarction in Bangladeshi population. On the other hand, the results showed weak association (OR, 1.50; 95% CI, 0.35-6.10, p >0.05) between the 4a allele and the risk of myocardial infarction compared with the b allele.

The risk of MI with the combination of ACE (I/D) gene and smoking status

The risk associated with the combination of ACE (I/D) genotypes and smoking status was estimated (Table 4). There were six combined groups while the non-smoker group with II genotypes was considered the reference group. The risk of having myocardial infarction was low for both smokers and non-smokers

with ID genotypes (OR, 1.1; 95% CI, 0.40-3.0, p>0.05; OR, 2.3; 95% CI, 1.0-8.50, p>0.05; respectively) and smokers with II genotypes and non-smoker with DD genotypes (OR, 1.1; 95% CI, 0.5-3.1, p>0.05; OR, 2.3; 95% CI, 0.7-7.2, p>0.05, respectively). On the other hand, the risk of occurrence of myocardial infarction was significantly higher among smokers (OR, 4.1; 95 % CI, 1.5-11.2, p<0.01) with DD genotypes.

Table 4. Risk of MI with the combination of ACE (I/D) gene and smoking status.

Genotypes	Non-Smoker	Smoker
II	**1 (19/09)**	1.1, 0.4 to 3.1 (25/13)[a]
ID	**2.3, 1.0 to 8.5 (18/20)[a]**	**1.1; 0.4 to 3.0 (32/17)[a]**
DD	2.3, 0.7 to 7.2 (11/12)[a]	4.1; 1.5 to 11.2 (15/29)*

Odds ratios (OR) and 95 % confidence interval (95 % CI), OR adjusted for ages and gender. Fisher's exact test was performed. p<0.05 was taken as level of significance. aNon-significant; *p<0.01.

Biochemical parameters and different genotypes (I/D, and G894T & 4a/b)

The Table 1 represents the significance levels of biochemical parameters. The serum Troponin I, AST and ALP levels were significantly (p<0.01, 0.001 and 0.001, respectively) higher among the patients compared to the control subjects whereas the albumin level was significantly (p<0.001) lower (Table 1). On the other hand, total protein and ALT levels were not significantly different. One way ANOVA tests were performed to estimate the levels of biochemical parameters for different genotypes and there were no significant differences exist.

4. Discussion

Myocardial infarction (MI) is a devastating disorder that evolved from the interaction of several genetic and environmental factors. These factors differ in various ethnic groups. Though numerous investigation have been performed to find the relation of environmental and genetic factors with MI, the underpinning mechanism of these disorders is still elusive. There are several candidate genes have been documented to be associated with MI worldwide (Cicoira et al., 2001; Cine et al., 2002; Oniki et al., 2008). Sequence variants of the components of the RAS system and the nitric oxide synthase are suggested to have significant influences on cardiovascular homeostasis. The polymorphisms of ACE and NOS3 genes have been investigated and reported to be involved with coronary artery disease and myocardial infarction in several studies (Cambien et al., 1992 ; Wang et al., 1996). In our present study, we have investigated the relationship between the most common polymorphisms of ACE (I/D) and NOS3 (G894T and 4a/b) genes and MI in Bangladesh.

In this study, the ACE (I/D) genotypes and allele frequencies were significantly different between two groups and D allele was more frequent in patients with MI than normal subjects. The risk of occurring MI was 3.28-fold (OR, 3.28; 95% CI, 1.60-6.70; p<0.01) in patients with DD allele while the patients with D allele (ID+DD), was 2.05 times more prone (OR = 2.05; 95% CI = 1.20-3.70; p < 0.05) to experience MI. Moreover, we found strong association of cigarette smoking with MI, where smokers with DD genotypes were 3.91-fold (OR, 3.91; 95% CI, 1.33-11.5, p<0.05) likely to develop cardiovascular diseases.

An association between the polymorphism in the ACE gene and the risk of MI was first reported by Cambien et al. (1992). Some follow-up studies have also shown a significant association between the ACE DD genotype and an increased risk of MI (Rigat et al., 1990; Seckin et al., 2006). A meta-analysis carried out by Samani et al. on 3,394 MI cases and 5,047 controls also showed a high frequency of DD genotypes in MI patients (Samani et al., 1996). Studies from different part of India have revealed that the ACE DD genotype is a risk factor for coronary artery disease and hypertension, which is also a potent risk factor for MI (Bhavani et al., 2004; Dalal et al., 2006). In addition, Firouzabadi et al. in Iran, and Uemura et al. in Japan have also shown association between DD variants of ACE gene and MI (Uemura et al., 2000; Firouzabadi et al., 2012).

However, some studies have not been able to establish an association between ACE gene polymorphism and MI (Keavney et al., 2000; Abdelhedi et al., 2013). A study conducted by Lindpaintner et al., showed no association between the ACE I/D polymorphism and the risk of CAD (Lindpaintner et al., 1995). In addition, Pandey et al. in India and Basol et al. in Turkey also reported lack of association between ACE I/D polymorphism and MI (Pandey et al., 2011; Basol et al., 2014). Therefore ethnic differences may be considered as contributing factor for these discrepancies.

Though several studies across the world have shown association between NOS3 (G894T and 4a/b) gene polymorphism and MI, we found no significant association in Bangladeshi population. Our findings are in agreement with two different studies conducted in South Indian population (Syed et al., 2010; Narne et al., 2013). Study conducted in Italian population also reported lack of association between NOS3 (G894T and 4a/b) genes and CAD (Colomba et al., 2008). In addition, Karvonen et al. also stated that the G894T variant of the NOS3 gene was not a major risk factor for cardiovascular alterations (Karvonen et al., 2002).

In contrast, several studies in different countries supported the hypothesis about the association between NOS3 (G894T and 4a/b) polymorphism and MI. Abolhalaj et al. (2013) found significant association between NOS3 4a/b gene polymorphism and CAD patients in Iran. Li et al. study also suggested that

NOS3 polymorphism is one of the contributing factors for the predisposition of hypertension in the Han population in southwestern China (Li et al., 2011). Moreover, Colombo et al. also reported the evidence of association between NOS3 (G894T) polymorphism MI (Colombo et al., 2003).

In this study, we have found increased levels of Troponin I, AST and ALP as well as decreased level of Albumin among the patients while we could not found any association between ALT and MI. A population based cohort study in the United State also reported no association between MI and ALT, while several investigations showed that ALT and AST are associated with myocardial infarction (Ruhl et al., 2009; Moon et al., 2014; Gao et al., 2017). A recent meta-analysis study conducted on over 9.24 million participants and reported contradictory results (Kunutsor et al., 2014).

However, our study was conducted on small number of samples which was one of the main limitations that necessitate careful interpretation of results. Studies on larger population would certainly be more conclusive. In conclusion, we found the association between ACE (I/D) gene polymorphism and MI in Bangladeshi population. The D allele was significantly higher in MI patients compared to control subjects. Thus, DD genotype would be considered as a risk factor and II genotype would be a molecular marker of reducing CAD. On the other hand, the G894T and 4a/b polymorphic genotypes of the NOS3 gene were not found to be a risk factor for MI in Bangladeshi population. Therefore, genotyping of ACE (I/D) gene would be a biomarker of early diagnosis of CAD and also be helpful to intervene personalized medicine as a novel treatment of CAD.

Conflict of interest

The authors reported that there is no conflict of interest.

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