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Original Article

Association of extracellular superoxide dismutase (EC-SOD) polymorphisms with risk of type 2 diabetes mellitus in a Chinese Han population

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Abstract: We aimed to investigate whether the EC-SOD rs2536512, rs8192291 and rs1799895 polymorphisms and haplotypes are associated with T2DM in a Chinese Han population. A total of 540 Chinese Han patients with T2DM and 562 healthy subjects were enrolled in our study since October 2013, and all of them had no blood relationship. An iPlex GLOD SNP genotyping analysis of the EC-SOD rs2536512, rs8192291 and rs1799895 was carried out in a 384 well plate format using the Sequenom MassARRAY® System (Sequenom, Inc. San Diego, USA). We observed that the CT (OR=1.58, 95% CI=1.20-2.08) and TT (OR=15.27, 95% CI=4.34-53.75) genotypes of rs8192291 were associated with T2DM susceptibility compared with the CC genotype. In dominant and recessive models, rs8192291 was correlated with a moderate statistically increased susceptibility of T2DM compared with the reference genotype. The GTC, GCC and GCG haplotypes were associated with risk of T2DM. In summary, rs8192291 polymorphism and haplotypes may become a useful biomarker for prediction of the susceptibility of this disease. Further experiments are necessary to validate our results.

Keywords: Type 2 diabetes mellitus, EC-SOD, polymorphism, haplotype, environmental factors

Introduction

Type 2 diabetes mellitus (T2DM), a chronic and complex disease, is the most common type of endocrine disease worldwide [1, 2], and its prevalence is increasing in recent decades and reaching epidemic proportions in China. Mortality from diabetes doubles during the last two decades increased to 1.3 million deaths worldwide [3]. It is estimated about 113.9 million adults with diabetes and 493.4 million with prediabetes in China [4]. The pathogenetic mechanisms of T2DM are still debated and require a long term process. Currently, many factors contribute to the pathogenesis of T2DM, including aging, family history impaired glucose tolerance, previous gestational diabetes, nourishment, obesity or overweight, and poverty [5, 6]. However, the prevalence of T2DM showed discrepancies in different population even when they exposed to similar risk factors, suggesting that genetic characteristics may be involved in the pathogenesis of T2DM. However, the exact molecular mechanism of developing T2DM is not fully understood.

Superoxide dismutase (SOD), an antioxidant enzyme with a high activity on catalytic dismutation of superoxide radical anion, is involved in many biological processes caused by oxidative stress (OS) [7]. SOD changes harmful reactive compounds into oxygen and water, and removes stress from oxidation state [7]. Three forms are presented in SOD in human body, including CuZn SOD or SOD1 in the plasma, Mn SOD or SOD2 in mitochondria, and EC-SOD or SOD3 in extracellular matrix. EC-SOD is primarily localized in the pancreas, skeletal muscles and blood vessels, and it is the main cleaner for oxygen free radicals. The genes encoding the EC-SOD are located on chromosome 4 (4p16.3-q21) with a length of 5900 bp and 720 bp in coding region, and consist of three exons and two introns.

Low EC-SOD activity induces high alloxan susceptibility of beta-cells, and then it attributes to a high susceptibility to superoxide radicals caused by activated inflammatory leukocytes and hyperglycemia [8]. An in vivo study has reported that EC-SOD is associated with the altered metabolic state in diabetic skin, which can increase the reactive oxygen species [9]. In this gene, three common SNPs have been observed on the 2 and 3 extrons, including rs2536512 (Ala40Thr), rs8192291 (Arg213-Gly) and rs1799895 (Leu53Leu). Currently, only a few studies reported the association between EC-SOD polymorphisms and risk of T2DM, but the results are inconsistent [10, 11]. Given that oxidative stress with an imbalance in the oxidant and antioxidant activity seems to contribute to the pathogenesis of T2DM [12, 13], the aim of this study was to investigate whether the EC-SOD rs2536512, rs8192291 and rs1799895 polymorphisms and haplotypes are associated with T2DM in a Chinese Han population.

Patients and methods

Ethics

The study protocol was approved by the ethics committee of Zhengzhou Central Hospital. All subjects involved in this study signed the informed consents prior to enrollment, and agreed to participate into our study voluntarily.

Patients and controls

A total of 540 Chinese Han patients with T2DM were selected from the department of dndocrinology of Zhengzhou Central Hospital since October 2013, and all of them had no blood relationship. All patients were primarily diagnosed by laboratory evaluations based on criteria from the World Health Organization-International Diabetes Federation (WHO-IDF) [14]. Exclusion criteria were those with autoimmune disease, malignant cancers, or serious liver or kidney diseases.

A total of 562 healthy subjects were enrolled from healthy volunteers attending the normal physical examination in Zhengzhou Central Hospital during the same period, and they formed the control group. The controls were confirmed to be free of T2DM, other metabolic disorders, autoimmune disease, malignant cancers and end-stage liver or kidney diseases. The mean age of patients and controls were 62.5±9.9 and 61.6±10.6, respectively. There were 206 (38.1%) males and 334 (61.9%) females in patients with T2DM, and 250 (44.5%) males and 312 (55.5%) females in controls.

Demographic and lifestyle characteristics were collected from self-reported questionnaires, including sex, age, family history of T2DM, history of tobacco smoking and alcohol drinking, physical activity, weight (kg) and height (m). The body mass index (BMI) is calculated by dividing weight in kilograms by height in meters squared.

Tobacco smoking was categorized into non-smokers and ever smokers. The definition of ever smokers was defined as those smoking at least one cigarette per day for more than six months. Alcohol drinking was divided into non-drinkers and ever drinkers. Ever drinkers were defined as those who consumed at least one alcoholic drink a day for at least six months.

The blood pressure, plasma glucose levels, serum urea, cholesterol, triglyceride, high-density lipoprotein-cholesterol (HDL-c) and low-density lipoprotein-cholesterol (LDL-c) were collected from medical records.

Genotype analysis

Blood samples (5 ml) were collected from each participant after 12 hour fast (between 7:00 and 9:00 am), and then stored in ethylenediaminetetraacetic acid (EDTA) containing tubes for DNA extraction and measurement of clinical variables. Genomic DNA was extracted from EDTA blood samples by a standard extraction procedure, using Tiangen DNA Blood Mini Kit (Tiangen Biotech Co., Ltd., Beijing, China). The primers and probes of polymerase chain reaction (PCR) amplification and single base extension assays for the EC-SOD rs2536512, rs8192291 and rs1799895 were designed using MassARRAY® Assay Design 3.1 Software (Sequenom, Inc. San Diego, USA). An iPlex GLOD SNP genotyping analysis of the EC-SOD rs2536512, rs8192291 and rs1799895 was carried out in a 384 well plate format using the Sequenom MassARRAY® System (Sequenom, Inc. San Diego, USA). The DNA fragment were amplified in a 5-μL mixture containing 2.8 μL HPLC grade water, 0.5 μL 10 × PCR buffer with

Table 1. Demographic, lifestyle and clinical characteristics of investigated subjects

Variables	Patients N=540		Controls N=562		t value or χ^2 value	*P* value
	N	%	N	%		
Age, years						
<60	203	37.59	247	43.95		
≥60	337	62.41	315	56.05	4.61	0.03
Gender						
Female	206	38.15	250	44.48		
Male	334	61.85	312	55.52	4.56	0.03
SBP, mmHg	137.63±18.43		131.38±16.75		5.90	<0.001
DBP, mmHg	83.56±11.18		77.20±10.55		9.72	<0.001
BMI, kg/m^2						
<24	193	35.74	364	64.77		
≥24	347	64.26	198	35.23	92.83	<0.001
Smoking						
Never	217	40.19	281	50.00		
Ever	323	59.81	281	50.00	10.71	0.001
Drinking						
Never	169	31.30	244	43.42		
Ever	371	68.70	318	56.58	17.26	<0.001
Physical activity						
Less	154	28.52	161	28.65		
Moderate	226	41.85	252	44.84		
Heavy	160	29.63	149	26.51	1.52	0.47
Family history of T2DM						
No	480	88.89	534	95.02		
Yes	60	11.11	28	4.98	14.08	<0.001
Serum urea, μnmol/L	330.99±105.27		326.60±104.48		0.70	0.49
FPG, mmol/L	8.50±2.11		4.95±1.02		35.75	<0.001
TC, mmol/L	4.53±1.07		4.54±1.08		-1.09	0.28
TG, mmol/L	1.76±0.93		1.41±0.97		6.09	<0.001
HDL-c, mmol/L	1.11±0.40		1.36±0.46		-9.58	<0.001
LDL-c, mmol/L	3.72±1.42		3.07±0.41		10.48	<0.001

SBP: Systolic blood pressure; DBP: Diastolic blood pressure; BMI: Body mass index; FPG: fasting plasma glucose; TC: total cholesterol; TG: triglyceride.

$MgCl_2$ of the 20 mM, 0.4 μL $MgCl_2$ of the 25 mM, 0.1 dNTP Mix of 25 mM, 0.5 μL forward and reverse primers Mix, 0.2 μL PCR Enzyme of the 5 U/μl, and 1 μL DNA samples of the 10 ng/μL. Then the SAP and iPLEX reactions were performed. The PCR samples are desalted, and then dispensed to a SpectroCHIP and analyzed with MALDI-TOF MS.

Statistical analysis

The continuous variables were expressed using mean ± standard deviation (SD), and categorical variables were displayed using percentages and frequencies (%). Comparison of continuous and categorical variables between the two study groups was carried out by Chi-square (χ^2) test or student t test. The genotype distributions of EC-SOD rs2536512, rs8192291 and rs1799895 between groups were compared by Chi-square (χ^2) test. Hardy-Weinberg equilibrium (HWE) of the effectiveness of the SNP allele frequency was evaluated by a goodness-of-fit Chi-square test. The association of EC-SOD rs2536512, rs8192291 and rs1799895 with risk of T2DM was analyzed by binary logistic regression analyses, adjusting for potential confounding factors. Three genetic models were used for analysis, including co-dominant, dominant and recessive models. The linkage disequilibrium and haplotype analyses of EC-SOD SNPs were analyzed by SHEsis software. Interaction between SNPs and environmental factors was analyzed by Chi-square test. Data analyses were carried out by IBM SPSS Statistics for Windows, Version 21.0. (IBM Corp, Armonk, NY, USA). Two-tailed and P<0.05 was adopted as statistical significance in this study.

Results

The demographic information of patients and controls were summarized in **Table 1**. When compared with non-diabetic normal controls, patients had significantly higher age (χ^2=4.61, P=0.03), SBP (t=5.90, P<0.001), DBP (t=9.72, P<0.001), BMI (χ^2=92.83, P<0.001), FPG (t=

Table 2. Genotype distributions of EC-SOD rs2536512, rs8192291 and rs1799895 between T2DM patients and controls

	Patients		Controls		χ^2 value	*P* value	Patients		Controls	
SNP	N=540	%	N=562	%			χ^2 for HWE	*P* value	χ^2 for HWE	*P* value
rs2536512										
GG	391	72.41	429	76.33						
GA	135	25.00	124	22.06						
AA	14	2.59	9	1.60	2.88	0.24	0.33	0.57	0.01	0.99
rs8192291										
CC	244	45.19	322	57.30						
CT	259	47.96	237	42.17						
TT	37	6.85	3	0.53	40.20	<0.001	8.37	<0.001	33.55	<0.001
rs1799895										
CC	509	94.26	501	89.15						
CG	39	7.22	33	5.87						
GG	14	2.59	6	1.07	3.33	0.29	81.59	<0.001	29.77	<0.001

Table 3. Association between EC-SOD rs2536512, rs8192291 and rs1799895 polymorphisms and the risk of T2DM

SNPs	β	S.E.	Wals	OR[1]	95% CI	*P* value
rs2536512						
Codminant						
GG				1.00	Reference	
GA	0.13	0.16	0.66	1.14	0.83-1.58	0.42
AA	-0.27	0.50	0.30	0.76	0.29-2.02	0.59
Dominant						
GA+AA vs GG	0.12	0.16	0.54	1.12	0.82-1.53	0.46
Recessive						
AA vs GG+GA	-0.32	0.49	0.42	0.73	0.28-1.90	0.52
rs8192291						
Codminant						
CC				1.00	Reference	
CT	0.46	0.14	10.69	1.58	1.20-2.08	0.001
TT	2.73	0.64	18.03	15.27	4.34-53.75	<0.001
Dominant						
CT+TT vs CC	0.56	0.14	16.65	1.76	1.34-2.30	<0.001
Recessive						
TT vs CC+CT	2.51	0.64	15.48	12.25	3.52-42.68	<0.001
rs1799895						
Codminant						
CC				1.00	Reference	
CG	-0.23	0.29	0.65	0.79	0.45-1.39	0.42
GG	-0.63	0.57	1.24	0.53	0.18-1.61	0.26
Dominant						
GC+GG vs CC	-0.29	0.25	1.35	0.75	0.45-1.22	0.25
Recessive						
GG vs CC+CG	-0.66	0.56	1.35	0.52	0.17-1.57	0.25

[1]Adjusted for age, sex, SBP, DBP, BMI, tobacco smoking, alcohol drinking and family history of T2DM.

35.75, P<0.001), TG (t=6.09, P<0.001) and LDL-c (t=10.48, P<0.001), and lower HDL-c (t=-9.58, P<0.001). Patients with T2DM were more likely to be males (χ^2=4.56, P=0.03), ever smokers (χ^2=10.71, P=0.001) and ever drinkers (χ^2=17.26, P<0.001), and have a family history of T2DM (χ^2=14.08, P< 0.001).

The genotypes of EC-SOD rs2536512, rs8192291 and rs1799895 were shown in **Table 2**. The frequencies of CC, CT and TT genotypes in rs8192291 were significantly different between patients and controls (χ^2=40.20, P<0.001). However, the genotype frequencies of EC-SOD rs2536512 and rs1799895 did not show significant differences between patients and control subjects. Moreover, the allele frequencies of rs2536512 met the requirements of HWE in both patients and controls (P>0.05), while rs8192291 and rs1799895 were not (P<0.001).

We observed that the CT (OR=1.58, 95% CI=1.20-2.08) and TT (OR=15.27, 95% CI=4.34-53.75) genotypes of rs8192291

Table 4. Haplotype analysis of EC-SOD rs2536512-rs8192291-rs1799895

Haplotype	Patients N=1080	%	Controls N=1124	%	*P* value	OR	95% CI
ACC	96	8.89	106	9.43	0.33	1.16	0.87-1.55
ATC	35	3.24	50	4.45	0.06	1.52	0.98-2.36
GCC	730	67.59	614	54.63	<0.001	0.70	0.59-0.84
GCG	45	4.17	24	2.14	0.01	0.53	0.32-0.88
GTC	196	18.15	266	23.67	<0.001	1.55	1.26-1.91

Gobal results: Total Chi-square =29.81, df=4, *P* value <0.001.

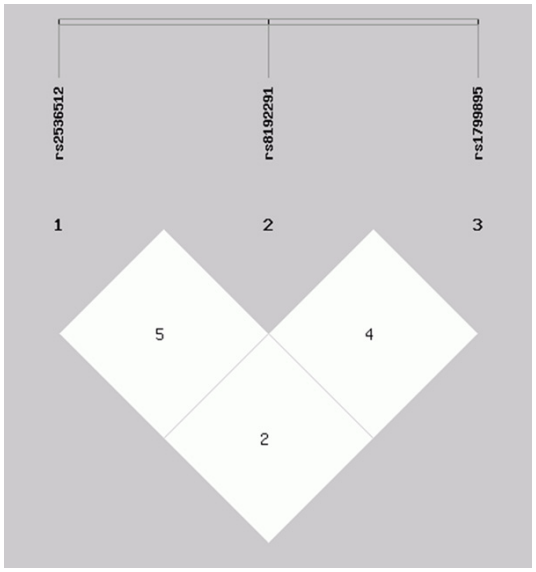

Figure 1. D' of the linkage disequilibrium tests for EC-SOD rs2536512, rs8192291 and rs1799895.

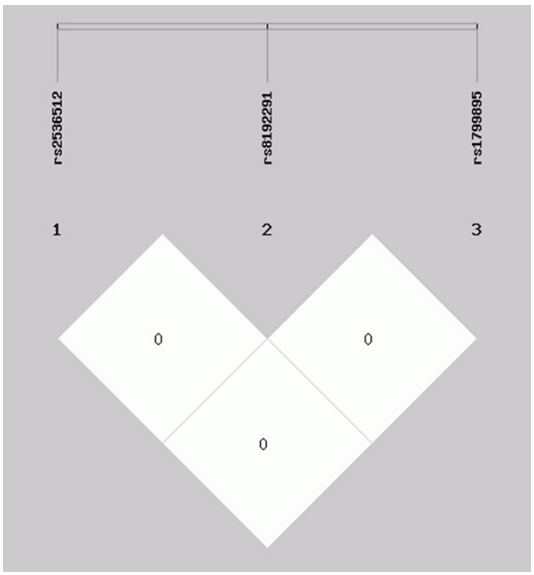

Figure 2. r^2 of the linkage disequilibrium tests for EC-SOD rs2536512, rs8192291 and rs1799895.

were associated with T2DM susceptibility compared with the CC genotype (**Table 3**). In dominant model, the CT+TT genotype of rs8192291 was correlated with a moderate statistically increased susceptibility of T2DM compared with the CC genotype (OR= 1.76, 95% CI=1.34-2.30). In recessive model, a heavy correlation was found between the TT genotype of rs8192291 and risk of T2DM (OR=12.25, 95% CI=3.52-42.68), when compared with the CC+CT genotype. However, there was no correlation between rs2536512 and rs1799895 polymorphisms and the risk of T2DM (P>0.05).

Haplotype comparison analysis indicated that GTC haplotype with the order of rs2536512, rs8192291 and rs1799895 polymorphisms in gene position significantly increased the risk of T2DM (OR=1.55, 95% CI=1.26-1.91) (**Table 4**). However, GCC (OR=0.70, 95% CI=0.59-0.84) and GCG (OR=0.53, 95% CI=0.32-0.88) haplotypes conferred a decreased risk to T2DM. We found no linkage disequilibrium among rs2536512, rs8192291 and rs1799895 (**Figures 1** and **2**).

We performed an interaction between rs8192291 and environmental factors (age, gender, tobacco smoking, alcohol drinking, physical activity, family history of T2DM and BMI). We observed that the TT+CT genotype was correlated with higher risk of T2DM in individuals with older age (OR=1.87, 95% CI=1.37-2.55), ever smokers (OR=1.81, 95% CI=1.31-2.51), ever drinkers (OR=2.02, 95% CI=1.35-3.01) and a family history of T2DM (OR=1.60, 95% CI=1.25-2.05) when compared with the reference groups (**Table 5**).

Discussion

T2DM is the most prevalent metabolic diseases worldwide, and it is multi-factorial disorder that results from the interaction of individual's hereditary factors with environmental factors. Therefore, understanding of susceptibility variants has become a critical way to investigate the etiology of T2DM. In this study, we demonstrated that EC-SOD rs8192291 was associated with susceptibility of T2DM in all genetic

Table 5. Interaction between EC-SOD rs8192291 polymorphism and environmental factors in T2DM risk

Variables	Patients		Controls		OR	95% CI	*P* value
	CC N=244	TT+CT N=296	CC N=322	TT+CT N=240	TT+CT vs CC		
Age							
<60	94	109	133	114	1.35	0.93-1.96	0.11
≥60	150	187	189	126	1.87	1.37-2.55	<0.001
Gender							
Female	92	114	142	108	1.63	1.12-2.36	0.01
Male	152	182	180	132	1.63	1.20-2.23	0.002
Tobacco smoking							
Never	102	115	157	124	1.43	0.98-2.04	0.06
Ever	142	181	165	116	1.81	1.31-2.51	<0.001
Alcohol drinking							
Never	69	100	142	102	1.40	0.97-1.87	<0.001
Ever	175	196	180	138	2.02	1.35-3.01	0.07
Physical activity							
Less	76	78	92	69	1.36	0.88-2.13	0.17
Moderate	109	117	140	112	1.34	0.94-1.92	0.11
Heavy	59	101	90	59	1.60	0.83-2.41	0.14
Family history of T2DM							
No	219	261	306	228	1.86	0.75-4.61	0.18
Yes	25	35	16	12	1.60	1.25-2.05	<0.001
BMI, kg/m^2							
<24	83	110	199	165	1.60	1.12-2.27	0.009
≥24	161	186	123	75	1.90	1.33-2.71	<0.001

models, and the G-C-C, G-C-G and G-T-C haplotypes could affect the risk of T2DM.

Expression of EC-SOD in vascular cells can be changed in response to a variety of stimuli in many pathological conditions such as hypertension, atherosclerosis and diabetes [15]. The EC-SOD rs8192291 substitution affects the heparin-binding domain of the enzyme. It damages the affinity for heparin and endothelial cell surface leading to an increased serum concentration, therefore, the blood vessels' wall might be deficient in EC-SOD activity [16]. The rs8192291 polymorphism was reported to be related to the development of many diseases, such as ischemic heart disease, familiar amyloidotic polyeuropathy type 1, diabetic polyneurophathy and atherosclerosis of hemodialysis patients [17-20]. Moreover, diabetic patients with rs8192291 polymorphism showed an elevated risk for ischemic heart and cerebrovascular disease [21].

Recently study has reported that serum EC-SOD concentration could be considered as a sensitive biochemical marker of insulin resistance [22]. Park H et al. reported serum concentration of EC-SOD was strongly related to HOMA-R concentrations (r=-0.452), and treatment method could improve insulin sensitivity through increasing EC-SOD [22]. Adachi T et al. found an inversely association of EC-SOD level with fasting plasma glucose, BMI and homeostasis model assessment-insulin resistance index [23]. The rs8192291 substitution is located in the amino-terminal domain of EC-SOD, where it is thought to have a function on the tetramerization of the enzyme. The rs8192291 polymorphism was found to be correlated with the susceptibility to insulin resistance and hypertension in T2DM patients [11]. The genetic mutation of EC-SOD rs8192291 could elevate 8-15 folds levels of EC-SOD in serum, and decrease the affinity towards heparin and endothelial cells [21], while the EC-SOD

rs2536512 and rs1799895 genetic polymorphisms could not alter the capacity of affinity to heparin and enzymatic activity.

Currently, several studies have reported the relationship between EC-SOD polymorphisms and risk of T2DM [10, 11, 19, 24, 25]. Ukkola et al. firstly carried out a study with 239 patients with T2DM and 245 control subjects in a Caucasian population, but they did not find a relationship between Cu/Zn SOD and EC-SOD polymorphisms and risk of macroangiopathy in T2DM patients [25]. Tamai et al. performed a study with 205 T2DM patients and 220 non-diabetic subjects, and they showed that EC-SOD rs2536512 was associated with insulin resistance and the susceptibility to T2DM [11]. In a recent study, they reported an positive association between EC-SOD rs2536512 and risk of T2DM in a Chinese population [10]. However, our study found that rs8192291 polymorphism was strongly associated with an increased risk of T2DM. Discrepancies of these results may be attributed to different sample size, ethnicities and study designs.

We found the EC-SOD G-C-C, G-C-G and G-T-C haplotypes were associated with the risk of T2DM. One previous study reported that EC-SOD haplotypes might be a genetic markers for susceptibility to cerebral infarction, essential hypertension and acute lung injury [26-28]. However, no study reported the relationship between EC-SOD haplotypes and T2DM susceptibility. Therefore, further studies with large sample sizes and detailed gene-environmental data are greatly required to confirm our findings.

Our study revealed that EC-SOD Arg213Gly polymorphism had interaction with environmental factors, such as age, smoking, drinking and family history of T2DM. Two previous studies suggested an interaction of EC-SOD polymorphism with smoking and drinking in the risk of oral cancer [29, 30]. Marklund et al. indicated that the plasma concentration of EC-SOD significantly increased with age and could be influenced by lifestyle factors, such as obesity and smoking [31]. Our results suggested that EC-SOD rs8192291 polymorphism had an interaction with age, smoking and drinking, which were in line with previous results.

There are two limitations in this study. First, since patients and controls were enrolled from only one place in China, which may bring in selection bias. Second, the sample size of this study was relatively small, which could cause low statistical power in determining the differences between groups.

In summary, this study suggests that EC-SOD rs8192291 was positively associated with the risk of T2DM under all genetic models in Han Chinese individuals, and the EC-SOD G-C-C, G-C-G and G-T-C haplotypes contribute to the susceptibility of T2DM. Therefore, rs8192291 polymorphism and haplotypes may become a useful biomarker for prediction of the susceptibility of this disease. Further experiments are necessary to validate our results.

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Disclosure of conflict of interest

None.

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