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Polymorphic variations in manganese superoxide dismutase (*MnSOD*) and endothelial nitric oxide synthase (*eNOS*) genes contribute to the development of type 2 diabetes mellitus in the Chinese Han population

J.Y. Li[1], F. Tao[1], X.X. Wu[2], Y.Z. Tan[1], L. He[3] and H. Lu[1]

[1]Diabetes Research Institute, Department of Endocrinology,
Shanghai Key Laboratory of Traditional Chinese Clinical Medice,
Shuguang Hospital Affiliated to Shanghai University of Traditional Chinese Medicine,
Shanghai, China
[2]Shanghai Technical Institute of Electronics & Information, Shanghai, China
[3]Bio-X Institutes, Shanghai Jiao Tong University, Shanghai, China

Corresponding author: H. Lu
E-mail: luhao403@163.com

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ABSTRACT. Impaired antioxidant defense increases the oxidative stress and contributes to the development of type 2 diabetes mellitus (T2DM). MnSOD and eNOS are important antioxidant enzymes. This aim of this study was to verify the association of *MnSOD* and *eNOS* tagSNPs with T2DM in a Chinese Han population. Four tagSNPs of *MnSOD* and eight tagSNPs of *eNOS* were detected using TaqMan technology in 1272 healthy controls and 1234 T2DM patients. All study participants were unrelated members of the Han ethnic group in China. In this study, the frequency of the rs4880 *MnSOD* single nucleotide polymorphisms (SNP)

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genotype differed significantly between T2DM patients and controls [allele: P = 0.03, genotype: P = 0.04, odd's ratio (OR) = 1.26; 95% confidence interval (CI) = 1.07-1.49]. The A-T haplotype and G-T haplotype remained significant in T2DM after Bonferroni correction (P = 1.58 x 10^{-6} and 8.00 x 10^{-4}, respectively) with a global p-value of 7.25 x 10^{-8}. The rs1799983 and rs891512 SNPs of *eNOS* differed significantly between T2DM patients and controls [rs1799983: corrected allele: P = 2.10 x 10^{-3}, corrected genotype: P = 6.30 x 10^{-3}, OR = 1.43 (95%CI = 1.18-1.73); rs891512, corrected allele: P = 3.50 x 10^{-3}, corrected genotype: P = 9.10 x 10^{-3}, OR = 1.70 (95%CI = 1.26-2.30)]. Following Bonferroni correction, none of the haplotypes of *eNOS* were significant in T2DM. These results indicate that common variants in *MnSOD* and *eNOS* increased the risk of T2DM in the Chinese Han population.

Key words: Type 2 diabetes mellitus; Genetic polymorphisms; MnSOD; eNOS; Chinese Han population

INTRODUCTION

T2DM is a chronic disease which, as a consequence of the overproduction of reactive oxygen species (ROS), is related to oxidative stress (Victor et al., 2011). Oxidative stress is developed as a result of an imbalance between free radical production, often increased because of dysfunctional mitochondria, and reduced antioxidant defense (Small et al., 2012). Manganese superoxide dismutase (MnSOD) and endothelial nitric oxide synthase (eNOS) eliminate the excess ROS and maintain the equilibrium between oxidative and anti-oxidative activity under normal physiological conditions (Chen et al., 2012). Therefore, impaired antioxidant defense increases the oxidative stress and contributes to the development of T2DM (Chen et al., 2012). MnSOD and eNOS are important antioxidant enzymes. Functional polymorphisms in these antioxidant enzymes are involved in the pathogenesis of T2DM (Banerjee and Vats, 2014). Low levels of antioxidant enzymes or their non-functionality result in the excessive production of ROS, which initiates stress-related pathways, thereby leading to insulin resistance and T2DM (Banerjee and Vats, 2014).

The *MnSOD* gene is located on chromosome 6q25 and includes 6 exons, spanning 14 kb. MnSOD decomposes toxic ROS to H_2O_2 and quenches the free radicals produced by the electron transport chain (Chen et al., 2012). Homozygous MnSOD knockout (Sod2-/-) mice are neonatal lethal, produce reduced levels of cellular ATP, have a lower O_2 consumption, and generate increased levels of superoxide (Zhang et al., 2010). A functional polymorphism in exon 2 of the *MnSOD* gene (causing a Val16Ala substitution; rs4880) resulted in structural alterations in the mitochondrial targeting domain, attributing the decreased antioxidant potential to limited post-transcriptional transport (Banerjee and Vats, 2014) . The *MnSOD* Val16Ala variation has been shown to increase the risk of oxidative stress-related pathological conditions, such as T2DM and diabetic microvascular disease Nakanishi et al., 2008).

The *eNOS* gene is located on chromosome 7q35-36 and includes 26 exons, spanning 21 kb (Ma et al., 2014b). Nitric oxide (NO) is produced as a result of the oxidation of L-arginine by eNOS (Moncada and Higgs, 1993). Superoxide, which is increasingly produced in endothelial cells, reacts directly with NO to form a more harmful molecule, peroxynitrite ($ONOO^-$), thereby

reducing the bioavailability of NO (Triggle and Ding, 2010). Elevated ROS production and reduced NO bioavailability, together with the intermediate product peroxynitrite, significantly account for apoptosis in the endothelial cells, and endothelial dysfunction in T2DM (Johansen et al., 2005). The presence of polymorphisms in *eNOS* might contribute to a decrease in eNOS activity, reduction in the NO level, and has been reported as a potential factor affecting the pathogenesis and development of T2DM (Ma et al., 2014b).

The genetic component of T2DM and its chronic complications are believed to be polygenic. Therefore, it is likely that a combination of genes may contribute to its overall susceptibility (Chen et al., 2012). In this study, we investigated the association of *MnSOD* and *eNOS* genetic polymorphisms with T2DM using a case-control study in the Chinese Han population.

MATERIAL AND METHODS

Subjects

The study was conducted in 2,506 unrelated subjects, including 1,234 T2DM patients and 1,272 healthy controls. All subjects were recruited between September 2011 and February 2014 from the Shuguang Hospital, affiliated to the Shanghai University of Traditional Chinese Medicine. All participants belonged to the Han Chinese sub-population. T2DM was diagnosed according to the 1999 World health organization (WHO) criteria. Those patients who had a history of ketoacidosis, required continuous insulin treatment, had exocrine pancreatic disease, had other types of diabetes, or displayed positive anti-glutamic acid decarboxylase (anti-GAD), anti-insulin auto-antibody (anti-IAA), or anti-islet cell antibody (anti-ICA) autoantibody responses were excluded from the study. The control group was recruited from subjects who came to the hospital for routine exams. The control participants were included based on the following criteria: normal glucose tolerance as assessed by a standard 75 g oral glucose tolerance test (OGTT) (fasting plasma glucose < 5.6 mM, 2 h plasma glucose < 7.8 mM), or HbA1c < 5.6%, and no known family history of diabetes (based on a questionnaire). Anthropometric data was collected at the time of recruitment. Written informed consent for this study was obtained from all participants and was approved by the Medical Ethics Committee of Shuguang Hospital.

Genotyping

Genomic DNA was extracted from peripheral blood samples using a standard phenol-chloroform extraction method. Tag SNP was selected using the Haploview software with pair-wise tagging, $r^2 \geq 0.5$, and minor allele frequency (MAF) of 0.05 (de Bakker et al., 2006). Four tagSNPs of the *MnSOD* gene (rs4480, rs8032939, rs5746105, rs5746136, and rs8031) and eight tagSNPs of the *eNOS* gene (rs1799983, rs1800779, rs2070744, rs3918184, rs3918186, rs3918227, rs7830, and rs891512) were randomly chosen from the different blocks. While rs7403531 was identified by GWAS to be positively associated with T2DM development (Li et al., 2013), a suitable rs4467032 Taqman assay could not be designed by Applied Biosystems. As such, all SNPs were genotyped using an ABI 7900 DNA detection system (Applied Biosystems, Foster City, CA) using the TaqMan® technology. All probes were designed by Applied Biosystems. A standard 5 µL PCR mixture, prepared using TaqMan® Universal PCR Master Mix (Applied Biosystems), was analyzed according to manufacturer guidelines.

Statistical analysis

All data was expressed as mean ± standard deviation (SD) (continuous variables) or as a percentage of the total (categorical variables). Intergroup significance was assessed by Student's *t*-test (continuous variables) and the χ^2 test (categorical variables). All parameter calculations, allele and genotype frequencies, Hardy-Weinberg equilibrium, pair-wise linkage disequilibrium (LD) analysis, haplotype analysis, and P value permutations were conducted using SHEsis Online Version (http://analysis.bio-x.cn) (Shi and He, 2005; Li et al., 2009), a robust and user-friendly platform with integrated analysis tools that are particularly suited to association studies. P values were corrected using the Bonferroni method. The significance level was set at $\alpha = 0.05$. Power calculations were performed using the G-power software (http://www.psycho.uni-duesseldorf.de/abteilungen/aap/gpower3/).

RESULTS

The clinical characteristics of the study subjects are summarized in Table 1. The age of the subjects at examination (P = 0.065), sex (P = 0.419), and low density lipoprotein (LDL) levels (P = 0.056) were comparable between the patients and controls. Meanwhile, significant differences were noted in the body mass index (BMI) (P < 0.001), presence of hypertension (P < 0.001), and high density lipoprotein (HDL) (P < 0.001), total cholesterol (p < 0.001), and triglyceride (P < 0.001) levels between patients and controls.

Table 1. Clinical characteristic of patients and controls.

Characteristic	Patients (1234)	Controls (1272)	P
Age (year)	53 ± 15	54 ± 12	0.065
Sex (M/F)	600/634	640/632	0.419
Body mass index (BMI) (kg/m2)	28.4 ± 5.1	23.0 ± 2.4	<0.001
SBP (mm Hg)	143.8 ± 20.8	121.7 ± 18.9	<0.001
DBP (mm Hg)	84.6 ± 9.9	78.4 ± 11.2	<0.001
FBG (mmol/l)	9.7 ± 2.9	4.8 ± 0.3	<0.001
HbA1c (%)	9.1 ± 2.2	5.3 ± 1.1	<0.001
Urea (mmol/l)	5.1 ± 1.5	9.5 ± 7.4	<0.001
HDL (mmol/l)	1.2 ± 0.9	1.4 ± 0.3	<0.001
LDL (mmol/l)	2.6 ± 1.5	2.5 ± 1.1	0.056
Total cholesterol (mmol/l)	5.2 ± 3.3	4.8 ± 2.6	<0.001
Triglycerides (mmol/l)	2.3 ± 1.7	1.5 ± 0.9	<0.001

For *MnSOD* analysis, all SNPs were in Hardy-Weinberg equilibrium in the control group. The association between *MnSOD* SNPs and T2DM in case-control subjects is summarized in Table 2. Rs5746136, rs5746105, and rs4880 were positively associated with T2DM in the allele and genotype distributions (rs5746136: allele, P = 0.025; genotype, P = 0.080; Odd's ratio (OR) = 1.14 [95% confidence interval (CI) = 1.016-1.275]; rs5746105: allele, P = 0.035; genotype, P = 0.022; OR = 1.14 [95%CI = 1.009-1.286]; rs4880: allele, P = 0.007; genotype, P = 0.009; OR = 1.26 [95%CI = 1.066-1.493]) (Table 2). After Bonferroni correction, the rs4880 was still significant in both allele and genotype distributions (allele: P = 0.027, genotype: P = 0.036, OR = 1.26 [95%CI = 1.066-1.493]). Power analysis revealed that the statistical power with which a significant association (P < 0.05) could be detected using our sample was approximately 90% in allele or genotype comparisons.

Table 2. Genotype and allelic frequencies of MnSOD SNPs in T2DM patients and control subjects.

SNP	Na	Allele		P[b]	OR [95%CI]	Genotype			P[b]	HWE
rs8031		A	T			AA	AT	TT		
Control	1244	305 (0.123)	2183 (0.877)	0.8787	0.99	23 (0.018)	259 (0.208)	962 (0.773)	0.9880	0.256420
Case	1205	292 (0.121)	2118 (0.879)		[0.831457~1.171067]	22 (0.018)	248 (0.206)	935 (0.776)		0.243542
rs5746136		A	G			AA	AG	GG		
Control	1240	1028 (0.415)	1452 (0.585)	0.0252 (0.1008[c])	1.14	220 (0.177)	588 (0.474)	432 (0.348)	0.0798	0.416843
Case	1192	1064 (0.446)	1320 (0.554)		[1.016268~1.275482]	240 (0.201)	584 (0.490)	368 (0.309)		0.763804
rs5746105		C	T			CC	CT	TT		
Control	1212	1586 (0.654)	838 (0.346)	0.0351 (0.1404[c])	1.14	525 (0.433)	536 (0.442)	151 (0.125)	0.0215 (0.0860[c])	0.434991
Case	1152	1574 (0.683)	730 (0.317)		[1.009137~1.286161]	525 (0.456)	524 (0.455)	103 (0.089)		0.085177
rs4880		C	T			CC	CT	TT		
Control	1236	285 (0.115)	2187 (0.885)	0.0068 (0.0272[c])	1.26	22 (0.018)	241 (0.195)	973 (0.787)	0.0091 (0.0364[c])	0.120283
Case	1204	340 (0.141)	2068 (0.859)		[1.065991~1.493176]	44 (0.037)	252 (0.209)	908 (0.754)		0.000002

[a]Numbers genotyped successfully. [b]Uncorrected P value. [c]Corrected P value, calculated as per the Bonferroni's method.

Linkage disequilibrium among the four tagSNPs of *MnSOD* is shown in Figure 1. The selection criteria for haplotypes analyses included two factors: adjacent SNPs with pairwise D' > 0.85 and SNPs in the same block. Haplotypes with frequencies > 0.03 were tested in these analyses. Based on the selection criteria, rs5746136 and rs5746105 were subjected to haplotype analysis. Haplotype analysis revealed that the A-T and G-T haplotypes of rs5746136-rs5746105 were positively associated with T2DM (P = 3.97 x 10^{-7} and 0.0002, respectively). The A-T and G-T haplotypes were still significant in T2DM (P = 1.58 x 10^{-6} and 0.0008, respectively) after Bonferroni correction (Table 3).

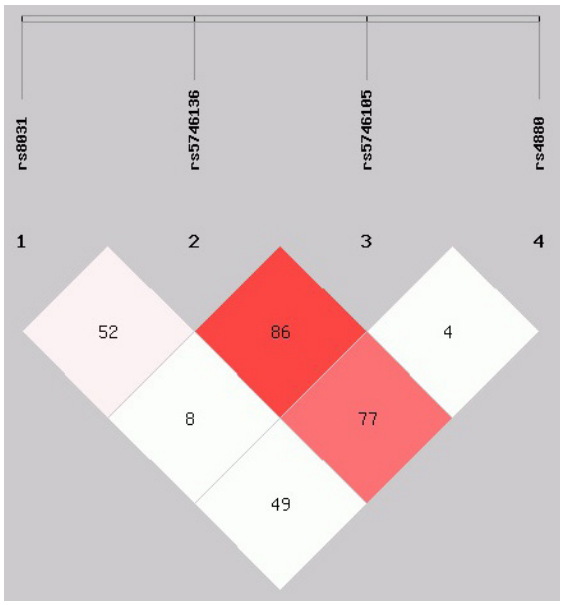

Figure 1. Linkage disequilibrium among the four tagSNPs of MnSOD.

Table 3. Haplotype frequencies of MnSOD SNPs in T2DM patients and control subjects.

Haplotype	Case	Control	P[a]	OR [95%CI]	Global P
rs5746136-rs5746105					
A-C	946.29 (0.414)	96.44 (0.401)	0.3620	1.056 [0.940-1.186]	7.25 x 10-8
A-T	69.71 (0.030)	23.56 (0.010)	3.97 x 10-7 (1.58 x 10-6[b])	3.180 [1.986-5.091]	
G-C	615.71 (0.269)	609.57 (0.253)	0.2132	1.086 [0.954-1237]	
G-T	656.29 (0.287)	810.43 (0.337)	0.0002 (0.0008[b])	0.793 [0.700-10.897]	

[a]Uncorrected P value. [b]Corrected P value, calculated as per the Bonferroni's method.

All SNPs in the control group subjected to the *eNOS* analysis were in Hardy-Weinberg equilibrium, excluding rs3918184. The association between *eNOS* tagSNPs and T2DM in case-control subjects is summarized in Table 4. rs1799983, rs3918227, rs3918186, and rs891512 were positively associated with T2DM in both allele and genotype distributions (rs1799983: allele, P = 0.0003; genotype, P = 0.0009; OR = 1.426 [95%CI = 1.176-1.728]; rs3918227: allele, P = 0.0128; genotype, P = 0.0155; OR = 0.729 [95%CI = 0.568-0.936]; rs3918186: allele, P = 0.0271;

genotype, P = 0.0862; OR = 0.814 [95%CI = 0.679-0.977]; rs891512: allele, P = 0.0005; genotype, P = 0.0013; OR = 1.698 [95%CI = 1.258-2.291]) (Table 2). The allele and genotype distributions of rs1799983 and rs891512 were still significant (rs1799983: allele, P = 0.0021; genotype, P = 0.0063; rs891512: allele, P = 0.0035; genotype, P = 0.0091) after Bonferroni correction. Power analysis revealed that the statistical power of our sample to detect a significant association (P < 0.05) was approximately 90% in allele or genotype comparisons.

Table 4. Genotype and allelic frequencies of eNOS SNPs in T2DM patients and control subjects.

SNP	N	Allele		P[b]	OR [95%CI]	Genotype			P[b]	HWE
rs1800779		A	G			AA	AG	GG		
Control	1220	2144 (0.879)	296 (0.121)	0.3391	0.920	944 (0.774)	256 (0.210)	20 (0.016)	0.6225	0.35
Case	1196	2080 (0.870)	312 (0.130)		[0.776-1.091]	908 (0.759)	264 (0.221)	24 (0.020)		0.58
rs2070744		C	T			CC	CT	TT		
Control	1240	296 (0.119)	2184 (0.881)	0.3637	1.082	16 (0.013)	264 (0.213)	960 (0.774)	0.6137	0.94
Case	1204	308 (0.128)	2100 (0.872)		[0.913-1.283]	20 (0.017)	268 (0.223)	916 (0.761)		0.65
rs1799983		G	T			GG	GT	TT		
Control	1244	2213 (0.889)	275 (0.111)	0.0003 (0.0021[c])	1.426	978 (0.786)	257 (0.207)	9 (0.007)	0.0009 (0.0063[c])	0.07
Case	1216	2237 (0.920)	195 (0.080)		[1.176-1.728]	1024 (0.842)	189 (0.155)	3 (0.002)		0.06
rs3918227		A	C			AA	AC	CC		
Control	1252	156 (0.062)	2348 (0.938)	0.0128 (0.0896[c])	0.729	4 (0.003)	148 (0.118)	1100 (0.879)	0.0155 (0.1085[c])	0.09
Case	1112	112 (0.046)	2312 (0.954)		[0.568-0.936]	0 (0.000)	112 (0.092)	1100 (0.908)		0.68
rs3918186		A	T			AA	AT	TT		
Control	1240	2240 (0.903)	240 (0.097)	0.0271 (0.1897[c])	0.814	1012 (0.816)	216 (0.174)	12 (0.010)	0.0862	0.94
Case	1204	2128 (0.884)	280 (0.116)		[0.679-0.977]	940 (0.781)	248 (0.206)	16 (0.013)		0.90
rs891512		A	G			AA	AG	GG		
Control	1252	72 (0.029)	2432 (0.971)	0.0005 (0.0035[c])	1.698	0 (0.000)	72 (0.058)	1180 (0.942)	0.0013 (0.0091[c])	0.44
Case	1212	116 (0.048)	2308 (0.952)		[1.258-2.291]	4 (0.003)	108 (0.089)	1100 (0.908)		0.29
rs7830		A	C			AA	AC	CC		
Control	1232	1043 (0.423)	1423 (0.577)	0.2001	0.928	238 (0.193)	567 (0.460)	428 (0.347)	0.3381	0.37
Case	1176	952 (0.405)	1400 (0.595)		[0.827-1.041]	200 (0.170)	552 (0.469)	424 (0.361)		0.04

[a]Numbers genotyped successfully. [b]Uncorrected P value. [c]Corrected P value, calculated as per the Bonferroni's method.

Linkage disequilibrium among the four tagSNPs of the *eNOS* gene is shown in Figure 2. rs3918227 and rs3918186 and rs1800779 and rs2070744 were subjected to haplotype analysis based on the selection criteria. The haplotype analysis revealed a positive correlation between the A-A and C-T haplotype of rs3918227-rs3918186 and T2DM (P = 30.0191 and P = 0.0278, respectively). After Bonferroni correction, none of the haplotypes were significant in T2DM (Table 5).

DISCUSSION

T2DM is a chronic disorder characterized by insulin resistance and β-cell dysfunction. The currently favored hypothesis is that oxidative stress leads to insulin resistance, β-cell dysfunction, impaired glucose tolerance, and ultimately T2DM. It is believed that therapies aimed at reducing oxidative stress would benefit patients with T2DM (Banerjee and Vats, 2014).

Mitochondrial oxidative damage is believed to play a key role in pancreatic β-cell failure in the pathogenesis of T2DM (Lim et al., 2011). MnSOD is mainly located in the mitochondrial

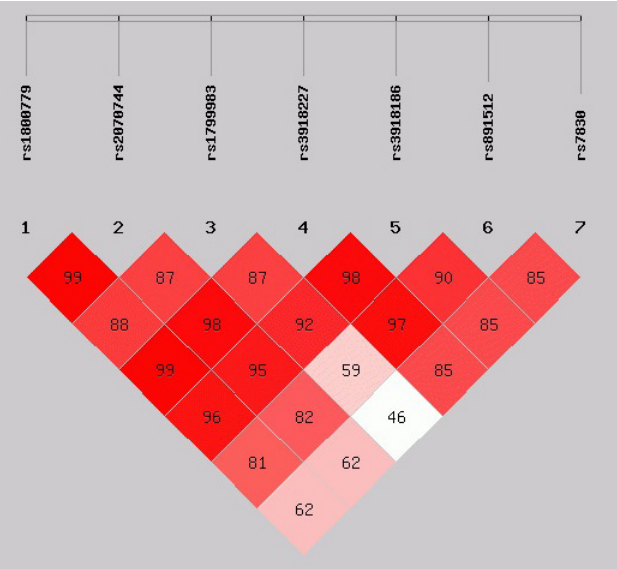

Figure 2. Linkage disequilibrium among the four tagSNPs of eNOS.

Table 5. Haplotype frequencies of eNOS SNPs in T2DM patients and control subjects.

Haplotype	Case	Control	P[a]	OR [95%CI]	Global P
Rs3918227-rs3918186					
A-A	107.66 (0.045)	147.93 (0.060)	0.0191 (0.0573[b])	0.738 [0.572-0.952]	0.0084
C-A	2020.34 (0.839)	2092.07 (0.844)	0.6690	0.967 [0.829-1.128]	
C-T	279.66 (0.116)	239.93 (0.097)	0.0278 (0.0.0834[b])	1.227 [1.022-1.472]	

[a]Uncorrected P value. [b]Corrected P value, calculated as per the Bonferroni's method.

matrix and is responsible for scavenging approximately 80% of the free radicals from the oxidative and phosphorylation process in mitochondria (Qiu et al., 2015). The activity levels of MnSOD were reported to be significantly lower in T2DM patients compared to healthy subjects (Fujimoto et al., 2008). Another study reported that MnSOD protected pancreatic β-cells against oxidative stress, promoting their survival, and increasing insulin secretion in cell models of glucotoxicity and glucolipotoxicity associated with T2DM (Lim et al., 2011). A functional polymorphism was identified in exon 2 of the *MnSOD* gene rs4880 (Ala16Val), which was associated with oxidative stress in T2DM, and its resultant complications. The substitution from valine to alanine was shown to induce a 30-40% increase in MnSOD activity in mitochondria, resulting in reduced risk of coronary artery disease and acute myocardial infarction (Fujimoto et al., 2008; Vanita, 2014). Nakanishi et al. (2008) identified an association between the *MnSOD* Ala16Val polymorphism and the development of T2DM among Japanese-Americans. In our study, we also discovered a significant correlation between rs4880 (Ala16Val) and T2DM in the Chinese Han population. After Bonferroni correction, the allele and genotype distributions of rs4880 remained significant (allele: P = 0.027, genotype: P = 0.036, OR = 1.26 [95%CI = 1.066-1.493]). These results suggested that insufficient reactive oxygen species scavenging might be associated with a susceptibility to T2DM.

Endothelial nitric oxide synthase (eNOS) produces NO from L-arginine. An increased production of reactive oxygen species, including superoxide anion (O^{2-}) may contribute to T2DM and diabetic complications (Ma et al., 2014a). O^{2-} effectively inactivates NO. Nitric oxide has been associated with insulin resistance and T2DM (Deli Muti et al., 2014). Previous studies have strongly suggested an association between polymorphisms in the *eNOS* gene and the bioavailability of eNOS and endothelial function; in addition, these polymorphisms have been implicated in the development of T2DM and diabetic nephropathy (Santos et al., 2011; Hermans et al., 2012; Santos et al., 2012; Rahimi et al., 2013). In addition, an association has been suggested between an SNP (Glu298Asp variant, rs1799983) in the *eNOS* gene and increased risk of T2DM (Rahimi et al., 2013). In our study, we also found a significant association between rs1799983 (Glu298Asp) and T2DM in the Chinese Han population. After Bonferroni correction, the allele and genotype distributions of rs1799983 remained significant (allele: P = 0.002, genotype: P = 0.006, OR = 1.426 [95%CI = 1.176-1.728]). We also discovered an association between other SNPs (such as rs891512, and not rs1799983) and T2DM. These results suggested that *eNOS* is a risk factor for T2DM and its chronic complications.

The genetic component of T2DM and its chronic complications are believed to be polygenic. MnSOD plays a key role in pancreatic β-cell failure in the pathogenesis of T2DM. eNOS has been associated with insulin resistance and T2DM. Insulin resistance and β-cell dysfunction are the major pathophysiological features of T2DM (Saisho, 2015). In addition, MnSOD and eNOS are important antioxidant enzymes that play major roles in regulating antioxidant stress. Therefore, a combination of the *MnSOD* and *eNOS* genes could contribute to the overall T2DM susceptibility.

Additional studies with larger sample sizes and stricter criteria for control and case inclusion are required to enhance the power of the conclusions of this study.

In conclusion, this study indicates that the *MnSOD*-rs4880 and *eNOS*- rs1799983 and rs891512 variants confer increased risk of T2DM to the Chinese Han population. Furthermore, functional studies are required to elucidate the precise mechanisms with which *MnSOD* and *eNOS* gene polymorphisms are associated with antioxidative stress function and T2DM.

Conflicts of interest

The authors declare no conflict of interest.

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