Genetic polymorphisms in extracellular superoxide dismutase Leu53Leu, Arg213Gly, and Ala40Thr and susceptibility to type 2 diabetes mellitus

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ABSTRACT. The most common type of endocrine disease is type 2 diabetes mellitus (T2DM); genetic factors contribute to the development to T2DM. In this study, we investigated the role of the Leu53Leu, Arg213Gly, and Ala40Thr polymorphisms in extracellular superoxide dismutase (*EC-SOD*) gene in the development of T2DM in a Chinese population. DNA was extracted from peripheral blood samples obtained from 256 T2DM patients and 324 control subjects recruited from our hospital between January 2013 and March 2015. DNA was genotyped by polymerase chain reaction-restriction fragment length polymorphism. The obtained data was then statistically analyzed. The chi-square test revealed a statistically significant difference in the

genotype frequencies of *EC-SOD* Ala40Thr (χ^2 = 13.26, P = 0.001) between the patients and controls. Unconditional regression analysis indicated that the GA and AA genotypes of *EC-SOD* Ala40Thr were associated with an increased risk of T2DM compared to the GG genotype {adjusted odds ratio (OR) [95% confidence interval (CI)] = 1.46 (1.01-2.11) and 2.67 (1.48-4.85), respectively}. In the dominant model, the GA+AA genotype of *EC-SOD* Ala40Thr was correlated with a higher risk of T2DM, in comparison with the GG genotype (OR = 1.64, 95%CI = 1.16-2.33). In the recessive model, AA of *EC-SOD* Ala40Thr showed a 2.19-fold higher risk of developing T2DM than the GG+GA genotype. In conclusion, people with the Ala40Thr polymorphism in *EC-SOD* are at a higher risk of developing T2DM; therefore, this may be utilized as a biomarker for early screening of T2DM in a Chinese population.

Key words: Extracellular superoxide dismutase; Type 2 diabetes mellitus; Single nucleotide polymorphism; Leu53Leu; Arg213Gly; Ala40Thr

INTRODUCTION

The most common type of endocrine disease is type 2 diabetes mellitus (T2DM), wherein the insulin secretion capacity and pancreatic beta cells are damaged, leading to insulin resistance (Goldstein, 2002). An estimated 300 million individuals suffered from T2DM until 2010, and about 90 million of them were Chinese (Yang et al., 2010). Therefore, early detection and diagnosis of T2DM is of great importance. The development of T2DM can be attributed to multiple environmental and lifestyle factors, such as high fat dietary, individuals aged 40 years and above suffering from dyslipidemia, high cholesterol and fat dietary, and obesity or excessive weight, combined with hypertension (Pearson, 2015; Uma Jyothi and Reddy, 2015). Recent twin study has reported a significant correlation between genetic and environmental factors in the risk of metabolic syndrome and diabetes (Song et al., 2015; Sung et al., 2015). Therefore, genetic factors may contribute to the risk of developing T2DM, and many genome-wide association studies have shown many genetic loci in the onset and development of T2DM (Imamura et al., 2016; Prabhanjan et al., 2016; Rao et al., 2016).

Extracellular superoxide dismutase (*EC-SOD*) is the main superoxide dismutase of the extracellular matrix (Kimura et al., 2003). EC-SOD is observed in the pancreas, skeletal muscles, and blood vessels (the latter in particular abundance), which is the main cleaner for extracellular oxygen free radicals. The heparin-binding domain of *EC-SOD* could bind to the extracellular surface of endothelial cells and blood vessels, but the lysine residues of heparin-binding domain are likely to be glycosylation under the condition of hyperglycemia, and its affinity to heparin is reduced (Góth and Nagy, 2012). Previous study has shown a lower EC-SOD protein expression in diabetic skin tissue in comparison to the normal tissue (Kim, 2013). Some studies have also indicated that the *EC-SOD* gene plays an important role in the development and complications of T2DM (Kimura et al., 2003; Tamai et al., 2006). A C-to-G substitution at position 213 of *EC-SOD* resulting in a Arg→Gly mutation in the resultant polypeptide chain, and its association with the onset of type 1 and 2 diabetes patients has been extensively studied (Kimura et al., 2003; Strokov et al., 2003; Tamai et al., 2006). Other studies

have analyzed a silent Leu53Leu mutation in Japanese and Mediterranean subjects; however, the implication of this mutation in T2DM has not been clarified so far (Tamai et al., 2006). In this study, we investigated the correlation between the Leu53Leu, Arg213Gly, and Ala40Thr polymorphisms in *EC-SOD* and the risk of developing T2DM in a sample Chinese population.

MATERIAL AND METHODS

Subjects

This study included 256 patients with T2DM, and they were recruited from the Baoji Traditional Chinese Medicine Hospital between January 2013 and March 2015. T2DM was confirmed based on the WHO-IDF criteria (American Diabetes Association, 2004). Patients with other endocrine diseases, acute or chronic infectious diseases, malignant tumors, or end-stage liver or kidney diseases were excluded from this study. The mean age of patients with T2DM was 52.65 ± 10.25 years. The T2DM group comprised 99 (38.67%) females and 157 (61.33%) males. T2DM patients showed mean fasting glucose and insulin levels of 9.43 ± 3.64 and 58.65 ± 16.22 mM, respectively.

Three hundred and twenty four subjects were recruited to the control group from among individuals who received regular health examinations at the Baoji Traditional Chinese Medicine Hospital. Lack of T2DM and other endocrine disorders was confirmed in the control subjects via a blood glucose examination. The mean age of control subjects was 53.91 ± 10.46 years. The comprised 141 females (43.52%) and 183 (56.48%) males. The control subjects revealed mean fasting glucose and insulin levels of 4.81 ± 2.62 and 50.74 ± 15.71 mM. Control subjects with acute or chronic infectious diseases, or end-stage liver or kidney diseases were excluded from this study.

The demographic and clinical data of patients with T2DM and control subjects were collected from an interview using a structured questionnaire, or the patient medical records. The demographic data included age, gender, body mass index (BMI), tobacco smoking and alcohol consumption status, and a history of hypertension. The clinical data included fasting glucose and fasting insulin levels. Signed informed consent forms were obtained from all willing patients and control subjects. The study design was approved by the Clinical Research Ethics Committee of our hospital.

Genotyping

Peripheral blood (5 mL) was collected from all individuals in ethylene diamine tetraacetic acid-coated tubes and stored at -20°C until further use. DNA was extracted from peripheral blood by salt extraction using the QIAamp DNA Blood Mini kit (Qiagen, Venlo, Netherlands) according to the manufacturer protocols. The *EC-SOD* Leu53Leu, Arg213Gly, and Ala40Thr polymorphisms were genotyped by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). The primers of the *EC-SOD* Leu53Leu, Arg213Gly, and Ala40Thr polymorphisms were designed using Primer 5.0 (Table 1). The PCR conditions were set as follows: initial denaturation at 94°C for 5 min; 38 cycles of denaturation at 94°C for 45 s, annealing at 58°C for 90 s, and extension at 72°C for 60 s; and a final extension at 72°C for 10 min. The restriction enzymes for digestion of *EC-SOD* Leu53Leu, Arg213Gly, and Ala40Thr were summarized in Table 1. The PCR products were separated by 1% agarose

gel electrophoresis and analyzed. The reproducibility of the results was ensured by repeating the genotyping process in 15% of the samples (blinded random sampling).

Table 1. Primers, restriction enzymes, and product sizes of theextracellular superoxide dismutase (*EC-SOD*) Leu53Leu, Arg213Gly, and Ala40Thr polymorphisms.

EC-SOD	Primers (5'-3')	Restriction enzyme
Leu53Leu	Forward: CGTGACTAAGCCTCACTCTGC	*Alu*I
	Reverse: ACTTCAGCAAAGGCGAAGGT	
Arg213Gly	Forward: GGCTGGCCTGCTGCGTGGTGG	*Eco*52I
	Reverse: CCTTGCACTCGCTCTCGCGCG	
Ala40Thr	Forward: GGCTGGCCTGCTGCGTGGTGG	*Hha*I
	Reverse: CCTTGCACTCGCTCTCGCGCG	

Statistical analysis

The data was statistically analyzed using the SPSS statistical software v.17.0 (SPSS Inc., Chicago, IL, USA). The demographic, lifestyle, clinical variables, genotype distributions, and the genotype frequencies of *EC-SOD* Leu53Leu, Arg213Gly, and Ala40Thr polymorphisms were analyzed using the chi-squared test. The Pearson chi-square test was used to assess deviations of the genotype distributions of *EC-SOD* Leu53Leu, Arg213Gly, and Ala40Thr from the Hardy-Weinberg equilibrium (HWE). The associations between the *EC-SOD* Leu53Leu, Arg213Gly, and Ala40Thr polymorphisms and risk of T2DM were estimated by multiple logistic regression analysis, and the results were expressed by odds ratios (ORs) and 95% confidence intervals (95%CI) adjusting for potential confounding factors.

RESULTS

The demographic and clinical characteristics of the patients and controls are summarized in Table 2. Analysis using the Student *t*-test or chi-square test revealed that the patients with T2DM were more likely to have higher BMI ($t = 15.59$, $P = 0.17$), fasting glucose levels ($t = 17.76$, $P < 0.001$) and fasting insulin levels ($t = 5.94$, $P < 0.001$), and have a history of hypertension status ($\chi^2 = 19.33$, $P < 0.001$).

Table 2. Demographic and clinical characteristics of type 2 diabetes mellitus patients and control subjects.

Characteristics	Patients (N = 256)	%	Controls (N = 324)	%	*t* test or χ^2 test	P value
Age (years)	52.65 ± 10.25		53.91 ± 10.46			
Gender					1.45	0.07
Female	99	38.67	141	43.52		
Male	157	61.33	183	56.48	1.38	0.24
BMI (kg/m^2)	27.54 ± 2.47		24.24 ± 2.58		15.59	<0.001
Tobacco smoking						
No	150	58.59	208	64.20		
Yes	106	41.41	116	35.80	1.90	0.17
Alcohol consumption						
No	168	65.63	222	68.52		
Yes	88	34.38	102	31.48	0.54	0.46
Hypertension						
No	158	61.72	254	78.40		
Yes	98	38.28	70	21.60	19.33	<0.001
Fasting glucose (mM)	9.43 ± 3.64		4.81 ± 2.62		17.76	<0.001
Fasting insulin (mM)	58.65 ± 16.22		50.74 ± 15.71		5.94	<0.001

The genotype distributions of the *EC-SOD* Leu53Leu, Arg213Gly and Ala40Thr polymorphisms are summarized in Table 3. We observed a statistically significant difference in the genotype frequencies of *EC-SOD* Ala40Thr (χ^2 = 13.26, P = 0.001) between T2DM patients and control subjects; however, the genotype distributions of *EC-SOD* Leu53Leu (χ^2 = 0.29, P = 0.59) and Arg213Gly (χ^2 = 0.79, P = 0.67) did not differ significantly between the study groups. The genetic distributions of *EC-SOD* Arg213Gly and Ala40Thr were in accordance with the HWE in both T2DM patients and controls; however, those of *EC-SOD* Leu53Leu polymorphism did not.

Table 3. Genotype frequencies of the extracellular superoxide dismutase (*EC-SOD*) Leu53Leu, Arg213Gly, and Ala40Thr polymorphisms in the patient and control groups.

EC-SOD	Patients (N = 256)	%	Controls (N = 324)	%	χ^2 test	P value	P value for Hardy-Weinberg equilibrium	
							Patients	Controls
Leu53Leu								
CC	178	69.53	232	71.60				
CT	78	30.47	92	28.40				
TT	0	0.00	0	0.00	0.29	0.59	0.004	0.002
Arg213Gly								
AA	104	40.63	141	43.52				
AG	121	47.27	150	46.30				
GG	31	12.11	33	10.19	0.79	0.67	0.64	0.45
Ala40Thr								
GG	91	35.55	154	47.53				
GA	124	48.44	144	44.44				
AA	41	16.02	26	8.02	13.26	0.001	0.91	0.34

The association between *EC-SOD* Leu53Leu, Arg213Gly and Ala40Thr polymorphisms and risk of T2DM was summarized in Table 4. Individuals carrying the GA (adjusted OR = 1.46; 95%CI = 1.01-2.11) and AA (adjusted OR = 2.67; 95%CI = 1.48-4.85) genotypes of *EC-SOD* Ala40Thr expressed an increased risk of T2DM, compared to the GG genotype (Table 4). In the dominant model, individuals with the GA+AA genotype of *EC-SOD* Ala40Thr was correlated with an elevated risk of onset of T2DM, compared to the GG genotype (OR = 1.64; 95%CI = 1.16-2.33), while in the recessive model, the AA genotype of *EC-SOD* Ala40Thr showed a 2.19-fold higher risk of developing T2DM compared to the GG+GA genotype. No significant association was observed between the *EC-SOD* Leu53Leu and Arg213Gly polymorphisms and the risk of T2DM in this population.

DISCUSSION

In this study, we investigated the association between the *EC-SOD* Leu53Leu, Arg213Gly, and Ala40Thr polymorphisms and susceptibility to T2DM in a Chinese population. The results of this study revealed that the mutant genotypes of the *EC-SOD* Ala40Thr polymorphism were associated with an elevated risk of developing T2DM compared to the wide-type genotype in the co-dominant, dominant, and recessive models.

The *EC-SOD* is located on the chromosome 4p16.3-q21 with 5900 bp in length, including 3 exons and 2 introns. Genetic mutation of *EC-SOD* Arg213Gly could reduce the affinity of EC-SOD towards heparin and the endothelial surface; the expression level of EC-SOD is reduced in blood vessels, and the cellular activity of Cu-Zn-SOD is decreased under the condition of hyperglycemia; reversely, the low expression of EC-SOD could promote

the glycosylation of proteins in blood vessels (Li et al., 2011; Sook Chung et al., 2012); polymorphism of EC-SOD gene is relative to the high EC-SOD level in serum (Yamada et al., 1997); therefore, this polymorphism could elevate the risk of T2DM (Kimura et al., 2003; Tamai et al., 2006; Naganuma et al., 2008; Kim, 2013; Cui et al., 2014). A G-to-A transition mutation at *EC-SOD* Ala40Thr damages the tetramerization of the SOD3 enzyme, and inhibits the secretion of the protein (Cui et al., 2014; Pongsavee, 2015). Therefore, these two genetic mutations may contribute to the development of T2DM.

Table 4. Relationship between the extracellular superoxide dismutase (*EC-SOD*) Leu53Leu, Arg213Gly, and Ala40Thr polymorphisms and development of type 2 diabetes mellitus.

EC-SOD	Patients (N = 256)	%	Controls (N = 324)	%	OR (95%CI)[1]	P value
Leu53Leu						
Co–dominant						
CC	178	69.53	232	71.60	1.0 (Ref.)	-
CT	78	30.47	92	28.40	1.11 (0.76-1.61)	0.59
TT	0	0.00	0	0.00	-	-
Dominant						
CC	178	69.53	232	71.60	1.0 (Ref.)	-
CT+TT	78	30.47	92	28.40	1.11 (0.76-1.61)	0.59
Recessive						
CC+CT	256	100.00	324	100.00	1.0 (Ref.)	-
TT	0	0.00	0	0.00	-	-
Arg213Gly						
Co–dominant						
AA	104	40.63	141	43.52	1.0 (Ref.)	-
AG	121	47.27	150	46.30	1.09 (0.76-1.57)	0.61
GG	31	12.11	33	10.19	0.5	0.78
Dominant						
AA	104	40.63	141	43.52	1.0 (Ref.)	-
AG+GG	152	59.38	183	56.48	1.13 (0.80-1.59)	0.48
Recessive						
AA+AG	225	87.89	291	89.81	1.0 (Ref.)	-
GG	31	12.11	33	10.19	1.21 (0.70-2.11)	0.46
Ala40Thr						
Co–dominant						
GG	91	35.55	154	47.53	1.0 (Ref.)	-
GA	124	48.44	144	44.44	1.46 (1.01-2.11)	0.04
AA	41	16.02	26	8.02	2.67 (1.48-4.85)	0.001
Dominant						
GG	91	35.55	154	47.53	1.0 (Ref.)	-
GA+AA	165	64.45	170	52.47	1.64 (1.16-2.33)	0.004
Recessive						
GG+GA	215	83.98	298	91.98	1.0 (Ref.)	-
AA	41	16.02	26	8.02	2.19 (1.26-3.84)	0.003

[1]Adjusted for gender, age, body mass index (BMI), hypertension, and fasting glucose and fasting insulin levels.

So far, previous studies have reported the role of the *EC-SOD* Leu53Leu, Ala40Thr, and Arg213Gly polymorphisms in the risk of T2DM in different ethnicities (Ukkola et al., 2001; Kimura et al., 2003; Strokov et al., 2003; Zotova et al., 2003; Chu et al., 2005; Tamai et al., 2006; Naganuma et al., 2008; Samoila et al., 2008). Some studies reported a significant correlation between *EC-SOD* polymorphisms and the onset of T2DM. Samoila et al. (2008), in a study conducted in 144 Romanian subjects, reported a correlation between the *EC-SOD* Thr40 allele polymorphism and risk of diabetes and hypertension. Chu et al. (2005), on the other hand, reported that the *EC-SOD* Arg213Gly polymorphism did not exert a significant

protective effect on the arterial pressure, vascular function, or vascular levels of oxidative stress. Tamai et al. (2006), Strokov et al. (2003) and Zotova et al. (2003) all indicated that *EC-SOD* Ala40Thr and *EC-SOD* Arg213Gly genetic variants could influence the susceptibility of developing T2DM. However, Ukkola et al. (2001) countered that polymorphisms in *EC-SOD* had no correlation with the onset of T2DM. The discrepancies in these studies may be attributed to the differences in populations, selection of study subjects, and sample content.

This study was subject to two limitations. Firstly, the study subjects were recruited from among the patients of only one hospital in China; this study design may result in selection bias. Secondly, the sample size included in this study was relatively small; this could account for the low statistical power in determining the differences between the patients and controls. Therefore, further studies in people belonging to other ethnicities, and with larger sample sizes, are required to evaluate the association between *EC-SOD* polymorphisms and the development of T2DM.

In conclusion, the results of our study indicated that the *EC-SOD* Ala40Thr polymorphism was significantly correlated with a higher risk of T2DM, but the *EC-SOD* Leu53Leu and Arg213Gly polymorphisms were not. Therefore, this may be utilized as a biomarker for early screening of T2DM in a Chinese population. These results must be validated by further studies with larger sample sizes.

Conflicts of interest

The authors declare no conflict of interest.

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