Genetic polymorphisms of the *TLR4* gene and their association with susceptibility to type 2 diabetes mellitus in the Chinese population

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ABSTRACT. Toll-like receptor 4 (*TLR4*) is potentially an important gene affecting the susceptibility to type 2 diabetes mellitus (T2DM). The objective of this study was to evaluate whether genetic polymorphisms of the *TLR4* gene are associated with T2DM susceptibility. This potential association was analyzed in 668 T2DM patients and 672 healthy controls by polymerase chain reaction-restriction fragment length polymorphism and DNA sequencing methods. Two novel genetic polymorphisms (g.12375A>G and g.14367G>A) were investigated, and our data support the idea that the g.14367G>A variant significantly increased susceptibility to T2DM in homozygote comparison (AA *vs* GG: OR = 2.396, 95%CI = 1.682-3.413, P < 0.0001), heterozygote comparison (GA *vs* AA: OR = 1.322, 95%CI = 1.050-1.664, P = 0.0175), dominant model (AA/GA *vs* GG: OR = 1.511, 95%CI = 1.217-1.876, P = 0.0002), recessive model (AA *vs* GA/GG: OR = 2.093, 95%CI = 1.496-2.927, P < 0.0001), and allele contrast (A *vs* G: OR = 1.503, 95%CI = 1.279-1.766, P < 0.0001). The allele A of g.14367G>A

variants may contribute to the susceptibility to T2DM. However, we failed to detect a similar significantly increased susceptibility to T2DM in the g.12375A>G variant. Our findings suggest that the g.14367G>A genetic polymorphism of the *TLR4* gene is associated with the susceptibility to T2DM in the population studied.

Key words: Association analysis; Genetic polymorphisms; Susceptibility; Toll-like receptor 4; Type 2 diabetes mellitus

INTRODUCTION

Type 2 diabetes mellitus (T2DM), a complex chronic disease, is a significant global health problem. The incidence of T2DM is increasing rapidly in the world. It is estimated that six people die from the disease worldwide every minute (Wild et al., 2004; Arora et al., 2011). Recently, studies reported that there is a very high T2DM incidence in China where diabetes has become a major public health problem (Yang et al., 2010), with approximately 92.4 million adults with the disease and 148.2 million with prediabetes (Jiang et al., 2013). A large number of previous studies have shown that the human Toll-like receptor 4 (*TLR4*) is potentially an important gene influencing the susceptibility to T2DM (Illig et al., 2003; Rudofsky et al., 2004; Creely et al., 2007; Kim et al., 2008; Buraczynska et al., 2009; Arora et al., 2011; Maldonado-Bernal et al., 2011; Jiang et al., 2013). To date, several single nucleotide polymorphisms (SNPs) have been identified in the human *TLR4* gene (Raby et al., 2002; Kolz et al., 2008; Arora et al., 2011; Jiang et al., 2013), such as Asp299Gly (Rudofsky et al., 2004; Kim et al., 2008; Buraczynska et al., 2009; Arora et al., 2011; Maldonado-Bernal et al., 2011; Jiang et al., 2013) and Thr399Ile (Rudofsky et al., 2004; Kim et al., 2008; Kolz et al., 2008; Buraczynska et al., 2009; Maldonado-Bernal et al., 2011; Jiang et al., 2013). Furthermore, several studies proved that the *TLR4* gene variants were associated with susceptibility to T2DM (Rudofsky et al., 2004; Kim et al., 2008; Kolz et al., 2008; Buraczynska et al., 2009; Arora et al., 2011; Maldonado-Bernal et al., 2011; Jiang et al., 2013). However, the data from these studies are inconsistent, and the exact mechanism is poorly understood. To the best of our knowledge, there are no related studies on the association between the g.12375A>G and g.14367G>A genetic variants in the *TLR4* gene and susceptibility to T2DM. Thus, the objective of the present study was to evaluate whether the g.12375A>G and g.14367G>A genetic variants of the *TLR4* gene influence susceptibility to T2DM in the Chinese population.

MATERIAL AND METHODS

Study population

The study involved 668 patients with T2DM and 672 healthy controls who were consecutively enrolled between January 2010 and August 2012 in the People's Hospital of Liaocheng (People's Hospital of Liaocheng, Liaocheng, Shandong Province, China). All subjects were unrelated Han Chinese. All the healthy controls were matched with the patient population in terms of age, gender, and residence area, excluding those with a history of diabetes or other medical diseases. The T2DM patients were diagnosed by doctors according to the 2003 American Diabetes Association criteria (Genuth et al., 2003). Demographic and general characteristics on gender, age, body

mass index, diabetes duration, systolic blood pressure, and diastolic blood pressure are summarized in Table 1. The laboratory analytical methods for measuring triglycerides, total cholesterol, fasting plasma glucose (FPG), low-density lipoprotein cholesterol, and high-density lipoprotein cholesterol were described previously (Jing et al., 2011). The study protocol was approved by the Ethics Committee of the People's Hospital of Liaocheng, and all subjects gave written informed consent.

Genotyping

Genomic DNA was isolated from peripheral blood of patients and controls using a QIAamp DNA kit (Qiagen, Hilden, Germany). The polymerase chain reaction (PCR) primers were designed by the Primer Premier 5.0 software. The primer sequences, annealing temperature, and fragment sizes and region are presented in Table 2. The *TLR4* genotype variants were detected by the PCR-restriction fragment length polymorphism (PCR-RFLP) technique and verified by DNA sequencing (ABI3730xl DNA Analyzer, Applied Biosystems, Foster City, CA, USA). PCRs were performed in a total volume of 20 µL, including 50 ng template DNA, 1X buffer (100 mM Tris-HCl, pH 8.3; 500 mM KCl), 0.25 µM primers, 2.0 mM $MgCl_2$, 0.25 mM dNTPs, and 0.5 U *Taq* DNA polymerase (Promega, Madison, WI, USA). PCR conditions were as follows: an initial denaturation at 94°C for 5 min, followed by 33 cycles at 94°C for 32 s, at the corresponding temperature (as presented in Table 2) for 32 s, at 72°C for 32 s, and a final extension at 72°C for 6 min. The PCR amplified products were digested with 5 U of restriction enzymes (MBI Fermentas, St. Leon-Rot, Germany; Table 2) at 37°C for 10 h following the manufacturer protocol. Digestion products were separated by electrophoresis on a 3% agarose gel containing 0.5 µg/mL ethidium bromide and visualized under UV light.

Statistical analysis

Statistical analyses were performed with the Statistical Package for Social Sciences software (SPSS, Windows version, release 15.0; SPSS Inc., Chicago, IL, USA). The chi-square (χ^2) test was used to evaluate Hardy-Weinberg equilibrium in allelic and genotypic frequencies, and to compare the differences of demographic and general characteristics between T2DM patients and healthy controls. Odds ratios (ORs) with their 95% confidence intervals (95%CIs) for the association analysis between allelic and genotypic frequencies and T2DM susceptibility were evaluated using multivariate logistic regression models. A P value < 0.05 was defined as a statistically significant difference.

RESULTS

General characteristics of subjects

A total of 1340 subjects were collected in this case-control study, including 668 T2DM cases and 672 healthy controls. The demographic and general characteristics of subjects are shown in Table 1. There was no significant difference between T2DM patients and healthy controls with regard to gender and age (P = 0.5146 and P = 0.3279, respectively). We found that the clinical characteristics and anthropometric data were significantly different between the T2DM patients and healthy controls, as expected (P < 0.001; Table 1).

Table 1. Demographic and general characteristics of type 2 diabetes mellitus (T2DM) cases and healthy controls.

Characteristics	T2DM cases (N = 668)	Healthy controls (N = 672)	P value
Gender (male/female)	342/326	356/316	0.5146
Age (years)	54.32 ± 10.21	55.28 ± 9.87	0.3279
Body mass index (kg/m²)	27.5 ± 4.1	24.31 ± 3.6	<0.001
Diabetes duration (years)	8.9 ± 6.8	NA	ND
Systolic blood pressure (mmHg)	124.6 ± 16.5	113 ± 12.8	<0.001
Diastolic blood pressure (mmHg)	78.5 ± 8.9	75.2 ± 6.7	<0.001
Fasting plasma glucose (mM)	9.21 ± 2.11	5.18 ± 0.48	<0.001
Total cholesterol (mM)	4.98 ± 1.21	4.29 ± 1.11	<0.001
Triglycerides (mM)	1.92 ± 1.62	1.33 ± 0.47	<0.001
High-density lipoprotein cholesterol (mM)	1.12 ± 0.18	1.36 ± 0.21	<0.001
Low-density lipoprotein cholesterol (mM)	2.93 ± 0.19	2.03 ± 0.31	<0.001

Data are reported as means ± SD or numbers (%). NA = not applicable; ND = not determined.

Genotyping of *TLR4* SNPs

Two novel genetic variants (g.12375A>G and g.14367G>A) were detected by PCR-RFLP and DNA sequencing methods in this study. According to the sequence analysis, we found that the g.12375A>G variant was an A to G mutation in intron 2 of the *TLR4* gene. As for the g.14367G>A variant, sequence analysis detected that this was a G to A mutation in exon 3 of the *TLR4* gene, and that this caused a glutamic (Glu) to lysine (Lys) amino acid replacement (p.Glu474Lys, GenBank ID: NG_011475.1, NM_138554.4, and NP_612564.1). The PCR products of the g.12375A>G polymorphism were digested with the *Tsp*EI restriction enzyme, electrophoresed, and divided into three genotypes: AA (177 and 50 bp), AG (227, 177, and 50 bp), and GG (227 bp; Table 2). Similarly, PCR products of the g.14367G>A polymorphism were digested with the *Taq*I restriction enzyme and divided into three genotypes: GG (180 and 78 bp), GA (258, 180, and 78 bp), and AA (258 bp, Table 2). Table 3 shows the allele and genotype frequencies of the g.12375A>G and g.14367G>A variants in both T2DM patients and healthy subjects. Alleles A and G were predominant alleles of g.12375A>G and g.14367G>A in the subjects studied, respectively. The genotype frequencies of the two variants in the populations studied did not significantly deviate from Hardy-Weinberg equilibrium (for T2DM patients, $\chi^2 = 0.1684$ and $\chi^2 = 4.8824$, respectively; for healthy controls, $\chi^2 = 0.5399$ and $\chi^2 = 0.0540$, respectively; all P values > 0.05). For the g.14367G>A variant, the allele frequencies of T2DM patients (G: 61.60%; A: 38.40%) were highly significantly different from healthy controls (G: 70.68%; A: 29.32%, $\chi^2 = 24.6864$, P < 0.0001). In addition, the genotype frequencies in T2DM patients were not consistent with the healthy controls, with the differences being statistically significant ($\chi^2 = 24.8269$, P < 0.0001, Table 3). For the g.12375A>G variant, the allele and genotype frequencies of T2DM patients were not consistent with healthy controls, but the differences were not statistically significant ($\chi^2 = 1.2491$, P = 0.2637, and $\chi^2 = 1.2775$, P = 0.5280; Table 3).

Table 2. Primer and PCR-RFLP analysis for genotyping the *TLR4* gene polymorphism.

SNPs	Primer sequences	Annealing temperature (°C)	Amplification fragment (bp)	Region	Restriction enzyme	Genotype (bp)
g.12375A>G	5'-CATTGGCTTGCTGTTTGCTGG-3' 5'-GGAGGAATCATGACAAATAGCTTCC-3'	64.0	227	Intron 2	*Tsp*EI	AA: 177, 50 AG: 227, 177, 50 GG: 227
g.14367G>A	5'-CATGAGTTCAAACTTCTTGGG-3' 5'-GTCAAGTTTCTCAGCTCTGTGAAG-3'	59.0	258	Exon 3	*Taq*I	GG: 180, 78 GA: 258, 180, 78 AA: 258

SNPs = single nucleotide polymorphisms; PCR-RFLP = polymerase chain reaction-restriction fragment length polymorphism.

Table 3. Genotype and allele frequencies of the *TLR4* gene polymorphism in cases and controls.

Group	g.12375A>G							g.14367G>A						
	Genotype frequencies			Allele frequencies				Genotype frequencies			Allele frequencies			
	AA	AG	GG	A	G	χ^2	P	GG	GA	AA	G	A	χ^2	P
Cases (N = 668)	256 (0.3832)	311 (0.4656)	101 (0.1512)	823 (0.6160)	513 (0.3840)	0.1684	0.9193	267 (0.3997)	289 (0.4326)	112 (0.1677)	823 (0.6160)	513 (0.3840)	4.8824	0.0871
Controls (N = 672)	277 (0.4122)	302 (0.4494)	93 (0.1384)	856 (0.6369)	488 (0.3631)	0.5399	0.7634	337 (0.5015)	276 (0.4107)	59 (0.0878)	950 (0.7068)	394 (0.2932)	0.0540	0.9734
Total (N = 1340)	533 (0.3978)	613 (0.4574)	194 (0.1448)	1679 (0.6265)	1001 (0.3735)	0.6792	0.7120	604 (0.4508)	565 (0.4216)	171 (0.1276)	1773 (0.6616)	907 (0.3384)	4.5699	0.1012
	$\chi^2 = 1.2775$, P = 0.5280			$\chi^2 = 1.2491$, P = 0.2637				$\chi^2 = 24.8269$, P < 0.0001			$\chi^2 = 24.6864$, P < 0.0001			

TLR4 variants and susceptibility to T2DM

Table 4 shows that the alleles and genotypes from the g.14367G>A variants were statistically associated with susceptibility to T2DM. There was significantly increased susceptibility to T2DM in the homozygote comparison (AA *vs* GG: OR = 2.396, 95%CI = 1.682-3.413, χ^2 = 24.18, P < 0.0001), heterozygote comparison (GA *vs* GG: OR = 1.322, 95%CI = 1.050-1.664, χ^2 = 5.65, P = 0.0175), dominant model (AA/GA *vs* GG: OR = 1.511, 95%CI = 1.217-1.876, χ^2 = 14.02, P = 0.0002), recessive model (AA *vs* GA/GG: OR = 2.093, 95%CI = 1.496-2.927, χ^2 = 19.19, P < 0.0001), and allele contrast (A *vs* G: OR = 1.503, 95%CI = 1.279-1.766, χ^2 = 24.69, P < 0.0001; Table 4). However, as shown in Table 4, for the g.12375A>G variant there was no significant difference in susceptibility to T2DM in any genetic model (P > 0.05; Table 4).

Table 4. Association between the risk of type 2 diabetes mellitus (T2DM) and *TLR4* gene polymorphisms.

SNPs	Comparisons	Test of association		
		OR (95%CI)	χ^2 value	P value
g.12375A>G	Homozygote comparison (GG *vs* AA)	1.175 (0.846-1.663)	0.93	0.3361
	Heterozygote comparison (AG *vs* AA)	1.114 (0.883-1.406)	0.83	0.3611
	Dominant model (GG/AG *vs* AA)	1.129 (0.907-1.405)	1.17	0.2787
	Recessive model (GG *vs* AG/AA)	1.109 (0.818-1.504)	0.44	0.5054
	Allele contrast (G *vs* A)	1.093 (0.935-1.279)	1.25	0.2637
g.14367G>A	Homozygote comparison (AA *vs* GG)	2.396 (1.682-3.413)	24.18	<0.0001
	Heterozygote comparison (GA *vs* GG)	1.322 (1.050-1.664)	5.65	0.0175
	Dominant model (AA/GA *vs* GG)	1.511 (1.217-1.876)	14.02	0.0002
	Recessive model (AA *vs* GA/GG)	2.093 (1.496-2.927)	19.19	0.0001
	Allele contrast (A *vs* G)	1.503 (1.279-1.766)	24.69	<0.0001

DISCUSSION

T2DM is a complex polygenic disease resulting from complex interactions between multiple environmental and genetic factors (Permutt et al., 2005; Freeman and Cox, 2006; Liu et al., 2011; Al-Daghri et al., 2012; Gan and Yang, 2012; Gonul et al., 2012). Genetic factors play key roles in the pathogenesis of T2DM. *TLR4* is one of the most important candidate genes for T2DM, and genetic variants in the *TLR4* gene have been reported to be associated with T2DM susceptibility (Illig et al., 2003; Rudofsky et al., 2004; Creely et al., 2007; Kim et al., 2008; Buraczynska et al., 2009; Arora et al., 2011; Maldonado-Bernal et al., 2011; Jiang et al., 2013). In this study, we demonstrated an association between the g.12375A>G and g.14367G>A genetic variants of the *TLR4* gene and the risk of T2DM in the Chinese Han population. Our data showed the distribution differences in the allele and genotype frequencies between T2DM patients and healthy controls (Table 3). In addition, the g.14367G>A genetic variant was strongly associated with increased susceptibility to T2DM in the Chinese Han population. The allele A

could contribute to the risk of occurrence of T2DM. In contrast, there was no significantly increased susceptibility to T2DM in the g.12375A>G variants (Table 4). To date, several studies have been investigating the role of *TLR4* gene polymorphisms on the risk of T2DM, but none included the g.12375A>G and g.14367G>A polymorphisms. Most studies have focused on two SNPs, Asp299Gly (Rudofsky et al., 2004; Kim et al., 2008; Buraczynska et al., 2009; Arora et al., 2011; Maldonado-Bernal et al., 2011; Jiang et al., 2013) and Thr399Ile (Rudofsky et al., 2004; Kim et al., 2008; Kolz et al., 2008; Buraczynska et al., 2009; Maldonado-Bernal et al., 2011; Jiang et al., 2013). However, the results from these studies are inconclusive. Our findings suggested that the g.12375A>G and g.14367G>A genetic variants in the *TLR4* gene are associated with increased susceptibility to T2DM in the Chinese population. Results from this study should assist in understanding the role of the *TLR4* gene in T2DM.

In summary, this is the first study evaluating the association between the g.12375A>G and g.14367G>A genetic variants of the *TLR4* gene and susceptibility to T2DM. Our findings suggest that the g.14367G>A genetic polymorphism of the *TLR4* gene is strongly associated with susceptibility to T2DM, and the *TLR4* genetic variants may serve as molecular marker for assessing the risk of T2DM. However, further studies are required to determine if these results apply to larger populations of different ethnicities.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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