Genetic analysis of the *ELOVL6* gene polymorphism associated with type 2 diabetes mellitus

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Abstract

Recent animal studies have indicated that overexpression of the elongation of long-chain fatty acids family member 6 (*Elovl6*) gene can cause insulin resistance and β-cell dysfunction. These are the major factors involved in the development of type 2 diabetes mellitus (T2DM). To identify the relationship between single nucleotide polymorphisms (SNP) of *ELOVL6* and T2DM pathogenesis, we conducted a case-control study of 610 Han Chinese individuals (328 newly diagnosed T2DM and 282 healthy subjects). Insulin resistance and islet first-phase secretion function were evaluated by assessment of insulin resistance in a homeostasis model (HOMA-IR) and an arginine stimulation test. Three SNPs of the *ELOVL6* gene were genotyped with polymerase chain reaction-restriction fragment length polymorphism, with DNA sequencing used to confirm the results. Only genotypes TT and CT of the *ELOVL6* SNP rs12504538 were detected in the samples. Genotype CC was not observed. The T2DM group had a higher frequency of the C allele and the CT genotype than the control group. Subjects with the CT genotype had higher HOMA-IR values than those with the TT genotype. In addition, no statistical significance was observed between the genotype and allele frequencies of the control and T2DM groups for SNPs rs17041272 and rs6824447. The study indicated that the *ELOVL6* gene polymorphism rs12504538 is associated with an increased risk of T2DM, because it causes an increase in insulin resistance.

Key words: Elongation of long-chain fatty acids family member 6; Single nucleotide polymorphism; Type 2 diabetes mellitus; Insulin resistance; β-cell function

Introduction

It is generally accepted that insulin resistance and β-cell dysfunction are major factors involved in the development of type 2 diabetes mellitus (T2DM) (1). One of the genes involved is the elongation of long-chain fatty acids family member 6 (*ELOVL6*) gene, located on chromosome 4q25. It encodes a microsomal enzyme that catalyzes the elongation of saturated and monounsaturated fatty acids with 12, 14, and 16 carbons (2,3). Recent studies have indicated that overexpression of *Elovl6* contributes to β-cell dysfunction (4). A deficiency in *Elovl6* increases insulin sensitivity and decreases insulin resistance (5). These facts indicate the possible involvement of *Elovl6* in the development of T2DM.

Studies on the role of *Elovl6* have been performed in animal models (3,6) and *in vitro* (7). To date, only one European study has been carried out to explore the possible association between insulin resistance and the genetic variation of *ELOVL6* in a human population (8).

There are no comparable data available on Asian populations and no study has demonstrated the relationship between *ELOVL6* and islet function.

In this study, we analyzed three single nucleotide polymorphisms (SNPs), rs12504538, rs17041272, and rs6824447, to identify the relationship between *ELOVL6* and T2DM pathogenesis. We have previously found that rs12504538 is associated with gout (Miao ZM, Li CG, Han L, Wang YG, unpublished data), based on the outcome of microarray analyses. A series of studies (9-11) have suggested that elevated serum uric acid levels potentially increase the risk of T2DM. We investigated whether genes or loci that contribute to gout could also play a role in the pathogenesis of T2DM. An additional aim was to verify that, in a Chinese Han population, SNPs rs17041272 and rs6824447 are associated with T2DM, as reported in a previous European study (8).

Material and Methods

Study population

A total of 610 age-matched subjects (328 newly diagnosed T2DM patients and 282 healthy control individuals) were recruited from the Affiliated Hospital of the Medical College, Qingdao University (Shandong, China), from January 2009 to June 2012. All patients and controls were natives of the Shandong Province. Geographic matching of patients and controls to the Shandong Province was the best method of ensuring the same ancestral origin of the two groups. This can effectively eliminate the adverse impact of population stratification (12). The study protocol conformed to the ethics guidelines of the 1975 Declaration of Helsinki and was approved by the Ethics Committee of the Affiliated Hospital of Medical College, Qingdao University. Informed consent was obtained from all participants. The diagnosis and classification of T2DM was based on the criteria published by the American Diabetes Association in 2010 (13). Patients with other endocrine diseases and acute, severe diabetic complications were excluded. Healthy individuals, with no personal or familial history of T2DM or any other serious illness, were recruited for the control group.

Metabolic testing

Subjects underwent metabolic testing after an overnight fast (8-12 h). All participants had plasma levels of fasting blood glucose (FBG), total cholesterol (TC), and triglycerides (TG) measured using a Sysmex Chemix-180 automatic biochemistry analyzer (Sysmex Corporation of America, USA). An arginine stimulation test was also performed as described below.

A dose of 5 g arginine hydrochloride (Shanghai Biochemical Pharmaceutical Factory, China) was infused into subjects within 30-60 s. Blood samples were collected at -10, 2, 4, and 6 min from infusion. Serum insulin was measured using a Roche Elecsys 2010 automatic electro-chemiluminescence immunoassay analyzer (Hoffmann-La Roche Ltd., Switzerland).

Laboratory methods

Blood samples were collected from the subjects and stored at -20°C. Genomic DNA was extracted from peripheral blood leukocytes using a whole blood genome extraction kit (Beijing Tiangen Biotech Corporation, China).

Information on the polymerase chain reaction (PCR) conditions for the selected SNPs is shown in Table 1. The reactions contained 0.5 μL genomic DNA, 0.8 μL dNTPs, 0.15 μL of each primer, 1.5 μL MgCl$_2$, and 0.15 μL Taq DNA polymerase (5 U/μL), in a total reaction volume of 15 μL. Amplification was performed using the following program: 95°C for 5 min, 20 cycles of 95°C for 30 s, 68°C for 45 s, and 72°C for 1 min, with the annealing temperature decreased by 0.5°C/cycle. This was followed by 20 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 40 s, with a final single extension step of 72°C for 6 min. The PCR products were digested with three different restriction enzymes (Fermentas Life Sciences, Lithuania): *Rca*I (rs12504538), *Msp*I (rs17041272), and *Alu*I (rs6824447). The fragments were separated by electrophoresis on a 3% ethidium bromide-stained agarose gel and visualized under ultraviolet illumination (Vilber Lourmat, France). In addition, all the samples were tested with DNA sequencing to confirm the genotype.

Calculations

We calculated the body mass index (BMI) values of the subjects to assess obesity. Insulin resistance was measured using a homeostasis model (HOMA-IR), as follows: HOMA-IR = FBG × fasting insulin (FIns)/22.5 (14). Basic insulin secretion was calculated using β-cell function of homeostasis model assessment (HOMA-β), as follows: HOMA-β = 20 × FIns/(FBG-3.5) (14). Early

Table 1. Information for the SNPs of the *ELOVL6* gene studied here.

SNPs	Forward primer sequence	Reverse primer sequence	PCR product size (bp)	Restriction enzyme (incubation temperature, °C)	RFLP (bp)
rs12504538	5′-CATACCCGACCCA TACAATACT-3′	5′-TTAGGAGGGTTT AGGGAAAGTTA-3′	295	*Rca*I (30°)	TT: 295 CC: 186, 109 CT: 295, 186, 109
rs17041272	5′-gcggtcccaaaagggtcagt TCTTTTTAAGGTTTATGC TCACTCC-3′	5′-gcggtcccaaaagggtcagt GAAGGACCCATTGGA GGCT-3′	150	*Msp*I (37°)	CC: 150 GG: 105, 45 CG: 150, 105, 45
rs6824447	5′-gcggtcccaaaagggtcagt TCCCCCAAGAGTTTGGA AA-3′	5′-gcggtcccaaaagggtcagt TCCACCTAATTTTTAA AAGCAGAG-3′	147	*Alu*I (30°)	AA: 147 GG: 103, 44 AG: 147, 103, 44

The underlined base in the forward primer is different from that of the original sequence and serves as the introduction of a recognition site for the restriction enzyme *Msp*I; gcggtcccaaaagggtcagt were inserted as protective bases, which could increase restriction enzyme cutting activity. SNP: single nucleotide polymorphism; RFLP: restriction fragment length polymorphism.

phase insulin secretion was assessed by acute insulin response to arginine (AIRarg) and was defined as the average of the three insulin values measured at 2, 4, and 6 min after, minus the value measured 10 min before, infusion with arginine hydrochloride (15).

Statistical analysis

The Statistical Package for Social Sciences (SPSS) version 17.0 was used for all the statistical analyses. The Student *t*-test was used to determine the statistical significance of differences between continuous variables. The chi-square test was used for analysis of the distribution of the allele and genotype frequencies, with the corresponding P values adjusted using the Bonferroni correction. Hardy-Weinberg equilibrium was calculated using the chi-square test to compare the observed genotype frequencies with the expected genotype frequencies. P values <0.05 were considered to be significant.

Results

Participant characteristics

Table 2 shows the clinical characteristics of the T2DM patients (n = 328) and control subjects (n = 282). The T2DM group presented a higher mean TG, FBG and FIns levels, and HOMA-IR value (P<0.05), and lower AIRarg

Table 2. Demographic and clinical characteristics of the study population.

| Parameter	Control (n = 282)	T2DM (n = 328)	P
Age (years)	50.6 ± 13.6	52.3 ± 10.9	0.197
BMI (kg/m^2)	24.7 ± 5.3	25.7 ± 3.4	0.162
Waist circumference (cm)	85.9 ± 8.7	83.7 ± 23.5	0.329
TC (mM)	5.4 ± 0.9	5.1 ± 1.0	0.113
TG (mM)	1.3 ± 0.7	1.9 ± 1.2	<0.001
FBG (mM)	5.6 ± 1.6	8.3 ± 2.7	<0.001
FIns (mU/L)	11.9 ± 8.7	21.6 ± 43.7	0.010
Ins (2 min; mU/L)	74.7 ± 56.1	67.5 ± 51.3	0.323
Ins (4 min; mU/L)	63.4 ± 51.8	58.5 ± 47.1	0.467
Ins (6 min; mU/L)	48.1 ± 41.3	46.1 ± 46.6	0.740
AIRarg (mU/L)	50.0 ± 41.7	35.7 ± 30.3	0.003
HOMA-IR	3.1 ± 3.0	8.5 ± 18.8	0.001
HOMA-β	142.6 ± 118.5	192.2 ± 281.8	0.067

Data are reported as means ± SD. BMI: body mass index; TC: total cholesterol; TG: triglycerides; FBG: fasting blood glucose; FIns: fasting insulin; Ins (2 min), Ins (4 min), and Ins (6 min): plasma insulin levels in response to bolus injections of arginine at 2, 4, and 6 min; AIRarg: acute insulin response to arginine; HOMA-IR: insulin resistance of homeostasis model assessment; HOMA-β: β-cell function of homeostasis model assessment; T2DM: type 2 diabetes mellitus. Statistical analysis was carried out with the Student *t*-test.

than the control group. There was no significant difference between the two groups in the following variables: age, BMI, waist circumference, TC, and HOMA-β (P>0.05).

Distribution of *ELOVL6* genotypes and alleles in T2DM patients and controls

The distribution of genotypes at the rs12504538, rs17041272, and rs6824447 loci in both groups showed no significant difference from the Hardy-Weinberg equilibrium values (P>0.05 in T2DM and control groups).

The possible genotypes at the rs12504538 locus were TT, CT, and CC (T is thymine, C is cytosine), but only TT and CT were detected in the samples (Figure 1). The T2DM group had higher frequencies of the C allele and CT genotype than the control group (23.8 *vs* 14.9% and 11.9 *vs* 7.4%, respectively). The differences were significant (P<0.017, 0.05/3) after Bonferroni's correction (Table 3). No statistical differences were observed in allele and genotype frequencies between the T2DM and control groups (Table 3) for SNPs rs17041272 (Figure 2) and rs6824447 (Figure 3).
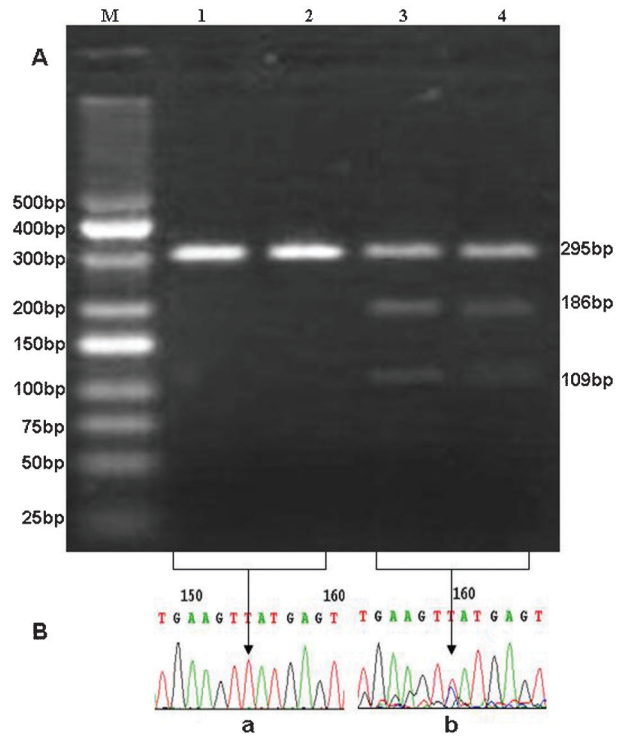
Figure 1. Genotypes of the *ELOVL6* single nucleotide polymorphism (SNP) rs12504538. *A*, Restriction fragment length polymorphism analyses for determination of the rs12504538 genotype (digested by *Rca*I). The TT genotype shows a band of 295 bp (*lanes 1* and *2*) and the CT genotype shows 3 bands of 295, 186, and 109 bp (*lanes 3* and *4*). *B*, Nucleotide sequences surrounding the SNP rs12504538. *a*, TT genotype; *b*, CT genotype. *Lane M*: DNA marker.

Table 3. Distribution of the *ELOVL6* genotypes and alleles in T2DM patients and controls.

SNP	n	Genotype frequency [n (%)]			Allele frequency [n (%)]	
rs12504538		TT	CT	CC	T	C
Control	282	240 (85.1)	42 (14.9)	0 (0)	522 (92.6)	42 (7.4)
T2DM	328	250 (76.2)	78 (23.8)	0 (0)	578 (88.1)	78 (11.9)
			$\chi^2 = 7.578$, P = 0.006		$\chi^2 = 6.752$, P = 0.009	
rs17041272		CC	CG	GG	C	G
Control	282	216 (76.6)	60 (21.3)	6 (2.1)	492 (87.2)	72 (12.8)
T2DM	328	244 (74.4)	80 (24.4)	4 (1.2)	568 (86.6)	88 (13.4)
			$\chi^2 = 1.503$, P = 0.472		$\chi^2 = 0.112$, P = 0.738	
rs6824447		GG	AG	AA	G	A
Control	282	102 (36.2)	162 (57.4)	18 (6.4)	366 (64.9)	198 (35.1)
T2DM	328	124 (37.8)	168 (51.2)	36 (11.0)	416 (63.4)	240 (36.6)
			$\chi^2 = 4.809$, P = 0.090		$\chi^2 = 0.288$, P = 0.591	

P<0.017 (0.05/3) were significant after Bonferroni's correction. T2DM: type 2 diabetes mellitus.

Association between the rs12504538 polymorphism of the *ELOVL6* gene and T2DM

The clinical characteristics of the enrolled subjects were subsequently compared with genotypes of the rs12504538 polymorphism (TT *vs* CT). Results (Table 4) showed that the CT genotype was significantly associated with higher TG (P = 0.041), TC (P = 0.034), FBG (P = 0.043), FIns (P = 0.046), and HOMA-IR (P = 0.040).

Discussion

To our knowledge, this is the first report to indicate that rs12504538 is relevant to the prevalence of T2DM. Our results showed a significant increase in the frequency of the C allele and the CT genotype for the T2DM group compared with the control group.

Recent murine studies have shown the importance of
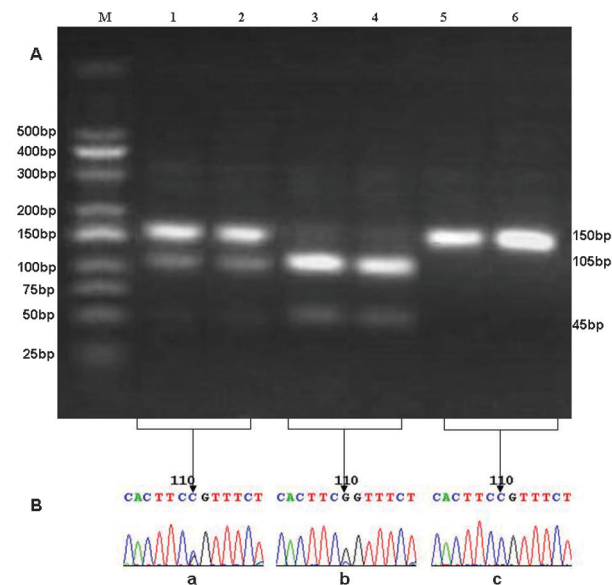
Figure 2. Genotypes of the *ELOVL6* single nucleotide polymorphism (SNP) rs17041272. *A*, Restriction fragment length polymorphism analyses for determination of the rs17041272 genotype (digested by *Msp*I). The GC genotype shows 3 bands of 150, 105, and 45 bp (*lanes 1* and *2*), the GG genotype shows 2 bands of 105 and 45 bp (*lanes 3* and *4*), and the CC genotype shows a band of 150 bp (*lanes 5* and *6*). *B*, Nucleotide sequences surrounding the SNP rs17041272. *a*, GC genotype; *b*, GG genotype; *c*, CC genotype. *Lane M*: DNA marker.
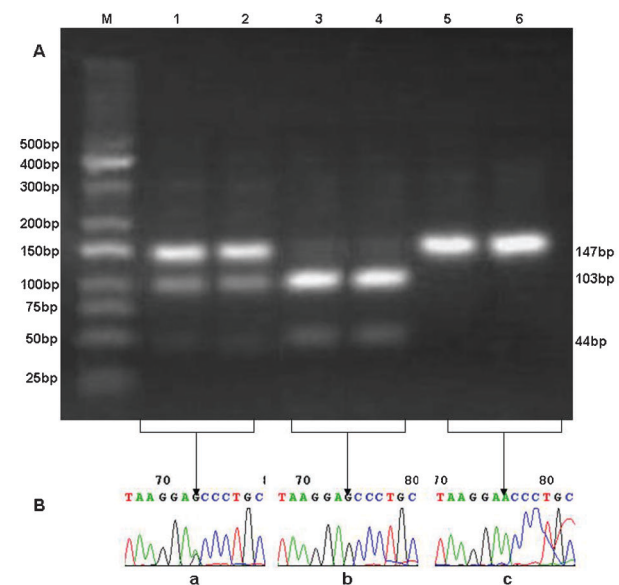
Figure 3. Genotypes of the *ELOVL6* single nucleotide polymorphism (SNP) rs6824447. *A*, Restriction fragment length polymorphism analyses for determination of rs6824447 genotype (digested by *Alu*I). The AG genotype shows 3 bands of 147, 103, and 44 bp (*lanes 1* and *2*), the GG genotype shows 2 bands of 103 and 44 bp (*lanes 3* and *4*), and the AA genotype shows a band of 147 bp (*lanes 5* and *6*). *B*, Nucleotide sequences surrounding the SNP rs6824447. *a*, AG genotype; *b*, GG genotype; *c*, AA genotype. *Lane M*: DNA marker.

Table 4. Clinical characteristics according to genotypes of the rs12504538 polymorphism of the *ELOVL6* gene.

Parameter	TT (n = 250)	CT (n = 78)
Age (years)	50.8 ± 12.3	51.9 ± 11.8
BMI (kg/m^2)	24.1 ± 3.2	25.7 ± 4.3*
Waist circumference (cm)	80.5 ± 19.0	84.4 ± 19.6*
TC (mM)	5.1 ± 0.9	5.4 ± 1.0*
TG (mM)	1.4 ± 0.8	1.8 ± 1.2*
FBG (mM)	6.8 ± 1.6	7.9 ± 2.7*
FIns (mU/L)	13.7 ± 13.1	32.2 ± 67.4*
Ins (2 min; mU/L)	66.6 ± 51.3	81.5 ± 57.6
Ins (4 min; mU/L)	57.9 ± 47.2	68.0 ± 53.4
Ins (6 min; mU/L)	43.4 ± 39.3	57.6 ± 58.1*
AIRarg (mU/L)	42.2 ± 36.3	36.2 ± 32.0
HOMA-IR	4.5 ± 6.0	12.6 ± 28.3*
HOMA-β	164.5 ± 229.9	200.8 ± 245.5

Data are reported as means ± SD. BMI: body mass index; TC: total cholesterol; TG: triglycerides; FBG: fasting blood glucose; FIns: fasting insulin; Ins (2 min), Ins (4 min), and Ins (6 min): plasma insulin levels in response to bolus injections of arginine at 2, 4, and 6 min; AIRarg: acute insulin response to arginine; HOMA-IR: insulin resistance of homeostasis model assessment; HOMA-β: β-cell function of homeostasis model assessment. *P<0.05 compared to TT (Student *t*-test).

Elovl6 in energy metabolism and insulin sensitivity. Mice with a targeted deletion of *Elovl6* do not develop diet-induced insulin resistance, despite being obese and showing hepatosteatosis. This is partially due to the restoration of hepatic insulin receptor substrate-2 and suppression of hepatic protein kinase Cε, which results in the restoration of Akt phosphorylation (6). Reduced *Elovl6* expression may play a protective role in β-cell function. Knockdown of *Elovl6* limits the elongation of palmitate to stearate, which instead allows palmitate to be desaturated to palmitoleate, a potentially less lipotoxic fatty acid. This results in the attenuation of palmitate-induced endoplasmic reticulum stress and apoptosis in pancreatic β-cells (4). Therefore, the *Elovl6* gene is closely associated with insulin resistance and β-cell dysfunction.

In order to test the function of the *ELOVL6* gene in humans, we conducted this study in a Chinese Han population. We calculated insulin resistance using a homeostasis model and observed that the CT genotype of rs12504538 was associated with higher FBG, BMI, TG, TC, FIns, and HOMA-IR values than the TT genotype. This indicated that the CT genotype has the potential to increase the risk of T2DM by increasing insulin resistance through an effect on fatty acid metabolism. However, no association was observed between the rs12504538 polymorphism and AIRarg. Therefore, the relationship between *ELOVL6* and β-cell function needs further investigation.

No statistical significance was observed between the genotype and allele frequencies of the control and T2DM groups for the rs17041272 and rs6824447 SNPs. This is contradictory to the Spanish data reported by Morcillo et al. (8), in which carriers of the minor allele of rs17041272 had a higher risk of having a high HOMA-IR value. Carriers of the minor allele of rs6824447 had a lower risk of being insulin resistant. This led to the conclusion that these *ELOVL6* gene polymorphisms are related to insulin sensitivity. The differences between the two studies are partially due to racial genetic differences. It is well known that there are differences between races in genetic background and living environment. It may even be that the same gene locus plays a different role in different races. Thus, the racial differences observed in these gene polymorphisms indicate that *ELOVL6* could provide a potential target for future directed therapies for T2DM, using geographic region for patient stratification.

In our study there are several limitations that should be considered. First, the data for some of the phenotypic disease characteristics, such as the duration of T2DM history, hemoglobin A1c, low-density lipoprotein cholesterol, and high-density lipoprotein cholesterol, were not described in detail. Additional details of the patients' clinical history will be needed. The sample size was relatively small and the study should be replicated in a much larger sample. Finally, to unravel the exact mechanism by which this chromosomal region is pathologically involved in T2DM, a more in-depth investigation of the locus, with functional experiments, is required.

Despite these limitations, this is the first study to test the hypothesis that *ELOVL6* genetic variation is associated with insulin sensitivity and β-cell function in a Chinese Han population. We have revealed an association between SNP rs12504538 and T2DM and identified *ELOVL6* as a novel candidate gene for T2DM therapy.

Acknowledgments

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