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ORIGINAL ARTICLE

The endothelial dysfunction in patients with type 2 diabetes mellitus is associated with IL-6 gene promoter polymorphism in Chinese population

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Abstract The purpose of this study is to examine the effects of IL-6 gene promoter −174G/C and −572G/C polymorphism on endothelial function of Chinese T2DM and normal glucose regulation (NGR) subjects. 512 newly diagnosed T2DM patients and 483 NGR subjects were recruited and Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) was performed for the IL-6 gene promoter −174G/C and −572G/C polymorphism. Flow-mediated dilation (FMD) was measured as a non-invasive indicator for endothelial function. The results show that the C allele and CC genotype at −174 of IL-6 gene promoter region was extremely rare in both T2DM and NGR groups; genotypes' and alleles' frequency at −572 of IL-6 gene promoter region is of no difference between T2DM and NGR groups; within T2DM group, higher plasma IL-6 concentration and lower FMD was found in patients with −572 GC (2.36 ± 0.69, 4.23 ± 3.82%) or GG (2.32 ± 0.74, 4.24 ± 3.67%) genotype, compared with patients with CC (2.15 ± 0.62, 5.28 ± 3.94%) genotype. The conclusion of the study is that in comparison with patients of CC genotype, the T2DM patients of −572 GC or GG genotype may have more aggravated endothelial dysfunction (ED) and be at higher risk for coronary artery disease (CAD).

Keywords Interleukin-6 · Polymorphism · Type 2 diabetes · Endothelial dysfunction · Flow-mediated dilation

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Abbreviations

IL-6	Interleukin-6
T2DM	Type 2 diabetes mellitus
NGR	Normal glucose regulation
CAD	Coronary artery disease
ED	Endothelial dysfunction
FMD	Flow-mediated dilation
BMI	Body mass index
SBP	Systolic blood pressure
DBP	Diastolic blood pressure
FPG	Fasting plasma glucose
2 h PPG	2 h Postloading plasma glucose
INS	Insulin
HOMA-IR	Homeostasis model assay of insulin resistance
TC	Total cholesterol
TG	Triglyceride
HDL-c	High-density lipoprotein–cholesterol
LDL-c	Low-density lipoproteincholesterol

Introduction

T2DM has been considered to be a risk equivalent of coronary artery disease (CAD) [1]. In the process of CAD initiation and development, the endothelial dysfunction (ED) is an early event and predate the clinical evidence of CAD for many years [2, 3]. ED also can be found in patients with T2DM [4], and is further deteriorated in T2DM patients concomitant with CAD [5].

ED could be a pathological state linking T2DM and CAD, and even potentially plays a causative role on initiation and development of CAD [3]. Many risk factors associated with ED have been reported such as age, sex, inflammation, obesity, insulin resistance, smoking, hypertension, and

hyperlipidemia [6–8], among which it is well established that chronic low-grade inflammation plays a central role in the pathogenesis of ED [9, 10].

IL-6, as a confirmed proinflammatory cytokine, plays an important role in acute phase response that is associated with insulin-resistant states and T2DM [11]. The main source of IL-6 is immune cells and adipose tissue, but endothelial cells and atherosclerotic plaques have also been identified as some of the other sources of IL-6 [12, 13], supporting a possible local role of inflammation in the initiation and progression of CAD. Furthermore, the elevated IL-6 in circulation has been found linked with CAD development [14, 15]. Some single nucleotide polymorphisms (SNPs) have been identified in IL-6 gene promoter region, among which, −174G/C and −572G/C have been reported to be associated with IL-6 expression [16, 17]. Many studies checking relationship of the polymorphisms of two sites and CAD had been conducted and results were discrepant [16–19], furthermore, there is scarce study about whether these polymorphisms are associated with ED. In the present study, we performed the association study in Chinese cohorts of T2DM patients and normal controls, and found a relationship between IL-6-572G/C polymorphism and ED in T2DM patients.

Materials and methods

Subjects

The 512 newly diagnosed T2DM patients and 483 normal glucose regulation (NGR) control were enrolled from clinical and health examination center in Hangzhou hospital, Nanjing medical university. Ethical approval was received from local research ethics committees and informed consent was obtained from all subjects. According to WHO 1999 criteria, all T2DM patients had been diagnosed, and NGR (FPG level less than 6.1 mmol/l and 2 h PPG level below 7.8 mmol/l) were confirmed by 75-g oral glucose tolerance test (OGTT). The exclusion criteria include a history of cardiovascular disease and abnormal ECG, peripheral artery disease and cerebral stroke, recently onset infection within 2 weeks, and liver or renal function impairment.

Anthropometric parameters and biochemical measurements

Body weight and height were measured unclothed and without shoes in the morning. Body mass index (BMI) was calculated as body weight in kilograms divided by height in square meters (kg/m^2). Blood pressure was read from the non-dominant arm of seated patients with mercury sphygmomanometer after 20 min of rest. For each subjects, measurement was taken three times and the mean was used in analysis.

Overnight fasting blood specimens were obtained for the measurements of plasma glucose, insulin, serum lipids, and IL-6 levels. FPG, 2 h PPG, total cholesterol (TC), triglyceride (TG), high-density lipoprotein cholesterol (HDL-c), and low-density lipoproteincholesterol (LDL-c) were measured using an automated biochemical instrument (Hitachi, 7600 2020, Japan). Serum insulin was measured by radioimmunoassay (Sangon Company, Shanghai, China). Insulin resistance was estimated using HOMA-IR, according to the formula HOMA-IR = fasting insulin (mU/l) × FPG (mmol/l)/22.5. Serum IL-6 was measured using ELISA kits (R&D Systems, Inc., USA). The intra- and inter-assay coefficients of variation were less than 10 and 15%, respectively.

Assessment of endothelial function

Flow-mediated dilation (FMD) was measured to assess arterial endothelial function non-invasively. After a 12-h fast, no smoking for 12 h, and no alcohol intake for 3 days, individuals were examined by same reader in a dark, temperature-controlled, quiet room after 20 min of rest. The right brachial artery was imaged in a longitudinal section between 10 and 15 cm above the antecubital fossa using a 15-MHz linear array transducer (Agilent 5500, Andover, MA). After baseline brachial artery diameter was measured, a reactive hyperemia was induced by inflation of a blood pressure cuff on the forearm to suprasystolic levels for 3 min. One minute after cuff deflation, the post-ischemic diameter of the brachial artery was measured. FMD was expressed as percentage change = 100 × [post-ischemic diameter minus the diameter at rest]/brachial artery diameter at rest. Three times of measurements were conducted at baseline and post-ischemic for each subjects, and the averages were used in the analysis.

Genotyping

Genomic DNA was prepared from peripheral blood samples using phenol–chloroform standard procedure. Two sites polymorphism were checked by PCR-RFLP. The fragment comprising site of restriction enzyme SfaNI at −174G/C was amplified using following primers: sense 5′-TGA CTTCAGCTTTACTCTTTGT-3′ antisense 5′-CTGATTG GAAACCTTATTAAG-3′; the fragment comprising site of restriction enzyme *Mbi*I at −572G/C was amplified using following primers: sense 5′-GGAGACGCCTTGAAGTA ACTGC-3′, antisense 5′-GAGTTTCCTCTGACTCCATC GCAG-3′. The condition of PCR and restriction enzyme digestion was described before [20, 21]. The primer was

synthesized by Biosun Biotech, shanghai. The restriction enzyme was bought from R&D Systems Inc.

Statistical analysis

Statistical analyses were performed using SPSS (Version 13.0). Data are given as means ± standard deviation [SD], unless otherwise stated. Allele frequencies were estimated by gene counting. The x^2 test was used to compare the observed numbers of each genotype among different group and with those expected for a population in Hardy–Weinberg equilibrium. The Student's t test was used to test differences between groups as appropriate. Associations between IL-6 genotypes and baseline demographic and clinical characteristics were evaluated by analysis of variance (ANOVA). Two-tailed P value less than 0.05 was considered statistically significant.

Results

Sample characteristics

Table 1 shows anthropometric and biochemical data of the sample. The age and male to female rate in type 2 diabetes (T2DM) group and normal glucose regulation (NGR) group were of no significant differences. Compared with samples in NGR group, the T2DM patients had a remarkably higher level of body mass index (BMI), systolic blood pressure (SBP), diastolic blood pressure (DBP), fasting plasma glucose (FPG), 2 h postloading plasma glucose (2 h PPG), homeostasis model assay of insulin resistance (HOMA-IR) and triglyceride (TG), but the fasting insulin level (INS) and high-density lipoprotein–cholesterol (HDL-c) were found lower in T2DM group. There were no significant change in total cholesterol (TC) and low-density lipoprotein–cholesterol (LDL-c) between two groups. Also in Table 1, the plasma IL-6 was 2.23 ± 1.84 pg/ml in T2DM group, which was significantly higher than 1.79 ± 1.54 pg/ml in NGR group, while the FMD was dramatically lower in T2DM group (4.81 ± 3.72%) than it in NGR group (5.81 ± 4.21%).

Genotype and T2DM

The genotype distributions and allele frequencies of two SNPs in T2DM group and NGR group were shown in Table 2. The C allele and GC genotype in IL-6-174G/C were extremely rare, with only two cases in T2DM group and one case in NGR group, and CC genotype in IL-6-174G/C was not found in any group. The genotype of IL-6-572G/C distributions was in accordance with Hardy–Weinberg equilibrium, and between T2DM and NGR groups, these distributions were of no significant difference, the odds ratio of GC to CC and GG to CC being 0.9727 (95% CI, 0.7483–1.264) and 0.8888 (95% CI, 0.5677–1.391),

Table 1 Clinical characteristics of subjects with T2DM and NGR

Variable	T2DM group (n = 512)	NGT group (n = 483)	P value
Men/women	295/217	276/207	
Age (years)	57.4 ± 10.6	56.8 ± 13.1	
BMI (kg/m^2)	24.5 ± 4.7	22.4 ± 2.8	<0.0001
SBP (mmHg)	142.5 ± 24.1	131.6 ± 20.7	<0.0001
DBP (mmHg)	92.3 ± 14.6	82.8 ± 13.9	<0.0001
FPG (mmol/l)	8.5 ± 5.6	4.6 ± 1.7	<0.0001
2 h PPG (mmol/l)	13.6 ± 5.6	5.7 ± 1.6	<0.0001
HbA1c (%)	8.7 ± 2.9	4.7 ± 1.3	<0.0001
INS (mIU/l)	5.4 ± 0.76	7.4 ± 0.37	<0.0001
HOMA-IR	2.04 ± 0.84	1.51 ± 0.57	<0.0001
TG (mmol/l)	2.69 ± 1.15	1.59 ± 0.34	<0.0001
TC (mmol/l)	4.96 ± 1.53	4.86 ± 1.37	
HDL-c (mmol/l)	1.04 ± 0.65	1.16 ± 0.78	0.0084
LDL-c (mmol/l)	2.51 ± 0.46	2.48 ± 0.45	
IL-6 (pg/ml)	2.23 ± 1.84	1.79 ± 1.54	<0.0001
FMD (%)	4.81 ± 3.72	5.81 ± 4.21	<0.0001

Data are expressed as means ± SD

BMI body mass index, *SBP* systolic blood pressure, *DBP* diastolic blood pressure, *FPG* fasting plasma glucose, 2 h *PPG* 2 h postloading plasma glucose, *INS* insulin, *HOMA-IR* homeostasis model assay of insulin resistance, *TC* total cholesterol, *TG* triglyceride, *HDL-c* high-density lipoprotein–cholesterol, *LDL-c* low-density lipoproteincholesterol, *IL-6* interleukin 6, *FMD* flow-mediated dilation

Table 2 The distribution of IL-6 gene promotor genotypes in T2DM and NGR

	T2DM (512)	NGT (483)	χ	*P* value	OR (95% CI)
−174 CC	0	0			
GC	2 (0.4%)	1 (0.2%)			
GG	510 (99.6%)	482 (99.8%)	0.2787	0.5976	0.5290 (0.04779–5.857)
C	2 (0.20%)	1 (0.1%)			
G	1022 (99.8%)	965 (99.9%)	0.2877	0.5917	0.5241 (0.04742–5.793)
−572 CC	253 (49.4%)	244 (50.5%)			
GC	210 (41%)	197 (40.8%)			0.9727 (0.7483–1.264)
GG	49 (9.6%)	42 (8.7%)	0.2717	0.8730	0.8888 (0.5677–1.391)
C	716 (69.9%)	685 (70.9%)			
G	308 (30.1%)	281 (29.1%)	0.2331	0.6290	0.9536 (0.7865–1.156)

Table 3 Clinical characteristics, plasma IL-6 and FMD of T2DM patients according to −572 genotypes

Variable	CC ($n = 253$)	GC ($n = 210$)	GG ($n = 49$)	*P* value
Men/women	142/111	131/79	29/20	
Age (years)	57.1 ± 11.6	57.6 ± 11.5	56.8 ± 10.5	
BMI (kg/m^2)	23.9 ± 3.1	24.8 ± 3.7	24.7 ± 3.9	0.0152
SBP (mmHg)	140.5 ± 14.9	143.5 ± 15.1	144.8 ± 13.8	0.0418
DBP (mmHg)	92.5 ± 13.3	91.4 ± 13.1	91.8 ± 14.1	
FPG (mmol/l)	8.2 ± 6.6	8.7 ± 5.8	8.9 ± 4.9	
2 h PPG (mmol/l)	12.9 ± 6.2	13.7 ± 4.9	13.4 ± 4.7	
HbA1c (%)	8.6 ± 2.5	8.7 ± 1.9	8.8 ± 2.5	
INS (mIU/l)	5.35 ± 0.57	5.45 ± 0.68	5.4 ± 0.65	
HOMA-IR	1.95 ± 0.64	2.11 ± 0.66	2.13 ± 0.62	0.0167
TG (mmol/l)	2.42 ± 1.34	2.84 ± 1.65	2.97 ± 1.72	0.0036
TC (mmol/l)	4.87 ± 1.26	4.99 ± 1.44	4.98 ± 1.21	
HDL-c (mmol/l)	1.02 ± 0.61	1.05 ± 0.54	1.05 ± 0.58	
LDL-c (mmol/l)	2.52 ± 0.35	2.50 ± 0.34	2.50 ± 0.35	
IL-6 (pg/ml)	2.15 ± 0.62	2.36 ± 0.69	2.32 ± 0.74	0.0025
FMD (%)	5.28 ± 3.94	4.23 ± 3.82	4.24 ± 3.67	0.0095

Data are expressed as means ± SD

BMI body mass index, *SBP* systolic blood pressure, *DBP* diastolic blood pressure, *FPG* fasting plasma glucose, 2 h *PPG* 2 h postloading plasma glucose, *INS* insulin, *HOMA-IR* homeostasis model assay of insulin resistance, *TC* total cholesterol, *TG* triglyceride, *HDL-c* high-density lipoprotein–cholesterol, *LDL-c* low-density lipoproteincholesterol, *IL-6* interleukin 6, *FMD* flow-mediated dilation

respectively. Also, we had not found significantly different allele frequencies between T2DM and NGR groups, odds ratio of G to C being 0.9536 (95% CI, 0.7865–1.156).

Genotype and plasma IL-6, FMD

Table 3 shows within T2DM group, in comparison with patients with −572CC genotype, the patients with GC and GG genotype had significantly higher plasma IL-6 levels (2.36 ± 0.69 and 2.32 ± 0.74 vs. 2.23 ± 1.84, $P = 0.0025$), BMI, SBP, HOMA-IR, and TG, while these patients had remarkably lower FMD (4.23 ± 3.82% and 4.24 ± 3.67% vs. 5.22 ± 3.94%, $P = 0.0095$). After further adjustments for age, sex, BMI, SBP, DBP, HOMA-IR, and TG, the statistically noticeable difference of plasma IL-6 levels and FMD still persisted ($P = 0.0136$ and $P = 0.0273$, respectively), while plasma IL-6 levels were adjusted solely, the difference of FMD between various genotypes attenuated to non-significance ($P = 0.2051$), the differences of BMI, SBP, HOMA-IR, and TG also disappeared.

We also did comparison of plasma IL-6 and FMD between different genotype within NGR group but failed to find any significant differences. For patients of genotype CC, GC, and GG of NGR, Plasma IL-6 was 1.76 ± 0.67, 1.81 ± 0.69, and 1.8 ± 0.84 pg/ml ($P = 0.7446$), FMD was 6.07 ± 4.86%, 5.72 ± 4.79%, and 5.77 ± 3.96% ($P = 0.7321$), respectively.

Discussion

In the present study, we found that plasma IL-6 level was higher and FMD was lower in patients with T2DM, compared with it in subjects with NGR. The C allele of

IL-6-174G/C is extremely rare in Chinese han population, CC genotype was not found in any group. As for IL-6-572G/C, in comparison with patients with CC genotype, T2DM patients with GG or GC genotype have lower FMD, and higher IL-6 concentration.

Extremely rare C allele at −174 found in our study was coincident with study from another Chinese han cohort which also found no CC genotype and in which C allele was very rare [22], while in Caucasians, reported C allele at −174 was more than 40% [17, 18, 20], these indicate Chinese and Caucasians have a different genetic background at this site. With respect to −572, our study failed to show the correlation between polymorphism and T2DM, which is either inconsistent with previous study conducted in Caucasians which found an association between −572 polymorphism and T2DM, but control group of that study was not in Hardy–Weinberg equilibrium [23], so we need further studies to testify this relationship.

T2DM is a disease predisposed by chronic inflammation, many studies have proved this pathogenic role of inflammation in diabetes [24]. One of the characterized pathophysiological states of metabolic syndrome and T2DM is insulin resistance which is thought to be caused by chronic low-grade inflammation in adipose tissue, liver, and muscle [25]. Many biomarkers of inflammation have been identified to be associated with insulin resistance, and predicted the development of type 2 diabetes, such as the tumor necrosis factor-α (TNF-α), interleukin 6 (IL-6), and C-reactive protein [26]. The present study shows that the patients with T2DM have higher IL-6 levels and HOMA-IR, which verified that there is a chronic inflammatory state in patients with insulin resistance and T2DM.

IL-6 expression in relation to genotype at position −572 has been assessed in peripheral mononuclear cells, and results showed that peripheral mononuclear cells of GC or GG genotype have higher IL-6 expression [27]. We found in T2DM group, but not in NGR group, in comparison with subjects with CC genotype. subjects with GC or GG genotype have higher plasma IL-6 levels. As IL-6 expression is highly inducible upon stimulus [28], we speculated that the differences in IL-6 production between −572G/C genotypes are significant only upon a stimulus, such as a chronic inflammation of T2DM in present study. In accordance with our finding, Anders also demonstrated among different −572 G/C genotypes, IL-6 levels were similar in healthy control, but significantly different in patients with acute coronary syndrome [16].

Previous studies have shown chronic low-grade inflammation which features obese, insulin resistance, and T2DM plays a central role in the pathogenesis of ED [4, 5, 29, 30], our data also show remarkable ED in patients with T2DM that indicated by decreased FMD, a non-invasive detection technique first described by Celermajer et al. [31]. As ED is one of the earliest events predicting the onset of overt CAD [2], with worse ED, there is a higher risk of concomitant CAD in T2DM patients. In the present study, compared with T2DM patients with −572 CC genotype, lower FMD was found in the T2DM patients with −572 GC or GG genotype, which indicates the T2DM patients with −572 GC or GG genotype had more aggravated ED than the patients with −572 CC genotype.

It is well known that through stimulating endothelial cells to upregulate the expression of adhesion molecules and depressing NO production, IL-6 causes ED [32, 33]. Also, a greater number of other factors have been previously reported to be associated with ED, such as age, sex, lower HDL-c, higher TG, and hypertension [6, 7, 8]. Our data also indicated that in comparison with −572 CC genotype, the T2DM patients with −572 GC or GG genotype had higher BMI, SBP, HOMA-IR, and TG, but after further adjustment of age, sex, BMI SBP, DBP, HOMA-IR, and TG, the different FMD among genotypes still persisted, while further adjustment of IL-6 concentration solely, the difference attenuated to non-significance, that means IL-6 is the main factor that influence genotype-dependent ED in the present study, and IL-6-572 G allele is an independent risk factor of ED among T2DM patients.

The limitation of the present study might be the sensitivity of the measurements of FMD in evaluating CAD risk, as only newly diagnosed T2DM without detectable cardiovascular disease had been recruited in the T2DM group. However, in the present cohort, established cardiovascular risk factors, such as BMI, SBP, HOMA-IR, and TG have been shown to be associated with these early markers of ED, suggesting the validity of our methods.

In conclusion, IL-6-174G/C is not a common polymorphism site in Chinese han population, IL-6-572G/C polymorphism is associated with ED among T2DM patients, in comparison with patients of CC genotype, the T2DM patients of −572 GC or GG genotype may have more aggravated ED and are at higher risk for CAD.

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Conflict of interest None.

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