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Genetic polymorphism c.1562C>T of the MMP-9 is associated with macroangiopathy in type 2 diabetes mellitus

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ABSTRACT

Objective: To determine whether the matrix metalloproteinase-9 (MMP-9) c.1562C>T polymorphism has an effect on the plasma MMP-9 levels and the macroangiopathic complications in type 2 diabetes mellitus (T2DM).

Methods: The genotypes and allelic frequencies of the MMP-9 c.1562C>T were examined with polymerase chain reaction and restriction fragment length polymorphism in 320 patients with T2DM and 160 unrelated healthy subjects. The plasma concentrations of MMP-9 were determined in all subjects.

Results: The mean plasma concentrations of MMP-9 of patients with T2DM were significantly higher than that of controls and the plasma levels of MMP-9 were higher in diabetic patients with macroangiopathy than in patients without macroangiopathy ($P < 0.05$). The genotype (CC, CT, and TT) distribution of c.1562C>T polymorphism of the MMP-9 gene was 60.0%, 31.3%, and 8.8% in diabetic patients with macroangiopathy, 76.3%, 21.3%, and 2.5% in patients without macroangiopathy, and 77.5%, 21.3%, 1.3% in controls, respectively, a significant difference was found between diabetic patients with and without macroangiopathy ($P < 0.05$). The frequency of the allele T was higher in patients with macroangiopathy than in patients without macroangiopathy (24.4% vs 13.1%; $P < 0.05$). Moreover, the plasma MMP-9 levels were markedly higher in patients with TT genotype than those with CC or CT genotype in patients with macroangiopathy ($P < 0.05$).

Conclusion: The MMP-9 c.1562C>T gene polymorphism associated with a predisposition to increased plasma MMP-9 levels could constitute a useful predictive marker for diabetic macroangiopathy.

Introduction

Type 2 diabetes mellitus (T2DM) is strongly associated with elevated mortality and morbidity from atherosclerotic vascular disease manifesting as coronary heart disease, cerebrovascular disease, and peripheral vascular disease [1,2]. Diabetic macroangiopathy, manifested by atherosclerosis of coronary arteries, cerebral arteries, and large arteries of the lower extremities, is the major cause of mortality and significant morbidity [3]. Predictors of susceptibility for development of macroangiopathy in diabetic subjects would help focus our treatment strategies.

Matrix metalloproteinase-9 (MMP-9) is one of a set of zinc-dependent endopeptidases capable of degrading components of the extracellular matrix (ECM) [4]. The MMP-9 gene has a C-to-T promoter polymorphism at position −1562, which affects transcription and leads to promoter low-activity (C/C) and high-activity (C/T, T/T) genotypes [5]. MMP-9 has three repetitive type II fibronectin domains, which allow it to bind to ECM components, such as gelatin, collagen, and laminin. Increased expression of this enzyme is seen in some neoplastic, cardiovascular, and respiratory diseases [6]. Recent studies revealed that the plasma level of MMP-9 was of both diagnostic and prognostic significance in coronary artery diseases and renal diseases [7–10]. MMP-9 has recently been identified in human atherosclerotic lesions [11,12]. It is active against denatured collagens and type IV, V, and XI collagens in addition to the proteoglycans and elastin also found in atherosclerotic lesions [13,14].

No data are as yet available on the impact of the MMP-9 genotypes at the vessel-wall level in respect to the atherosclerotic plaque rupture and thrombosis. Nor has it hitherto been sought to establish whether MMP-9 genotypes affect development and progression of diabetic macroangiopathy. In the present study, we investigated the association between MMP-9 c.1562C>T polymorphism and the macroangiopathy in T2DM.

Materials and methods

Study subjects. The study was approved by the Clinical Research Ethics Committee of Harbin Medical University, and all subjects gave written informed consent. Macroangiopathy was diagnosed by the

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presence of cardiovascular disease or cerebrovascular disease and peripheral vascular disease. Cardiovascular disease was defined as a history of myocardial infarction, angina pectoris or ischemic heart disease, ongoing treatment with drugs prescribed for cardiovascular disease, or the presence of a pathological electrocardiogram according to the Minnesota code. A cerebrovascular lesion was considered present if diagnosed according to medical records or if a pathological finding by computed tomography of the brain had been registered. Peripheral vascular disease was defined as clinical macroangiopathy (no pulses) present during a foot examination or a medical history of symptoms typical of intermittent claudication. Overt nephropathy was defined as albuminuria $\geqslant$300 mg/l or serum creatinine >100 μmol/l for women and >110 μmol/l for men. Incipient nephropathy was defined as albuminuria 30–299 mg/l. Hypertension was defined as $\geqslant$140/90 mmHg at examination or presence of antihypertensive treatment.

One hundred and sixty diabetic patients with macroangiopathy, 160 diabetic patients without macroangiopathy, and 160 unrelated healthy subjects were recruited from the physical examination station of the local district. Blood sampling was performed between 9:00 and 10:00 h after an overnight fast. Fasting serum lipids were determined by an autoanalyzer. Plasma glucose levels were measured with the glucose oxidase method on a Beckman Glucose Analyzer (Beckman, Fullerton, CA, USA); plasma insulin concentrations were measured by commercial radioimmunoassay kits (Radim, Rome, Italy); HbA_1c was measured by a commercial kit (Bio-Rad, Richmond, CA). The intra-assay and inter-assay coefficients of variation were <7% and 14%, respectively. Serum MMP-9 concentrations were measured by enzyme-linked immunosorbent assay using monoclonal antibodies as previously reported [15].

Analysis of the polymorphism c.1562C>T of the MMP-9. Genomic DNA was extracted from peripheral blood leukocytes. The c.1562C>T polymorphism of the MMP-9 gene was determined using a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) based protocol as described by Koh et al. [16]. A 435-bp region spanning nucleotides −1809 to −1374 in the MMP-9 promoter was amplified using forward primer 5′-GCCTGGCACATAGTAGGCCC-3′ and reverse primer 5′-CTTCCTAGCCAGCCGGCATC-3′. The amplification reaction mixture (7.5 μl) contained 20 ng of genomic DNA, 0.2 mM of each dNTP, 0.5 μM of each primer, 0.5 unit of Prozyme DNA polymerase, and 1× PCR buffer. The PCR reaction was performed for 5 min at 95 °C and then for 35 cycles of 30 s at 95 °C, 30 s at 60 °C, and 90 s at 72 °C with a final extension at 72 °C for 10 min. Aliquots of the PCR product were mixed with 10 μl of reaction solution containing 1 μl of 10× enzyme buffer, 6 μl of $3dH_2O$, and 0.5 μl of the *SphI* restriction enzyme for the restriction fragment length polymorphism analysis. Each digestion reaction was incubated at 37 °C for 6 h and analyzed with 2% agarose gel electrophoresis (Fig. 1).

Statistical analyses. All frequencies were estimated by gene counting. A Chi-square test was used to compare genotype and gene frequencies between the groups. ANOVA was used to compare average values of biochemical parameters between genotypes. All data were expressed as the mean ± SD. A two-tailed *P*-value <0.05 was considered to be statistically significant for all analyses. All statistical analyses were performed with SPSS software (version 13.0; SPSS, Chicago, IL, USA).

Results

Association of the MMP-9 c.1562C>T polymorphism with diabetic macroangiopathy

The relationship between distribution frequency of genotypes and alleles of MMP-9 was shown in Table 1. The genotype

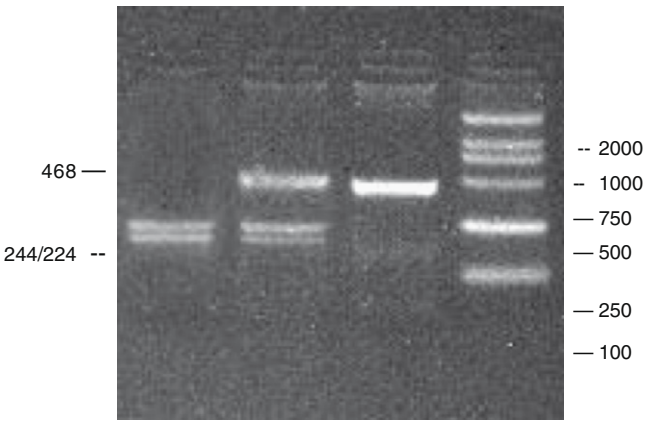

Fig. 1. Genotypes of 5-HT2AR c.1438A>G promoter region by PCR-RFLP. Lane 1: homozygote GG; lane 2: heterozygote AG; lane 3: homozygote AA.

frequency of the c.1562C>T polymorphism of the MMP-9 gene were as follows: the genotype (CC, CT, and TT) distribution of c.1562C>T polymorphism of the MMP-9 gene was 60.0%, 31.3%, and 8.8% in diabetic patients with macroangiopathy, 76.3%, 21.3%, and 2.5% in diabetic patients without macroangiopathy, and 77.5%, 21.3%, and 1.3% in controls, respectively. The genotype frequency for this polymorphism was found to be in Hardy–Weinberg equilibrium. The genotype and allele frequencies of the MMP-9 c.1562C>T polymorphism were similar between the control subjects and diabetic patients without macroangiopathy ($P >$ 0.05). There were significant differences of genotype and allele frequencies in MMP-9 gene between the control subjects and diabetic patients with macroangiopathy ($P <$ 0.05).

Association of the genotypes and alleles of the MMP-9 c.1562C>T with the risk of diabetic macroangiopathy

The genotype TT was associated with the risk of macroangiopathy with an OR of 2.16 (95% CI 1.875–3.036) and allele T was associated with the risk of macroangiopathy with an OR of 1.03 (95% CI 1.01–3.26) in patients with T2DM (Table 2).

Biochemical and anthropometric measurements of the three group

Compared with the control group, the patients with T2DM had higher body mass index (BMI), HbA_1c, plasma glucose, insulin, CÙpeptide, total cholesterol, triglyceride (TG), and MMP-9. The diabetic patients with macroangiopathy had significantly higher concentrations of serum MMP-9 than those without macroangiopathy (Table 3).

Association of biochemical parameters and the MMP-9 c.1562C>T genotype with T2DM

The homozygotes TT patients with macroangiopathy had significantly higher triglyceride (TG), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), insulin, and CÙpeptide than those with the CT heterozygotes and CC homozygotes ($P <$ 0.05). While the body mass index, HbA_1c, plasma glucose were not significantly different across the MMP-9 c.1562C>T genotype groups (Table 4).

Influence of the c.1562C>T polymorphism of the MMP-9 gene on serum MMP-9 levels

The patients with TT homozygotes had significantly higher MMP-9 levels (CC: 110.25 ± 28.06 ng/ml, TC: 118.20 ± 32.15 ng/

Table 1
Distribution frequency of genotypes and alleles of MMP-9 c.1562C>T.

Group	Genotype			Allele	
	CC (%)	TC (%)	TT (%)	C (%)	T (%)
Control (n = 160)	124 (77.5)	34 (21.3)	2 (1.3)	282 (88.1)	38 (11.9)
The patients without macroangiopathy (n = 160)	122 (76.3)	34 (21.3)	4 (2.5)	276 (86.9)	42 (13.1)$^{\#}$
The patients with macroangiopathy (n = 160)	96 (60.0)	50 (31.3)	14 (8.8)	242 (75.6)	78 (24.4)*

Values are expressed as mean ± SD. Compared with the control group, $^{*}P < 0.05$; compared with the patients without macroangiopathy, $^{\#}P < 0.05$.

Table 2
Distribution of genotypes and alleles of MMP-9 c.1562C>T in patients with the risk of macroangiopathy.

	The patients without macroangiopathy	The patients with macroangiopathy	P	Odds ratio	(95% CI)
CC genotype	122 (76.3)	96 (60.0)	0.064	2.39	(2.128–4.697)
TT genotype	4 (2.5)	14 (8.8)	0.037	2.16	(1.875–3.036)
C allele	276 (86.9)	42 (13.1)	0.059	1.47	(1.142–2.985)
T allele	242 (75.6)	78 (24.4)	0.041	1.03	(1.365–2.968)

P-value and odd ratio were obtained by χ^2 test.

Table 3
Biochemical and anthropometric measurements for the three groups.

	Control (n = 160)	The patients without macroangiopathy (n = 160)	The patients with macroangiopathy (n = 160)
Age (years)	53.1 ± 8.22	53.11 ± 10.73	52.23 ± 10.74
BMI (kg/m^2)	22.61 ± 1.32	24.35 ± 1.76*	25.18 ± 2.67*
Total cholesterol (mmol/l)	4.13 ± 0.42	5.02 ± 0.60*	5.65 ± 0.79*
Low-density lipoprotein cholesterol (mmol/l)	2.20 ± 0.62	3.12 ± 0.88*	3.54 ± 1.06*
High-density lipoprotein cholesterol (mmol/l)	1.39 ± 0.17	1.17 ± 0.20*	1.04 ± 0.28$^{\#,*}$
Triglycerides (mmol/l)	1.15 ± 0.41	2.37 ± 0.88*	2.90 ± 1.28$^{\#,*}$
Fasting glucose (mmol/l)	5.14 ± 0.34	7.32 ± 1.24	8.46 ± 1.73
HbA$_1$c (%)	5.49 ± 0.42	8.03 ± 1.38*	9.32 ± 1.86*
Fasting serum insulin (mU/l)	13.89 ± 1.86	11.87 ± 1.77	13.48 ± 2.04
Fasting serum C-peptide (μg/l)	1.50 ± 0.18	1.28 ± 0.22	1.49 ± 0.29
MMP-9 (ng/ml)	43.47 ± 27.93	84.21 ± 35.27*	116.20 ± 30.04$^{\#,*}$

Values are expressed as mean ± SD. Compared with the control group, $^{*}P < 0.05$; compared with the patients without macroangiopathy, $^{\#}P < 0.05$.
BMI: body mass index; MMP-9: matrix metalloproteinase 9.

ml, TT: 122.48 ± 33.57 ng/ml, $P < 0.05$ for difference between CC and TT) in diabetic patients with macroangiopathy. There were no statistical differences in MMP-9 levels in the control subjects and patients without macroangiopathy ($P > 0.05$) (Table 4).

Discussion

In the present study, we raised the question of whether the polymorphism of the MMP-9 gene may be associated with diabetic macroangiopathy. Specifically, we hypothesized that the allele frequencies of the different polymorphisms in diabetic patients might be different from those in nondiabetic subjects and that the different polymorphisms could be related to various forms of macroangiopathy and its risk factors in patients with type 2 diabetes. Our results suggested that genetic polymorphism c.1562C>T of the MMP-9 was significantly associated with diabetic macroangiopathy.

How to explain the association of the genotype TT which is associated with diabetic macroangiopathy. On the one hand, extensive vascular remodeling is likely to take place in atherogenesis and lesion progression [13]. Lesion growth in the early stage of atherosclerosis may result mainly from lipid deposition, because in the late stages of atherosclerosis in more advanced lesions, connec-

Table 4
Demographic characteristics according to the MMP-9 c.1562C>T genotype.

	The patients without macroangiopathy (n = 160)			The patients with macroangiopathy (n = 160)		
	CC	TC	TT	CC	TC	TT
BMI (kg/m^2)	24.37 ± 1.71	24.34 ± 1.79	24.34 ± 1.77	25.14 ± 2.65	25.16 ± 2.69	25.21 ± 2.68
Total cholesterol (mmol/l)	5.06 ± 0.61	5.01 ± 0.57	4.99 ± 0.63	5.57 ± 0.77	5.62 ± 0.76	5.73 ± 0.81*
Low-density lipoprotein cholesterol (mmol/l)	3.11 ± 0.85	3.13 ± 0.89	3.12 ± 0.87	3.49 ± 1.04	3.53 ± 1.07	3.63 ± 1.09*
High-density lipoprotein cholesterol (mmol/l)	1.15 ± 0.22	1.19 ± 0.17	1.16 ± 0.20	1.05 ± 0.27	1.04 ± 0.29	1.03 ± 0.28
Triglycerides (mmol/l)	2.32 ± 0.84	2.41 ± 0.92	2.37 ± 0.89	2.81 ± 1.28	2.91 ± 1.28	3.12 ± 1.28*
Fasting glucose (mmol/l)	7.32 ± 1.21	7.34 ± 1.26	7.30 ± 1.23	8.46 ± 1.75	8.44 ± 1.71	8.48 ± 1.74
HbA$_1$c (%)	8.01 ± 1.36	8.04 ± 1.37	8.02 ± 1.41	9.29 ± 1.84	9.33 ± 1.87	9.34 ± 1.89
Fasting serum insulin (mU/l)	11.88 ± 1.75	11.85 ± 1.79	11.89 ± 1.74	13.40 ± 2.02	13.47 ± 2.01	13.65 ± 2.11*
Fasting serum C-peptide (μg/l)	1.25 ± 0.25	1.30 ± 0.21	1.27 ± 0.23	1.41 ± 0.24	1.52 ± 0.28	1.60 ± 0.32*
MMP-9 (ng/ml)	84.22 ± 35.21	84.25 ± 35.31	84.19 ± 35.28	110.25 ± 28.06	118.20 ± 32.15	122.48 ± 33.57*

Values are expressed as mean ± SD. Compared with the CC genotype $^{*}P < 0.05$.
BMI: body mass index; MMP-9: matrix metalloproteinase 9.

tive tissue accumulation and smooth muscle cell proliferation are the prominent contributors to plaque growth [13]. MMP-9 alters postinjury vascular remodeling and, like MMP-3 and MMP-1, evinces proteolytic activity against several proteins associated with complicated plaques (e.g., type IV collagen, a major component in the basement membrane) and facilitates vascular smooth muscle cell migration and proliferation [13]. We assumed that remodeling of the arterial extracellular matrix and subsequent plaque vulnerability or smooth muscle cell migration associated with the MMP-9 T allele contributes to the pathogenesis of atherosclerosis. On the other hand, MMP-9 is expressed in the shoulder region of the atherosclerotic lesion and is regulated at three levels: transcription, activation of proenzymes, and specific inhibition by endogenous tissue inhibitors of MMPs, which, in turn, like the MMPs themselves, are regulated by various cytokines and growth factors through cis elements in their gene promoters [13]. An increase in MMP-9 expression in the plaque shoulder areas, especially in MMP-9 promoter T-allele carriers, may promote the development of atherosclerotic lesions, leading to diabetic macroangiopathy [17–19].

A functional cytosine (C) to thymidine (T) single nucleotide polymorphism at position −1562 in the MMP-9 promoter was reported [20–22]. Transient transfection and DNA–protein interaction assays indicated that T allele-associated promoter activity (due to the preferential binding of a putative transcriptional repressor protein) was higher than the C allele-associated promoter activity [23,24]. Many studies further showed that this functional polymorphism was correlated to increased susceptibility to certain diseases [25–27]. In our population the prevalence of macroangiopathy differs according to the MMP-9 c.1562C>T genotype in patients with T2DM. Moreover, in our study we found that the patients with TT genotype had significantly higher TG, LDL-C, insulin, and CÙpeptide than those with CT or TT genotypes, an effect that disappears in the control subjects. The fact that in type 2 diabetes the prevalence of macroangiopathy increased and reached the maximum value in 1562T homozygous may suggest that this allele could be associated with macroangiopathy in diabetic patients but not in control subjects. Our results suggested that the genetic polymorphism c.1562C>T of the MMP-9 was significantly associated with diabetic macroangiopathy and the patients with macroangiopathy had significantly higher levels of MMP-9 compared to those without macroangiopathy. Other studies have also shown that dinucleotide repeat polymorphism of MMP-9 gene is associated with diabetic nephropathy [28]. In addition, some studies have indicated a role for the variant c.1562C>T of the MMP-9 in the pathogenesis of coronary artery disease [13].

In the present study, the genetic polymorphism c.1562C>T of the MMP-9 was statistically significantly associated with diabetic macroangiopathy. Also, we found that the c.1562C>T of the MMP-9 gene did affect on the plasma level of MMP-9 in patients with T2DM. This is the first report demonstrating that the plasma level of MMP-9 is modified by the genetic polymorphism c.1562C>T of the MMP-9 in Chinese patients with T2DM. The present study showed that the genetic polymorphism c.1562C>T of the MMP-9 was statistically significantly associated with diabetic macroangiopathy. This study provided new clinically relevant information regarding the genetic determinants modulating diabetic macroangiopathy and the potential underlying mechanisms. Further studies with larger sample sizes are needed to confirm these findings.

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