Association of Genetic Polymorphisms in Matrix Metalloproteinase-9 and Coronary Artery Disease in the Chinese Han Population: A Case–Control Study

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Objective: Matrix metalloproteinase-9 (MMP-9) plays an important role in inflammation and matrix degradation involved in atherosclerosis and plaque rupture. The T allele of rs3918242 has been reported to lead to a high promoter activity and associate with the extent of coronary artery disease (CAD). And some studies have reported that the G allele of rs17576 might be associated with CAD. The aim of this study was to assess the association between the polymorphisms of the *MMP-9* gene and CAD in the Chinese Han population. **Methods:** This case–control study comprised 258 CAD cases and 153 controls from the Chinese Han Population. The genomic DNA of *MMP-9* was isolated from whole blood. Polymerase chain reaction-based restriction fragment length polymorphism was used to determine the rs3918242 and rs17576 genotypes in the *MMP-9* gene and the total serum levels of MMP-9 were measured using enzyme-linked immunosorbent assay in both case and control groups. **Results:** Analysis of *MMP-9* gene polymorphisms showed that the frequencies of the T allele and CT + TT genotypes of rs3918242 were significantly higher in the case group than in the control group ($p < 0.05$). However, the distribution of variant genotypes of rs17576 did not differ between the case and control groups ($p > 0.05$). The total serum level of MMP-9 was significantly higher in the case group than in the control group ($p < 0.05$). The subjects carrying T alleles in the CAD group had higher average serum MMP-9 levels compared with CC genotypes ($p < 0.05$). **Conclusions:** Our results suggest that the single-nucleotide polymorphism of rs3918242 in the *MMP-9* gene is associated with CAD and high serum levels of MMP-9 are also associated with CAD in the Chinese Han population. Therefore, genetic variation of rs3918242 may participate in the development of CAD through influencing MMP-9 expression.

Introduction

ATHEROSCLEROSIS IS THE major cause of coronary artery disease (CAD), which is a multifactorial disorder with both genetic and environmental components. However, the extent of the genetic basis in the occurrence of CAD remains incompletely understood. Atherosclerosis is characterized by artery intima damage in the blood vessel wall. Extracellular matrix (ECM) synthesis and degradation is in balance with the normal vascular intima place, but the degradation of the matrix dominates in the vascular intima of atherosclerosis, especially the vulnerable plaque areas (Heeneman *et al.*, 2003). The accumulation of lipids and fibrous elements in the large arteries promotes smooth muscle cell (SMC) migration and proliferation. Because vascular SMC is surrounded by a

basement membrane, SMC migration and proliferation necessitate breaking down the ECM (Newby and Zaltsman, 1999). Matrix metalloproteinases (MMPs) are a family of Zn^{2+} -dependent endopeptidases, which are considered to be the most potent proteases in the metabolism of the ECM and are present in atherosclerotic plaques and appear to be more active in unstable lesions (Galis and Khatri, 2002; Visse and Nagase, 2003). Changed expression of MMPs has been implicated in the pathogenesis of several cardiovascular disorders, including atherosclerosis (Newby, 2005). Recent observations suggest that genetic variation affects the expression of MMPs, which may contribute to the occurrence of cardiovascular disease (Henney *et al.*, 2000).

MMP-9, also known as gelatinase B or 92-kDa type IV collagenase, is one of the important members of MMPs that

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may contribute to the breakdown of the ECM. MMP-9 is characterized by broad substrates, such as gelatin, type IV collagen, and elastin, but it possesses specialized proteolytic activity against type IV collagen, a major component of the coronary artery basement membrane underlying the endothelium and surrounding each vascular SMC (Birkedal-Hansen *et al.*, 1993). MMP-9 is secreted from macrophages in the fibrous cap and has been suggested to be involved in the remodeling processes associated with atherosclerosis and plaque rupture (Loftus *et al.*, 2000; Nanni *et al.*, 2007). The expression of MMP-9 is regulated primarily at the transcriptional level. MMP-9 expression is increased after vascular injury (Bendeck *et al.*, 1994) and is particularly evident in inflammatory atherosclerotic lesions. High expression of MMP-9 has been associated with coronary plaque destabilization (Galis and Khatry, 2002).

The human *MMP-9* gene, which is located on chromosome 20q12.2–13.1, contains 13 exons and 12 introns. Common variants in the promoter and exon polymorphisms have been reported to be associated with CAD in recent years. Zhang *et al.* (1999b) reported that there was a functional single-nucleotide polymorphism (SNP) of rs3918242, the nucleotide variation from C to T gave rise to a twofold increase in the promoter activity and the T allele tended to have a high risk of severe CAD. And the other common SNP A/G polymorphism of rs17576 in the *MMP-9* gene leading to an amino acid substitution in the catalytic domain of the MMP-9 enzyme has also been described (Zhang *et al.*, 1999a), although few functional data are available. The SNP may presumably be involved in the binding of the enzyme to its substrate elastin (Shipley *et al.*, 1996).

It has been observed that the levels of serum MMP-9 were elevated in patients with coronary atherosclerosis (Spurthi *et al.*, 2012) and that there was an association between the levels of circulating MMP-9 and cardiovascular events and mortality (Blankenberg *et al.*, 2003; Weiss *et al.*, 2010). Opstad *et al.* (2012) reported that in T-allele carriers, mRNA levels were 1.7-fold lower compared to wild-type CC. The carriers with the minor T-allele of rs3918242 had higher MMP-9 serum levels compared to those with the major C-allele (Blankenberg *et al.*, 2003).

The question whether SNPs in the *MMP-9* gene are associated with CAD in the Chinese Han population has no consistent answers. Our objectives in the present report, therefore, were (1) to investigate a genetic association between SNPs in the *MMP-9* gene and risk of CAD, (2) to evaluate the relationship between the serum level of MMP-9 and CAD, (3) to evaluate the relationship of SNP and serum MMP-9 in the Chinese Han population through a case-control design.

Subjects and Methods

Study subjects

This hospital-based case-control study included 258 unrelated patients with CAD and 153 unrelated controls to undertake a genetic analysis for association between the *MMP-9* gene polymorphisms and CAD. All the subjects used for this study were the Chinese of Han descent. Patients with CAD (146 males and 112 females) were admitted to the First Hospital of Jilin University, Changchun, China from 2009 to 2011. Control subjects (83 males and 70 females) were randomly selected from the same geographical area during routine

arrival checkup. The study was approved by the ethics committee of the First Hospital of Jilin University, Changchun China, and written informed consent was given by every subject.

Diagnosis was made independently by at least two well trained cardiologists based on the following criteria. All patients recruited had evidence of CAD documented by unstable angina or myocardial infarction. Unstable angina and myocardial infarction were confirmed per Chinese guidelines (Chinese Medical Cardiology Subcommittee, Chinese Editorial Committee of Cardiology Journal, 2007, 2010). Patients with nonatherosclerotic vascular diseases, congenital heart disease, cardiomyopathy, valvular disease, renal or hepatic disease, and cancer were excluded. All control subjects had ECG, chest X-ray, and serum analysis. They were classified as healthy subjects based on their physical examination coupled with the absence of personal or family history and reasons to suspect CAD.

We recorded clinical data, including age, gender, weight, height, smoking habit, and the number of cigarettes consumed per day by each smoker, the presence or absence of hypertension (defined as diastolic blood pressure >90 mmHg and/or systolic blood pressure >140 mmHg and/or if subjects were receiving antihypertensive medication), the presence or absence of type I or type II diabetes (fasting blood glucose ≥ 7.0 mM, and/or using glucose-lowering medication, including insulin), and the presence or absence of hyperlipidemia (defined as cholesterol >5.2 mM and/or triglyceride >1.7 mM, current medications particularly the use of lipid-lowering drugs). Overnight fasting venous blood samples were collected from each subject for total cholesterol (TC), triglyceride (TG) levels, glucose, genomic DNA extraction, and serum extraction.

Genotyping

To perform genetic analysis, we selected rs3918242 (SphI site)—the functional C1562T polymorphism located in the promoter region and rs17576 (MspI site)—the functional R279Q polymorphism located in exon 6. SNP information was taken from NCBI dbSNP Build 132 (www.ncbi.nlm.nih.gov/SNP/). The selected SNPs were restricted to a minor allele frequency of >15% in the HapMap-CHB database (www.hapmap.org). Genomic DNA used for PCR amplification was extracted from whole blood samples using a DNA extraction kit (Promega, Beijing, China). SNPs were genotyped using standard polymerase chain reaction and restriction fragment length polymorphism analysis. Primers and conditions for amplification follow. The *MMP-9* rs3918242 polymorphism was amplified from 100 ng of genomic DNA in a 15- μ L PCR reaction using the following primers: forward GCCTGGCAC ATAGTAGGCCC; reverse CTCCTAGCCAGCCGGCATC. PCR conditions included denaturation at 95°C for 5 min followed by 35 cycles of 94°C for 35 s, 56°C for 30 s, and 72°C for 45 s, and a final extension at 72°C for 5 min. The *MMP-9* rs17576 polymorphism was amplified from 100 ng of genomic DNA in a 15- μ L PCR reaction using the following primers: forward TCACCCTCCCGCACTCTGG; reverse CGGTGCGTA GTTGCGGTGG. PCR conditions included denaturation at 95°C for 5 min followed by 30 cycles of 94°C for 30 s, 58°C for 1 min, and 72°C for 1 min, and a final extension at 72°C for 10 min.

TABLE 1. GENERAL CHARACTERISTICS OF PATIENTS AND CONTROLS INCLUDED IN OUR STUDY

Subject characteristics	Case (n=258)	Control (n=153)	p
Age (year)	63.97±12.32	63.61±11.8	>0.05
Male (n/%)	146/56.59%	83/54.25%	>0.05
BMI (kg/m ²)	24.390±5.86	21.07±4.17	<0.05
TC (mM)	5.03±1.12	4.42±1.07	<0.05
TG (mM)	1.71±1.18	1.69±1.01	>0.05
MMP-9 level (ng/mL)	174.36±35.09	123.73±28.53	<0.05
Smokers (n/%)	69/26.74%	12/7.80%	<0.05
DM (n/%)	65/25.19%	38/24.84%	>0.05
Hypertension (n/%)	146/56.59%	26/16.99%	<0.05

BMI, body mass index; TC, total cholesterol; TG, triglyceride; DM, diabetes mellitus.

Measurement of serum MMP-9

The extracted sera were stored at -80°C for MMP-9 concentration detection. The total serum MMP-9 levels were measured by enzyme-linked immunosorbent assay (ELISA) using a commercially available kit (MMP-9 Human ELISA Kit; Sangon Biotech, Shanghai, China) in both groups.

Statistical analysis

All statistical analyses were conducted using SPSS (version 17.0). Data are expressed as percentages of total for categorical variables, and mean±SD for continuous variables. The statistical analyses on the characteristics of the subjects were performed with the Pearson χ^2 test for categorical variables such as sex, smokers, diabetes, and hypertension and with the Student's *t* test for the continuous variable of age, BMI, TC, and TG with normal distribution. The Hardy-Weinberg equilibrium for the genotypic distributions of SNPs was tested using the chi-square (χ^2) goodness-of-fit test. *p* Values of <0.05 were set as a significant level. Logistic regression analysis was performed in genotypic analysis with the adjustment for confounding factors.

Results

Characteristics of study subjects

The demographic and clinical characteristics of the 258 CAD patients and 153 control subjects are listed in Table 1. Compared with the control group, the CAD group had bigger BMI, higher TC levels, higher MMP-9 concentrations, more

smokers, and more individuals with hypertension although there was no significant difference in the mean age, sex, TG levels, and prevalence of diabetes mellitus between the CAD and control groups.

Hardy-Weinberg equilibrium

The χ^2 goodness-of-fit test showed that the genotypic distributions of rs3918242 and rs17576 did not deviate from the Hardy-Weinberg equilibrium in both the CAD and control groups (*p*>0.05). The subjects in both groups were representative.

MMP-9 gene polymorphism analysis

For the polymorphism analysis of rs3918242, the size of the amplicon was 662 bp, we saw three genotypes after digested by SphI, cut homozygosity: TT (469 bp, 193 bp), cut heterozygosity: CT (662 bp, 469 bp, 193 bp), and uncut homozygosity: CC (662 bp) (Fig. 1).

For the polymorphism analysis of rs17576, the size of the amplicon was 300 bp, we got three genotypes after digested by MspI, cut homozygosity: GG (170 bp, 130 bp), cut heterozygosity: AG (300 bp, 170 bp, 130 bp), and uncut homozygosity: AA (300 bp) (Fig. 2).

Allele and genotype analysis

The frequency of the T allele in the *MMP-9* promoter was 14.3% in the CAD group compared with 7.2% in the control group. The rs3918242 T allele was associated with CAD ($\chi^2=9.524$, *p*<0.05) and survived 1000 permutations (global *p*<0.05) (Table 2). Furthermore, while there were no TT homozygotes in the control group, we calculated the CT plus TT genotype, and the frequencies were 25.2% in the CAD group compared with 14.4% in the control group (*p*<0.05). After being adjusted for confounding factors, the logistic regression test revealed genotypic associations between rs 3918242 and CAD ($\chi^2=6.732$, *p*<0.05) (Table 3). However, the frequencies of the G allele, AG plus GG genotype of rs17576 were similar (*p*>0.05) (Table 3).

The serum MMP-9 analysis

The total serum level of MMP-9 was higher in the CAD group compared with the control group (174.36±35.09 ng/mL vs. 106.73±28.53 ng/mL, *p*<0.05) (Fig. 3). The MMP-9 level was still an independent risk for CAD after being adjusted for covariates ($\chi^2=4.183$, *p*<0.05). There were also

FIG. 1. Enzymolysis electrophoretic of Matrix metalloproteinase-9 (*MMP-9*) gene rs3918242 polymorphism.

FIG. 2. Enzymolysis electrophoretic of *MMP-9* gene rs17576 polymorphism.

significant differences in the serum MMP-9 levels between the two groups with and without the SNP in CAD subjects. The subjects carrying T alleles (TT homozygotes and CT heterozygotes) had higher average serum MMP-9 levels compared with CC genotypes (207.10 ± 39.43 ng/mL vs. 158.07 ± 26.96 ng/mL, $p < 0.05$) (Fig. 4). A multiple logistic regression analysis with a forward stepwise selection was also performed. The results revealed that the TC level, history of smoking, hypertension, and the rs3918242 C allele and MMP-9 concentration were independently associated with CAD. The most predictive risk factor for CAD was hypertension (odds ratio [OR] = 12.107, 95% confidence interval [CI] 4.180–20.202, $p < 0.05$), followed by the T allele in the *MMP-9* promoter (OR = 5.036, 95% CI 2.554–8.205, $p < 0.05$).

Discussions

CAD is one of the main causes of death in both developed and developing countries. Atherosclerosis is apparently initiated in response to arterial endothelial injury before CAD happens, which allows increased permeability to lipids, monocytes, and lipid-laden macrophages. The MMP-9 secreted from macrophages apparently helps medial smooth muscles to migrate into the intima by degrading the ECM (Robertson *et al.*, 2007). The *MMP-9* gene is located on chromosome 20. It encodes the gelatinase enzyme for ECM metabolism. The aim of this study was to investigate the association between the common variants of the *MMP-9* gene and CAD, the relationship between the serum level of MMP-9 and CAD and the relationship of SNP and serum level of MMP-9. The selected SNPs are a promoter and an exon, they are useful to find the disease-related variants because they are highly informative based on the CHB dbSNP database.

The present study showed that the promoter genetic variation in the *MMP-9* gene was associated with CAD in the Chinese Han population, the frequencies of minor allele T and CT+TT genotype of rs3918242 were significantly higher in case than in control subjects ($\chi^2 = 9.524$, $p < 0.05$; $\chi^2 = 6.732$, $p < 0.05$). The allele frequency of rs3918242 in controls, observed in our study, was similar to that in the HapMap-CHB reference population. Our results also suggest that individuals carrying the minor allele T of rs3918242 may have a higher risk for CAD. This study is in agreement with several prior investigations indicating that genetic variation in the *MMP-9* gene may affect risk of CAD. It has been reported that the *MMP-9* T allele was associated with increased risk of developing CAD in patients younger than 55 years in the Tehran population of Iran (Saedi *et al.*, 2012). Koh *et al.* suggested that the rs3918242 polymorphism in the *MMP-9* promoter had a significant and independent role in the development of AMI due to plaque rupture in the Korean population (Koh *et al.*, 2008). Zhang *et al.* performed a study in 374 patients with available angiographic data, the result revealed that the rs3918242 T allele was associated with complicated coronary lesions, 26% of those carrying 1 or 2 copies of the T allele had >50% stenosis in three coronary arteries, whereas only 15% of C/C homozygotes had triple-vessel disease (Zhang *et al.*, 1999a). These results suggested that the *MMP-9* gene rs3918242 polymorphism might play an important role in the development of CAD due to ECM degradation. The mechanism whereby the SNP of rs3918242 in the *MMP-9* gene contributes to CAD is probably because the SNP exerts an allelic effect on *MMP-9* promoter strength, such that the T allelic promoter has a higher transcriptional activity than the C allelic promoter, which is likely to be attributed to binding a transcriptional repressor to the C allele (Zhang *et al.*, 1999a; Morgan *et al.*, 2003).

TABLE 2. DISTRIBUTION OF ALLELE FREQUENCIES OF SINGLE-NUCLEOTIDE POLYMORPHISMS IN CASE AND CONTROL GROUPS

SNPs	Group	HWE ^a	Allele numbers (%)		χ^2	P	OR	95% CI
rs3918242	Case	0.061	C	T	9.524	0.002 ^a	0.463	0.281–0.762
	Control	0.875	442/85.7	74/14.3				
rs17576	Case	0.267	A	G	3.313	0.069	1.419	0.972–2.072
	Control	0.431	73/14.1	443/85.9				
			58/19.0	248/81.0				

^aThe adjusted p -value was 0.001 from 1,000 permutations.

HWE, Hardy-Weinberg equilibrium; SNP, single-nucleotide polymorphism; OR, odds ratio; CI, confidence interval.

TABLE 3. DISTRIBUTION OF GENOTYPE FREQUENCIES OF SNPs IN CASE AND CONTROL GROUPS

SNPs	Genotype	Case (%)	Control (%)	χ^2	p	OR	95% CI
rs3918242	CC	193/74.8	131/85.6	6.732	0.009	2.005	1.178–3.414
	CT+TT	65/25.2	22/14.4				
rs17576	AA	3/1.2	4/2.6	0.497	0.481	2.282	0.504–10.335
	AG+GG	255/98.8	145/97.4				

However, our study did not show any significant association between the genetic variation of rs17576 and CAD. There was no significant difference in the rs17576 genotype frequency between the CAD and control groups (Zhi *et al.*, 2010). Opstad *et al.* (2012) showed that the reduced endogenous MMP-9 activity was obtained in the patients with stable CAD measured by the fluorometric quantitative assay. Our result was similar to these studies. There was neither an allelic nor genotypic association between rs17576 and CAD ($p < 0.05$) in the Chinese Han population. rs17576 did not show any association with CAD mainly because the replacement from wild type (A) to mutant type (G) and the subsequent amino acid switch from arginine to glutamine induced lower gelatinolytic activity of the zymogen.

Associations between the rs3918242 polymorphism and higher circulating concentrations of the protein have previously been reported (Blankenberg *et al.*, 2003; Yasmin *et al.*, 2006; Gai *et al.*, 2009; Wu *et al.*, 2010). Overexpression of MMP-9 was found in human atherosclerotic plaques and involved in the rupture of the plaques (Brown *et al.*, 1995). In this study, we also study the MMP-9 activity association with CAD and gene polymorphism. We found that the patients in the CAD group had higher levels of MMP-9, and CAD patients who carried the T allele also had a significantly higher MMP-9 activity in the serum than those who carry the C allele. These findings support the hypothesis that connective tissue remodeling after artery intima damage is mediated by MMP-9, which plays an important role in atherogenesis. 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 inhibitor of MMPs. The key step is transcriptional regulation because most MMP genes are expressed only when active physiological or pathological tissue remodeling takes place (Matrisian, 1990). This association may be explained by the enhanced ability of vascular SMCs to migrate and proliferate during atherogenesis in individuals carrying the T allele with higher transcriptional activity (Ye, 2000).

Although we got positive results in our study, the association between the *MMP-9* gene polymorphism and CAD are still inconsistent in different studies. Therefore, further studies on a larger population in cohort studies will be required to confirm this polymorphism as a potential novel genetic marker for CAD or plaque rupture, and prospective studies are needed to determine whether patients who experience fatal coronary events differ from those who experience non-fatal events.

Conclusions

This randomized clinical trial showed that the SNP rs3918242 in the *MMP-9* gene is very likely to be associated with CAD and that high serum levels of MMP-9 are strongly associated with CAD in the Chinese Han population. Genetic variation of rs3918242 may influence MMP-9 expression in T allele carriers. MMP-9 could be a significant risk factor for CAD and might play an important role in CAD involving the development of arteriosclerosis.

FIG. 3. The serum MMP-9 level between the coronary artery disease group and control group.

FIG. 4. The serum MMP-9 level between the two groups with and without single-nucleotide polymorphisms.

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Author Disclosure Statement

All authors have no conflict of interests.

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