Haplotype analysis of the matrix metalloproteinase 3 gene and myocardial infarction in a Chinese Han population

The Beijing atherosclerosis study

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Summary

Matrix metalloproteinase (MMP) 3 plays an important role in the pathogenesis of myocardial infarction (MI). Up to now, there has been conflicting data regarding the possible contribution of the MMP3 -1612 5A/6A promoter polymorphism to MI. In this study, we have investigated the possible association of three polymorphisms (-1612 5A/6A, -376C/G, Glu45Lys) in the MMP3 gene with MI in a Chinese Han population. The polymorphisms were analyzed in 509 patients with MI, and in 518 healthy controls. The frequency of the 5A allele was 14% in the healthy controls, which is less than in Western populations (40%-52%). Logistic regression analyses of individual polymorphisms indicated that individuals carrying the -1612 5A allele had an increased risk of MI (odds ratio [OR] 1.75, 95% confidence interval [CI] 1.28 to 2.40), as did those carrying the -376 G allele (OR 1.78, 95% CI 1.33 to 2.38). The three polymorphisms studied were found to be in strong linkage disequilibria. Haplotype analyses showed that the 5A-G-Lys haplotype (-1612 5A, -376G and 45Lys) was independently associated with susceptibility to MI. Taken together, the effect of the MMP3 polymorphisms studied may be attributable to the -1612 5A/6A polymorphism. We conclude that the MMP3 -1612 5A/6A polymorphism is associated with MI in our population, implying that individuals of the 5A allele carriers have an increased risk of suffering MI.

Keywords

Metalloproteinase, myocardial infarction, polymorphism, haplotype

Thromb Haemost 2004; 92: 867–73

Introduction

Myocardial infarction (MI) is one of the leading causes of death worldwide. Approximately 90% of MI results from an acute thrombus that obstructs an atherosclerotic coronary artery. Plaque rupture is considered to be the major trigger of coronary thrombosis (1, 2). Although the mechanism underlying plaque rupture is unclear, it has been shown that lipid-rich plaques express higher levels of matrix metalloproteinases (MMPs) than fibrotic plaques, and MMPs may contribute to weakening of the cap and subsequent rupture (3, 4). MMP3 is a key member of the MMPs family expressed in atheromas, which has broad substrate specificity, such as gelatin, fibronectin, vitronectin, laminin, and type IV collagen. Moreover, it can activate other enzymes in the MMP family (5, 6).

Over the past few years, the most studied polymorphism of MMP3 gene has been a functional promoter polymorphism (6A/5A) at position -1612 (7). *In vitro* assays revealed that the 5A allele had 2-fold higher promoter activity than the 6A allele (8). Recently, two reports from Japan showed that the transcrip-

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Received March 27, 2004
Accepted after revision July 4, 2004

Financial support:
This work was funded by grants 2002BA711A05, 2002BA711A08 and 2002BA711A10 of The National Tenth Five-Year Plan Key Programs from Ministry of Science and Technology of The People's Republic of China and H020220030130 Biomedical Project from the Council of Science and Technology, Beijing

Prepublished online September 7, 2004 DOI: 10.1160/TH04-03-0192

tionally more active 5A allele was over-presented in patients with acute MI compared with healthy controls (9, 10). By contrast, another even larger study also in Japan found that individuals homozygous for the 6A allele were at an increased risk of MI in women (11). More recently, a study in English patients indicated that individuals carrying the 5A allele might be predisposed to developing MI, whereas those carrying the 6A6A genotype might be predisposed to developing atherosclerotic plaques with significant stenosis (12).

In the present study, we compared the distributions of the -1612 5A/6A, -376G/C, and Glu45Lys polymorphisms alone or combined between patients with MI and controls in order to determine whether the polymorphisms are clinically useful for predicting MI in the Chinese Han populations.

Material and methods

Study subjects

The study population comprised 1027 unrelated Han Chinese from Beijing (819 men and 208 women). A total of 509 patients with MI (415 men and 94 women) were recruited from hospitalized patients of Fu Wai Hospital (Beijing, P. R. China) between October 1997 and December 2001. Eligible patients were those who met the WHO criteria for definite acute MI and survived the latest event more than 3 months. 518 gender- and age-matched healthy control subjects (404 men and 114 women) were recruited during the years of 2000 to 2001 from community participants of the International Collaborative Study of Cardiovascular Disease in Asia (InterASIA) in Beijing metropolitan area population (13). InterASIA selected a nationally representative sample of the general population aged 35 to 74 years in China. A total of 15,838 persons completed the survey and examination. All control subjects were judged to be free of coronary artery disease (CAD) by history, clinical examination, electrocardiography and the Rose questionnaire. Subjects with congenital heart disease, cardiomyopathy, valvular disease, and renal or hepatic diseases were excluded from the study. The present study has been carried out in accordance with the Declaration of Helsinki (2000) of the World Medical Association, and has been approved by the local Ethics Committee. All patients and control subjects gave informed consent.

A set of questionnaires was completed that included details of medical history, family history of CAD, drug intake, cigarette smoking, and alcohol consumption. Blood pressure, weight, height, waistline, and hip circumference were recorded, and the body mass index (BMI) and waist-to-hip ratio were calculated. The subjects were considered to have diabetes if they had ever been diagnosed as having type I or II diabetes by a physician or fasting blood glucose ≥7.0 mM. Hypertension was defined as an average systolic blood pressure (SBP) ≥ 140 mmHg, an average diastolic blood pressure (DBP) ≥ 90 mmHg, and/or self-reported current treatment for hypertension with antihypertensive medication. Hyperlipidemia was defined as a fasting total serum cholesterol level of > 220mg/dL (5.72mM), a fasting triglyceride level of > 150mg/ dL 1.70mM), and/or the intake of lipid-lowering drugs. Smoking status was defined as smoking 1 or more cigarettes per day for 3 months preceding the examination. Drinking was defined as usually drinking alcohol at least once a month during the year ahead of the interview.

Venous blood was drawn from all subjects after an overnight fast. Blood, serum and plasma were separated immediately and stored at –70°C.

Genotyping

Genomic DNA was isolated from white blood cells by the standard salt precipitation method (14). Genotyping for the -1612 5A/6A, -376C/G, and Glu45Lys polymorphisms were carried out by polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) analysis. Briefly, the DNA sequence containing the relevant polymorphic site was amplified by PCR, and the amplicon was digested with an

Table 1: Primers and restriction enzymes used for detection of the sequence variants.

Polymorphisms	Primers (5'→3')	Restriction enzymes(T)	Restriction fragments genotype, n (bp)
-1612 5A/6A	TTCTCCATTCCTTTGATGGGGGGAAAGA(F) TTATCTGTTGGGCTCCACTGTTTCTTCC(R)	Tth 111I (37°C)	6A6A,153 5A5A,123/30 5A6A,153/123/30
-376 C/G	CCACTTACATCTTTTATTTGCTTTTATCACC(F) ATCACCCGCAGCTTGACTCAT(R)	Eco72 I (37°C)	CC, 358 GG,328/30 GC,358/328/30
Glu45Lys	TTGCGCATCACCTCCAGAGT(F) GGGGCTTAAGGCACATGAGTA(R)	Taq I (65°C)	Glu45Glu,293/133 Lys45Lys,426 Glu45Lys,426/293/133

F indicates forward primer; R, reverse primer; T, incubation temperature.

* Underline represents mismatched base pairs.

appropriate restriction enzyme that cleaves only 1 of the 2 alleles. The sequences of PCR primers and conditions for RFLP are described in Table 1. Samples were subjected to denaturation at 94°C for 5 min, followed by 32 to 35 cycles of 94°C for 40 s, annealing at the corresponding temperature for 30 s, then extension at 72°C for 40 s, and a final step at 72°C for 8 min. Each 10-μl reaction consisted of approximately 25 ng of genomic DNA, 2.0 mM $MgCl_2$, 1.5 pmol of each primer, 0.2 mM of each deoxynucleoside triphosphate, 0.5 units of Taq polymerase (TaKaRa) and 1μl 10 × Mg^{2+} free reaction buffer. Restriction enzymes were obtained from MBI Fermentas and digests (10 μl) containing 5 units of restriction enzyme were incubated at 37°C overnight or at 65°C for 6 hours (Table 1). The digests were then separated on a 2% agarose gel and visualized by ethidium bromide staining.

Statistical analysis

Statistical analysis was conducted using the SPSS 10.0 version for Windows, 2LD program and EH program (http://www.iop.kcl.ac.uk/IoP/Departments/PsychMed/GEpiBSt/software.shtml), in conjunction with the haplo.score and haplo.glm programs in the haplo.stas package (http://www.mayo.edu/hsr/people/schaid.html).

Measurable variables were presented as mean ± SD and compared between patients with MI and controls with the use of the unpaired Student's t-test. Hardy-Weinberg equilibrium was assessed by the χ^2 test. Univariate analysis, used to measure the association of each single polymorphism with MI, was tested by the χ^2 test. Multivariate analysis, applied to measure the association between genotypes and MI, was performed by the logistic regression model. Pairwise linkage disequilibrium coefficients were calculated with estimated haplotype frequencies using the 2LD program, and the extent of disequilibrium was expressed in terms of D' (15, 16). Haplotype frequencies for various polymorphism combinations were estimated using the EH program. The EH program also performed an omnibus likelihood ratio test to examine the differences in haplotype frequency profiles between the cases and the controls (17). Moreover, multiple analyses between haplotypes and MI were performed using the haplo.score program (18). The interactions between haplotypes and gender were evaluated using the haplo.glm program (19). All statistical tests were 2-tailed, and $p < 0.05$ was considered statistically significant.

Power calculations were performed using a Normal Power calculator (http://calculators.stat.ucla.edu/powercalc/). Briefly, on the basis of frequencies of the lower allele in the control group being 13.7%, 26.9% and 32.7% for the -1612 5A/6A, -376C/G, and Glu45Lys polymorphisms, respectively, the sample size in this study would allow detecting a relative risk by allele of 1.6 for the 5A/6A polymorphism and a relative risk of 1.5 for the -376 C/G and Glu45Lys polymorphisms, with a power of 80% at the 0.05 significance level (2-sides).

Results

General characteristics

The main baseline characteristics of patients with MI and healthy controls are shown in Table 2. Controls and patients were matched by age and sex. Briefly, compared to the control group, the MI group had a greater proportion of smokers, more patients with hypertension, diabetes or stroke, and had a larger average BMI, and waist-hip ratio. The MI group also had significantly higher serum triglyceride levels, fasting glucose levels, and lower HDL-C (high density lipoprotein cholesterol) than the control group did. There were no significant differences in SBP, total cholesterol or LDL-C (low density lipoprotein cholesterol) between cases and controls. Moreover, DBP was significantly lower in patients than in controls, which could be the result of medication of diagnosed patients.

Association analyses of single polymorphism and pairwise linkage disequilibrium

The data on the MMP3 genotypic distributions within the studied population were consistent with the distribution predicted by the Hardy-Weinberg equilibrium, and showed that the frequencies of 5A and -376G alleles were significantly higher in cases than in controls (Table 3). Univariate analyses indicated that the 5A/6A and -376C/G polymorphisms were significantly associated with MI. Further gender-stratified analyses showed that the 5A/6A and -376C/G polymorphisms were only associated with an increased risk of MI in men (results not shown). Mutivariate logistic regression analysis, with adjustment for

Table 2: Clinical characteristics of study subjects.

	Cases (n=509)	Controls (n=518)	*P*
Male	415 (81.5)	404 (78.0)	NS
Average age	54.28±9.40	54.64±9.33	NS
BMI (kg/m^2)	26.59±3.18	24.82±4.42	<0.001
Waist-hip ratio	0.92±0.05	0.87±0.07	<0.001
TC (mM)	5.17±1.06	5.18±0.96	NS
HDL-C (mM)	1.07±0.25	1.27±0.31	<0.001
TG (mM)	1.85±1.27	1.44±0.88	<0.001
LDL-C (mM)	3.25±1.04	3.25±0.88	NS
Glucose (mM)	6.07±2.23	5.49±1.54	<0.001
SBP (mmHg)	129.84±20.67	128.71±19.06	NS
DBP (mmHg)	76.17±11.06	79.79±10.34	<0.001
Hypertension (%)	260 (51.1)	109 (21.0)	<0.001
Diabetes (%)	117 (23.0)	25 (4.8)	<0.001
Stroke (%)	61 (12.0)	14 (2.7)	<0.001
Smokers (%)	339 (66.6)	292 (56.4)	0.001

BMI indicates body mass index; TC, total cholesterol; TG, triglyceride; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; SBP, systolic blood pressure; DBP, diastolic blood pressure.
Average age in the case group represents the first onset age of MI.

Genotypes	Cases n=509	Control n=518	Genetic Model	Adjusted OR(95% CI)	*P*
5A/6A					
6A6A	330	385	Dominant	1.75(1.28-2.40)	<0.001
6A5A	163	124	Recessive	2.17(0.83-5.69)	0.114
5A5A	16	9	Additive	1.67(1.26-2.21)	<0.001
Freq. of 5A Allele (%)	19.2	13.7			<0.001
-376C/G					
CC	200	279	Dominant	1.78(1.33-2.38)	<0.001
CG	251	199	Recessive	1.49(0.91-2.45)	0.117
GG	58	40	Additive	1.53(1.22-1.91)	<0.001
Freq. of G allele (%)	36.1	26.9			<0.001
Glu45Lys					
Glu45Glu	202	235	Dominant	1.16(0.87-1.55)	0.302
Glu45Lys	240	227	Recessive	1.19(0.76-1.85)	0.452
Lys45Lys	67	56	Additive	1.13(0.91-1.40)	0.262
Freq. of Lys Allele (%)	36.7	32.7			0.056

OR indicates odds ration; CI, confidence interval. ORs were adjusted for sex, age, hypertension, diabetes, smoking, body mass index, and hyperlipidemia. Genetic models are based on the presumption that the lower allele of each polymorphism is risk allele.

Table3: Genotypic distribution and allele frequencies of 3 MMP3 polymorphisms in the Study Subjects.

Polymorphism Combinations	Haplotypes	Overall	Cases	Controls	*P*
6A/5A-C/G-Glu/Lys					
	6A-C-Glu	0.631	0.595	0.666	0.019
	5A-C-Glu	0.007	0.010	0.005	NS
	6A-C-Lys	0.035	0.023	0.046	0.047
	5A-C-Lys	0.013	0.011	0.014	NS
	6A-G-Glu	0.011	0.020	0.001	0.006
	5A-G-Glu	0.004	0.007	0.001	NS
	6A-G-Lys	0.160	0.170	0.150	NS
	5A-G-Lys	0.140	0.163	0.117	0.037
	Ln (L)	-1912.72	-991.62	-896.42	<0.01

Table 4: Estimates for haplotype frequencies and comparison between cases and controls, using the EH program.

Table 5: Score tests for association between haplotypes and MI, adjusted for nongenetic covariates by the haplo.score program.

Haplotype	Frequency	Score	*P*	
			χ^2	Simulation
6A-C-Glu	0.631	-0.248	0.013	0.013
6A-G-Lys	0.160	0.411	0.681	0.688
5A-G-Lys	0.140	3.396	0.001	<0.001

Haplotypes with frequency <0.05 are not included in this table. Covariates adjusted include age, sex, hypertension, diabetes, smoking status, body mass index, total cholesterol, triglyceride, and high density lipoprotein cholesterol.

age, gender, BMI, hypertension, hyperlipidemia, diabetes, and smoking, still revealed that the 5A/6A and -376C/G polymorphisms were related to MI in both dominant and additive genetic models (Table 3). In particular, in the dominant model, the -1612 5A/6A and -376C/G variants showed the strongest association, with an adjusted odds ratio for MI of 1.75 for individuals carrying the 5A allele, and of 1.78 for individuals carrying the -376G allele.

There were strong linkage disequilibria between the -376C/G and Glu45Lys polymorphism (D' = 0.93, $p < 0.001$), between the 5A/6A and Glu45Lys polymorphism (D' = 0.89, $p < 0.001$), and between the 5A/6A and -376C/G polymorphism (D' = 0.82, $p < 0.001$).

Table 6: Major results from selected studies between the 5A/6A polymorphism and MI in different populations.

Study population (reference)	Sample Size	5A-allele Frequency	Associated Genotype	Adjusted OR (95% CI)
Japanese	330 MI		5A6A/5A5A	2.25(1.51-3.35)
(9)	330 Controls	18%		
Japanese(female)	590 MI		5A6A/6A6A	4.7(2.0-12.2)
(11)	704 Controls	21%		
Chinese Taiwanese	150 MI		5A6A/5A5A	2.19(1.21-3.98)
(26)	150 Controls	20%		
English	135 MI		5A6A/5A5A	1.48(1.07-2.06)
(12)	1105 non-MI CAD	50%		
Japanese	group 1: 164 MI		5A6A/5A5A	1.67(1.02-2.74)
(10)	group 1: 302 MI		5A6A/5A5A	1.61(1.12-2.23)
	335 Controls	12%		
Chinese	509 MI		5A6A/5A5A	1.75(1.28-2.40)
(this study)	518 Controls	14%		

OR indicates odds ratio; 95%CI, 95% confidential interval.

Haplotype analysis

Table 4 shows the results of the haplotype analyses using the EH program. Omnibus haplotype profile tests showed a significant association with MI in this population ($p < 0.01$). It was found that the most common haplotype among the Chinese was 6A-C-Glu (63.1%) of the -1612 5A/6A, -376C/G, and Glu45Lys polymorphisms. There were another 2 haplotypes with a frequency ≥ 5%, i.e. 6A-G-Lys (16.0%) and 5A-G-Lys (14.0%), along with 6A-C-Glu accounting for 93% of all existing haplotypes. Among the three common haplotypes, univariate analysis showed that the frequency of 5A-G-Lys was significantly higher ($p = 0.037$), while that of 6A-C-Glu was lower ($p = 0.019$) in patients than in healthy controls, indicating that in contrast to the 6A-C-Glu haplotype, the 5A-G-Lys haplotype was associated with an increased risk of MI.

Table 5 shows the results of a score test using the haplo.score program. Contrary to 6A-C-Glu, the 5A-G-Lys haplotype maintained a significant association with MI, even after adjustment for conventional risk factors. Moreover, the results were in accordance with the univariate analysis of haplotypes using the EH program, as well as the single polymorphism analyses using the χ^2 test.

Although the single polymorphism analyses indicated some gender-specific effects of the 5A/6A and -376C/G polymorphisms, the additional haplotype-gender interaction analysis, using a haplo.glm program, did not provide evidence to suggest that gender modify the association between the MMP3 haplotypes and MI significantly (results not shown).

Discussion

This study indicates that individuals carrying the -1612 5A allele of the MMP3 gene are disposed to developing MI, and that the 5A-allelic frequency was 14% in our healthy controls, a frequency similar to those published in the Chinese Taiwanese (20%) and Japanese (12%-21%) populations of Mongolian origin, but much lower than the 40%-52% published for Caucasians (8-12, 20-26; Table 6). The MMP3 5A/6A promoter polymorphism was firstly identified by Ye S et al. (7). *In vitro* transient transfection studies have shown an approximately two-fold difference in expression driven by the two alleles, with the 5A allele being the more active (8). The 6A allele was preliminarily associated with an increased rate of progression of coronary atherosclerosis in an English population (7). Subsequently, several studies have reported that the 6A6A genotype was associated with an increased intima-media thickness and positive remodeling of the carotid artery in Finnish, Italian and American, respectively (20-23). Together, these data suggested that individuals homozygous for the 6A allele would have lower MMP3 levels in their arterial walls because of reduced gene transcription, and this lower enzyme activity might therefore favour the accumulation of extracellular matrix and the progression of atherosclerosis. Consistent with this hypothesis, analyses of genetic association in two clinical trials (LOCAT and REGRESS) showed that the 6A allele predisposed to greater lesion progression as assessed by quantitative coronary angiography (24, 25). However, two recent studies indicated that the 5A allele was associated with an increased risk of acute

MI in both Japanese and Chinese Taiwanese populations (9, 26; Table 6). In addition, a more recent study in English showed evidence of CAD patients carrying the 5A allele being at a higher risk of MI compared with CAD patients not carrying the 5A allele, while individuals with the 6A6A genotype had a greater extent of coronary atherosclerosis compared with those with other genotypes (12, Table 6). Considering these rather confusing findings, it should be noted that coronary atherosclerotic plaque rupture is the most common cause of MI (1), and that plaque progression and plaque rapture are two distinct processes that recognize different pathogenetic mechanisms (27). It is now becoming increasingly clear that local overexpression of activated MMP3, especially in the vulnerable shoulders, may promote atherosclerotic plaque rupture favouring a pathological development towards MI (4, 28). Accordingly, these findings support the notion that matrix accumulation in the arterial wall is enhanced in individuals carrying the transcriptionally less active 6A allele of the MMP3 gene, whereas matrix degradation and atherosclerotic plaque instability are promoted by the more active 5A allele (9, 12). In the present study, we provided further evidence that carriers for 5A allele are also more susceptible to MI in a Chinese population.

Haplotype analysis is frequently more powerful than the analyses using single polymorphism (29, 30). Recently, Schaid et al. (18) presented a haplo.score method to study haplotype associations in unrelated individuals, which global and haplotype-specific tests of association are based on the score function and can be adjusted for environment covariates. In the present study, we applied this method and identified an association between the 5A-G-Lys (-1612 5A, -376G, 45Lys) haplotype and an increased risk of MI, compared with the most common haplotype (i.e. 6A-C-Glu). The haplotype contains the 5A allele and this finding was thus in agreement with the above-mentioned data from analyses of the 5A/6A polymorphism, as well as the study performed by Beyzade et al. (12), in which three haplotypes containing the 5A allele were related to MI susceptibility. Therefore, the effect of the MMP3 gene variant may be attributable to the 5A/6A polymorphism. This could also be inferred from comparison of the effects of 5A-G-Lys and 6A-G-Lys haplotypes, which only differed at the -1612 site.

In contrast to the report by Beyzade et al. (12), the single polymorphism analyses in our study also showed that carriers with -376G allele were independently associated with an increased risk of MI. The interpretation of the measures of effects for candidate genes in an association study must be made with caution because alternative explanations need to be considered. An explanation that the study is affected by a population structure bias whereby many ethnic subgroups with different allelic distributions are pooled in the analysis is unlikely because all genotyped subjects were ethnically homogeneous. Although the -376G allele in controls was less frequent in our population (26.9%) than in the English (53.0%) (12), and the selection criteria were different between the two studies, the discrepancy may in part be due to genetic heterogeneity across ethnicities and sample selection bias. However, when we also take into consideration the fact that the common MMP3 haplotype 6A-G-Lys, also containing -376G and 45Lys, did not show the same association with MI as the 5A-G-Lys haplotype, and on the other hand, a gel shift assay did not detect any differential binding of transcription factors to the -376G/C promoter polymorphism (12), it seems likely that the -376G/C polymorphism confers, if any, only a modestly increased MI risk. Besides, strong linkage disequilibria among the three investigated polymorphisms were also observed in this study. Taken together, the best possible explanation is that the functional effect on susceptibility to MI of the 5A/6A polymorphism was marked by the -376G/C polymorphism and the 5A-G-Lys haplotype through linkage disequilibrium.

It must be pointed out that in a large-scale association study in Japan, the 6A allele was found to be a risk factor for MI in women (11). In contrast, we observed an association between the MMP3 high-activity 5A allele and MI in men by the univariate analyses. It is well known that the incidence of MI is low in women, especially among premenopausaul women; they are probably protected by their higher serum level of estrogen (31). These observations seemed to suggest that there may be a gender-specific association of the 5A/6A polymorphism with MI. In this study, however, further gender-specific interaction analysis did not yield a significant interaction between the haplotype and gender (Table 6). This needs further confirmation by prospective studies based on different populations.

This study did have some limitations. First, no association between the 5A/6A polymorphism and MI in women was likely due to the small sample size of female subjects. Second, the study subjects were not recruited prospectively. Thus, a survival bias and selection bias could not be excluded. Third, although age and sex were not significant confounding variables, other risk factors of cardiovascular disease were not controlled. Another limitation was that plasma levels of MMP3 were not determined.

In conclusion, the present study suggests that the 5A allele was related to a significantly increased risk of MI in the Chinese Han population, and that the frequencies of the MMP3 polymorphisms vary considerably among different races and ethnic groups.

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