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Haplotype Effect of the Matrix Metalloproteinase-1 Gene on Risk of Myocardial Infarction

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Abstract**—**Myocardial infarction (MI) is commonly caused by atherosclerotic plaque rupture following excessive degradation of collagen fibers in the atherosclerotic lesion. We investigated whether interindividual variability in risk of MI was related to polymorphisms in the gene encoding matrix metalloproteinase (MMP)-1, a key fibrillar collagen–degrading enzyme. Several single nucleotide polymorphisms in the MMP1 gene promoter were identified following sequencing DNA samples from 30 individuals. An analysis of the polymorphisms in a cohort of British whites with coronary atherosclerosis, including 639 patients with MI and 538 non-MI subjects, revealed a haplotype effect of the −519A>G and −340T>C polymorphisms on risk of MI, with the A_{-519}-C_{-340} and G_{-519}-T_{-340} haplotypes being protective (odds ratio=0.70 [0.57 to 0.86]; *P*=0.0007), whereas the G_{-519}-C_{-340} haplotype increased MI risk (odds ratio=1.94 [1.15 to 3.28]; *P*=0.013). This finding was replicated in a subsequent analysis of 387 Swedish MI patients and 387 healthy controls (odds ratio=0.70 [0.55 to 0.89], *P*=0.003, for A_{-519}-C_{-340} and G_{-519}-T_{-340}; odds ratio=1.54 [0.97 to 2.46], *P*=0.07, for G_{-519}-C_{-340}). In vitro assays showed that compared with the A_{-519}-T_{-340} haplotype, the A_{-519}-C_{-340} and G_{-519}-T_{-340} haplotypes had lower promoter activity, whereas the G_{-519}-C_{-340} haplotype had greater promoter strength, in driving gene expression in human macrophages. Haplotype-specific differences in MMP1 mRNA level in atherosclerotic tissues were also detected. The data indicate that MMP1 gene variation is a genetic factor contributing to interindividual differences in MI risk. **(*Circ Res*. 2005;97:1070-1076.)**

Key Words: matrix metalloproteinase ■ genetics ■ polymorphism ■ haplotype analysis ■ atherosclerosis

Myocardial infarction (MI) is predominately caused by coronary atherosclerotic plaque rupture, a mechanical breakdown of the fibrous cap overlaying the lipid core of the plaque.[1,2] Rupture of the plaque results in exposure of thrombogenic material (eg, tissue factor and lipids) located in the lipid core to the blood and leads to thrombus formation and, consequently, to the ischemic event.[1,2] The biomechanical strength of the fibrous cap largely depends on its content of fibrillar collagens, type I and III in particular, which are cross-linked to provide structural support.[3] Excessive degradation of these collagen fibers has been recognized as an important mechanism underlying atherosclerotic plaque rupture.[4–6]

Matrix metalloproteinase (MMP)-1 (or interstitial collagenase) can degrade fibrillar collagens, especially types I and III, which are resistant to most proteinases.[7,8] Pathological studies have shown that MMP1 is expressed by macrophages in atherosclerotic plaques and colocalizes with degraded fibrillar collagens in the shoulder region, which is prone to rupture.[9–11] MMP1 expression is significantly increased in vulnerable atherosclerotic plaques, characterized by having a thin fibrous cap overlaying a large lipid core, compared with stable atherosclerotic plaques, characterized by having a thick fibrous cap.[11,12] Thus, it has been postulated that increased MMP1 expression promotes atherosclerotic plaque rupture.[11]

In this study, we investigated whether interindividual variability in risk of MI was related to MMP1 gene polymorphisms that might influence MMP1 expression or activity. We first performed a search for polymorphisms in this gene by sequencing DNA samples from a group of unrelated individuals. The polymorphisms were then studied in relation to MI in a cohort of coronary artery disease patients. The findings were verified in an independent sample from another population. We then performed functional analyses of the polymorphisms found to be associated with MI risk.

Subjects and Methods

Identification of MMP1 Gene Variants

The MMP1 gene promoter (1.9 kb), all 10 exons, and the intron–exon junctions were amplified by PCR using primers described in Table I in the online data supplement available at http://circres.ahajournals.org. The PCR amplicons were sequenced using ABI

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TABLE 1. Characteristics of Subjects

	British Sample			Swedish Sample		
	MI Group (n=639)	Non-MI Group (n=538)	*P*	MI Group (n=387)	Control Group (n=387)	*P*
Age, y	62.9 (10.0)	63.8 (9.9)	0.21	52.5 (5.6)	53.0 (4.9)	0.16
Gender, male (%)	79	73	0.02	82	82	1.00
Body mass index, kg/m^2	27.6 (4.3)	27.5 (4.1)	0.75	27.4 (4.0)	25.9 (3.2)	<0.01
Smokers, current and previous, %	77	71	0.02	75	60	<0.01
Statin treatment, %	60	52	<0.01	35	0	<0.01
Total cholesterol, mmol/L	5.0 (1.0)	5.3 (1.1)	<0.01	5.1 (1.1)	5.4 (1.0)	<0.01
HDL-cholesterol, mmol/L	1.2 (0.3)	1.3 (0.3)	0.04	1.1 (0.3)	1.4 (0.4)	<0.01
Triglyceride, mmol/L	1.8 (1.2)	1.9 (1.1)	0.19	1.9 (1.2)	1.4 (0.7)	<0.01
Hypertension, %	42	49	0.01	34	6	<0.01
Type 2 diabetes, %	10	10	0.95	11	0*	<0.01

Means (SD) are shown for quantitative variables. Lower cholesterol level in the MI groups is likely attributable to the higher percentage of patients on statin treatment in these groups. *No control subjects had a previous diagnosis of manifest type 2 diabetes at the time of recruitment to the study. However, 3 individuals had a fasting plasma glucose concentration >7.0 mmol/L at the time of blood sampling. HDL indicates high-density lipoprotein.

sequencers and BigDye terminator cycle sequencing reagents. The PCR and sequencing were performed using DNA templates from 30 unrelated whites, which provided >90% power to detect sequence polymorphisms with an allele frequency of >5%.[13]

Subjects in Studies of MMP1 Gene Polymorphisms and Haplotypes in Relation to MI Risk

Analyses of MMP1 polymorphisms and haplotypes in relation to MI risk were performed in 2 population samples. The first was a cohort of British whites with coronary artery disease, documented angiographically as having >50% diameter stenosis in at least 1 coronary artery, as described previously.[14] It consisted of 2 groups, namely 639 patients who had experienced an MI and 538 non-MI subjects. The characteristics of the 2 groups are summarized in Table 1. The second sample consisted of 387 Swedish patients with an MI at the age of <60 years and 387 gender- and age-matched healthy Swedish individuals participating in a program aimed at identifying novel biomarkers and susceptibility genes for MI. The characteristics of the 2 groups are summarized in Table 1 and have been described in detail elsewhere.[15] In both studies, MI was diagnosed according to standard clinical criteria, including electrocardiographic and/or enzymatic changes. Both studies were approved by the respective local research ethics committees, and all subjects gave informed consent.

Determination of Genotypes

For each polymorphism, a DNA sequence containing the polymorphic site was amplified by PCR using primers described in supplemental Table I. The amplicons were digested with an appropriate restriction endonuclease that specifically cleaved 1 of the 2 alleles. The restriction endonucleases used were *Xmn*I (for −1607GG>G), *Hind*III (for −839G>A), *Msp*I (for −755G>T), *Kpn*I (for −519A>G), *Ban*I (for −422T>A), *Afl*II (for −340T>C), and *Hae*III (for −320T>C), respectively. Digests were subjected to gel electrophoresis, and DNA was detected by poststaining of the gel with Vistra Green and visualized using a fluorimager to determine genotypes.

Statistical Analyses of MMP1 Polymorphisms and Haplotypes in Relation to MI Risk

Allele and genotype frequencies were calculated by gene counting. Analyses of MMP1 polymorphisms in relation to MI were performed, first, by examining the polymorphisms individually and then, second, by studying haplotypes. Individual polymorphisms in relation to MI were examined by χ^2 analysis. In the haplotype analyses, a systematic analysis of all possible combinations of 1 to k polymorphisms to select the most informative and parsimonious haplotype configuration in terms of predicting disease status was performed using a previously described method that calculates the Akaike's Information Criterion (AIC) values for each haplotype model and then subtracts the minimum AIC value obtained for each model over all models explored, giving a rescaled AIC value for each haplotype model.[16] As described previously,[16] the models with a rescaled AIC ≤2 are considered equivalent to the most informative model, and among these models, the most parsimonious model with the fewest polymorphisms is considered the best model. Because the choice of models is based on AIC values, it circumvents problems associated with methods based on null-hypothesis testing, such as the requirement of multiple testing correction. After identifying the best model, the haplotype effects of the polymorphisms in this model on MI risk were analyzed using the THESIAS program (www.genecanvas.org), which implements the stochastic-EM (Expectation–Maximization) algorithm.[17] Haplotype effects on MI risk were adjusted for age, gender, body mass index, smoking, cholesterol levels, statin treatment, hypertension, and diabetes.

Transient Transfection and Reporter Assays

Transient transfection and reporter assays were performed to compare the strengths of MMP1 promoter of different haplotypes in driving gene expression. For each haplotype, the corresponding MMP1 promoter (from −1870 bp to +42 bp relative to the transcriptional start site) was generated by PCR using genomic DNA as template and then cloned into a TOPO PCR cloning vector (Invitrogen). The cloned MMP1 promoter was sequenced to verify that there was no misincorporation during PCR and then subcloned into a plasmid (pGL3-Basic Vector; Promega) containing a firefly luciferase reporter gene. The resultant construct was mixed with a plasmid (pRL-TK, Promega) containing a *renilla* luciferase gene under the control of a thymidine kinase promoter and transferred into cultured THP-1 human monocytic cells using the lipofection method with FuGENE 6 transfection reagent. The transfected cells were treated with phorbol 12-myristate 13-acetate to induce differentiation of the cells into macrophages. At 36 hours after transfection, the cells were lysed and the activities of the firefly luciferase and *renilla* luciferase in the lysates measured with the use of a dual-luciferase assay kit (Promega). The ratio of firefly luciferase level to *renilla* luciferase level was used as a measure of the MMP1 gene promoter activity. Three independent experiments in duplicate were performed for each construct.

(A)

Single nucleotide Polymorphisms		Sequences
SNP1	-1607 G ins/del	TAATTAGAAA [GG/G] ATATGACTTA
SNP2	-839 G>A	CTGTTGCCTA [G/A] GCTGGAGTGC
SNP3	-755 G>T	GCCTCTTCAG [G/T] GACTAGGACT
SNP4	-519 A>G	GCAATAGGGT [A/G] CCAGGCAGCT
SNP5	-422 T>A	TCAGTACAGG [T/A] GCCGAACAGC
SNP6	-340 T>C	CCTGTAGCAC [T/C] TTATGACCAT
SNP7	-320 T>C	TCAGAACCAG [T/C] CTTTCAAAAA

(B)

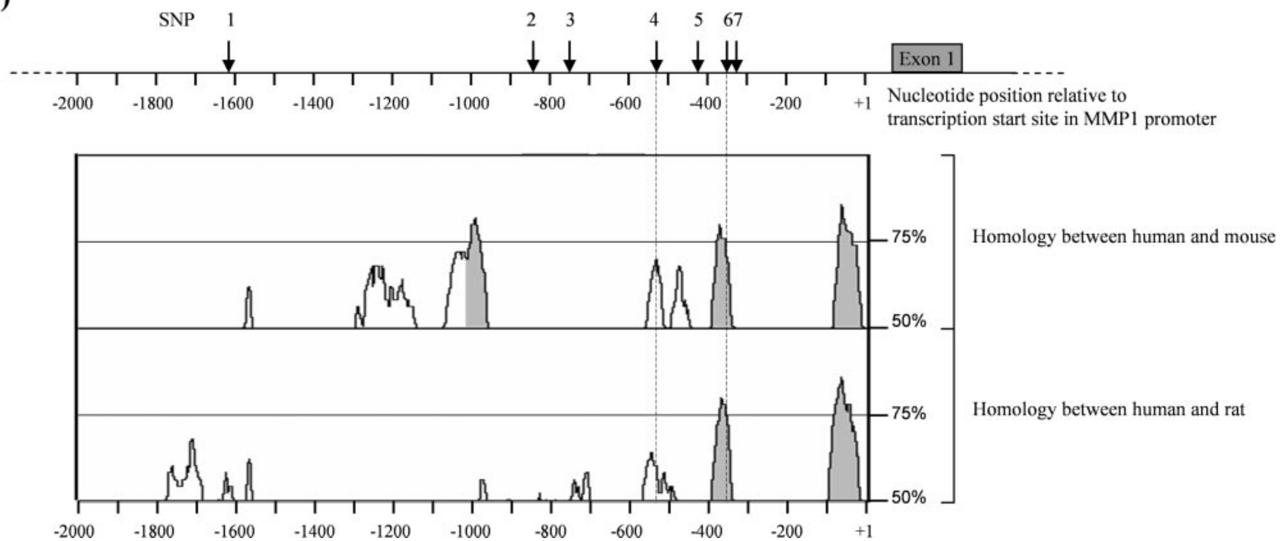


Figure 1. MMP1 gene polymorphisms identified. A, Summary of the position (relative to the transcription start site) and nucleotide changes of the promoter polymorphisms identified. B, Schematic presentation of the locations of the MMP1 polymorphisms in relation to conserved regions identified using the mVistra program.[34] The −519A>G polymorphism is located in a human MMP1 gene promoter region that shares >60% homology with orthologous sequences in mouse and rat. The −340T>C polymorphism is situated in a human MMP1 gene promoter region that shares >75% homology with orthologous sequences in the other 2 species. The other polymorphisms are not located in regions conserved in all of these species.

Electrophoretic Mobility-Shift Assays

For each allele, a double-stranded oligonucleotide corresponding to the sequence at and surrounding the polymorphic site was generated and used as a probe. The sequences of these probes are described in supplemental Table I. Each probe was labeled with ^{32}P at the 5′ terminus. The labeled probe was incubated with nuclear protein extracts from cultured human macrophages derived from THP-1 cells, followed by nondenaturing polyacrylamide gel electrophoresis and autoradiography, as described.[18,19] Three independent experiments were performed for each polymorphism.

Real-Time RT-PCR

RNA was extracted from atherosclerotic plaques removed from patients (n=26) undergoing carotid endarterectomy and reverse transcribed to cDNA. Real-time PCR was performed in duplicates in an ABI Prism 7700 Sequence Detection System. PCR primers (described in supplemental Table I) were designed with the use of the Primer Express program (Applied Biosystems), with the forward and reserve primers placed in different exons. The MMP1 real-time RT-PCR results of different samples were standardized for the amounts of RNA template and efficiencies of reverse transcription using the housekeeping gene 36B4 (acidic ribosomal phosphoprotein PO subtype) as a reference[20] and applying the $2^{-\Delta CT}$ method.[21] The haplotype effects of the MMP1 −519A>G and −314C>T polymorphisms on MMP1 mRNA levels were analyzed using the THESIAS program, adjusting for statin treatment and atherosclerotic plaque types defined according to the revised American Heart Association classification system,[22] which takes into account macrophage density, smooth muscle cell density, the size of lipid core, and the proportion of fibrous tissue.

Results

Identification of Polymorphisms in the MMP1 Gene

To search for naturally occurring, common sequence variants of the MMP1 gene, we sequenced the promoter, exons, and intron–exon junctions of the gene in 30 unrelated white individuals. In the promoter region, single-nucleotide polymorphisms were identified at positions −1607 (GG/G, ie, G insertion/deletion), −839 (G/A), −755 (G/T), −519 (A/G), −422 (T/A), −340 (T/C), and −320 (T/C) (Figure 1A). Two of these 7 promoter polymorphisms, namely −519A>G and −340T>C, were found to be located in regions conserved in human, mouse, and rat (Figure 1B). In the coding region, only 2 polymorphisms were identified, neither of which changes the amino acid sequence. The subsequent studies were focused on the promoter polymorphisms.

MMP1 Haplotype Effects on Risk of MI

To investigate whether there was a genotypic or haplotypic effect of MMP1 on MI risk, we first studied the polymorphisms in a sample of British whites with hemodynam-

TABLE 2. Haplotype Effects of the MMP1 −519 and −340 Polymorphisms on Risk of MI

Haplotype	Haplotype Frequency		Haplotype Effects on Risk of MI*	Haplotype Effects on Risk of MI* (With Homogenous Effects of A_{-519}-C_{-340} and G_{-519}-T_{-340})
	MI Group	Control Group		
British sample				
A_{-519}-T_{-340}	0.43	0.36	Reference haplotype	Reference haplotype
A_{-519}-C_{-340}	0.19	0.24	OR=0.68 (95% CI=0.52–0.88), *P*=0.0039	OR=0.70 (95% CI=0.57–0.86), *P*=0.00067
G_{-519}-T_{-340}	0.31	0.37	OR=0.71 (95% CI=0.56–0.89), *P*=0.0027	
G_{-519}-C_{-340}	0.07	0.03	OR=1.94 (95% CI=1.15–3.28), *P*=0.013	OR =1.93 (95% CI=1.14–3.26), *P*=0.014
Swedish sample				
A_{-519}-T_{-340}	0.40	0.34	Reference haplotype	Reference haplotype
A_{-519}-C_{-340}	0.22	0.27	OR=0.72 (95% CI=0.54–0.94), *P*=0.016	OR=0.70 (95% CI=0.55–0.89), *P*=0.0032
G_{-519}-T_{-340}	0.27	0.34	OR=0.68 (95% CI=0.52–0.89), *P*=0.0049	
G_{-519}-C_{-340}	0.11	0.05	OR=1.54 (95% CI=0.97–2.46), *P*=0.070	OR=1.53 (95% CI=0.96–2.4), *P*=0.074

The analyses showed that there was no significant difference between the G_{-519}-T_{-340} and A_{-519}-C_{-340} haplotypes in relation to MI risk. *Haplotype effects were analyzed using the THESIAS program, adjusting for age, gender, body mass index, smoking, cholesterol levels, statin treatment, hypertension and diabetes. CI indicates confidence interval.

ically significant coronary atherosclerosis, consisting of 639 patients who had experienced an MI and 538 non-MI subjects. Frequencies of alleles, genotypes, and haplotypes derived from the 7 polymorphisms are shown in supplemental Table II. No significant difference in genotype distribution of individual polymorphisms between the 2 groups was detected.

Data of a systematic analysis of all possible combinations of the polymorphisms in relation to MI are presented in supplemental Table III. The analysis showed that the best model in terms of predicting MI status was 1 that consisted of the −519A>G and −340T>C polymorphisms. An analysis of haplotypes based on these 2 polymorphisms showed that the haplotype frequencies were significantly different between the MI and non-MI groups (χ^2=16.48 with 3 degrees of freedom, $P<10^{-3}$; Table 2). Compared with the A_{-519}-T_{-340} haplotype, both the A_{-519}-C_{-340} and G_{-519}-T_{-340} haplotypes conferred a protective effect against MI (odds ratio [OR]=0.68 [0.52 to 0.88], *P*=0.004, for A_{-519}-C_{-340}; OR=0.71 [0.56 to 0.89], *P*=0.003, for G_{-519}-T_{-340}; the common OR associated with these 2 haplotypes being OR=0.70 [0.57 to 0.86], *P*=0.0007; Table 2), whereas the G_{-519}-C_{-340} haplotype was associated with an increased risk of MI (OR=1.94 [1.15 to 3.28], *P*=0.013; Table 2).

To verify the findings above, a Swedish sample consisting of 387 MI patients and 387 healthy controls was genotyped for the polymorphisms. As in the British study, the Swedish study showed a protective effect of the A_{-519}-C_{-340} and G_{-519}-T_{-340} haplotypes (OR=0.72 [0.54 to 0.94], *P*=0.02, for A_{-519}-C_{-340} versus A_{-519}-T_{-340}; OR=0.68 [0.52 to 0.89], *P*=0.005, for G_{-519}-T_{-340} versus A_{-519}-T_{-340}; and OR=0.70 [0.55 to 0.89], *P*=0.003, for A_{-519}-C_{-340}+G_{-519}-T_{-340} versus A_{-519}-T_{-340}; Table 2) and an increased risk for the G_{-519}-C_{-340} haplotype (OR=1.54 [0.97 to 2.46], *P*=0.07, for G_{-519}-C_{-340} versus A_{-519}-T_{-340}; Table 2).

Effects of MMP1 Polymorphisms on Promoter Activity in Driving Gene Expression

To investigate whether the −519A>G and −340T>C polymorphisms had a functional effect, we examined the promoter activity of MMP1 haplotypes in driving the expression of a luciferase reporter gene in transiently transfected THP-1 cell–derived human macrophages. Compared with the haplotypes encompassing both the A_{-519} and T_{-340} alleles, the haplotypes encompassing both the A_{-519} and C_{-340} alleles and those encompassing both the G_{-519} and T_{-340} alleles had lower promoter activity (*P*<0.01), whereas the haplotype containing both the G_{-519} and C_{-340} alleles had higher promoter activity (Table 3).

Effects of MMP1 Polymorphisms on Nuclear Protein Binding to the MMP1 Promoter

To investigate whether the −519A>G and −340T>C polymorphisms were located in DNA sequences recognized by nuclear proteins, and if so, whether the polymorphisms affected the DNA-protein interactions, we performed electrophoretic mobility shift assays with oligonucleotide probes corresponding to the different alleles and nuclear protein extracts from THP-1 human macrophages. For the −519A>G polymorphism, a DNA–protein complex (indicated by an arrow in Figure 2A) was detected in assays using a probe corresponding to the A_{-519} allele but not in assays using a probe corresponding to the G_{-519} allele. For the −340T>C polymorphism, a DNA–protein complex (indicated by an arrow in Figure 2B) was detected in assays using a probe corresponding to the C_{-340} allele but not in assays using a probe corresponding to the T_{-340} allele.

MMP1 Expression Levels in Atherosclerotic Plaques From Patients of Different MMP1 Haplotypes

We also examined MMP1 expression levels in atherosclerotic plaques from patients of different haplotypes using the real-time RT-PCR method. Compared with the A_{-519}-T_{-340} haplotype, the A_{-519}-C_{-340} and G_{-519}-T_{-340} haplotypes were associated with lower MMP1 expression in atherosclerotic plaques (*P*=0.033; Figure 3). The G_{-519}-C_{-340} haplotype, which was rare, with a frequency of <6% in British whites, was not present in the patients (n=26) from whom the

TABLE 3. Promoter Activity of MMP1 Haplotypes

Haplotype	Mean (SD) Luciferase Activity	Individual Haplotype Tested	Mean (SD) Luciferase Activity
$A_{-519}\text{-}T_{-340}$	5.83 (1.70)	$GG_{-1607}\text{-}G_{-839}\text{-}G_{-755}\text{-}\mathbf{A}_{-519}\text{-}A_{-422}\text{-}\mathbf{T}_{-340}\text{-}T_{-320}$	5.37 (0.44)
		$GG_{-1607}\text{-}G_{-839}\text{-}G_{-755}\text{-}\mathbf{A}_{-519}\text{-}T_{-340}\text{-}\mathbf{T}_{-340}\text{-}C_{-320}$	5.78 (1.47)
		$GG_{-1607}\text{-}G_{-839}\text{-}G_{-755}\text{-}\mathbf{A}_{-519}\text{-}T_{-340}\text{-}\mathbf{T}_{-340}\text{-}T_{-320}$	7.00 (2.37)
		$GG_{-1607}\text{-}G_{-839}\text{-}T_{-755}\text{-}\mathbf{A}_{-519}\text{-}T_{-340}\text{-}\mathbf{T}_{-340}\text{-}C_{-320}$	5.16 (2.22)
$A_{-519}\text{-}C_{-340}$	3.17 (1.13)	$GG_{-1607}\text{-}G_{-839}\text{-}G_{-755}\text{-}\mathbf{A}_{-519}\text{-}A_{-340}\text{-}\mathbf{C}_{-340}\text{-}T_{-320}$	4.03 (0.95)
		$G_{-1607}\text{-}G_{-839}\text{-}T_{-755}\text{-}\mathbf{A}_{-519}\text{-}A_{-340}\text{-}\mathbf{C}_{-340}\text{-}T_{-320}$	2.31 (0.20)
$G_{-519}\text{-}T_{-340}$	3.53 (1.13)	$G_{-1607}\text{-}A_{-839}\text{-}T_{-755}\text{-}\mathbf{G}_{-519}\text{-}A_{-340}\text{-}\mathbf{T}_{-340}\text{-}T_{-320}$	2.54 (0.25)
		$G_{-1607}\text{-}A_{-839}\text{-}T_{-755}\text{-}\mathbf{G}_{-519}\text{-}T_{-340}\text{-}\mathbf{T}_{-340}\text{-}T_{-320}$	4.52 (0.50)
$G_{-519}\text{-}C_{-340}$	6.73 (0.42)	$G_{-1607}\text{-}A_{-839}\text{-}T_{-755}\text{-}\mathbf{G}_{-519}\text{-}A_{-340}\text{-}\mathbf{C}_{-340}\text{-}T_{-320}$	6.73 (0.42)

Data shown are means (SD) of activity of luciferase with expression that was driven by MMP1 promoters of different common haplotypes (frequency $>$0.03) in THP-1 cell–derived human macrophages in 3 independent experiments. Nucleotides at the −519 and −340 sites are shown in bold letters.

atherosclerotic plaques were obtained for MMP1 expression level measurement.

Discussion

The study provides evidence of differences in MI risk between individuals of different MMP1 gene haplotypes, which were observed initially in a sample from Southern England and then in an independent sample from the Swedish population. In both studies, the A_{-519}-C_{-340} and G_{-519}-T_{-340} haplotypes were associated with a reduction in MI risk, whereas the G_{-519}-C_{-340} haplotype was associated with an increase in MI risk. The data suggest a contribution of MMP1 gene variation to interindividual variability in risk of MI, which is known to have a multifactorial etiology.

The reporter assays showed that compared with the A_{-519}-T_{-340} haplotype, the A_{-519}-C_{-340} and G_{-519}-T_{-340} haplotypes had lower promoter activity, whereas the G_{-519}-C_{-340} haplotype had higher promoter activity, in driving gene expression in human macrophages. The mRNA assays in atherosclerotic tissues showed that compared with the A_{-519}-T_{-340} haplotype, the A_{-519}-C_{-340} and G_{-519}-T_{-340} haplotypes were associated with reduced MMP1 expression in atherosclerosis plaques. These analyses suggest that differences in MMP1 gene promoter activity and expression might, perhaps in part, explain the differences in MI risk according to MMP1 haplotypes, as observed in the British and Swedish studies. Although the activity of MMPs is influenced not only by the level of MMP expression but also by MMP activators and inhibitors, previous studies have shown a strong relationship between MMP expression and MMP activity in atherosclerotic lesions.[9,11]

The electrophoretic mobility-shift assays showed that the −519A>G and −340T>C polymorphisms have an allele-specific effect on binding of nuclear proteins from human macrophages. This could potentially explain the haplotype effect of these 2 polymorphisms on MMP1 promoter activity

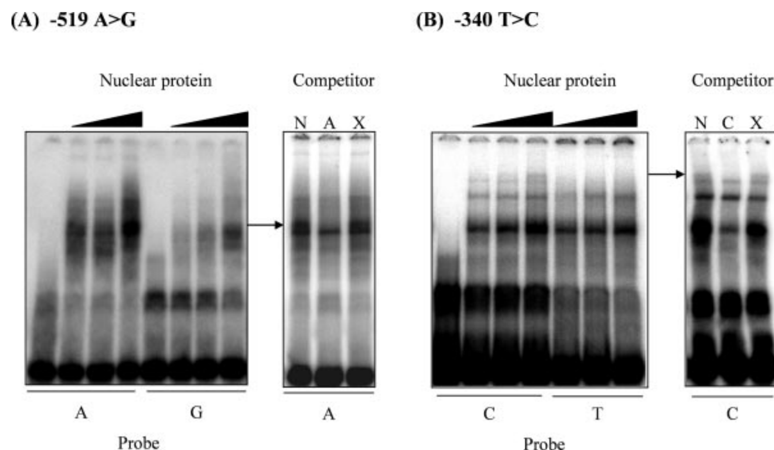
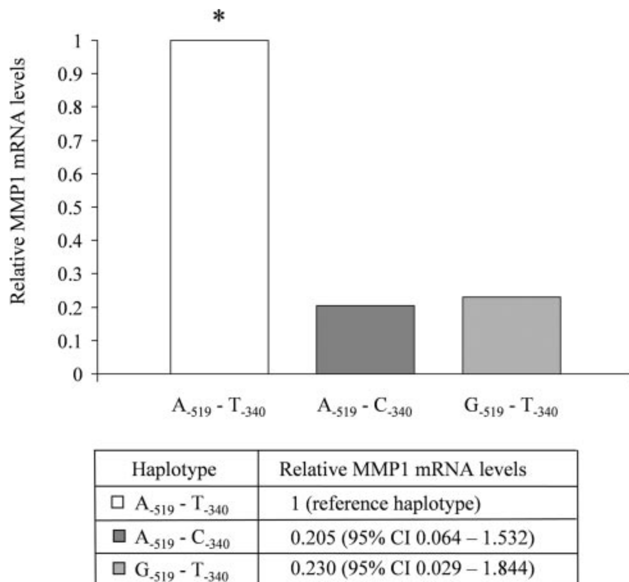


Figure 2. Effects of MMP1 polymorphisms on interactions of the MMP1 gene promoter with nuclear proteins. A, Representative results of electrophoretic mobility shift assays in which radiolabeled probes corresponding to the A_{-519} allele (lanes 1 through 4 in the left panel and lanes 1 through 3 in the right panel) or the G_{-519} allele (lanes 5 through 8 in the left panel) were incubated with nuclear protein extracts derived from THP-1 human macrophages in the absence or presence of competitors. Arrow indicates a DNA–protein complex detected in assays using the A_{-519} probe but not in assays using the G_{-519} probe. N indicates noncompetitor; A, competitor corresponding to the A_{-519} allele; X, nonspecific competitor. B, Representative results of electrophoretic mobility shift assays with radiolabeled probes corresponding to the C_{-340} allele (lanes 1 through 4 in the left panel and lanes 1 through 3 in the right panel) or the T_{-340} allele (lanes 5 through 7 in the left panel). Arrow indicates a DNA–protein complex detected in assays using the C_{-340} probe but not in assays using the T_{-340} probe. N indicates noncompetitor; C, competitor corresponding to the C_{-340} allele; X, nonspecific competitor.



Haplotype	Relative MMP1 mRNA levels
□ A_{-519} - T_{-340}	1 (reference haplotype)
■ A_{-519} - C_{-340}	0.205 (95% CI 0.064 – 1.532)
■ G_{-519} - T_{-340}	0.230 (95% CI 0.029 – 1.844)

Figure 3. Levels of MMP1 expression in atherosclerotic plaques. RNA was extracted from carotid atherosclerotic plaques removed from patients (n=26) undergoing carotid endarterectomy and reverse transcribed into cDNA. The cDNAs were used as templates in real-time PCR assays of the MMP1 gene and the housekeeping gene 36B4.[20] The relative level of MMP1 mRNA in each sample, standardized against the housekeeping gene, was calculated using the $2^{-\Delta Ct}$ method.[21] The haplotype effects of the MMP1 −519G>A and −314T>C polymorphisms on MMP1 mRNA levels were analyzed using the THESIAS program,[17] adjusting for statin treatment and atherosclerotic plaque types defined according to the revised American Heart Association classification system,[22] which takes into account macrophage density, smooth muscle cell density, the size of lipid core, and the proportion of fibrous tissue. The asterisk indicates a significant difference (P=0.033) comparing A_{-519}-T_{-340} with G_{-519}-T_{-340} and A_{-519}-C_{-340}. The G_{-519}-C_{-340} haplotype was not present in the patients studied.

detected in the reporter assays, but a complete explanation awaits analyses to characterize the nuclear proteins involved and their interactions. The −519A>G and −340T>C polymorphisms are located in conserved regions in the MMP1 promoter, as shown in Figure 1B. It has been suggested that conserved sequences often harbor transcription factor–binding sites.[23] In silico examination showed that the −340C allele (but not the −340T allele) might contain a binding site (CACCT on the reverse strand) for transcription factor TCF8 (also known as AREB6). We performed supershift assays with a TCF8 antibody to examine whether TCF8 was present in the DNA–protein complexes detected in the electrophoretic mobility-shift assays. The supershift assays showed that none of the DNA–protein complexes was affected by the antibody, suggesting that TCF8 is probably not involved in these complexes. The in silico examination did not reveal other transcription factor–binding site in the DNA sequences surrounding −519A>G or −340T>C, suggesting that the nuclear proteins binding to these regions are not in the current transcription factor databases, which only include factors that have been characterized. Therefore, identifying the proteins binding to these polymorphic sites will likely require cloning techniques such as the 1-hybrid assay.

An increasing number of studies have shown that a disease phenotype can be associated with a haplotype made up of polymorphisms that are not individually associated with the phenotype.[24–29] The present study provides another such example. The study showed that MI risk was associated with haplotypes derived from the −519A>G and −340T>C polymorphisms, although there was no association between the disease and the polymorphisms individually. In a systematic analysis of all different combinations of the 7 MMP1 polymorphisms using an AIC-based method (described in Subjects and Methods) that circumvents problems associated with methods based on null-hypothesis testing, such as the requirement for multiple testing correction, the haplotype model consisting of the −519A>G and −340T>C polymorphisms was shown to be the best model in terms of predicting the disease status.

It appears that there are at least 2 reasons that might explain why a phenotype can be associated with a haplotype but not with the individual polymorphisms that make up the haplotype. First, a functional effect on gene expression can be dependent on the interaction between 2 or more polymorphisms, as demonstrated in a study by Terry et al, which showed a cooperative influence of several polymorphisms on interleukin-6 gene transcriptional regulation and that studying a single polymorphism in isolation would not uncover the overall functional effect of the polymorphism in combination with other functional polymorphisms.[30] Second, haplotypes generally have a higher probability than individual polymorphisms of showing useful linkage disequilibrium with an unknown causal variant.[31]

It has been suggested that genetic factors may be classified into 2 broad categories: (1) susceptibility genes that affect certain initial events in the disease process and thus the susceptibility of an individual to the disease; and (2) disease modifying genes that affect the course of the disease process after it has been initiated and thus influence the disease outcome.[32] The results of the British study suggest that the MMP1 gene can have a modifying effect on coronary artery disease and that subjects in this study had coronary atherosclerosis, and in these patients, there was an association between MMP1 haplotypes and MI risk. It is possible that atherosclerotic lesions in individuals carrying higher MMP1 expression haplotypes are more likely to develop into vulnerable plaques because of excessive degradation of fibrillar collagens.

In summary, this study has shown a haplotype effect of the MMP1 gene on risk of MI. The results are consistent with the notion that MMP1 plays an important role in the pathogenesis of atherosclerosis and its clinical complications[1] and the notion that regulatory polymorphisms constitute an important type of genetic variant that influences susceptibility to complex diseases.[33] The data might have utility in future development of approaches for identifying at-risk individuals and strategies to optimize treatment for this common, complex disease.

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