Yonsei Medical Journal
Vol. 43, No. 4, pp. 473 - 481, 2002

Original Article

The Roles of Stromelysin-1 and the Gelatinase B Gene Polymorphism in Stable Angina

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Matrix metalloproteinases contribute to vascular remodeling by breaking down extracellular-matrix while new matrix is synthesized. Of the variety of MMPs, stromelysin-1 and gelatinase B may have key roles in coronary artery atherosclerosis. Moreover, The 5A/6A polymorphism in the promoter region of the stromelysin-1 gene may be a pathogenetic risk factor for acute myocardial infarction. Gelatinase B (92-kDa type IV collagenase and MMP-9) is one of the MMPs found to be highly expressed in the disruption-prone regions of atherosclerotic plaques. C- to T substitution at the promoter site (-1562) resulted in the higher promoter activity of the T-allelic promoter. The R279Q polymorphism in exon 6 led to the substitution of adenosine by guanine, and was a common polymorphism in the general population. We evaluated the relation between these polymorphisms and stable angina, the severity of atherosclerosis in coronary artery disease, and instent restenosis after percutaneous coronary angioplasty.

The study population was composed of 131 patients with stable angina (mean age 61.3 years, 89 males) and 117 control subjects (mean age 59.3 years, 59 males). Coronary angiographies were performed in all cases at Yonsei University Cardiovascular Hospital from February 1998 to June 2000. The genotype for each polymorphism was determined using a SNaPshotTM kit and by restriction fragment length polymorphism (RFLP).

The prevalence of 5A containing a polymorphism of the stromelysin-1 gene was higher in the stable angina group than in control patients, but no difference in the two polymorphisms of the gelatinase B gene was found between the two groups. By multiple logistic analysis, the 5A-allele of the stromelysin-1 gene was found to be an independent risk factor of stable angina with an odds ratio of 2.29 (95% CI; 1.19-4.38). However, the severity of atherosclerosis in coronary artery or in stent restenosis was not related to any polymorphism of stromelysin-1 or gelatinase B.

Our results show that functional genetic variation of stromelysin-1 could be a significant risk factor for stable angina, and might play an important role in coronary atherosclerosis involving vascular remodeling.

Key Words: Stable angina, polymorphism, stromelysin-1, gelatinase B

Received February 9, 2002
Accepted May 28, 2002

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INTRODUCTION

Matrix metalloproteinases contribute to vascular remodeling by breaking down extracellular-matrix while new matrix is synthesized. By breaking down extracellular matrix, MMPs may allow smooth muscle cells to invade and migrate, which contributes to the pathologic processes of atherosclerosis and restenosis after angioplasty.[1] Recently common variants in the promoter polymorphism of the stromelysn-1 and the gelatinase B MMP genes have been reported to be associated with atherosclerosis.[2-4]

The Stromelysin gene may play a key role in the rupture of atherosclerotic plaque, and the 5A/6A polymorphism in the promoter region of the stromelysin-1 gene may be a pathogenetic risk factor for acute myocardial infarction.[5] The polymorphism is located 600 bp upstream from the start of transcription in which one allele has a run of 6 adenosines (6A), whereas the other has only 5 adenosines (5A).[2,6] *In vitro* assays of promoter activity have revealed that the 5A allele has a 2-fold higher promoter activity than the 6A allele.[7]

The gelatinase B gene (92-kDa type IV collagenase and MMP-9) is one of the MMP genes found to be highly expressed in the disruption-prone regions of atherosclerotic plaques.[8-10] Zhang et al. suggested that a 9-bp sequence (GCGCAC/GCC, -1567 to -1559) containing the C-1562T polymorphic site is an important regulatory element, and is a binding site for a transcription repressor protein. Moreover, this DNA-protein interaction was abolished by a C-to T- substitution at the polymorphism site (-1562), resulting in a higher promoter activity T-allelic variant.[4] The R279Q polymorphism in exon 6 led to substitution of adenosine (A) by guanine (G) and was a common polymorphism in the population. Unlike the C to T substitution polymorphism, no study has been undertaken to address the possible functional effects of this polymorphism on atherosclerosis.[11]

The aims of this study were:- 1) to evaluate the relationship between polymorphisms of the stromelysin-1 promoter gene, and the gelatinase B gene in stable angina, 2) to evaluate the relationship of any polymorphism to the severity of coronary artery disease, and 3) to evaluate the relationship of any polymorphism to in stent restenosis.

MATERIALS AND METHODS

Subjects

The study populations were composed of 131 patients with stable angina and 117 control subjects. Coronary angiography was performed upon all patients at Yonsei University Cardiovascular Hospital from February 1998 to June 2000.

The inclusion criteria of stable angina were coronary artery disease proven by coronary angiography, without 1) a history of myocardial infarction or unstable angina, 2) previous coronary angioplasty, 3) peripheral or cerebral artery disease, 4) recent infection, and 5) atrial fibrillation. The inclusion criteria of control subjects were: 1) no evidence of coronary artery disease proven by coronary angiography, 2) no peripheral or cerebral arterial disease, and 3) freedom from recent infection and 4) atrial fibrillation.

All subjects enrolled in this study were Korean and gave written informed consent.

Assessment of angiographic data

Quantitative computer-assisted angiographic measurements (QCA) were performed on end-diastolic frames before balloon angioplasty, after stenting, and follow-up coronary angiography, using an on-line quantitative coronary angiographic system (Hicor, Siemens). Angiography was routinely performed in at least 2 projections, which were recorded in our database, and follow-up angiography was performed using the same projections. Operators were unaware of the patients' genotype. Minimal luminal diameter (MLD), reference diameter, percent diameter of stenosis, and lesion length were obtained from QCA. The severity of coronary artery atherosclerosis was evaluated by coronary angiography and graded as 1-vessel, 2-vessel, or 3-vessel disease using the number of $> 50\%$ occluded coronary arteries. Instent restenosis was defined as the classic criteria of more than 50% diameter stenosis during follow-up angiography.[12,13] Multivessel disease included 2-vessel and 3-vessel disease.

Baseline data

Baseline data was obtained from medical records and questioning. Lipid profiles (total cholesterol, low-density lipoprotein (LDL) cholesterol, triglyceride and high-density lipoprotein (HDL) cholesterol), lipoprotein (a), other inflammatory markers (ESR, fibrinogen) were obtained immediately after admission and before angiography in stable angina and in control subjects.

DNA analysis

Gelatinase B gene promoter polymorphism

Sense and antisense sequences of the primers were 5'- GCC TGG CAC ATA GTA GGC CC-3' and 5'-CTT CCT AGC CAG CCG GCA TC-3', respectively. After an initial denaturation at 95℃ for 10 minutes, samples were cycled 35 times, as follows:- 1 minute at 95℃, 1 minute at 62℃, and 1 minute at 72℃. Final extension was performed for 5 minutes at 72℃. After amplification, with

annealing at 62℃, the PCR product was restricted with 5 U of SphI restriction endonuclease over 2 hours at 37℃, and the final product was electrophoresed in 1.5% agarose gel and visualized directly by ethidium bromide staining. The C-allele lacks the SphI site that is present in the T- allele and gives rise to a fragment of 435bp rather than one of 247bp or 188bp (Fig. 1A).

Stromelysin-1 gene polymorphism

Genomic DNA was extracted from 300 μL of whole blood using a QIAmp Blood Kit (QIAGEN). Single nucleotide polymorphism (SNP) analysis was performed using the ABI Prism SNnapShot ddNTP Primer Extension Kit (ABI 310), according to the manufacturer's instructions. Sense and antisense sequences of the primer were 5′- GAT TAC AGA CAT GGG TGA CG-3′ and 5′-CAT CAC TGC CAC CAC TCT G-3′ respectively. After an initial denaturation at 95℃ for 10 minutes, samples were cycled 35 times as follows:- 1 minute at 95℃, 1 minute at 62℃, and 1 minute at 72℃. Final extension was performed for 5 minutes at 72℃. After amplification the PCR products were treated with 0.164 U/(l of shrimp alkaline phosphatase (USB Corp, Cleveland, Ohio, USA) and 0.164 U/(ll exonuclease (USB Corp, Cleveland, Ohio, USA) for one hour at 37℃, and this was followed by the enzyme deactivation for 15 minutes at 72℃. This template was mixed with primer 5′- CCT TTG ATG GGG GGA AAA A-3′, which was designed to complement the DNA sequence up to but not including the polymorphic nucleotide. In the presence of fluorescently labeled ddNTPs and Taq polymerase, the 3′ terminus of the primer was extended by a single ddNTP. This labeled product was then detected after electrophoresis using an ABI PRISMTM 310 genetic analyzer (Fig. 1B).

Gelatinase B gene exon 6 R279Q polymorphism

The sense and antisense sequences of the primer used were:- 5′- CTC GCC CCA GGA CTC TAG AC-3′ and 5′-GTG GAG GTA CCT CGG GTC GGG-3′, respectively. After an initial denaturation at 95℃ for 10 minutes, samples were cycled 35 times for:- 1 minute at 95℃, 1 minute at 62℃, and 1 minute at 72℃. Final extension was then performed for 5 minutes at 72℃. After amplification with annealing at 62℃, SNP analysis was performed using the same method as described above using an ABI Prism SNapShot ddNTP Primer Extension Kit (ABI PRISMTM 310) with 5′-GCG-AGA-GAC-TCT-ACA-CCC-3′ as primerb (Fig. 1C).

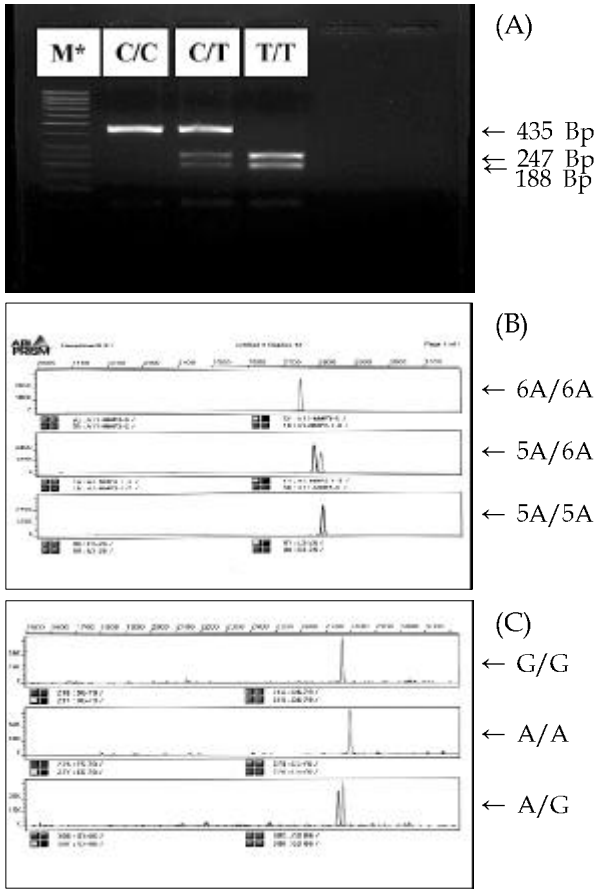

Fig. 1. (A) Polymorphisms of the gelatinase B gene promoter were analyzed using the restriction fragment length polymorphism (RFLP). The stromelysin-1 gene (B) and the gelatinase B gene exon 6 (C) were analyzed by using a single base pair extension assay using a SNaPshotTM kit. *M: Marker (50 bp).

Statistical analysis

Data was analyzed using SPSS 10.0 statistical software. Differences between groups of continuous variables, and discrete variables were analyzed using the independent t-test, and χ^2 test, respectively. χ^2 analysis was used to test for deviation in the genotype distribution from the Hardy-Weinberg equilibrium and to determine whether allele or genotype frequencies were

significantly different between stable angina and control subjects. Logistic multiple regression was carried out to determine genotype distribution differences between stable angina and control subjects. Adjusted estimates of conditioned relative risk and 95% confidence intervals were also calculated. A value of $p < 0.05$ was taken to indicate statistical significance.

RESULTS

Baseline characteristics

The baseline characteristics of patients with stable angina and control subjects are shown in Table 1. No significant differences were observed in terms of demographic characteristics, except age. In an analysis of coronary artery risk factors, total cholesterol and LDL - cholesterol levels were found to be significant higher in stable angina patients than in the control subjects. However, other risk factors, such as, smoking, hypertension, diabetes and HDL-cholesterol were not significantly for the two groups.

Distributions of stromelysin-1 and gelatinase B genotypes

In distribution of stromelysin-1 promoter gene, 6A/6A, 5A/6A, 5A/5A genotypes were 76.2% (n=189), 22.6% (n=56), and 1.2% (n=3), respectively. Distributions of the gelatinase B promoter gene, CC, CT and TT genotypes were 74.2% (n=184), 24.2% (n=60), and 1.6% (n=4), respectively. In cases of gelatinase B gene polymorphisms, the frequencies were 14.1% (n=35), 44.0% (n=109), and 41.9% (n=104) for genotypes GG, GA and AA, respectively. The distributions of genotypes of all three genes polymorphisms were compatible with the Hardy-Weinberg equation.

Comparing the stable angina group with the control group, the frequencies of the 6A homozygote were higher in the control group and the 5A/6A heterozygote was higher in the stable angina group. However, the distributions of the

Table 1. Baseline Clinical Characteristics of Subjects

	Stable angina (n=131)	Control (n=117)	*p*-value
Age (years)	61.3 ± 7.9	59.3 ± 8.5	p=0.04
Gender (M/F)	89/42	59/58	p=NS
BMI (kg/m^2)	25.7 ± 3.5	25.2 ± 4.3	p=NS
Smoking (n)	40 (30.8%)	39 (34.2%)	p=NS
Hypertension (n)	62 (47.3%)	52 (45.6%)	p=NS
Diabetes (n)	31 (23.7%)	18 (15.5%)	p=NS
Total Cholesterol[1]	198.0 ± 35.8	176.7 ± 33.3	$p<0.001$
LDL-cholesterol[1]	120.0 ± 27.6	102.0 ± 27.3	$p<0.001$
HDL-cholesterol[1]	42.6 ± 12.9	45.8 ± 12.9	p=NS
Triglyceride[1]	166.6 ± 107.7	142.6 ± 75.1	p=NS
Fibrinogen[1]	367.8 ± 93.5	381.1 ± 127.5	p=NS
ESR (mm/hr)	19.9 ± 14.9	20.0 ± 17.1	p=NS
Lipoprotein (a)[1]	28.2 ± 23.0	27.2 ± 20.8	p=NS

p=NS.
LDL, low density lipoprotein; HDL, high density lipoprotein.
[1]mg/dl.
*Student t-test (for continuous variables) and χ^2 test (for discrete variable) were used to compare the values for stable angina and control patients.
†Continuous variables are presented as mean ± SD.

genotypes at the two polymorphisms of gelatinase B were not significantly different in the two groups.

Comparison of genotype frequencies of the stromelysin-1 and the gelatinase B gene polymorphisms in stable angina and control patients

Genotype and allele frequencies of the stromelysin-1 gene and of the gelatinase B gene are summarized in Table 2. The distribution of each genotype was compatible with the Hardy-Weinberg equilibrium. The prevalence of the 5A/5A + 5A/6A genotype was significantly more greater in stable angina patients, and the 5A allele was more frequent in patients with stable angina. However, the prevalence of the C/T + T/T genotype and of the A/A + G/A genotype in gelatinase B was similar in the two groups (Table 2). By multivariate logistic analysis, the 5A/6A + 5A/5A genotype of the stromelysin-1 promoter was identified as an independent risk factor with an odds ratio of 2.28 (95% CI; 1.19 - 4.38) (Table 3).

Comparison of genotype frequencies in stable angina subjects with single-vessel or multi-vessel disease

In 131 stable angina cases for which coronary angiographic data were available, no significant association was found between gene polymorphism and the severity of coronary atherosclerosis (Table 4).

Table 2. Genotype and Allele Frequencies in Patients with Stable Angina and in Control Subjects

Variables	Stable angina (n=131)	Control (n=117)	*p* value
Stromelysin-1			
6A/6A	92 (70.2%)	97 (82.9%)	*p*=0.01
5A/6A + 5A/5A	39 (29.8%)	20 (11.8%)	
5A allele (frequency)	0.24	0.15	
Gelatinase B exon			
AA + GA	75 (57.3%)	69 (59.0%)	*p*=0.44
GG	56 (42.7%)	48 (41.0%)	
A allele (frequency)	0.4	0.4	
Gelatinase B promoter			
CC	99 (75.6%)	85 (72.6%)	*p*=0.35
CT + TT	32 (24.4%)	32 (27.4%)	
T allele (frequency)	0.21	0.22	

χ^2 test were used to compare the values for stale angina and control patients.

Table 3. Multiple Logistic Analysis of Risk Factors for Stable Angina

Predictors	Odds ratio (95% CI)	*p* value
Stromelysin-1, 5A/6A+ 5A/5A	2.28 (1.19 - 4.38)	0.01
Male gender	2.26 (1.29 - 3.93)	0.04
High LDL-cholesterol[1]	3.65 (1.79 - 7.39)	< 0.001

CI, Confidence interval.
[1]Higher LDL-cholesterol was defined when the LDL-cholesterol was higher than 130 mg/dl.

Comparison of genotype frequencies in stable angina patients with instent restenosis or patency

Genotype frequencies were analyzed in 48 patients that received percutaneous coronary angioplasty, and for whom 6-month follow-up coronary angiographic data were available. No significant association was found between polymorphisms in the stromelysin-1 or gelatinase B genes and in stent restenosis (Table 5).

DISCUSSION

This study provides evidence of an association between the stromelysin promoter 5A/6A polymorphism and stable angina. However, genetic polymorphisms of the gelatinase B C-1562T polymorphism in the promoter region and the R279Q polymorphism in the exon region, were not found to be associated with stable angina.

Stromelysin-1 is a key regulator of matrix remodeling and has broad substrate specificity; it can also activate other MMPs.[14-18] The stromelysin-1 promoter gene 5A/6A polymorphism has been

Table 4. Genotype Frequencies between Single and Multi-vessel Disease

Variables	Single vessel (n=48)	Multiple vessel (n=83)	*p* value
Stromelysin-1			
6A/6A	33 (68.8%)	59 (71.1%)	
6A/5A + 5A/5A	15 (31.2%)	24 (28.9%)	*p*=0.46
Gelatinase B exon			
AA + GA	27 (56.3%)	48 (57.8%)	
GG	21 (43.7%)	35 (42.2%)	*p*=0.50
Gelatinase B promoter			
CC	34 (70.8%)	65 (78.3%)	
CT + TT	14 (29.2%)	18 (21.7%)	*p*=0.23

χ^2 test was used to compare the values for single-vessel and multi-vessel disease.

Table 5. Genotype Frequencies between Patients with in stent Restenosis and Patency

Variables	In stent restenosis (n=14)	patency (n=34)	*p* value
Stromelysin-1			
6A/6A	22 (64.7%)	10 (71.4%)	
5A/6A + 5A/5A	12 (35.3%)	4 (28.6%)	*p*=0.46
Gelatinase B exon			
AA + GA	16 (47.1%)	8 (57.1%)	
GG	18 (52.9%)	6 (42.9%)	*p*=0.38
Gelatinase B promoter			
CC	25 (73.5%)	10 (71.4%)	
CT + TT	9 (26.5%)	4 (28.6%)	*p*=0.57

χ^2 test was used to compare the values for patients with in stent restenosis and patients with patency after 6-month follow-up coronary angiography.

previously examined in relation to atherosclerosis in a number of epidemiological studies. Terashima et al. reported that the frequency of 5A allele carriers was significantly higher in acute myocardial infarction (AMI) patients than in control subjects (48.8% vs. 32.7%, $p< 0.0001$). 5 Theses data indicates that individuals carrying the 5A allele are genetically predisposed to clinical events commonly caused by the rupture of coronary atherosclerotic plaque.[5,19] A common polymorphism (6A/6A genotype) in the promoter region for stromelysin-1 has been associated with the faster progression of coronary atherosclerosis in man after coronary bypass surgery, and in subjects with coronary artery disease.[3,20] However, this finding was only seen on comparing those with least stenosis (< 20%) and higher serum LDL-cholesterol (>160 mg/dl),[2] and more study is warranted. In our study, the distribution of the 5A allele was higher in the stable angina group than in the control group, which contrasts with previous results, though no data has been published for stable angina. Until recently, the conventional view of atherosclerosis has been of an indolent lesion, gradually accumulating cells and lipid and eventually stenosing the affected vessel. In contrast, accumulated evidence emphasizes that plaque has a dynamic structure, and undergoes a continual cycle of erosion and repair. Recent postmortem studies indicate that plaque rupture and erosion are frequent phenomena, and that the majority of such events are clinically silent. Moreover, this cycle of erosion and repair is also responsible for plaque growth.[21-23] We believed that 5A allele might play a role in the silent plaque rupture and growth associated with coronary artery luminal narrowing in stable angina. In this case, the 5A allele may effect not only major rupture leading to myocardial infarction,[5] but also lead to silent plaque rupture and the progression of atherosclerosis.

The gelatinase B gene possesses proteolytic activity against type IV collagen, a major component of the basement membrane, and has been known to facilitate vascular smooth muscle cell migration.[24,25] Zang et al. reported that the C-1562T polymorphism is related to the severity of coronary artery atherosclerosis.[4] One explanation for this finding is that the higher T-allele frequency associated higher gelatinase B expression would enhance smooth muscle migration during atherosclerosis. However, the relationship between the C-1562T polymorphism and the severity of coronary artery atherosclerosis is controversial. Wang et al. reported that the C-1562T polymorphism is not related with the presence or severity of coronary atherosclerosis.[26]

However, in a recent autopsy-based study, which compared gelatinase B promoter with high-activity genotypes versus the promoter with low-activity genotype, found that results was associated with a large area of complicated coronary plaque.[27]

The R279Q polymorphism in the exon 6 region was not evaluated in coronary artery disease, though it might be related to gelatinase activity. The evidence is as follows:- First, it was located in the catalytic domain of MMP-9 enzyme. Second, the polymorphism led to a substitution of a positively charged amino acid (arginine) by an uncharged amino acid (glutamine). Third, the polymorphism was common in the population.[11,28] Our data showed allele frequencies that were similar to those reported previously.[4,11] However, the frequency of both polymorphisms were not found to be significantly different in the two groups. On analyzing for combined effects of the two polymorphisms, no additive effect was observed. Nevertheless, a certain amount of controversy surrounds the gelatinase B polymorphism and the issue warrants further investigation.

Regarding the severity of coronary artery atherosclerosis, Zhang et al. reported that carriers of the T-allele had higher frequencies of severe disease than C/C homozygotes in the case of 3-vessel disease.[4] However, no significant difference was observed between single-vessel and multivessel disease in our study. Though it is possible that a combination of the genetic and environmental factors of coronary artery disease is responsible for coronary atherosclerosis.

Percutaneous transluminal coronary angioplasty is a widely used treatment for angina pectoris. Smooth muscle cells in an artery for post-percutaneous transluminal coronary angioplasty are in a strikingly different environment than those present during the development of de novo

atherosclerotic plaque. Main pathophysiology of instent restenosis is neointimal hyperplasia. MMPs may play a central role in the migration of medial smooth muscle cells to the intima. This theory is supported by increased gelatinase B synthesis and activity in the vessel 1 day after balloon injury, and its continued presence at 6 days after injury.[29] However, in our study, no difference in the genetic frequencies of above genes was observed between patients with in stent restenosis and patency. This finding can be explained by the fact that in stent restenosis is influenced by combinations of MMPs, platelet-derived growth factor and other environmental factors. However, in the present study, the study population was small and there were many limitations, thus further randomized study is required.

Finally, our understanding of factors and pathways leading to plaque rupture and growth remain incomplete, and the current status of the MMP hypothesis certainly does not provide all of the answers; continued investigation is warranted.

Our study demonstrates that the 5A/6A polymorphism in the stromelysin-1 gene is associated with stable angina proven atherosclerosis, and shows that connective tissue remodeling mediated by MMP-3 plays a possible role in atherosclerosis. Moreover, the study suggets that genetic variations influencing MMP-3 expression might influence disease phenotype.

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