Association of blood active matrix metalloproteinase-3 with carotid plaque score from a community population in Taiwan

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Key Words: Blood active matrix metalloproteinase-3; carotid plaque; MMP-3 genotype

Abstract

Objective- Matrix metalloproteinases (MMPs) are implicated in the pathogenesis of atherosclerosis. However, the relationship between blood levels of MMPs and the extent of carotid atherosclerosis remains uncertain. We assessed blood levels of active MMPs in relation to the extent of carotid plaque formation and intima-media thickness (IMT) in a community population in Taiwan.

Methods- In 433 subjects from a community primary stroke prevention program, blood levels of active MMP-1, total and active MMP-3 and active MMP-9 were determined using enzyme-linked immunosorbent assays, carotid plaque score (PS) and IMT by high-resolution B-mode ultrasonography and the common MMP-1, MMP-3, and MMP-9 promoter low- and high-activity genotypes by polymerase chain reaction and restriction fragment length polymorphism.

Results- Study subjects were separated into 3 groups based on PS: group 1 (PS=0), group 2 (PS=1 to 2) and group 3 (PS≧3). Blood levels of active and total MMP-3 bear a significant relationship with PS ($p<0.0001$ for both active and total MMP-3). A multiple ordinal logistic regression analysis revealed that blood levels of active MMP-3 are correlated with PS (OR, 1.4; 95% CI, 1.1 to 1.8; $p=0.0038$) but not IMT. MMP-3 -1612 6A6A is a dominant genotype in this population, which is associated with higher levels of blood active MMP-3.

Conclusion- Blood levels of active MMP-3 are associated with the extent of carotid atherosclerosis based on PS but not IMT in this community population in Taiwan and the MMP-3 -1612 6A6A genotype is associated with higher levels of blood active MMP-3.

Introduction

Carotid atherosclerosis, which can be detected with non-invasive methods, is a marker for the development of cardiovascular diseases.[1] The severity of carotid atherosclerosis can be assessed by intima-media thickness (IMT) and plaque score derived from high-resolution B-mode ultrasonography or Doppler measurement of blood flow velocity.[2-4] Carotid plaques and plaque-free IMT are biologically and genetically distinct entities, which represent different phenotypes of atherosclerosis. Plaques are focal manifestations of atherosclerosis while increased IMT represents mainly hypertensive medial hypertrophy. Both should be measured when atherosclerosis is assessed.[5] Atherosclerosis is a chronic inflammatory process in the arterial wall. However, the relationship between atherosclerosis and inflammatory markers such as hs-CRP remains uncertain.[6]

Matrix metalloproteinases (MMPs) are a family of endopeptidases implicated in the degradation of connective tissue and extracellular matrix (ECM) in inflammatory processes leading to atherosclerosis.[7-9] Increased expression of MMP-1, -3, -9 and tissue inhibitors of metalloproteinases (TIMP)-1 at the protein level has been shown in advanced carotid plaques.[9] The MMP-3 gene is overexpressed in smooth muscle cells and macrophages based in atherosclerotic plaques by in situ hybridization studies.[10] MMP-3 is capable of degrading a wide range of ECM components and

activating other MMPs (e.g. pro-MMP-1, -8, -9 and -13) as well as its own proenzyme, pro-MMP-3.[4]

The common MMP-1 and MMP-3 promoter low- and high-activity genotypes have been related to carotid plaque formation or IMT.[11, 12] Inconsistent results from different populations have been reported in regard to the relationship between the blood MMP levels and the extent of carotid plaque or IMT.[8, 12-14] Total blood MMP-9 levels were reported to have either positive[14] or negative[8, 13] correlation with carotid plaque or IMT. Total blood MMP-3 levels bear a positive correlation with carotid plaque among hyperlipidemia subjects[8], but a negative one in another study from a small sample of Caucasians.[12] There have no prior reports concerning the relationship between the blood MMP levels and the extent of carotid atherosclerosis in Asians.

In the present study, we determined blood levels of active MMPs in relation to the extent of carotid atherosclerosis based on carotid plaque/plaque-free IMT and the promoter common polymorphism of MMPs in a population of adults followed in a community primary stroke prevention program.

1. Materials and Methods

1.1. Study Participants

The study subjects were a community-based adult cohort of 433 subjects, age 30 or older in the coverage area of the Shin Kong Wu Ho-Su Memorial Hospital (SKH).

Subjects with history of stroke, cancer or inflammatory diseases (e.g., arthritis or infections) were excluded. The characteristics of this study population have been reported in details earlier.[15] In brief, subjects in the present study were recruited at SKH in a community primary stroke prevention program sponsored by the Department of Health and underwent physical examination including Duplex ultrasonographic examination of the carotid arteries and laboratory tests. This study was approved by the institutional review board for human subjects at SKH and each subject provided written informed consent.

1.2. Data Collection

Personal medical history, medication history, family history of diseases, dietary habits, and smoking status were entered in a structured questionnaire upon enrollment. Data including blood pressure, height, weight, waist and hip circumferences, total cholesterol, triglyceride, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, glucose, uric acid, and hs-CRP from fasting blood samples were collected. Body mass index (body weight (kg)/square height (m^2)) and waist-to-hip ratio were also determined.

Subjects were considered to have hypertension if they were on anti-hypertensive medications or with an average systolic blood pressure $\geqq$140 mmHg or diastolic

blood pressure ≧90 mmHg, to have diabetes if on medications for diabetes or with fasting glucose ≧7.0 mmol/L and to be smokers with a history of smoking more than 100 cigarettes based on a simplified definition from CDC, USA.

1.3. Carotid Ultrasonography

A SONOS 5500 ultrasound system (Philips, USA), equipped with a 3 to 11-MHz real-time B-mode scanner and a 3.6-MHz pulsed-Doppler mode scanner was used. All scans were videotaped for off-line analysis. The plaque scoring method has been reported previously.[4] In brief, carotid artery segments, including the proximal common carotid artery (>10 mm proximal to the bulb bifurcation), distal common carotid artery, bulb, internal carotid artery, and external carotid artery were measured bilaterally by the sonographer who was blinded to subjects' health status, risk factors and laboratory results. The presence of plaques was defined as localized echo structures encroaching into arterial lumen of at least 50% of the surrounding IMT value.[16] Plaque score (PS) was rated as grade 0 for normal or without plaque, 1 point for one small plaque with diameter stenosis <30%, 2 points for one medium plaque with 30% to 49% diameter stenosis or multiple small plaques, 3 points for one large plaque with 50% to 99% diameter stenosis or multiple plaques with ≥1 medium plaques, and 4 points for 100% occlusion.[2, 4] This is mainly for the quantification of the extent of carotid atherosclerosis rather than for the selection of patients for

intervention. PS were computed by summation of the points from the 10 carotid artery segments. Reproducibility of carotid plaque scoring was reflected by the agreement among raters with a Kappa value of 0.619. The study subjects were separated into 3 groups based on PS: group 1 (PS=0), group 2 (PS=1 to 2) and group 3 (PS≧3).[4]

Carotid plaque-free IMT at the far wall of both left and right CCA located 0 to 10 mm proximal to the carotid bifurcation were quantitatively determined as the distance from the leading edge of the first echogenic line (lumen-intima interface) to the leading edge of the second line (media-adventitia interface) based on a semiautomated edge-detection algorithm (Q-lab 4, Philips Medical System).[17] A region of interest (10 mm in length) was placed perpendicular to the vessel wall. The arithmetic average of these measurements was taken as the carotid IMT. The IMT value for each person was the mean of IMT from right and left CCA. The correlation coefficients of duplicate reading from a single reader were 0.95 to 0.96, and the interobserver correlation coefficients were 0.86 to 0.9 for both sides of CCA IMT measurements. The study subjects were separated into 3 groups based on tertiles of IMT.

1.4. Enzyme-linked Immunosorbent Assay

The plasma levels of active MMP-3 and the serum levels of active MMP-1 and MMP-9 were measured with commercially available assay kits (Activity Biotrak,

Amersham, Little Chalfont, Bucks, UK) using enzyme-linked immunosorbent assay (ELISA). The plasma levels of total MMP-3 were measured with commercially available ELISA kits (Quantikine, R&D Systems, Abingdon, UK). The assay was performed by a researcher who was blind to the clinical data. Samples were handled in identical and blinded fashion throughout the study. Samples were analyzed in duplicate and in random order in order to reduce systemic biases and interassay variations. The average interassay coefficients of variation were 12.7 % for active MMP-1, 12.3% for active MMP-3, 12.6 % for active MMP-9 and 8% for total MMP-3.

1.5. DNA Extraction and Determination of Genotypes

DNA was extracted from the buffy coat using the salting-out method. Genotyping was carried out by polymerase chain reaction (PCR) and restriction fragment length polymorphism method (RFLP). The primers and restriction enzymes of MMP-1 -1607 1G/2G[18], MMP-3 -1612 5A/6A[19], and MMP-9 -1562C/T[20] were adapted from previous studies. Sequence validation and duplicate genotyping from 5% to 10% of blinded samples were done. The concordance rate was 100%. In addition, all three polymorphisms were in Hardy-Weinberg disequilibrium.

1.6. Statistical Analyses

Continuous data were expressed as mean ± standard deviation and analyzed by Student's t test and general linear model. Categorical data were characterized by percentage and were compared by Cochran-Armitage trend test. Multiple ordinal logistic regression analysis was employed to determine potential risk factors in 3 groups based on carotid plaque scores in Model 1 and tertiles of IMT in Model 2. Statistical analysis was carried out using SAS Version 9.1 software (SAS Institute, Cary, NC, USA). Statistical significance was set at $P < 0.05$.

2. Results

A total of 433 study subjects were divided into 3 groups based on PS: group 1 (PS=0, n=238), group 2 (PS=1 to 2, n=136) and group 3 (PS ≧3, n=59) or on tertiles of IMT: T1 (≦0.64mm, n=146), T2 (0.64 to 0.74mm, n=139) and T3 (>0.74mm, n=147). Effects of age, gender, smoking, diabetes, obesity, hypertension, total cholesterol, LDL and triglyceride were statistically significant on PS and IMT (Table 1). A significant trend defined by the Cochran-Armitage trend test was observed between blood levels of active MMP-3 or total MMP-3 and PS or IMT, respectively.

Table 2 presents those factors that are significantly associated with risk of higher carotid plaque scores and thicker IMT by the multiple ordinal logistic regression model after adjustment for potential covariates, including age, gender, hypertension,

diabetes mellitus, cigarette smoking , hypercholesterolemia (≧5.18 mmol/L), hypertriglyceridemia (≧2.26 mmol/L), BMI (≧27 Kg/m^2), and blood active MMP-3 level. Age (OR=2.0, p<0.0001), hypertension (OR=2.2, p=0.0005), and blood active MMP-3 level (OR=1.4, p=0.0038) were independently associated with increased risk of higher PS. Age, male, hypertension, hypercholesterolemia and hypertriglyceridemia were independently related with increased risk of higher IMT values. We also replaced the blood levels of active MMP-3 with total MMP-3 and reanalyzed the multiple ordinal logistic regression model after adjustment for the same potential covariates. Age (OR=2.2, p<0.0001) and hypertension (OR=2.0, p=0.0019), but not blood total MMP-3 level (OR=1.1, p=0.279), were independently associated with increased risk of higher carotid plaque score (data not shown).

The MMP-1 -1607 1G/2G or MMP-9 -1562 C/T polymorphisms were not correlated with PS (data not shown). The genotypes of MMP-3 -1612 5A/6A polymorphism are illustrated in Table 3. Approximately 90% of the study population carries the MMP-3 -1612 6A6A genotype. MMP-3 -1612 6A6A genotype is associated with higher levels of blood active MMP-3 (P for trend=0.0012) and total MMP-3 (P for trend=0.0505).

Since there is a positive correlation between carotid PS and blood active MMP-3 level and the 6A-allele was associated with an increase in blood levels of active

MMP-3, the combination of PS and MMP-3 5A/6A polymorphism in relation to blood levels of active MMP-3 was therefore examined as shown in Figure 1. For subjects with the 6A6A genotype, the blood active MMP-3 levels were higher in those with carotid plaque (7.4±4.6 ng/ml) than without carotid plaque (5.5±3.4 ng/ml) ($p<0.001$). For subjects with the 5A5A or 5A6A genotypes, the blood active MMP-3 levels were 5.3±2.2 and 3.3±2.1 ng/ml for subjects with carotid plaque and without carotid plaque, respectively ($p<0.01$). The highest mean blood active MMP-3 concentrations (7.4±4.6 ng/ml) were noted in subjects carrying the MMP-3 -1612 6A6A genotype with carotid plaque while the lowest (3.3±2.1 ng/ml) were in subjects carrying either the MMP-3 -1612 5A5A or 5A6A genotype without carotid plaque.

A question can be raised about the validity in categorizing PS into 3 groups as presented above. To ascertain that such a PS-based classification is not an artifact, we also applied dichotic division of the subjects into 2 groups: with (PS> 0, n=238) and without plaque (PS=0, n=195). Results based on the simpler categorization of carotid atherosclerosis are essentially similar to the findings described above based on the 3-group classification (data not shown).

3. **Discussion**

Two major findings in this study are as follows: (1) A dose-response relationship was found between the blood active and total MMP-3 levels and the severity of carotid atherosclerosis based on PS and (2) the MMP-3 -1612 6A6A genotype found in nearly 90% of our study subjects is associated with higher blood active and total MMP-3 levels. The highest mean blood active MMP-3 levels were noted in subjects carrying the MMP-3 -1612 6A6A genotype with carotid plaque. To the best of our knowledge, this is the first study to demonstrate a dose-response relationship between blood levels of active and total MMP3 and the extent of carotid atherosclerosis.

Carotid plaques and plaque-free IMT are two different phenotypes, which may have common and distinct determinants.[4, 16] Our studies found age, hypertension and MMP-3 play significant roles in carotid plaque (later stage of atherosclerosis) formation. Our studies support a significant impact of age, male, hypertention, hypercholesterolemia and hypertriglyceridemia, but not MMP-3, on plaque-free IMT (an early predictor of atherosclerosis). Hypercholesterolemia, male and hypertriglyceridemia are not associated with carotid plaque formation in our study, which is in agreement with the previous studies in Taiwan.[4] Though the odds ratio is only 1.4 ($p=0.0038$) for blood active MMP-3 level, the impact of blood active MMP-3 level on PS is only next to age and hypertension and is even more significant than other traditional vascular risk factors such as diabetes mellitus, smoking and

hypercholesterolemia, while hs-CRP, MMP-1 or MMP-9 showed no significant impact.

Increased expression of MMP-1, -3, -9 at the protein level has been shown in advanced carotid plaques.[9] Technical limitations restrict clinical studies to measure the content of MMPs in carotid plaques. Results from the present study suggest that blood levels of active MMP-3, but not MMP-1 or MMP-9, bear a significant relationship with the extent of carotid atherosclerosis based on PS. Rauramaa et al. found no association between blood total MMP-3 and carotid IMT.[12] We elected to place our primary focus on the active MMPs than the total MMPs. Total MMPs are composed of pro-MMPs, active MMPs and MMPs bound to proteins including tissue inhibitors of metalloproteinases and may not accurately reflect the levels of active MMPs. Active MMPs may be better indicators of ongoing vascular remodeling activities than the total MMPs. Though our results show a positive correlation between active MMP-3 and total MMP-3 ($r=0.768$, $p<0.0001$), after adjusting potential covariates by a multiple ordinal logistic regression analysis, only blood levels of active MMP-3 (OR, 1.4; 95% CI, 1.1 to 1.8; $p=0.0038$) and, not total MMP-3, are correlated with carotid plaque scores.

Total blood MMP-3 and hs-CRP levels were both positively associated with the presence of carotid plaques in asymptomatic hyperlipidemic subjects.[8] However,

hypercholesterolemia itself can explain high serum levels of total MMPs.[21] The results relating both total MMP-3 and hs-CRP levels positively to carotid plaques were restricted to cases with hypercholesterolemia.[8] Hs-CRP concentrations fail to discriminate between the presence and absence of significant carotid atherosclerosis.[22] We found blood levels of active MMP-3, but not hs-CRP, correlated well with carotid atherosclerosis.

MMP-3 has been considered to be important in determining the composition and turnover of the fibrous cap to promote atherosclerosis progression.[23] The present study provides a novel finding that blood levels of active MMP-3 positively correlate with the increment of PS.

The common 5A/6A polymorphism in the promoter region of the MMP-3 gene, located 1612 base pair upstream of the transcription starting site has been shown in vitro[24] and in vivo[25] to affect the level of gene expression. The MMP-3 -1612 6A6A genotype shows an association with carotid plaques or IMT in 4 Caucasian studies with 6A6A genotype frequency ranging from 31 to 48%.[11, 12, 26, 27] It is note worthy that the frequency of the MMP-3 -1612 6A6A genotype (89%) in this study population is substantially higher than that reported in Caucasians. The 5A-allele is rare in Asians [28], while the 5A- and 6A-alleles are evenly distributed in Caucasians.[11] It is probably because of such a high frequency of the MMP-3 -1612

6A6A genotype in this population, its impact on phenotype, namely carotid atherosclerosis, is not apparent.

Though in vitro assays of promoter activity revealed that the 5A allele had 2-fold higher promoter activity than the 6A allele,[29] MMP-3 -1612 6A6A genotype in our study population on the contrary is associated with higher levels of blood active MMP-3 (P for trend=0.0012) and total MMP-3 (P for trend=0.0505). Samnegard et al. also found that the 6A-allele was associated with a graded increase in blood levels of total MMP-3 from patients with myocardial infarction and stable coronary artery disease.[23] A similar finding was noted in patients with rheumatoid arthritis.[30] Blood MMP-3 is speculated to be secreted from many types of cells that distribute throughout the body.[12] The reason for the discrepancy between in vitro study and the present in vivo results are not clear. Further studies are needed.

The clinical importance of serum biomarkers reflecting these inflammatory processes may distinguish unstable from stable carotid artery stenosis. Selected biomarkers such as blood active MMP-3 may be potentially useful in future selection of patients who may benefit from carotid interventions. Our finding that blood active MMP-3 is a more sensitive marker than hs-CRP may be an initial step toward the delineation of a biomarker profile for predicting unstable carotid artery stenosis beyond carotid ultrasound.

Romero et al found that the relation of circulating MMP-9 was stronger with more severe changes of carotid atherosclerosis.[14] However, Olson et al found that MMP-9 concentration and activity in plasma did not reflect the severity of carotid artery atherosclerosis.[13] The present study also showed no correlation of active MMP-9 with the severity of carotid atherosclerosis. The possible explanations of a negative impact of active MMP-9 are: (1) circulating tissue inhibitors of metalloproteinases such as TIMP-1, that were not measured, may preferentially suppress active MMP-9 and (2) possible ethnic difference. Further studies are needed to prove or disprove these 2 possibilities.

In conclusion, the present study shows that blood active MMP-3, but not MMP-1, MMP-9 or hs-CRP, is associated with carotid atherosclerosis in a community population enrolled in a primary stroke prevention program. The level of blood active MMP-3 bears a dose-response relation with the extent of carotid atherosclerosis based on PS. The increased blood active MMP-3 can partly be influenced by the MMP-3 -1612 6A6A homozygous genotype, which is found in nearly 90% of this study population. Blood active MMP-3 may be a useful marker to predict carotid plaques in Taiwan.

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References

[1] O'Leary DH, Polak JF, Kronmal RA, et al. Carotid-artery intima and media thickness as a risk factor for myocardial infarction and stroke in older adults. Cardiovascular Health Study Collaborative Research Group. N Engl J Med 1999;340:14-22.

[2] Crouse JR, Harpold GH, Kahl FR, Toole JF.McKinney WM. Evaluation of a scoring system for extracranial carotid atherosclerosis extent with B-mode ultrasound. Stroke 1986;17:270-275.

[3] Sutton-Tyrrell K, Wolfson SK, Jr., Thompson T.Kelsey SF. Measurement variability in duplex scan assessment of carotid atherosclerosis. Stroke 1992;23:215-220.

[4] Su TC, Jeng JS, Chien KL, et al. Hypertension status is the major determinant of carotid atherosclerosis: a community-based study in Taiwan. Stroke 2001;32:2265-2271.

[5] Spence JD. Measurement of intima-media thickness vs. carotid plaque: uses in patient care, genetic research and evaluation of new therapies. Int J Stroke 2006;1:216-221.

[6] Blackburn R, Giral P, Bruckert E, et al. Elevated C-reactive protein constitutes an independent predictor of advanced carotid plaques in dyslipidemic subjects. Arterioscler Thromb Vasc Biol 2001;21:1962-1968.

[7] Koenig W, Khuseyinova N. Biomarkers of atherosclerotic plaque instability and rupture. Arterioscler Thromb Vasc Biol 2007;27:15-26.

[8] Beaudeux JL, Giral P, Bruckert E, et al. Serum matrix metalloproteinase-3 and tissue inhibitor of metalloproteinases-1 as potential markers of carotid atherosclerosis in infraclinical hyperlipidemia. Atherosclerosis 2003;169:139-146.

[9] Orbe J, Fernandez L, Rodriguez JA, et al. Different expression of MMPs/TIMP-1 in human atherosclerotic lesions. Relation to plaque features and vascular bed. Atherosclerosis 2003;170:269-276.

[10] Henney AM, Wakeley PR, Davies MJ, et al. Localization of stromelysin gene expression in atherosclerotic plaques by in situ hybridization. Proc Natl Acad Sci U S A 1991;88:8154-8158.

[11] Ghilardi G, Biondi ML, DeMonti M, et al. Matrix metalloproteinase-1 and matrix metalloproteinase-3 gene promoter polymorphisms are associated with carotid artery stenosis. Stroke 2002;33:2408-2412.

[12] Rauramaa R, Vaisanen SB, Luong LA, et al. Stromelysin-1 and interleukin-6 gene promoter polymorphisms are determinants of asymptomatic carotid artery atherosclerosis. Arterioscler Thromb Vasc Biol 2000;20:2657-2662.

[13] Olson FJ, Schmidt C, Gummesson A, et al. Circulating matrix metalloproteinase 9 levels in relation to sampling methods, femoral and carotid atherosclerosis. J Intern Med 2008;263:626-635.

[14] Romero JR, Vasan RS, Beiser AS, et al. Association of carotid artery atherosclerosis with circulating biomarkers of extracellular matrix remodeling: the Framingham Offspring Study. J

Stroke Cerebrovasc Dis 2008;17:412-417.

[15] Hsieh YC, Hung CT, Lien LM, et al. A significant decrease in blood pressure through a family-based nutrition health education programme among community residents in Taiwan. Public Health Nutr 2009;12:570-577.

[16] Touboul PJ, Hennerici MG, Meairs S, et al. Mannheim carotid intima-media thickness consensus (2004-2006). An update on behalf of the Advisory Board of the 3rd and 4th Watching the Risk Symposium, 13th and 15th European Stroke Conferences, Mannheim, Germany, 2004, and Brussels, Belgium, 2006. Cerebrovasc Dis 2007;23:75-80.

[17] Lim TK, Lim E, Dwivedi G, Kooner J.Senior R. Normal value of carotid intima-media thickness--a surrogate marker of atherosclerosis: quantitative assessment by B-mode carotid ultrasound. J Am Soc Echocardiogr 2008;21:112-116.

[18] Fang S, Jin X, Wang R, et al. Polymorphisms in the MMP1 and MMP3 promoter and non-small cell lung carcinoma in North China. Carcinogenesis 2005;26:481-486.

[19] Zhou X, Huang J, Chen J, et al. Haplotype analysis of the matrix metalloproteinase 3 gene and myocardial infarction in a Chinese Han population. The Beijing atherosclerosis study. Thromb Haemost 2004;92:867-873.

[20] Jones GT, Phillips VL, Harris EL, et al. Functional matrix metalloproteinase-9 polymorphism (C-1562T) associated with abdominal aortic aneurysm. J Vasc Surg 2003;38:1363-1367.

[21] Sundstrom J.Vasan RS. Circulating biomarkers of extracellular matrix remodeling and risk of atherosclerotic events. Curr Opin Lipidol 2006;17:45-53.

[22] Wu YW, Yang WS, Chen MF, et al. High serum level of matrix metalloproteinase-1 and its rapid surge after intervention in patients with significant carotid atherosclerosis. J Formos Med Assoc 2008;107:93-98.

[23] Samnegard A, Silveira A, Tornvall P, et al. Lower serum concentration of matrix metalloproteinase-3 in the acute stage of myocardial infarction. J Intern Med 2006;259:530-536.

[24] Ye S, Eriksson P, Hamsten A, et al. Progression of coronary atherosclerosis is associated with a common genetic variant of the human stromelysin-1 promoter which results in reduced gene expression. J Biol Chem 1996;271:13055-13060.

[25] Zhu C, Odeberg J, Hamsten A, et al. Allele-specific MMP-3 transcription under in vivo conditions. Biochem Biophys Res Commun 2006;348:1150-1156.

[26] Rundek T, Elkind MS, Pittman J, et al. Carotid intima-media thickness is associated with allelic variants of stromelysin-1, interleukin-6, and hepatic lipase genes: the Northern Manhattan Prospective Cohort Study. Stroke 2002;33:1420-1423.

[27] Djuric T, Zivkovic M, Radak D, et al. Association of MMP-3 5A/6A gene polymorphism with susceptibility to carotid atherosclerosis. Clin Biochem 2008;41:1326-1329.

[28] Terashima M, Akita H, Kanazawa K, et al. Stromelysin promoter 5A/6A polymorphism is associated with acute myocardial infarction. Circulation 1999;99:2717-2719.

[29] Ye S. Influence of matrix metalloproteinase genotype on cardiovascular disease susceptibility and outcome. Cardiovas Res 2006;69:636-645.

[30] Mattey DL, Nixon NB, Dawes PT, et al. Association of matrix metalloproteinase 3 promoter genotype with disease outcome in rheumatoid arthritis. Genes Immun 2004;5:147-149.

Table 1 Characteristics of study subjects

	Carotid Plaque Score				Tertiles of Carotid IMT (mm)			
	0	1-2	≧3		T1(≦0.64)	T2	T3(＞0.74)	
Variables	N=238	N=136	N=59	P for trend	N=146	N=139	N=147	P for trend
Age, years	53.7±10.7	58.8±8.1	67.3±9.0	<0.0001	50.0±9.8	58.0±9.2	63.5±8.6	<0.0001
Males, %	37.0	56.6	62.7	<0.0001	37.7	44.6	57.1	0.0008
Systolic blood pressure, mmHg	120.9±18.2	130.0±19.0	138.8±20.6	<0.0001	115.5±16.2	126.8±18.6	136.0±18.8	<0.0001
Diastolic blood pressure, mmHg	77.1±11.0	82.3±11.3	82.1±12.6	<0.0001	74.7±10.5	79.8±10.9	83.5±11.2	<0.0001
Body mass index, kg/m^2	23.8±3.1	24.7±3.0	24.9±3.2	0.0033	23.2±3.1	24.4±3.0	25.1±2.9	<0.0001
Waist-to-hip ratio, cm	0.83±0.07	0.86±0.06	0.88±0.06	<0.0001	0.82±0.07	0.85±0.07	0.87±0.06	<0.0001
Hypertension, %	26.5	45.6	71.2	<0.0001	17.1	37.4	60.5	<0.0001
Diabetes mellitus, %	8.0	11.8	25.4	0.0037	7.5	8.6	18.4	0.0037
Cigarette smoking, %	14.3	27.2	37.3	<0.0001	19.2	17.3	27.9	0.0692
Cholesterol, mmol/L	5.1±0.9	5.3±0.9	5.4±0.9	0.0114	5.0±0.9	5.3±0.9	5.4±0.9	0.0002
LDL, mmol/L	3.2±0.8	3.4±0.8	3.4±0.7	0.0038	3.1±0.8	3.3±0.8	3.4±0.7	0.0008
HDL, mmol/L	1.3±0.4	1.3±0.3	1.2±0.4	0.0537	1.3±0.4	1.3±0.3	1.2±0.4	0.0452
Triglyceride, mmol/L	1.3±0.8	1.4±0.8	1.9±1.6	0.0008	1.2±0.7	1.4±0.8	1.7±1.3	<0.0001
Uric acid, mmol/L	288.9±82.7	310.8±75.0	316.1±93.6	0.0117	286.3±84.7	295.3±72.7	315.2±85.5	0.0084
Fasting glucose, mmol/L	5.5±0.9	5.9±1.7	5.9±1.0	0.0099	5.4±0.7	5.6±1.0	6.0±1.7	0.0004
Hs-CRP, mg/L	2.3±3.6	2.0±2.0	2.5±3.8	0.4485	2.1±3.1	2.1±3.2	2.6±3.3	0.3501
Active MMP-1, ng/ml	9.6±4.0	9.5±3.8	8.8±3.5	0.3505	9.6±4.2	9.4±3.8	9.4±3.6	0.8687
Active MMP-3, ng/ml	5.2±3.3	6.6±3.9	8.5±5.4	<0.0001	5.2±3.2	6.0±3.9	7.1±4.4	0.0002
Total MMP-3, ng/ml	9.3±7.0	11.0±7.8	16.1±15.6	<0.0001	9.2±6.9	9.7±6.1	12.2±10.4	0.0035
Active MMP-9, ng/ml	4.7±2.3	5.1±2.3	4.6±2.1	0.1768	4.5±2.1	4.8±2.4	5.1±2.2	0.1215

IMT: carotid intima-media thickness; T: tertile; LDL: low-density lipoprotein; HDL: high-density lipoprotein; hs-CRP: high sensitivity C-reactive protein; MMP: matrix metalloproteinases.

Table 2 Multiple ordinal logistic regression: risk factors associated with carotid plaque score and plaque-free IMT after adjustment for covariates

	Model I			Model II		
	OR	95% CI	p-value	OR	95% CI	p-value
Age (per SD increase)	2.0	1.6-2.5	<0.0001	2.8	2.2-3.6	<0.0001
Male	1.2	0.7-2.0	0.5813	1.7	1.0-2.8	0.0490
Hypertension	2.2	1.4-3.5	0.0005	2.3	1.5-3.5	0.0002
Diabetic mellitus	1.5	0.8-2.8	0.1828	1.5	0.8-2.7	0.6788
Cigarette smoking	1.5	0.9-2.6	0.1355	0.8	0.5-1.4	0.5033
Cholesterol≧5.18 mmol/L	1.4	0.9-2.2	0.0912	1.7	1.1-2.5	0.0148
Triglyceride≧2.26 mmol/L	1.1	0.6-2.0	0.7103	2.4	1.3-4.4	0.0062
BMI ≧27 Kg/m^2	1.2	0.7-2.0	0.5754	1.2	0.7-2.0	0.5953
Blood Active MMP-3 (per SD increase)	1.4	1.1-1.8	0.0038	1.0	0.8-1.3	0.7945

SD: standard deviation;

Model I: Dependent variable was defined as three groups according to plaque score =0, 1 to 2, and ≧3;

Model II: Dependent variable was defined as three groups according to the tertiles of IMT.

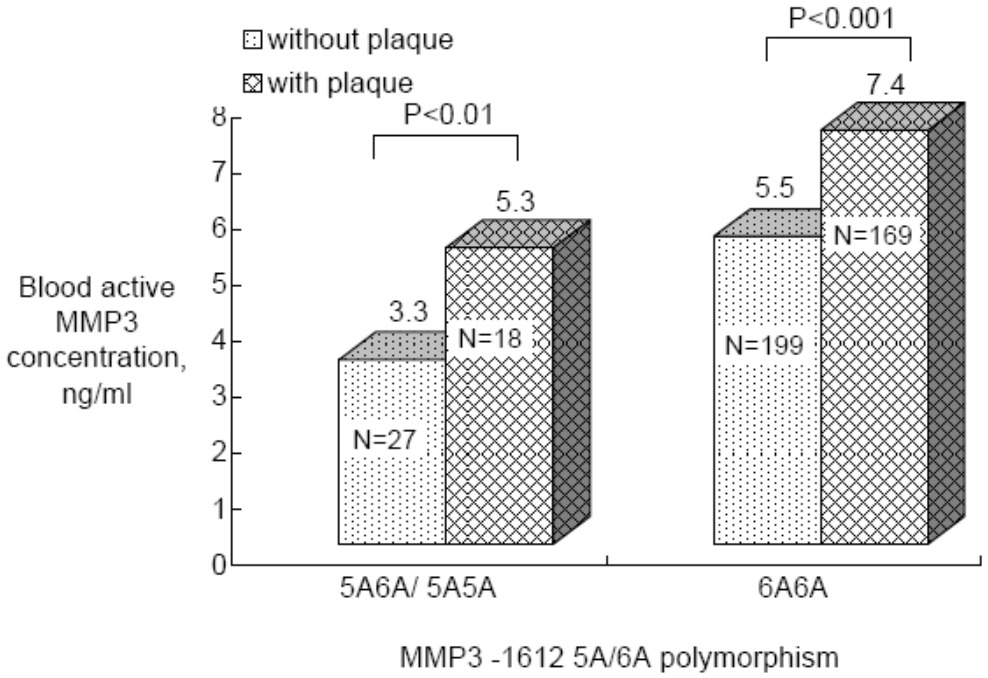
Table 3 Correlation between carotid plaque scores, blood active MMP-3 levels, blood total MMP-3 levels and MMP-3 -1612 5A/6A genotypes

Variable	Carotid plaque score, n (%)			Active MMP-3 level, ng/ml	Total MMP-3 level, ng/ml
	0	1-2	≥3	mean (SD)	mean (SD)
MMP-3 Gene					
5A5A (n=2)	1(0.4)	1(0.8)	0	2.3(0.5)	5.8(1.5)
5A6A (n=44)	27(11.6)	12(9.0)	5(8.6)	4.2(2.4)	7.7(4.7)
6A6A (n=378)	204(87.9)	121(90.3)	53(91.4)	6.4(4.1)	10.9(9.0)
		P=0.8484		P for trend =0.0012	P for trend =0.0505

MMP-3: matrix metalloproteinases-3; SD: standard deviation

Figure Legends

Figure 1 Blood active MMP-3 levels between subjects with or without carotid plaque based on different genotypes.


without plaque
with plaque
P<0.01
P<0.001
7.4
5.3
5.5
3.3
N=18
N=169
N=199
N=27
Blood active MMP3 concentration, ng/ml
0
1
2
3
4
5
6
7
8
5A6A/ 5A5A
6A6A
MMP3 -1612 5A/6A polymorphism