MMP-9 Microsatellite Polymorphism and Susceptibility to Carotid Arteries Atherosclerosis

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Objective—The aims of this study were to compare a microsatellite polymorphism (PM) of matrix metalloproteinase (MMP)-9 in patients with carotid atherosclerosis and control population, and to assess the relationship between this PM and plaque structure.

Methods and Results—One hundred fifty patients referring to vascular diagnostic centers for suspected carotid atherosclerosis (at ultrasound examination: 110 positive, 40 negative) and controls (n=110) have been genotyped for MMP-9 PM. After controlling for risk factors, allelic and genotype frequencies were significantly different among the groups, with significant prevalence of long microsatellites in patients with carotid atherosclerosis. Long microsatellites (settled as 22 to 27 repeats) were associated with carotid atherosclerosis (odds ratio [OR], 5.2; 95% confidence interval [CI], 2.9 to 9.2), compared with controls; an independent case control study on patients with coronary atherosclerosis confirmed such result. Binary logistic regression showed that hypertension, long microsatellites in MMP-9 PM and smoking habits were variables accounting for the difference between ultrasound-positive patients and controls. Long microsatellites were also associated to plaques with thin fibrous cap and echolucent core (OR, 13.1; 95% CI, 1.6 to 100). These alleles were slightly more represented in female patients (χ^2 test=0.019; OR, 2.7; 95% CI, 1.2 to 6) but not associated with other risk factors. Plasma MMP-9 levels were related neither to MMP-9 PM nor to plaque type, and were related to gender and extension of atherosclerosis in carotid arteries.

Conclusions—The number of repeats (≥22 CA) in the microsatellite of MMP-9 promoter, but not MMP-9 plasma levels, is associated to carotid atherosclerosis and particularly to plaques with a thin fibrous cap. (***Arterioscler Thromb Vasc Biol*. 2006;26:000-000.)**

Key Words: atherosclerosis ■ carotid arteries ■ MMP-9 ■ polymorphism ■ stroke

Carotid echolucent plaques with a thin fibrous cap are generally associated to elevated risk of acute cerebrovascular events.[1–3] Normal vessels remodeling and some features of plaques originating acute vascular events[4] are associated to the increased expression of members of the family of matrix metalloproteinases (MMPs).[5] These enzymes consist of a group of ≈20 neutral zinc-dependent endopeptidases, with different patterns in substrates specificity.[6] MMPs were identified in atherosclerotic tissue, and MMP-9 in particular was observed in unstable and, to a lesser extent, in stable atherosclerotic plaques.[4,7] MMP-9 activity in atherosclerotic plaques might induce monocyte[8] and smooth muscle cell (SMC)[9–11] migration and proliferation, degrade extracellular matrix, therefore contributing to thinning and fissuring of the fibrous cap of plaques.[12–14] Modulation of MMP-9 expression occurs through transcription factors and, likely, a genetic polymorphism (PM) in the promoter, namely the microsatellite (Cytosine-Adenine, CA)$_{13–27}$ at ≈−90 position,[15] affects transcription and, putatively, plays a role in the susceptibility to atherosclerosis. This microsatellite was proposed to facilitate the transition of B-DNA in Z-DNA, thus assisting the opening of the double strand and transcription. Studies on mice showed that even small differences between alleles (from 20 to 24 CA repeats) can account for a 30-fold increase in MMP-9 expression.[16] The majority of the studies agreed that an increasing number of repeats is associated with increased expression.[15–17] Therefore, a high number of repeats, putatively accounting for higher expression, could promote plaque growth and possibly influence the structure of plaque.

This study aimed to assess the association between microsatellites in MMP-9 promoter and carotid atherosclerosis and

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the prevalence of plaques with thin a fibrous cap and homogeneously echolucent core, identified as type 1 of the Gray Weale classification. Also, MMP-9 plasma levels were performed and analyzed.

Materials and Methods

Patients

Consecutive in-patients and out-patients visiting 2 noninvasive vascular diagnostic centers were studied. The control population consisted mainly of a group of blood donors attending the Blood Transfusion Centre of the Hospital of Trieste (Italy).

Ultrasound Assessment of the Carotid Arteries

Extra-cranial carotid arteries were examined with color Doppler ultrasound sonography. The scanning protocol entailed the longitudinal scanning of the near/far walls of the right and left carotid arteries at the distal common carotid artery, carotid bifurcation, and proximal internal carotid artery. Plaque was classified according to Gray Weale et al.[18]

Laboratory Determinations

The details of the method for the MMP-9 genotyping polymerase chain reaction (PCR) amplification and capillary analysis have been previously published.[19] Plasma MMP-9 levels have been determined with an R&D System Quantikine ELISA kit according to the instructions provided by the manufacturer.

Confirmatory Study

A group of patients with coronary atherosclerosis was compared with a control population. Patients with coronary atherosclerosis were selected among those admitted to the Emergency Unit of Trieste (Italy) for acute coronary syndrome.[20] The control population consisted of healthy volunteers attending the Blood Transfusion Center of the Trieste Hospital (Italy) or subjects referring for minor transient problems to the Outpatients Clinics of the Internal Medicine Department of the University of Trieste.

Results

At the end of enrollment, 110 consecutive ultrasound-positive patients, 40 ultrasound-negative patients and 110 control subjects were considered for the study. All genotyping was successful, with an interobserver concordance of 100%. Demographic data, risk factors, reasons for ultrasound evaluation, associate comorbidities, and medical therapy at the moment of examination are reported in Table I (please see http://atvb.ahajournals.org). A difference in allelic frequencies was found among the 3 groups (χ^2 test $P<0.0001$) and directly comparing control group with ultrasound-positive patients ($P<0.001$), controls with ultrasound-negative patients ($P=0.004$), and ultrasound-positive and ultrasound-negative patients ($P=0.019$) (Figure 1). Although the first mode was set, for all 3 groups, at 14 repeats, the second mode was at 21 repeats for controls and ultrasound-negative patients, and at 22 for the ultrasound-positive patients. Ultrasound-positive women had a different allelic distribution than men (χ^2 test $P=0.019$), with approximately twice the prevalence of microsatellites with 22 or more repeats (odds ratio [OR], 2.66; 95% confidence interval [CI], 1.16 to 6).

Multiple comparisons have been performed to detect exactly the alleles (or the group of alleles) more prevalent in patients with carotid atherosclerosis, compared with controls.

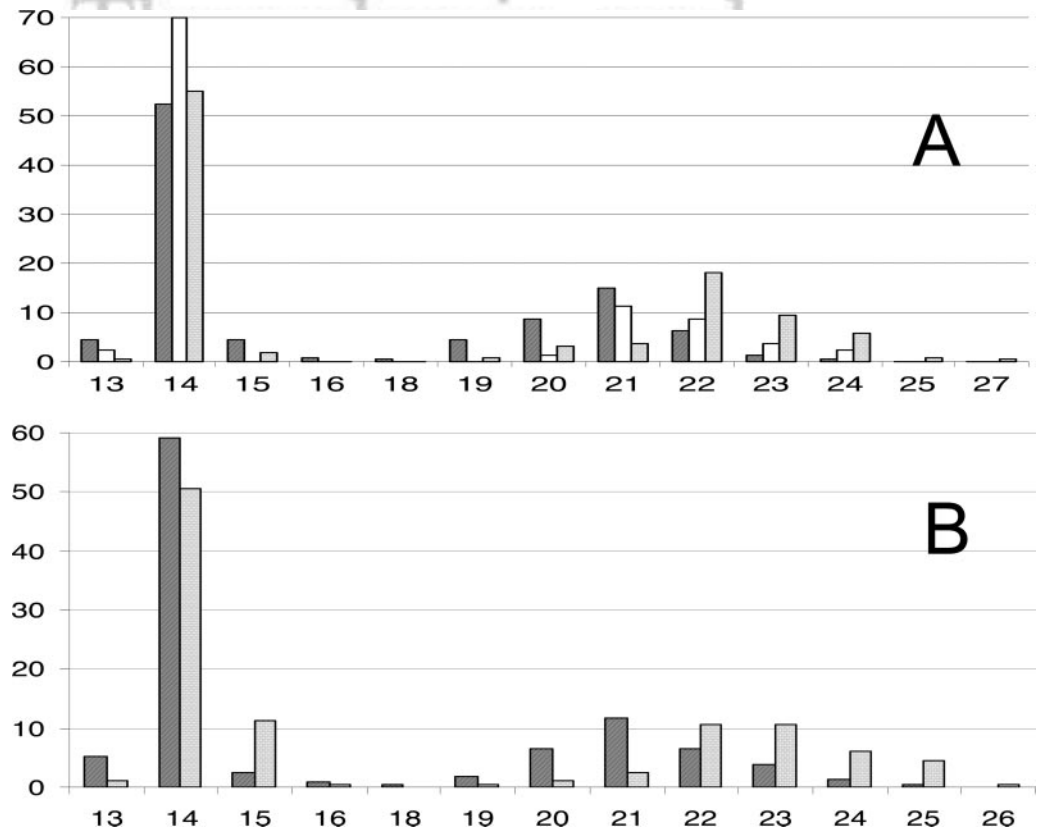

Figure 1. Allelic frequencies (in percentage) of the MMP-9 microsatellite polymorphism. A, Study population: patients with carotid atherosclerosis. Controls (dashed), ultrasound (US)-negative patients (empty), and US-positive patients (dotted). B, Independent replication study: patients with coronary atherosclerosis. Controls (dashed) and patients with coronary atherosclerosis (dotted).

Taking into consideration the Bonferroni correction, alleles with 21 or more repeats were significantly associated with carotid plaques. However, the prevalence of alleles with 21 repeats was higher in controls than in patients and, more relevant, the 95% CI or the OR of 21 or more repeats was significantly lower than that of 22 or more, as seen in the gap between the confidence intervals (Table 1). Standardization for gender and age, to account for gender difference in MMP-9 PM, did not change the results. A receiver-operator curve (ROC) (Figure 2A) was plotted to verify the changes in sensitivity and specificity of each midpoint. ROC confirmed the weak statistical association of 21 repeat allele with carotid atherosclerosis, compared with longer microsatellites.

Genetic frequency, considering only the length of the longest microsatellite, gave similar results (Table 2). When the cut-off was set between 20 and 21 repeats, the OR was 2.2 (95% CI, 1.3 to 3.7) and when set between 21 and 22, the OR was 7.6 (95% CI, 4.0 to 14.5). Both ways of grouping were effective in identifying alleles associated with carotid atherosclerosis but, similar to allelic frequencies, the CIs were substantially different, making carriers of 21 repeats as the longest microsatellite at the lowest risk for carotid plaques. This point was also confirmed by direct comparison of 21 versus 22 repeats (OR, 10.1; 95% CI, 3.8 to 27.4; and OR, 18; 95% CI, 4.6 to 70.5 for allelic and genetic frequency, respectively).

None of the risk factors analyzed in this study was associated with this polymorphism when analyzed as an ordinal or dichotomous variable (data not reported), and association of carotid plaques with MMP-9 PM was observed in both sexes (χ^2 test $P=0.624$). Binary logistic regression (stepwise method) showed that the variables accounting for the difference between cases and controls were hypertension, smoking habit and MMP-9 (Table II).

MMP-9 PM and Type 1 Plaques

Fifteen type 1 plaques were detected throughout the ultrasound assessments; none of the risk factors was associated with this type of plaque. The allelic frequency was significantly different in patients with type 1 plaques, compared with patients with other type of plaques or controls. Within positive ultrasound patients, the risk of presenting a type 1 plaque is increased by >3 times in carriers of 22 or more repeats (OR, 3.33; 95% CI, 1.508 to 7.356). Analyzing the genetic frequency, except for 1 patient with a genotype with 14/14, all patients carried at least 1 allele with 22 or more repeats (Table 2). No significant association was detected with the other types of plaque; however, there was a trend in reduction of OR from echolucent to echogenic plaques (Table 3; χ^2 test $P=0.007$).

TABLE 1. Odds Ratio (95% CI) of Groupings of the MMP-9 Alleles (ie, 13 Repeats Versus 14 or More, Then ≤14 Versus ≥15, and so on) Between the US Positive Patients and Controls (Unstandardized and Standardized for Sex and Age)

	Carotid Atherosclerosis		Coronary Atherosclerosis	
CA Repeats	Unstandardized	Standardized	Unstandardized	Standardized
13	0.1 (0.01–0.8)	0.1 (0.01–0.7)	0.2 (0.04–0.8)	0.2 (0.04–1.0)
14	1.1 (0.7–1.5)	0.9 (0.6–1.3)	1.7 (1.1–2.5)	1.7 (1.2–2.6)
15	1.2 (0.8–1.8)	1.1 (0.7–1.6)	1.2 (0.8–1.8)	1.3 (0.8–1.9)
16	1.3 (0.9–1.9)	1.1 (0.8–1.7)	1.2 (0.8–1.8)	1.3 (0.9–1.9)
18	1.3 (0.9–1.9)	1.1 (0.8–1.7)	1.2 (0.8–1.9)	1.3 (0.9–2.0)
19	1.5 (1.0–2.3)	1.4 (0.9–2.0)	1.3 (0.9–1.9)	1.4 (0.9–2.1)
20	2.1 (1.4–3.2)	1.9 (1.3–2.9)	1.7 (1.1–2.7)	1.9 (1.2–2.9)
21	6.0 (3.5–10.5	5.2 (3.0–9.2)	3.5 (2.1–5.8)	3.3 (2.0–5.3)
22	10.9 (3.8–31.2)	9.9 (3.5–28.4)	4.7 (2.4–9.1)	4.4 (2.3–8.4)
23	17.2 (2.3–130.7)	15.6 (2.1–118.8)	7.2 (2.4–21.3)	6.7 (2.3–19.8)
24			12.4 (1.5–97.4)	11.6 (1.5–91.1)
25				

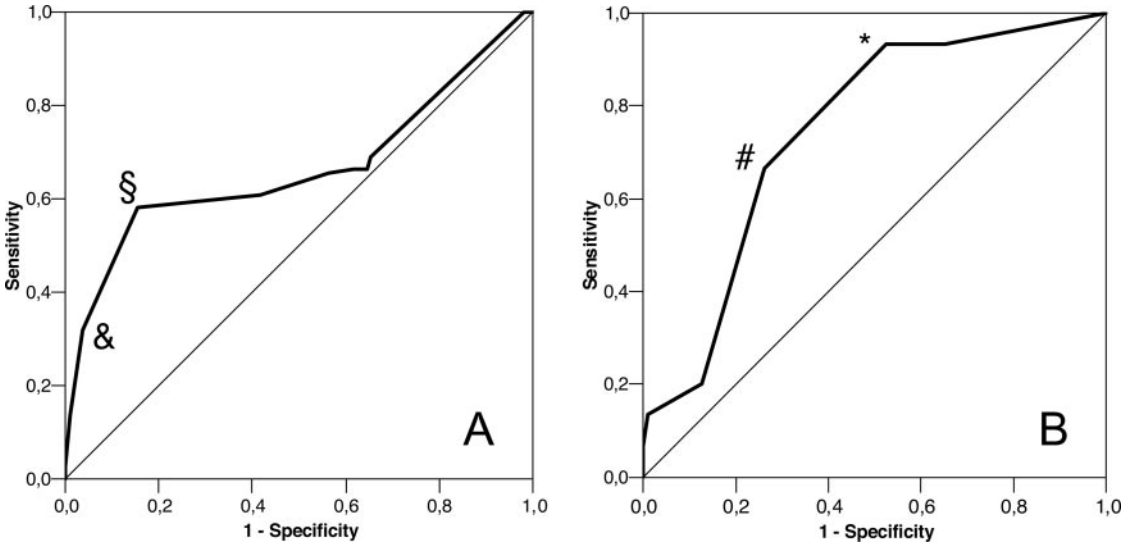

Figure 2. ROC curves of MMP-9 genotype. A, Genotype of MMP-9 in discrimination between US-positive patients and controls (area under curve, 0.664; SE, 0.038; asymptotic 95% CI, 0.589–0.739; $P<0.00001$). B, Within US-positive patients in discrimination between patients with type I plaque and patients with other types of plaques (area under curve, 0.744; SE, 0.061; asymptotic 95% CI, 0.624–0.864; $P=0.002$). Legend of midpoints (in number of repeats): §21.5; &22.5; *21.5; #22.5. Sensitivity/specificity of midpoints: §21.5 indicates 0.582/0.845; &22.5, 0.318/0.964; *21.5, 0.933/0.474; #22.5, 0.667/0.747.

Plasma MMP-9 Levels

No difference was detected between patients and controls (controls 41 [35–85 ng/mL]; negative ultrasound patients 34 [22–71 ng/mL]; and positive ultrasound patients 44 [24–88 ng/mL]; $P=0.887$). No relationship was noticed between MMP-9 plasma levels and genetic polymorphism using linear univariate correlation (Pearson $R=-0.036$, $P=0.716$) or when patients were categorized according to the number of repeats in carriers of ≥ 22 or <22 ($P=0.913$). Within all patients, no differences were detected between plaque types and plasma values of MMP-9 (Table 3) or between MMP-9 plasma level and number of areas affected by atherosclerosis (Kruskal-Wallis test $P=0.418$). A significant, but weak, correlation (Pearson test) was demonstrated, however, only for the relationship between MMP-9 plasma levels and number of areas affected by atherosclerosis (Figure 3). Within patients, multivariate stepwise analysis showed that only sex (men 54 [32–114]; women 36 [19–57]) and the number of affected areas were the determinants of plasma MMP-9 levels (cumulative R^2 0.134, $P=0.026$).

TABLE 2. Genetic Frequency in Patients and Controls Considering Only the Longest Microsatellite (χ^2 test, $P<0.0005$). All Direct Comparisons Among Genetic Frequencies of Controls, Type 1, and Type 2–4 Plaques Are <0.01 (range 0.009 to <0.0005)

	Carotid Atherosclerosis					Coronary Atherosclerosis	
			US Positive Patients				
CA Repeats	Controls	US Negative Patients	Total	Type 2–4 Plaques	Type 1 Plaque	Controls	Patients
13	2					3	0
14	36	20	34	33	1	48	30
15	1		3	3		0	9
16	2					2	1
18	1					1	0
19	6		1	1		2	1
20	16	1	5	5		13	2
21	29	7	3	3		22	4
22	13	7	29	25	4	13	15
23	3	3	20	13	7	8	17
24	1	2	12	11	1	3	12
25			2	1	1	1	6
26							1
27			1		1		
Total	110	40	110	95	15	116	98

TABLE 3. Odds Ratio of Plaque Types According to the MMP-9 PM (≥22 Repeats in One Allele of the Promoter) and MMP-9 Plasma Levels (Median [IQR], Kruskal-Wallis Test *P*=0.516)

	n	Odds Ratio	95% CI	MMP-9 (ng/mL)
Type 1	15	13.2	1.7–100	35 (28–45)
Type 2	30	1.5	0.4–5.3	47 (36–87)
Type 3	36	0.8	0.3–1.7	49 (22–112)
Type 4	29	0.5	0.2–1.4	46 (25–149)

Independent Replication of the Study

The genotyping was successful in all patients (n=98, men=58%, age 69±12 years) and controls (n=116, men=60%, age 68±13 years), and the results of the replication are reported in Figure 1B. The data confirm the pattern observed in carotid atherosclerosis: compared with controls, patients have an increased prevalence of longer microsatellites ($P<0.0001$). Also, when the proportion of alleles with >21 repeats was considered, according to the evidence from carotid atherosclerosis, the results were similar (32 versus 12%, c^2 test $P<0.0001$; OR, 3.3; 95% CI, 2.0 to 5.3). Data on genetic frequency were quite similar (c^2 test $P<0.0001$; OR, 3.9; 95% CI, 2.2 to 7.1).

Discussion

This study demonstrates a relationship between the MMP-9 microsatellite PM and carotid atherosclerosis. Patients with documented plaques have longer microsatellites than control population and, to a lesser extent, ultrasound-negative patients. It was also achievable to show a precise profile of the risk associated with each group of alleles. In the present study, ≈60% of patients were carriers of at least 1 allele putatively at high risk and ultrasound-negative patients had a 30% prevalence, whereas the prevalence in controls was <15%. A remarkable similarity was observed between the allelic frequency of coronary and carotid patients. This is not surprising, because the majority of patients with carotid atherosclerosis lesions are also carriers of coronary heart disease.

Several studies have documented a prominent role of MMP-9 in atherosclerosis development and complication. These studies used different approaches: genome-wide scans,[23] genetically engineered mice,[24] autopsy,[25,26] and pharmacology studies.[27] Nevertheless, the role of MMP-9 PM in susceptibility for atherosclerosis has still to be established. Although −1562 C/T PM has provided contradictory results, we have focused on the microsatellite PM, showing its association with progression of IMT and of constrictive remodeling.[19]

The results of the present study confirm the evidences from genetically engineered animal models and clinical evidences in humans. Whereas extreme effects in knockout mice, ie, either full or absent expression, documented the role of MMP-9, in humans, functional polymorphisms might help understanding how the different expression of the same protein accounts for genetic susceptibility for atherosclerosis. The absence of MMP-9 in knockout mice accounts for a reduced atherosclerotic burden into aortas of mice, whereas little is known on how the MMP-9 expression affects the development of carotid atherosclerotic plaque.

Another similarity between animal and human atherosclerosis is that the MMP-9 expression in both is not responsible in itself for atherosclerosis, being that classical risk factors are required: hyperlipidemia in knockout mice and hypertension or smoke in patients. The very same risk factors have been also

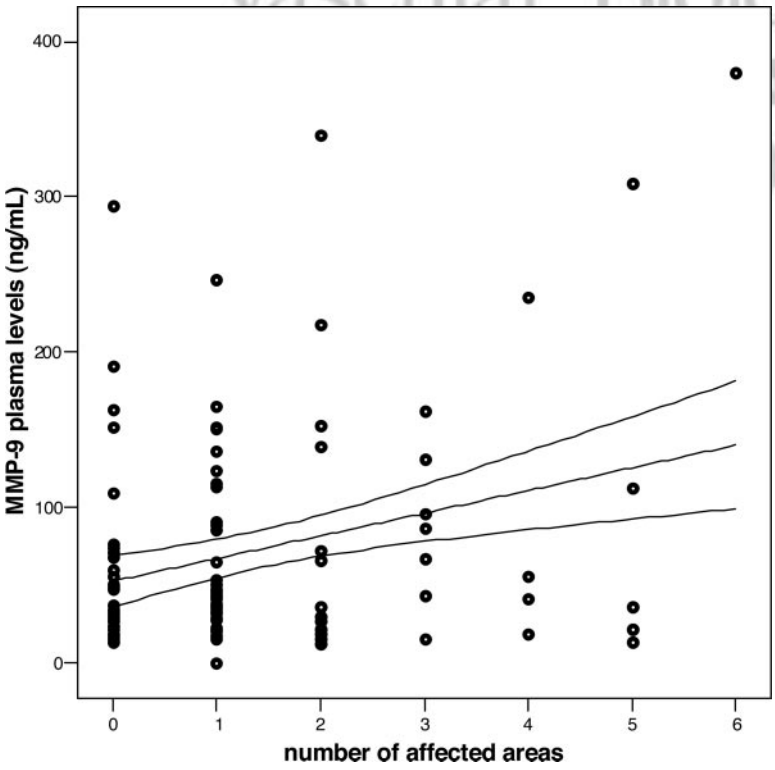

Figure 3. Relationship between the MMP-9 plasma levels and the number of affected areas ($R=0.276$, $P=0.005$). 0 indicates US-negative patients; number of areas, 1 to 6 areas among left and right common carotid artery, bifurcation, and internal carotid artery.

identified by other studies.[28,29] Interestingly, smoke[30] and hypertension[31] can induce the MMP-9 expression, but not by hyperglycemia,[32] whereas data on hypercholesterolemia are not conclusive.

Another important finding is that the very same long microsatellites seem to be associated with ultrasound features of carotid plaques, namely, the thin fibrous cap and echolucent core characterizing the type 1 plaque of the Gray Weale classification.

These plaques, associated to a higher prevalence of vascular events and alleged to be prone to rupture or erosion, might link this MMP and its polymorphism with the development of atherosclerosis events.

In our study, 22 or more CA repeats in 1 allele identifies 93% of plaques with thin fibrous cap classified as type 1, an increased risk of >10 times. The mechanism linking MMP-9 PM with the plaque structure can only be inferred. It involves increased expression of the MMP, increased degradation of the matrix, apoptosis,[33] and, therefore, shaping of a thinner fibrous cap. Also, Pollanen showed a relationship between genetic PM of MMPs and structure of fibrous cap. At least the size of a plaque rupture is different according to the haplotype of MMP-9 and MMP-3.[26]

The same factors, matrix degradation and SMC apoptosis, might also be effective within the plaques. Formation of a lipid core of a plaque is determined by the confluence of extracellular lipid into a unique core. The amounts of matrix and SMC within the core determine the echogenicity.[34] Our data are in good agreement with this hypothesis. The fact that in multivariate analysis MMP-9 PM is the main factor accounting for type 1 plaque adds to a possible role in determining the ultrasound characteristics of the plaques.

Plasma levels of MMP-9 in our study are not different in patients and controls and, in the positive ultrasound patients, in carriers of different plaque type. Also, MMP-9 PM did not account for a different plasma level. A study from Blankenberg et al showed that MMP-9 plasma levels predict the occurrence of future vascular events and were associated to the −1562 C/T genetic PM.[35] Our results, on the contrary, do not support a role of MMP-9 plasma level in atherosclerosis susceptibility, or in plaque type or in relationship with polymorphism. The possible explanation for such discrepancy could be in the different study size and in the different end points.

Other factors, related to the difficulties in assessment of MMP-9 plasma levels, might further explain our contradicting results. An important issue in analyzing plasma MMP-9 levels is that enzyme-linked immunosorbent assay (ELISA) technique for this protein measures at once pro MMP-9, the active form, and complexes MMP-9/TIMP; therefore, a genuine assessment of the relationship between circulating level and expression as well as of other variables might be blunted by this low specificity, especially considering the high TIMP-1 levels in patients with atherosclerosis. Another possible explanation of the correlation of MMP-9 plasma levels with disease extension is that MMP-9 is contained in tertiary granules of neutrophil granulocytes and increased levels might reflect their priming during contact with plaques. The results of multivariate analysis showing that only number of areas affected by plaques in the carotid arteries account for the plasma levels seem to support this explanation. To overcome these pitfalls, large numbers of participants are required: with the data obtained from this study, ≈1200 subjects and patients had to be enrolled to observe a statistical difference between patients and controls.

Study Limitations

Whereas the difference was significant between patients with and without carotid atherosclerosis, the latter group was also significantly different from healthy controls. This result can have some explanations, such as either patients with symptoms requiring carotid ultrasound assessment have either atherosclerosis in other arteries or, maybe, other conditions requiring the MMP-9 activity.

The controls were not evaluated for carotid atherosclerosis with ultrasound. Some false-negative controls might have been introduced, although this should have resulted in a conservative estimate of the risk. This analysis studies only the longest microsatellite, assuming it accounts for an expression more than double than that observed in shorter ones.[16] We do not think it resulted in an oversimplification, because comparison of allelic and genetic frequencies gave quite similar results. One has to consider that the number of possible genotypes is very high, and there are likely minimal differences in both the MMP-9 expression and prevalence of atherosclerosis.

In conclusion, the polymorphism of MMP-9 is a further tool for investigation of matrix remodeling during atherosclerosis. The advantages of assessing MMP-9 PM is that it allows the study of the human disease (rather than of animal models) and, in the clinical setting, is associated to a ten-fold increased risk of developing a thin fibrous cap plaque in the carotid arteries.

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Arteriosclerosis, Thrombosis, and Vascular Biology

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MMP-9 Microsatellite Polymorphism and Susceptibility to Carotid Arteries Atherosclerosis

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METHODS

Patients

Consecutive in- and out-patients visiting two non-invasive vascular diagnostic Centers were studied and then divided in ultrasound (US) positive and US negative patients. The assessment of risk factors, as well as the family and personal history of vascular events were collected at enrolment. The risk factors assessed included: smoking habits (smoker: any subject smoking more than 1 cigarette/day), hypercholesterolemia (total cholesterol > 200 mg/dL or HDL-cholesterol < 40 for women, or <35 mg/dL for men), hypertension (systolic and/or diastolic pressure > 140/90 mm/Hg on two or more measurements), diabetes (fasting blood sugar > 116 mg/dL), and overweight or obesity (BMI > 25). The control population consisted mainly of a group of blood donors attending the Blood Transfusion Centre of the Hospital of Trieste (Italy). Healthy volunteers (on the basis of accurate physical examination and history) willing to participate were enrolled. In order to avoid any confounding, Italian, Slavic and Interethnic population variables (coexisting in Trieste), patients were matched to controls by ethnicity (±4%). Gender (max 5 % discordance) and age (±5 years) matching has been carried out although, to reach this target, some subjects referred for minor transient problems to the Outpatients Clinics of the Internal Medicine Unit of the University of Trieste had to be enrolled. All patients had been informed about the purposes of the study, and signed a written consent. The study had been approved by the Ethical Committee of the Region F.V.G. and complies with the declaration of Helsinki.

Ultrasound assessment of the carotid arteries.

Extra cranial carotid arteries were examined with color–Doppler ultrasound sonography (Toshiba Power Vision 6000, Otawara-Shi, Japan, 7.5 MHz linear-array transducer). The scanning protocol entailed the longitudinal scanning of the near /far walls of the right and left carotid arteries at the distal common carotid artery, carotid bifurcation, and proximal

internal carotid artery. The common carotid artery has been defined as the portion 10 mm below the dilatation of the bulb; the bifurcation has been defined proximally by the dilatation and distally by the tip of the flow divider; the internal carotid artery has been defined as the portion 10 mm above the tip of the flow divider. All focal widening of the vessel wall relative to the adjacent wall, protruding > than 1.5 mm into the lumen have been considered as plaques. Plaque was classified according to Gray Weale et al.[18] Class 1, uniformly anechogenic (echolucent) plaque bound by a thin highly reflecting line, were considered as most likely associated to long MMP-9 promoter microsatellite. All the measurements were performed in real time. Each B-scan exam was performed and interpreted by two investigators (M.F., N.C.), blinded to the genotype results. Twenty percent of the ultrasound assessments were randomly assigned to a reproducibility/variability evaluation within - and between - operators. The inter operator reproducibility was 97%, while Kappa statistics for inter-operator variability was 94%.

DNA extraction and polymorphism analysis.

Venous blood samples from all participants were taken during an ultrasound assessment for patients or blood donation for controls. DNA was extracted from white blood cells with the Wizard genomic DNA purification kit from Promega (Madison, WI) according to the instructions of the manufacturer. To avoid patient's privacy violations, DNA vials of patients were labeled with a number identifying each patient in the institutional database.

The details of the method for the MMP-9 genotyping (PCR amplification and capillary analysis have been previously published [19]. Two investigators (C.G. and L.U.), blind to the clinical status of patients or type of plaque, carried out the interpretation of genotyping. Non-fluoresceinated amplicons from patients homozygous for 14 or 22 CA repeats were sequenced to ascertain the correspondence with the known sequence (Nucleotide accession number AF 538844).

Plasma MMP-9.

Plasma MMP-9 levels have been determined from a blood sample used for genotyping with an R&D System Quantikine ELISA kit according to the instructions provided by the manufacturer. In our hands, intra and inter assay variability were within the limits reported by the manufacturer (< 3% and < 8%, respectively).

Independent replication of the study.

The relatively small size of the sample required a different set of data to confirm the findings. Then, a group of patients with coronary atherosclerosis was compared with a control population. The former group (with coronary atherosclerosis) consisted of patients of both sexes admitted to the Emergency Unit of Trieste (Italy) for acute coronary syndrome, according the guidelines of the ACC/AHA guidelines [20] and confirmed by coronary angiography. The independent replication control population consisted of healthy volunteers; they were either blood donors attending the Blood Transfusion Center of the Trieste Hospital (Italy) or subjects referring for minor transient problems to the Outpatients Clinics of the Internal Medicine Department of the University of Trieste. Such population was selected in order to match patients by ethnicity (max 4% discordance), gender (max 5 % discordance) and age (±5 years).

The study and the replication control groups were independent. The comparison was carried out in the same way of the population study.

Study population and statistical analysis.

Allelic frequency of MMP-9 PM in Caucasian population shows two modes, the first one at 14 and the second at 21 repeats.[21-22] Available data agree that expression increases with the length of the microsatellite. The hypothesis that patients and controls have different allelic and genetic frequencies has been assessed with the χ^2 test. We also sought what alleles showed an association with carotid atherosclerosis dividing alleles into two groups, according to the number of repeats (e.g., 13 and ≥ 14 repeats, then ≤ 14 and ≥ 15 and so on), and calculating multiple χ^2 tests and Odds Ratios for association with carotid

atherosclerosis. The same tests were carried out for genotype frequencies of patients and controls taking into consideration only the longest allele. According to Bonferroni, to reduce the chances of type I statistic error due to multiple testing, the standard P value (0.05) has been divided by the number of comparisons (10) to obtain the final P value ($P < 0.005$). According to previous evidences,[19] the study was sized on the assumption that the group of alleles associated to atherosclerosis was 15% or more prevalent among patients or, otherwise, that the average number of repeats in the two populations would have differed by 1.5 repeats with a σ value of 4. This returned a number of a minimum of 150 alleles per group in the allelic frequency analysis. Genetic frequency was analyzed only with the longest allele in each patient and assuming a difference of 1.5 repeats with a σ value of 5, the number of observations had to be 100 or more per arm.

In sizing the study for the association between type 1 atherosclerotic plaques and MMP-9 polymorphism, we postulated that the prevalence of alleles with high number of repeats between patients with/without unstable plaques would have differed by 40% or more. Such hypothesis returned a figure of 15 patients (or more) with unstable atherosclerotic plaques and 110 total patients; enrolment continued until both these figures have been achieved. For all the calculations we set a power of 80% and an alpha value of 0.05.

The association of type 1 plaques with MMP-9 polymorphisms has been studied with χ^2 test and Odds Ratio (O.R. and 95% C.I.). In assessing the differences between the two Centers, MMP-9 plasma levels, and the relationship between carotid atherosclerosis and vascular risk factors, non-parametric statistics and χ^2 test (with O.R.) were used as appropriate. A P value <0.05 (two tailed) or, in the evaluation of O.R., a 95% confidence interval not encompassing the 1.00 value were considered statistically significant. All statistical analyses were conducted with SPSS 12.0.1 (Statistical Package for Social Sciences, Il) software on PC.

Table I, General data, risk factors, reasons for US assessment and therapy of study population. The number per each group is reported and, in parenthesis, the percentage (except for age).

	Carotid atherosclerosis			Coronary atherosclerosis	
	Controls	US Negative Patients	US Positive Patients	Controls	Patients
Number	110	40	110	116	98
Male	59 (54)	22 (55)	63 (57)	70 (60)	57 (58)
Age (yrs)	66 ± 10	67 ± 11	68 ± 9	68±13	69±12
Current smoker	22 (20)	16 (40)	34 (31)	28 (24)	40 (41)
Diabetes	4 (4)	6 (15)	17 (15)	6 (5)	18 (18)
Hypercholesterolemia	15 (14)	8 (20)	47 (43)	10 (9)	39 (40)
Hypertension	26 (24)	34 (85)	81 (74)	29 (25)	82 (75)
BMI>25	22 (20)	20 (50)	43 (39)	24 (21)	40 (41)
		Reasons for US assessment		Past history	
TIA/Stroke/amaurosis fugax		6 (15)	39 (35)		20 (20)
Coronary heart disease		0	18 (16)		60 (61)
Peripheral Arterial Disease		0	8 (7)		12 (12)
Syncope		10 (25)	6 (5)		0
Dizziness		4 (10)	33 (30)		0
Memory disturbance		3 (8)	4 (4)		0
Bruit at physical examination		0	8 (7)		0
2 or more risk factors		17 (42)	24 (22)		22 (22)
Therapy					
Aspirin	3 (3)	3 (8)	35 (32)	4 (3)	33 (34)
Ticlopidine	0	0	7 (6)	0	13 (13)
ACE inhibitor/ARB	8 (7)	22 (55)	23 (21)	6 (5)	35 (36)
β - blocker	5 (5)	2 (5)	13 (12)	5 (4)	45 (46)
Antihypertensive drugs (others)	2 (2)	9 (22)	4 (4)	3 (3)	20 (20)
Calcium channel blockers	0	0	11 (10)	0	9 (9)
Statin	3 (3)	5 (12)	11 (10)	3 (3)	29 (30)
Lipid lowering drugs	0	0	3 (3)	1 (1)	3 (3)
Anti diabetic drugs	0	2 (5)	1 (1)	1 (1)	10 (10)
No pharmacological treatment	90 (82)	3 (8)	24 (22)	98 (84)	10 (10)

Table II. Binary logistic regression (stepwise method) for US positive patients (compared to controls). Independent variables: hypertension, diabetes, smoke, dislipidemia, overweight, gender, and MMP-9 polymorphism. Results of the last step (3) are reported.

	Odds Ratio	S.E.	*P*.
Hypertension	2.7	0.426	<0.0001
MMP-9 PM	2.2	0.400	<0.0001
Smoke	1.0	0.453	0.028
Constant	-0.1	0.352	0.693