Matrix Metalloproteinase-1 and Matrix Metalloproteinase-3 Gene Promoter Polymorphisms Are Associated With Carotid Artery Stenosis

Giorgio Ghilardi, MD, RC; Maria Luisa Biondi, MD; Marco DeMonti, MD; Olivia Turri, PhD; Emma Guagnellini, MD; Roberto Scorza, MD, PO

Background and Purpose—The matrix metalloproteinases (MMPs) are a family of enzymes that are important in the resorption of extracellular matrix and are involved in atherogenesis. Recently, 2 common polymorphisms on MMP-1 (1G/2G) and MMP-3 (5A/6A) gene promoters have been described. The aim of this study was to investigate a possible association between MMP polymorphisms and increased risk of internal carotid artery (ICA) stenosis.

Methods—We studied 91 patients consecutively recruited for ICA stenosis who had undergone carotid endarterectomy and 133 subjects without ICA stenosis (controls). Polymorphic genotypes were determined by polymerase chain reaction and sequencing analysis.

Results—The frequency of the 6A allele was significantly different between cases and controls: 0.62 and 0.50, respectively (odds ratio [OR], 1.58; 95% CI, 1.08 to 2.33; \(P=0.017\)). The frequency of 6A/6A genotype was significantly higher in cases with involvement of both carotids (OR, 3.13; 95% CI, 1.14 to 8.5; \(P=0.026\)) and in patients with stenosis \(>70\%\) (OR, 2.55; 95% CI, 1.07 to 6.07; \(P=0.033\)). No significant differences were observed in MMP-1 distribution. Patients who were homozygous for both the 6A and 2G alleles had an elevated relative risk of ICA stenosis (OR, 2.66; 95% CI, 1.23 to 5.72; \(P=0.016\)). Multiple logistic regression analysis using the common risk factors and the 6A and 2G allele variants revealed that the 6A allele was an independent risk factor for ICA stenosis (\(P=0.049\)). When 6A/6A and 2G/2G were combined, the risk factor for ICA stenosis was 3-fold higher (OR, 3.31; 95% CI, 1.48 to 7.42; \(P=0.004\)).

Conclusions—Homozygosity for the 6A allele of the MMP-3 promoter is associated with carotid stenosis and, in association with MMP-1 2G homozygosity, predicts an increased risk of ICA stenosis. Even if obtained from a relatively limited patient series, these results might have relevant implications for treatment of ICA stenosis and possibly prevention of carotid-related stroke. (\textit{Stroke.} 2002;33:2408-2412.)

Key Words: atherosclerosis ■ carotid stenosis ■ gene expression ■ metalloproteinases ■ polymorphism

Mature atherosclerotic plaques typically consist of 2 main components: soft, lipid-rich atheromatous “gruel” and hard, collagen-rich sclerotic tissue. The sclerotic component (fibrous tissue) usually is by far the more voluminous component of the plaque, constituting \(>70\%\) of an average stenosing plaque.\(^1\)–\(^3\) Sclerosis, however, is relatively less dangerous than “soft plaques” because fibrous tissue appears to stabilize plaques, protecting them against disruption. In contrast, the soft component is more dangerous because it destabilizes plaques, making them prone to rupture, whereby the highly thrombogenic gruel is exposed to the flowing blood, leading either to thrombosis or to embolization of plaque debris to distal organs.

Over the last few years, matrix metalloproteinases (MMPs) have been increasingly implicated in connective tissue remodeling during atherogenesis. Extensive expression of the MMP-3 gene was localized particularly to plaque regions prone to rupture, such as the fibrous cap and its adjacent tissues.\(^4\) Recently, a common variant in the promoter of the MMP-3 gene has been described.\(^5\) In vitro assays of promoter activity revealed that the 5A allele had 2-fold higher promoter activity than the 6A allele.\(^6\)

Interstitial collagenase (MMP-1) is the only MMP that can cleave native collagen types I and III, which are major structural components of the fibrous plaque cap. MMP-1 might play a significant role in fibrous plaque disruption by contributing to the degradation of interstitial collagens and thinning of the fibrous cap.\(^7\) A common insertion polymorphism (an additional guanine) in the nucleotide sequence of the MMP-1 gene promoter has been reported. The 2G homozygotes show increased transcription activity compared with 1G homozygotes and controls.\(^8\)–\(^10\)
Internal carotid artery (ICA) stenosis has been recognized as a major cause of stroke, and carotid endarterectomy has been validated by several large trials as effective in prevention of stroke secondary to severe ICA stenosis.11–16 Interactions within the ICA stenosing plaque between connective tissue and the cells embedded in the fibrous cap overlying the inner core filled with lipids and necrotic debris appear to determine the history of the ICA stenosis, including complications that are recognized cause of stroke, such as plaque rupture or ulceration, intraplaque hemorrhage, and luminal thrombosis.17,18

The aim of this study was to investigate whether the MMP-3 and/or the MMP-1 promoter polymorphisms are associated with the presence of severe ICA stenosis in patients subjected to carotid endarterectomy.

Subjects and Methods
We studied 91 consecutive patients with ICA stenosis who underwent carotid endarterectomy at our vascular surgery service and 133 subjects (controls) with no evidence of ICA stenosis at ultrasound examination.

All 224 participating subjects underwent basic vascular evaluation, including clinical vascular examination, thorough color-coded echo flow imaging of the accessible arterial tree, and an ECG at rest. Patients underwent additional neurological evaluation and cerebral CT to assess symptoms and/or cerebral infarction related to ICA stenosis. Hypertension was defined according to the American Heart Association Scientific Statement on hypertension.19 Smoker definition included both ex-smokers and active smokers. Hypercholesterolemia was defined as elevated total serum cholesterol levels >200 mg/dL.

Carotid stenosis was assessed by color-coded echo flow imaging and confirmed by angiography, and patients were assigned to surgery according to the American Heart Association Scientific Statement on carotid endarterectomy.11 Preoperative evaluation included ultrasound assessment of plaque density and presence of ulceration. Carotid endarterectomies were performed by standard eversion technique.

Whole blood (3 mL) from patients and controls was collected into potassium EDTA. DNA was prepared with the Instagen Matrix extraction kit (Bio-Rad Laboratories). The polymerase chain reaction starts with 5 minutes of incubation at 94°C to activate the enzyme, followed by 35 cycles of 20 seconds at 94°C, 20 seconds at 55°C, and 30 seconds at 72°C. The amplification was verified on an agarose gel (2%) followed directly by sequencing with an automatic sequencer in fluorescent DNA capillary electrophoresis (ABI Prism 310; Applied Biosystems).

Differences between groups were examined by χ² test. Odds ratios (ORs) (approximate relative risk) were calculated as an index of the association of the MMP-1 and MMP-3 genotype with each phenotype. For each OR, 2-tailed probability values and 95% CIs were calculated.

Multiple logistic regression analysis was used to calculate the OR of ICA stenosis and its 95% CI in subjects exposed to specific risk factors. Only factors that were significantly associated with the development of carotid atheroma on univariate analysis were included in the logistic regression analysis. All statistical analyses were 2-sided and were performed with Stata Statistical Software (Stata Corporation).

Statistical analysis was assumed significant for a probability value of <0.05.

Results
Allele frequencies in both control and patient populations were in Hardy-Weinberg equilibrium for both MMPs. While sex frequency was similar between the 2 groups (P = 0.35), controls were slightly younger (mean ages: patients, 68 ± 7 years; controls, 64 ± 7 years; P = 0.01) (Table 1).

The 6A allele frequency was significantly different between patients and controls (P = 0.017). In contrast, the 2G allele frequency was different, although this was not significant (P = 0.085) (Table 2).

Ten patients had had transient ischemic attack, 31 had had stroke or silent cerebral infarction, and 50 were asymptomatic.

### TABLE 1. Characteristics of Patients and Control Subjects

<table>
<thead>
<tr>
<th></th>
<th>Patients (n = 91)</th>
<th>Controls (n = 133)</th>
<th>OR (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>68 ± 7</td>
<td>64 ± 7</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>Sex, M/F</td>
<td>54/37</td>
<td>87/46</td>
<td>0.35</td>
<td></td>
</tr>
<tr>
<td>Hypertension</td>
<td>54/90 (60%)</td>
<td>51/130 (39%)</td>
<td>2.32 (1.34–4.01)</td>
<td>0.002</td>
</tr>
<tr>
<td>Cigarette smoking</td>
<td>68/90 (75%)</td>
<td>74/130 (57%)</td>
<td>2.39 (1.29–4.21)</td>
<td>0.004</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>24/90 (27%)</td>
<td>21/131 (16%)</td>
<td>1.90 (0.98–3.66)</td>
<td>0.05</td>
</tr>
<tr>
<td>Hypercholesterolemia</td>
<td>66/89 (74%)</td>
<td>78/130 (60%)</td>
<td>1.91 (1.06–3.43)</td>
<td>0.03</td>
</tr>
</tbody>
</table>

### TABLE 2. Prevalence of Genotypes and Allele Frequencies in Patients and Controls

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Patients (n = 91)</th>
<th>Controls (n = 133)</th>
<th>OR (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1G/1G</td>
<td>12 (13%)</td>
<td>33 (25%)</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>1G/2G</td>
<td>54 (59%)</td>
<td>70 (53%)</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>2G/2G</td>
<td>25 (28%)</td>
<td>30 (22%)</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>2G allele frequency</td>
<td>0.57</td>
<td>0.49</td>
<td>1.39 (0.95–2.03)</td>
<td>0.085</td>
</tr>
<tr>
<td>MMP-3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5A/5A</td>
<td>13 (14%)</td>
<td>36 (27%)</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>5A/6A</td>
<td>43 (47%)</td>
<td>59 (44%)</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>6A/6A</td>
<td>35 (38%)</td>
<td>38 (28%)</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>6A allele frequency</td>
<td>0.62</td>
<td>0.50</td>
<td>1.58 (1.08–2.33)</td>
<td>0.017</td>
</tr>
</tbody>
</table>
TABLE 3. Clinical Characteristics of Patients According to Polymorphism of MMP-3 Gene Promoter

<table>
<thead>
<tr>
<th>Symptom</th>
<th>5A/5A</th>
<th>5A/6A</th>
<th>6A/6A</th>
<th>OR (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of ICAs involved</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Monolateral</td>
<td>5</td>
<td>17</td>
<td>6</td>
<td>3.13 (1.14–8.5)</td>
<td>0.026</td>
</tr>
<tr>
<td>Bilateral</td>
<td>8</td>
<td>26</td>
<td>29</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Stenosis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;70</td>
<td>8</td>
<td>24</td>
<td>12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;70</td>
<td>15</td>
<td>19</td>
<td>23</td>
<td>2.55 (1.07–6.07)</td>
<td>0.033</td>
</tr>
<tr>
<td>Stroke</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>7</td>
<td>26</td>
<td>17</td>
<td>1.51 (0.65–3.52)</td>
<td>0.334</td>
</tr>
<tr>
<td>Yes</td>
<td>6</td>
<td>17</td>
<td>18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plaque ulceration</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>9</td>
<td>24</td>
<td>16</td>
<td>1.70 (0.73–3.96)</td>
<td>0.218</td>
</tr>
<tr>
<td>Yes</td>
<td>4</td>
<td>19</td>
<td>19</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

ORs for genotype were calculated with 6A/6A vs 5A/6A and 5A/5A.

at the time of operation. No differences in clinical signs and symptoms (e.g., transient ischemic attacks and stroke or silent cerebral infarction) were found in the 1G/2G genotype, while 6A/6A homozygosity was significantly more frequent in patients with bilateral severe ICA stenosis and higher grade of stenosis, expressed as percentage of ICA occlusion (Table 3). No correlation was observed between 6A frequency and stroke or plaque ulceration. The 21 patients who were homozygous for both the 6A and 2G alleles had an elevated relative risk of ICA stenosis (OR, 2.66; 95% CI, 1.23 to 5.72; P = 0.016).

Multiple logistic regression analysis was performed to identify possible independent risk factors for ICA stenosis among patients and controls. The following variables were considered: hypertension, cigarette smoking, diabetes mellitus, hypercholesterolemia, and 6A and 2G alleles. Hypercholesterolemia, diabetes, and the presence of the 2G allele were not found to be independent risk factors for ICA stenosis in our series. The other 3 variables considered were found to be independent risk factors, including the presence of the 6A allele (P = 0.049; OR, 2.21; 95% CI, 1.00 to 4.88).

Multiple logistic regression analysis was then calculated for the association of both homozygosities 6A and 2G, matched against the aforementioned risk factors (Table 4). Homozygosity for both 6A and 2G resulted an independent risk factor for ICA stenosis (OR, 3.31; 95% CI, 1.48 to 7.42; P = 0.004).

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Multiple logistic regression analysis was then calculated for the association of both homozygosities 6A and 2G, matched against the aforementioned risk factors (Table 4). Homozygosity for both 6A and 2G resulted an independent risk factor for ICA stenosis (OR, 3.31; 95% CI, 1.48 to 7.42; P = 0.004).

Discussion

The results of the present study suggest that a genetic polymorphism of MMP-3, which modulates its enzymatic activity, is associated with ICA stenosis and the degree of stenosis. Moreover, the association of 6A/6A and 2G/2G (a functional polymorphism of MMP-1 promoter) was an independent risk factor for ICA stenosis (OR, 3.31; 95% CI, 1.48 to 7.42; P = 0.004).

MMPs play an important role in connective tissue remodeling during tissue repair, cell migration, angiogenesis, tissue morphogenesis, and growth. These physiological processes require a tightly controlled balance between the MMPs and specific tissue inhibitors of metalloproteinase. Disruption of this balance, however, could lead to several pathological states, such as atherosclerosis.

Previous studies have identified local expression of several MMPs in the context of atherosclerotic plaque, such as MMP-3 in coronary plaque and MMP-1 and MMP-9 in carotid plaque, especially in the so-called shoulder of unstable lesions.

MMP-3 is a key member of the MMP family, with broad substrate specificity. It can degrade types II, IV, and IX collagen, proteoglycans, laminin, fibronectin, gelatin, and elastin. In addition, MMP-3 can also activate other MMPs such as collagenase, matrilysin, and gelatinase B, rendering MMP-3 crucial to connective tissue remodeling process. Expression of MMP-3 is primarily regulated at the level of transcription, where the promoter of the gene responds to various stimuli, including growth factors, cytokines, tumor promoters, and oncogene products.

MMP-3 production is regulated in response to several stimuli. A common variant in the promoter region of the human MMP-3 gene, with 1 allele having a run of 5 adenosines (5A) and the other having 6 adenosines (6A), has been reported. In vitro experiments demonstrated that the 6A allele has lower promoter and transcriptional activity. The same investigators also demonstrated that the 6A allele of the MMP-3 5A/6A polymorphism was associated with a more rapid progression of coronary stenosis due to atherosclerosis. Therefore, subjects carrying the 6A allele might accumulate extracellular matrix because of decreased degradation.

The relationship between MMP-3 polymorphism and common carotid geometry in healthy subjects and in asymptomatic carotid artery atherosclerosis was recently described. Both studies showed an association with 6A/6A homozygosity and intima-media thickness.

Our study, performed on patients undergoing surgery for ICA stenosis, confirmed the association between the severity of stenosis and the 6A allele (P = 0.033). The presence of the latter was a weak risk factor for ICA stenosis, and thus reduced MMP-3 gene transcriptional activity seems to be associated with more rapid progression of stenosis in ICAs; 6A/6A homozygosity was higher in patients with both ICAs involved (21% versus 46%; P = 0.026) (Table 3). Reduced levels of MMP-3 in the 6A homozygotes compared with other genotypes might affect the balance between synthesis and degradation during matrix turnover to favor increased deposition of matrix, leading to more rapid growth of the atherosclerotic plaque.

Plaque instability, manifesting as ulceration of the fibrous cap, plaque rupture, or intraplaque hemorrhage, is
responsible for most of the complications of atherosclerosis.\textsuperscript{17,18,30} Interstitial collagens are the only known MMPs that can cleave native collagen types I and III,\textsuperscript{31} which are major structural components of the fibrous plaque.\textsuperscript{32} The major human interstitial collagenase, MMP-1, is secreted by a variety of mesenchymal and epithelial cell types.\textsuperscript{33} MMP-1 could play a significant role in fibrous plaque disruption by contributing to the degradation of interstitial collagens and thinning of the fibrous cap.\textsuperscript{23}

Nikkari et al\textsuperscript{7} have demonstrated that, in human carotid arteries, the matrix-degrading enzyme MMP-1 is expressed in advanced atherosclerotic plaques but not in nonatherosclerotic intima. This enzyme could contribute to plaque expansion, disruption, and thrombosis.

Rutter et al\textsuperscript{8} have recently reported that the insertion of a guanine nucleotide (G) at −1607 bp in the nucleotide sequence of the MMP-1 gene promoter generates a new 5′-GGA-3′ sequence that corresponds to a core recognition sequence of the binding site for members of the Ets family of transcription factors.\textsuperscript{9,10} The 2G homozygous polymorphism of the gene promoter results in an increased transcription activity in melanoma cell lines and in normal fibroblasts compared with 1G homozygotes and controls.\textsuperscript{8}

Many studies of MMP-1 expression and human carotid atherosclerosis have been performed;\textsuperscript{7} to our knowledge, however, this is the first study focusing on MMP-1 promoter polymorphism and ICA stenosis in operated subjects. Our results showed a high, but not significant, correlation ($P=0.085$) between the 2G allele and ICA stenosis. However, when 2G/2G and 6A/6A homozygotes were combined, the risk factor for ICA stenosis was 3-fold higher (OR, 3.31; $P=0.004$). This finding seems to suggest a synergistic effect between the lower proteolytic activity of the 6A homozygotes for MMP-3 gene promoter (stenosing effect) and the higher proteolytic activity of the 2G homozygotes for MMP-1 gene promoter (greater degradation effect of the atherosclerotic plaque core, leading to higher instability of the whole lesion). The net effect could be an increased growth velocity of the plaque in subjects homozygous for both 6A and 2G.

In conclusion, our results confirm the role of the 6A allele in the pathogenesis and progression of ICA stenosis. Although no uniform results were observed for MMP-1 gene promoter polymorphism, the presence of 6A and 2G homozygosity in the same subject shows a strong association with ICA stenosis.

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