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Plaque Rupture is a Determinant of Vascular Events in Carotid Artery Atherosclerotic Disease: Involvement of Matrix Metalloproteinases 2 and 9

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Background and Purpose Unstable carotid atherosclerotic plaques are characterized by cap rupture, leading to thromboembolism and stroke. Matrix metalloproteinases (MMPs) have been implicated in the progression of atherosclerosis and plaque rupture. The aim of this study was to assess the relationship between the expressions of MMP-2 and MMP-9 and carotid plaque instability.

Methods Eighty atherosclerotic plaques were collected from 74 patients undergoing carotid endarterectomy. Clinical information was obtained from each patient, and plaque morphology was examined at the macroscopic and microscopic levels. The immunohistochemical expressions of MMPs were graded using semiquantitative scales.

Results Macroscopic ulceration (84.6% versus 63.4%, p=0.042) and microscopic cap rupture (79.5% versus 51.2%, p=0.010) were more common in symptomatic than in asymptomatic patients. Immunoreactivities of MMP-2 and MMP-9 were increased in 40 and 36 atheromatous plaques, respectively. Macroscopic ulceration was strongly correlated with the expressions of MMP-2 (p<0.001) and MMP-9 (p=0.001). There were significant correlations between increased MMP-2 expression and cap rupture (p=0.002), intraplaque hemorrhage (p=0.039), and a thin fibrous cap (p=0.002), and between increased MMP-9 expression and cap rupture (p=0.010) and a large lipid core (p=0.013).

Conclusions Plaque rupture was significantly associated with the development of vascular events in carotid atherosclerotic disease. MMP-2 and MMP-9 are strongly correlated with plaque instability. **J Clin Neurol 2011;7:69-76**

Key Words metalloproteinase, carotid plaque, instability.

Introduction

Stroke is the second most frequent cause of death in Korea. Moreover, the prevalence of extracranial carotid artery atherosclerotic disease is increasing.[1-3] The degree of carotid artery stenosis is known to be strongly associated with stroke risk in symptomatic patients with carotid atherosclerotic disease.[4-6] It is becoming increasingly apparent that the acute disruption of atherosclerotic plaques precedes the onset of clinical syndromes.[7-9] Unstable plaques, characterized by cap rupture, lead to thromboembolism and stroke. The vulnerability of atherosclerotic plaques depends on many factors including endothelial cell function, inflammatory cells, cytokine production, smooth-muscle cell content, and cell death (including necrosis and apoptosis).[10,11]

Matrix metalloproteinases (MMPs), which are zinc-dependent physiologic regulators of the extracellular matrix (ECM), are produced by macrophages in atherosclerotic lesions.[12] These enzymes are capable of degrading various matrix proteins and may play an important role in the development and progression of these lesions. Overexpression of these enzymes in advanced atherosclerotic lesions may contribute to thinning

of the plaque cap and the development of ischemic events as a result of plaque rupture.[13]

It has been demonstrated that MMP-2 (72 kD, gelatinase A) and MMP-9 (92 kD, gelatinase B) cleave intact fibrillar collagen, in addition to nonfibrillar and fragmented interstitial collagen, and may be more important for matrix remodeling than previously thought.[14-16] Previous reports have described that the degradation of plaque collagen is significantly associated with increased expressions of MMP-2 and MMP-9 within the vulnerable regions of human atheroma.[12,17,18]

In the present study, we classified morphological characteristics according to clinical manifestation in patients with carotid atherosclerotic plaques. Furthermore, we evaluated differences in the patterns of expressions of MMP-2 and MMP-9 between stable and unstable carotid plaques.

Methods

Study population

We collected carotid plaques in consecutive patients undergoing carotid endarterectomy (CEA) at Kyung Hee University Medical Center Between January 2003 and November 2008. Detailed clinical information was obtained from each patient, with particular reference to carotid territory ischemic events. All patients were divided into two groups based on the timing of the most recent symptom. Patients with neurological symptoms referable to the ipsilateral carotid territory within the 12 months before surgery were classified as symptomatic, and the other patients were classified as asymptomatic.

This study was approved by an independent ethics committee at Kyung Hee University Medical Center (KMC IRB 0876-03), and informed consent to participate was obtained from all patients before specimen collection.

Preparation of the specimens

All operations were performed using standard surgical techniques and with minimal manipulation of the specimen. Carotid plaque tissues were obtained immediately after surgical resection and were digitally photographed and then stored in 0.2 M phosphate buffer solution (4°C). The tissue was cut into 5-mm-thick blocks from the central part of the tissue where stenosis was maximal along the length of the plaque, using methods similar to those reported by Redgrave et al.[9] (Fig. 1A). The specimen was fixed in 4% paraformaldehyde for 24 hours at 4°C, and then rinsed with 30% sucrose and stored in 30% sucrose at 4°C for 2 days. The specimens were then embedded separately into an optimum cutting temperature (OCT) compound (LEICA, 020108926) and stored at -80°C.

Histological analysis

All of the plaque tissues were photographed in the operating room just after surgical resection to allow examination of their gross morphology. For histological observation, 10-μm-thick sections were taken from each block and stained with hematoxylin and eosin (Fig. 1B). Histological observations were recorded independently by two observers who were blinded to clinical information. Any discrepancies between the two observers were resolved by discussion with an experienced pathologist (J.H. Lee).

As in previous reports,[9,19-21] the following features were measured: cap rupture, any thrombi, intraplaque hemorrhage (IPH), cap thinning, a large lipid core, and calcifications (Fig. 1C, D and E and Supplementary Fig. 1). Cap rupture was recorded if there was clear communication between the lipid core and the lumen with a break in the fibrous cap that did not appear to have been created during surgery. Any thrombus was defined as an organized collection of fibrin and red blood cells in the lumen.

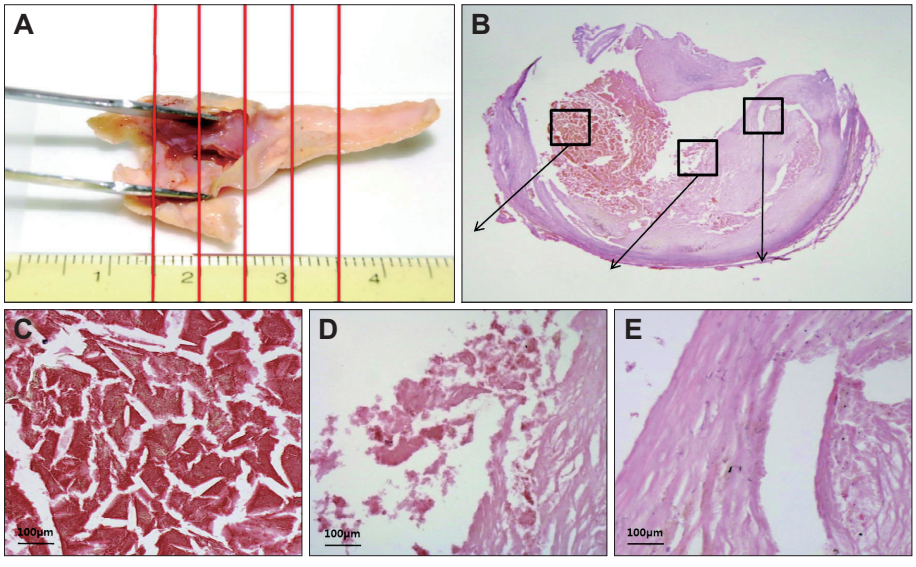

Fig. 1. Gross and histological morphology of a carotid specimen. A: Transverse sections were taken at 5-mm intervals and divided into central, shoulder, and peripheral regions. B: Results of hematoxylin and eosin staining (×10). C: Thrombus (×100). D: Rupture (×100). E: Necrotic lipid core (×100).

IPH was recorded if there was an area of erythrocytes within the plaque causing disruption of the plaque architecture.[22] Cap thinning was defined as fibrous-cap atheromas having lesions with a thin (<200 μm) fibrous cap and an underlying necrotic core.[23] A lipid core was defined as amorphous material containing cholesterol crystals. A large lipid core was defined as a lipid core measuring >50% of the thickness of the plaque or >25% of the total cross-sectional area.[24] Calcification was considered to be large when nodular deposits were seen.[25]

Immunohistochemistry and immunofluorescence microscopy

Frozen sections were cut at a thickness of 10 μm and mounted on microscope slides. Tissues were fixed on silane-coated microscope slides with 99% acetone (stored at -20°C). Glycine was mixed in phosphate-buffered saline (PBS, pH 7.4). After washing three times with PBS, the microscope slides were incubated with BLOTTO (100 mL of Tris-saline, 1 ml of skim milk, 1 ml of horse serum, and 0.02 g of azide) for 20 min.

Primary antibody, and antibodies raised against MMP-2 (sc-71595, Santa Cruz Biotechnology) and MMP-9 (sc-21733, Santa Cruz Biotechnology) were diluted to 1:100, and a peroxidase Vector ABC kit (PK-6102, Dako) was used for their detection. Colocalization studies for macrophages and smooth-muscle cells were performed using antibodies to CD-68 (m0851, Dako; 1:100) and smooth- muscle actin (SMA; m0876, Dako; 1:100), respectively. Sections were incubated in the primary antibodies for 2 hours at 37°C in an incubator. For immunohistochemistry, they were incubated in the secondary antibody, antimouse immunoglobulin G (IgG) from the ABC kit, for 30 minutes at 37°C in an incubator. Diaminobenzidine was used as a chromogen to detect the antibodies. For immunofluorescence, the sections were incubated with the secondary antibody, antimouse IgG-TR (sc-2781, Santa Cruz Biotechnology), to detect MMP-2 and MMP-9, at 37°C for 45 min in an incubator. After washing with PBS, the sections were mounted in Vectashield solution containing 4,6'diamidino-2-phenylindole to stain the nuclei (H-1500, Vector Laboratories, Burlingame, CA, USA).

The immunohistochemical expressions of MMP-2 and MMP-9 were graded using the following semiquantitative scale: 0=no stained cells, 1=occasional scattered cells or 1 group of ≥20 cells, 2=several groups (<5) of ≥20 cells, and 3=many groups (≥5) of ≥20 cells or 1 group of ≥100 cells (Supplementary Fig. 2). There was a high interobserver agreement for MMP-2 and MMP-9 (Pearson's r=0.704 and 0.659, respectively). A score of 2 or more on the rating scale was classified as advanced expression.

Western blot analysis

Frozen tissue samples from four atherosclerotic plaques were individually homogenized in RIPA buffer (with 50% glycerol).

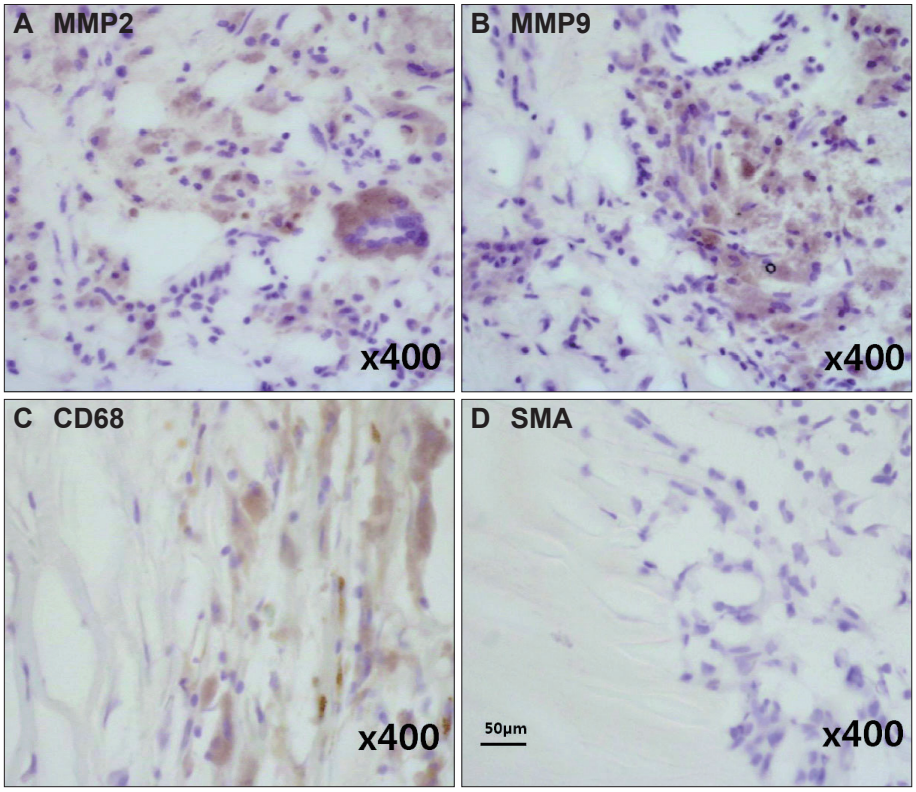

Fig. 2. Carotid specimens that were immunohistochemically positive for MMP-2 (A) and MMP-9 (B) were also strongly positive for CD68 (C) but negative for SMA (D). MMP: matrix metalloproteinase, SMA: smooth- muscle actin.

The concentration of protein was measured by enzyme-linked immunosorbent assay. Tissue lysate was applied to 8% sodium dodecylsulfate-polyacrylamide gel electrophoresis. After blocking with bovine serum albumin (5%) for 90 minutes, the primary antibodies raised against MMP-2 (sc-80201, Santa Cruz Biotechnology) and constitutive β-actin (sc-47778, Santa Cruz Biotechnology) were added (diluted to 1:500), and the gel was incubated overnight in a cold room (4°C) and then for a further hour at room temperature. The gel was then incubated with a secondary antibody (goat antimouse IgG; sc-2005, Santa Cruz Biotechnology) for 30 minutes. The produced bands were detected by a Western blotting detection system (luminal reagent; sc-2048, Santa Cruz Biotechnology) with photographic film (X-Omat, Kodak).

Statistical analysis

Patients whose most recent event was a stroke, cerebral transient ischemic attack, or amaurosis fugax were compared with asymptomatic patients for baseline characteristics and plaque histological features. Baseline demographic data are expressed as mean±SD values for continuous variables and as frequencies for categorical variables by *t*-test and χ^2 test. In all tests, the level of statistical significance was set at $p<0.05$. The interrater agreement was calculated by Pearson's rho for continuous ratings and by Cohen's Kappa for binominal gradings. All statistical analyses were conducted using the SPSS 13.0 package for Windows (SPSS, Chicago, IL, USA).

Table 1. Baseline characteristics of the subjects. Data are *n* (%) values except where stated otherwise

Characteristic	*n* (%)
Age (years; mean±SD)	66.3±8.4
Sex (*n*, male/female)	57/17
Hypertension	64 (86.5)
Diabetes	28 (37.8)
Hyperlipidemia	21 (28.4)
Smoking	37 (50.0)
Coronary artery disease	20 (27.0)
Bilateral carotid operation	6 (8.1)
Clinical presentation*	
Asymptomatic	41 (51.3)
Symptomatic	39 (48.8)
TIA	8 (10.0)
Amaurosis fugax	1 (1.3)
Stroke†	30 (37.5)
Location of lesion (*n*, right/left)	41/39
Stenosis degree (%, mean±SD)	73.2±15.1
Time since most recent ischemic event in symptomatic patients	
<3 months	32 (82.1)
3-6 months	5 (12.8)
6-12 months	2 (5.1)
mRS	1.2±1.2

*A total of 80 plaques from 74 patients, †Smoking: present history of smoking or quitting smoking within the previous 5 years.
TIA: transient ischemic attack, mRS: modified Rankin scale.

Results

In total, 108 CEAs were performed at Kyung Hee University Medical Center. Twenty-four specimens from atherosclerotic plaques were not obtained because of a lack of availability of immediate deliveries or the absence of informed consent. Four further specimens were excluded because they were too fragmented for morphological assessment. Eighty plaques from 74 patients undergoing CEA were analyzed. Six patients underwent bilateral CEA for separate ipsilateral symptomatic events or severe stenosis (>60%). Of 80 plaques, 41 were classified as asymptomatic and 39 were classified as symptomatic (transient ischemic attack=8, amaurosis fugax=1, and ischemic stroke=30). The baseline demographic features are listed in Table 1.

The age and risk factors did not differ significantly between

Table 2. Comparison of symptomatic and asymptomatic groups in terms of macroscopic, microscopic, and immunohistochemical features of carotid plaques. Data are *n* (%) values except where stated otherwise

	Symptomatic (*n*=39)	Asymptomatic (*n*=41)	*p*
Clinical			
Age (years; mean±SD)	64.7±8.3	67.8±7.9	0.096
Sex (*n*, male/female)	29/10	34/7	0.418
Hypertension	36 (92.3)	33 (80.5)	0.194
Diabetes	15 (38.5)	16 (39.0)	>0.999
Hyperlipidemia	10 (25.6)	12 (29.3)	0.805
Smoking*	22 (56.4)	19 (46.3)	0.382
Coronary artery disease	10 (25.6)	10 (24.4)	>0.999
Stenosis degree (%, mean±SD)	77.0±15.1	69.8±14.4	0.034
Macroscopic			
Plaque ulceration	33 (84.6)	26 (63.4)	0.042
Microscopic			
Rupture	31 (79.5)	21 (51.2)	0.010
Thrombus	23 (56.1)	18 (43.9)	0.189
IPH	26 (66.7)	22 (53.7)	0.261
Cap thinning	27 (69.2)	23 (56.1)	0.255
Large lipid core	20 (51.3)	18 (43.9)	0.655
Calcification	33 (84.6)	30 (73.2)	0.277
Immunohistochemistry†			
MMP-2	23 (56.1)	17 (41.5)	0.179
MMP-9	19 (48.7)	17 (41.5)	0.653

*Smoking: present history of smoking or quitting smoking within the previous 5 years, †Immunohistochemistry data are the numbers of plaques with advanced expression.
IPH: intraplaque hemorrhage.

the symptomatic and asymptomatic groups. Macroscopic ulceration (84.6% vs. 63.4%, p=0.042) and microscopic rupture (79.5% vs. 51.2%, p=0.010) were more frequent in symptomatic plaques than in asymptomatic plaques (Table 2). Microscopic findings suggestive of marked atherosclerosis such as cap rupture (p<0.001), any thrombus (p=0.005), IPH (p=0.021), and cap thinning (p=0.038) were significantly more frequent in ulcerated plaques than in nonulcerated plaques. The degree of calcification did not differ significantly between the groups (p=0.363)(Table 3). For interobserver agreement, the kappa values for rupture (0.688), any thrombus (0.697), and IPH (0.697) were substantial, while those for cap thinning (0.436) and a large lipid core (0.506) were moderate, and that for calcification (0.317) was fair.

No direct relationship was found between MMPs and clinically relevant manifestations. Nevertheless, macroscopic ulceration was strongly correlated with the expressions of MMP-2 (p<0.001) and MMP-9 (p=0.001), although the expressions of MMP-2 and MMP-9 did not differ significantly between the symptomatic and asymptomatic patient groups. Highly expressed MMP-2 and MMP-9 cells were strongly positive for CD68 and negative for SMA (Fig. 2). The findings of Western blot analyses support our immunohistochemical findings (Supplementary Fig. 3). Immunofluorescence staining revealed that the expressions of MMP-2 and MMP-9 were colocalized with macrophages and stained mainly the cytoplasm (Fig. 3). In addition, there were significant correlations between increased MMP-2 expression and cap rupture (p=0.002), IPH (p=0.039), and a thin fibrous cap (p=0.002), and between increased MMP-9 expression and cap rupture (p=0.010) and a large lipid core (p=0.013)(Table 4).

Discussion

We found that only plaque rupture was significantly associated with the development of vascular events in carotid atherosclerotic disease. In addition, the expressions of MMP-2 and MMP-9 were strongly related to plaque instability. The histological features of symptomatic carotid plaques were recently established in large clinical trials,[9,26] which found that cap rupture was the only morphological feature that was significantly associated with the occurrence of clinical events. We confirmed this result in our study. In addition, the degree of stenosis was higher in the symptomatic than the asymptomatic group. This might be attributable to selection bias for surgery, that was because the degree of stenosis remained important for relevant ischemic events.

It has been widely accepted that plaque rupture plays a crucial role in the pathogenesis of vascular events and that the destabilization of atherosclerotic plaques is mediated by a series of enzymes called MMPs, which are the main physiological regulators of the ECM. Several experimental and clinical studies have established the importance of metalloproteinases in the critical balance between ECM breakdown and synthesis that determines plaque instability, leading to plaque rupture and other aspects of vascular remodeling.[27,28] Cell-surface activation of MMPs is considered to be an important step in the pericellular degradation of the ECM during cell migration.

Increased expressions of MMP-1, -2, -3, -7, -8, -9, -10, -12, and -13 were found in macrophages and smooth-muscle cells in carotid atherosclerotic plaques.[12,23,27-32] Among these, MMP-9 has been highlighted as one of the most important enzymes, and its immunostaining mostly colocalizes with macrophages and relates to unstable carotid plaques.[12,29] In our study, CD68

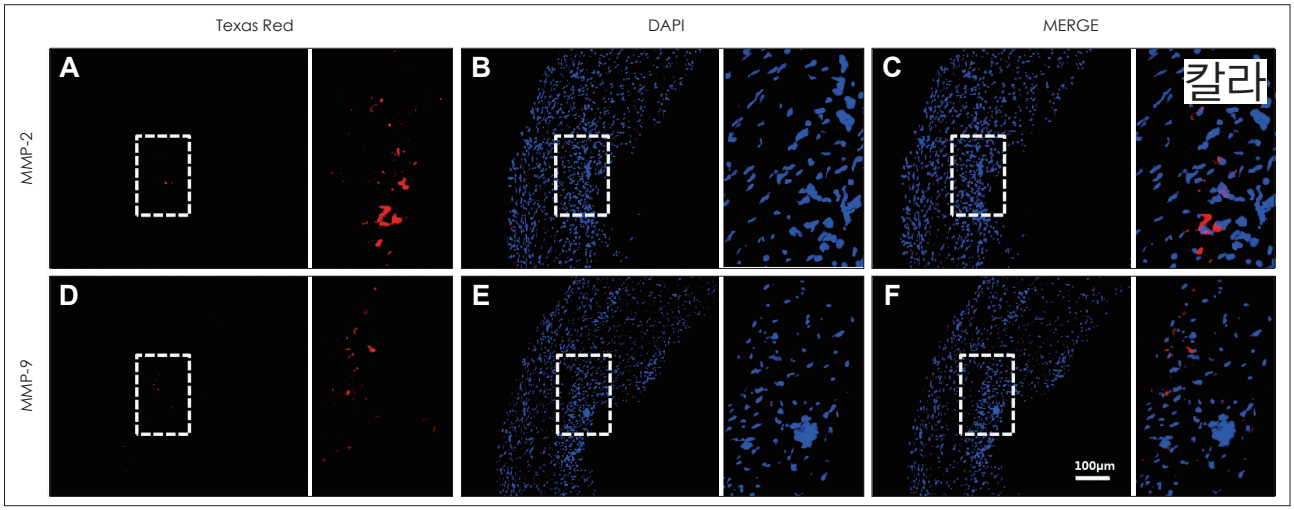

Fig. 3. Immunofluorescence staining illustrating the expressions of MMP-2 and MMP-9 produced by macrophages in carotid plaques. A and D: MMP-2 and MMP-9 were visualized with secondary antibodies conjugated to Texas Red (red). B and E: The nuclei were stained with DAPI (blue). C and F: MMPs colocalized with the cytoplasm in the macrophages. The white boxes in each image are magnified. Scale bar=100 μm. MMP: matrix metalloproteinase.

cells were positive for MMPs, which suggests that macrophages play a role in plaque instability. These findings are consistent with previously published results.[27-32] A significantly higher serum concentration of MMP-9 has been reported in patients with previous neurologic symptoms and unstable plaques, as determined by histological analysis, and a strong correlation was found between MMP-9 overexpression and the presence of macrophages in the plaques. However, the concentration of another gelatinase, MMP-2, was only slightly higher in the symptomatic group than in the asymptomatic group, but there was no association with any of the cell types studied immunohistochemically.[30] Several previous studies have revealed that MMP-2 is not related to carotid instability, and that increased MMP-2 activity is associated with the presence of smooth-muscle cells, suggesting a stable lesion phenotype. [29,31,33-35] Therefore, the relationship between MMP-2 expression and unstable plaques has been controversial.

We examined the expressions of MMP-2 and MMP-9 along with the characteristic histological plaque findings, including plaque rupture, any thrombus, IPH, a large lipid core, cap thinning, and calcification. The findings of our study show that both MMP-2 and MMP-9 are significantly associated with plaque rupture. One previous study showed that locally produced MMP-2 is activated by thrombin and therefore increases local matrix-degrading activity to complicated atherosclerotic plaques, such as IPH.[36] Another study revealed that human monocyte-derived macrophages induce collagen breakdown in the fibrous cap of atherosclerotic plaques, thereby contributing to cap thinning and weakening by MMP-1 and MMP-2.[18] Moreover, a large lipid core was related only to MMP-9. By measuring the MMP-9 level as a marker of plaque instability, recent studies have demonstrated that increased levels of oxidized low-density lipoprotein are markedly associated with MMP-9 activation, and that statins reduce inflammatory responses.[37,38]

Our study has several clear advantages. First, even though there are only a few previous reports on the histopathological characteristics of carotid plaques in Asian patients,[34,39] we performed careful clinicohistopathological correlations, and confirmed that plaque morphology determines the propensity to provoke clinical manifestations. Second, we described the histological findings and the association with MMPs in detail. We performed a histological analysis, satisfying the recommendations for the performance and reporting of studies of carotid plaque imaging with histology proposed by Lovett et al.[40]

Our study was subject to some limitations. First, we analyzed our results semiquantitatively by immunohistochemistry, and hence the results do not readily allow accurate quantification. Nevertheless, our estimates were performed by two observers, and the interobserver reliability was high. Second, the target site that we selected from the experimental specimens was the central (cap and core) region of the carotid plaque, because the focus of this study was plaque rupture. According to previous studies, MMPs are expressed primarily by macrophages in the shoulder regions of the atherosclerotic plaque and in the border between the lipid core and the overlying fibrous area.[12,32] Thus, it is possible that we excluded the regions of the plaques where inflammation is likely to take place (i.e., the shoulder).

These results have important implications not only with respect to our understanding of the processes that lead to acute cerebral ischemia due to carotid plaques, but also with regard to the interpretation of the topographic appearance of plaques. Our results confirm the associations of MMP-2 and MMP-9 with plaque instability. In the future, the use of high-through-

Table 3. Prevalence of histological features in plaques with macroscopic ulceration. Data are *n* (%) values except where stated otherwise

	Ulceration (*n*=59)	No ulceration (*n*=21)	*p*
Rupture	47 (79.7)	5 (23.8)	<0.001
Thrombus	36 (61.0)	5 (23.8)	0.005
IPH	40 (67.8)	8 (38.1)	0.021
Cap thinning	41 (69.5)	9 (42.9)	0.038
Large lipid core	32 (54.2)	6 (28.6)	0.074
Calcification	48 (81.4)	15 (71.4)	0.363
MMP-2	38 (64.4)	2 (9.5)	<0.001
MMP-9	33 (55.9)	3 (14.3)	0.001

Table 4. Prevalence of histological features in plaques with immunohistochemistry results. Data are *n* (%) values except where stated otherwise

	MMP-2		*p*	MMP-9		*p*
	Positive (*n*=40)	Negative (*n*=40)		Positive (*n*=36)	Negative (*n*=44)	
Rupture	33 (82.5)	19 (47.5)	0.002	29 (80.6)	23 (52.3)	0.010
Thrombus	22 (55.0)	19 (47.5)	0.655	21 (58.3)	20 (45.5)	0.271
IPH	29 (72.5)	19 (47.5)	0.039	23 (63.9)	25 (56.8)	0.647
Cap thinning	32 (80.0)	18 (45.0)	0.002	25 (69.4)	25 (56.8)	0.353
Large lipid core	23 (57.5)	15 (37.5)	0.117	23 (63.9)	15 (34.1)	0.013
Calcification	33 (82.5)	30 (75.0)	0.586	30 (83.3)	33 (75.0)	0.420

put techniques will potentially identify novel patterns of biomarkers that, along with traditional risk factors and imaging techniques, could help to target vulnerable patients and monitor the beneficial effects of pharmacological agents.

Conflicts of Interest

The authors have no financial conflicts of interest.

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