Pathological Quantification of Carotid Artery Plaque Instability in Patients Undergoing Carotid Endarterectomy

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Background: Unstable atherosclerotic carotid plaques cause cerebral thromboemboli and ischemic events. However, this instability has not been pathologically quantified, so we sought to quantify it in patients undergoing carotid endarterectomy (CEA).

Methods and Results: Carotid plaques were collected during CEA from 67 symptomatic and 15 asymptomatic patients between May 2015 and August 2016. The specimens were stained with hematoxylin-eosin and elastica-Masson. Immunohistochemistry was performed using an endothelial-specific antibody to CD31, CD 34 and PDGFRβ. The histopathological characteristics of the plaques were studied. By multiple-variable logistic regression analysis, plaque instability correlated with the presence of plaque rupture [odds ratio (OR), 9.75; P=0.013], minimum fibrous cap thickness (OR per 10 μm 0.70; P=0.025), presence of microcalcifications in the fibrous cap (OR 7.82; P=0.022) and intraplaque microvessels (OR 1.91; P=0.043). Receiver-operating characteristics analyses showed that these factors combined into a single score diagnosed symptomatic carotid plaques in patients with carotid artery stenosis with a high level of accuracy (area under the curve 0.92; 95% confidence interval 0.85–0.99 vs. asymptomatic).

Conclusions: This analysis of carotid plaque instability strongly suggested that the diagnostic scoring of carotid plaque instability improves the understanding and treatment of carotid artery disease in patients undergoing CEA.

Key Words: Carotid artery disease; Carotid endarterectomy; Unstable plaque

Ischemic strokes and transient ischemic attacks (TIA) are usually caused by unstable carotid lesions, which result in thrombus formation and occlusion of the artery. The histological characteristics of unstable carotid plaques have been described in several studies. In studies of coronary arteries, unstable plaques have been identified as ruptured, inflammatory plaques with thin fibrous caps (TFC) that were often the cause of acute coronary syndromes rather than stable angina. In the cerebral circulation, however, most ischemic strokes are caused by distal embolization originating from atherosclerotic plaques, or by an acute, instead of chronic, occlusion of a carotid artery. The interest in the morphology and functional characteristics of carotid plaques, using various imaging techniques and biochemical markers, has been growing. The progression of carotid plaques accelerated by hemorrhages developing inside the plaque has been observed in several imaging studies, and has been associated with predictors of future ischemic cerebrovascular events (CVE). In previously asymptomatic patients presenting with 50–79% carotid artery stenoses, thin or ruptured fibrous caps, hemorrhages within a plaque, and large, lipid-rich and necrotic cores (NC) have been associated with the development of adverse CVE. However, there is no comprehensive pathological measure of carotid plaque instability that can be used to predict the risk of ischemic CVE. We performed this study in patients who underwent carotid endarterectomy (CEA) to describe in detail the pathology of carotid atherosclerosis and develop a diagnostic pathological scoring of unstable plaques.

Methods

Sample Population
We analyzed data from 74 men and 8 women aged >30 years who, between May 2015 and August 2016, consecutively...
underwent CEA in the departments of neurosurgery of Nakamura Memorial Hospital, Kashiwaba Neurosurgical Hospital and Hokkaido Neurosurgical Memorial Hospital in Japan. The indications for surgery were >70% asymptomatic or symptomatic carotid artery stenosis. Patients were considered symptomatic if they had experienced an ischemic stroke or a TIA. Atherosclerotic plaques associated with symptoms were referred to as symptomatic or unstable plaques. The mean age of the 67 symptomatic patients was 73.2±6.9 years and that of the 15 asymptomatic patients was 71.3±7.6 years (NS). Patients whose excised CEA specimens were severely damaged were excluded from this analysis. This study, approved by the ETHICS COMMITTEE of each participating medical institution, complied with the Declaration of Helsinki on ethical principles for medical research involving human subjects, and all patients gave written informed consent to participate.

**Histological Examinations**

CEA was performed using standard surgical techniques with minimal handling of the specimens. The plaques were removed en bloc, fixed in 10% buffered formalin, transected transversely in 5-mm specimens, and embedded in paraffin. After hematoxylin-eosin and elastica-Masson (which stains elastin black and collagen and proteoglycans green) staining, 3–4 sections per specimen were examined. The sections with ulcerated plaques or thrombi, the most stenotic segments, or both, were retained for further analysis. The sections were examined by 2 independent observers, 1 of whom was an experienced histopathologist unaware of the clinical status and identity of the patient. The sections were probed with anti-CD31, anti-CD34 and PDGF receptor β antibodies, which recognize endothelial cells, to confirm the presence of microvessels in the plaque. The factors we chose as potential predictors of unstable carotid plaque are listed in Appendix S1.

**Definitions**

Plaque rupture: an area of fibrous cap disruption, where the overlying thrombus is in continuity with the underlying NC. 17

Plaque erosion: luminal thrombosis without communication between thrombus and NC. 17

Thrombus: laminated platelets or fibrin, with or without interspersed red and white blood cells.

Calcified nodule: a plaque with luminal thrombi containing a calcific nodule protruding into the vessel lumen through a disrupted TFC. 18

Intraplaque hemorrhage: microscopically visible blood and thrombus inside the plaque.

Intraplaque microvessels: presence of neovascularization in a carotid plaque.

Foamy macrophage within cap is a foam cell in the superficial intima.

Immature fibrillization: plaque stained light-green by elastica-Masson staining.

Necrotic core: acellular lipid pool within the intima, near the media where smooth muscle cells are scarce and proteoglycans and lipid deposition are abundant. 17

Microcalcifications: calcifications 0.5–15 μm in diameter. 19,20

Fragmented or sheet calcification: aggregation of microcalcifications ≥15 μm in diameter. 19,20

Nodular calcifications: breaks in calcified plates with fragments of calcium separated by fibrin. 19,21

**Semiquantification of Pathological Observations**

Because one of the characteristics of an unstable plaque is the presence of ≥25 macrophages per high-power (0.3-mm diameter) field (HPF), 22 and because immunohistochemical staining has revealed that most of the inflammatory cells at the site of plaque rupture are macrophages, 23 we used ≥25 inflammatory cells/HPF as a threshold for plaque instability. Furthermore, because some studies have shown that (1) matrix metalloproteinase-12 (MMP-12) promotes atherosclerosis, 24,25 (2) the main source of MMP-12 is a foamy cap macrophage, 26,27 and (3) in patients undergoing CEA, the proportion of MMP-12 macrophages is approximately 10% of all macrophages, 28,29 we used ≥3 foamy or hemosiderin-laden macrophages/HPF as a threshold for plaque instability.

Cellular infiltration (i.e., ≥25 inflammatory cells/HPF) was scored semiquantitatively as absent (=0), limited to the fibrous cap or the shoulder of the cap (=1), or extending to the fibrous cap and the shoulder of the cap (=2) in >1 cross-sectional image of the lesion. Similarly, infiltration by ≥3 foamy or hemosiderin-laden macrophages/HPF was scored semiquantitatively as absent (=0), limited to the fibrous cap or the shoulder of the cap (=1), or abundant (=2) in the fibrous cap and the shoulder of the cap in ≥1 cross-sectional image of the lesion. The distribution of calcification was scored semiquantitatively as absent (0), 1–30% (1), 91–180% (2), or >180% (3) in ≥1 cross-sectional image of the lesion. Intraplaque hemorrhages were scored semiquantitatively as ≤1 (0), 2 (1), or ≥3 (2) cross-sections of the lesion.

**Statistical Analysis**

Continuous variables are reported as mean±standard deviation (SD) and categorical variables as counts and percentages. Between-group differences were analyzed using Pearson’s chi-square or Fisher’s exact test for categorical variables and Student’s t-test or Mann-Whitney U-test for continuous variables, as appropriate. The diagnostic value of the morphological characteristics of the plaque as predictors of symptomatic adverse cerebral events was examined by single-variable logistic regression analysis. Characteristics that emerged with P values (Wald statistics) <0.05 in the single-variable analysis were entered in the multiple variable regression analysis. Characteristics not defined for non-lipid plaques, such as TFC, were assigned a score of 0.

The score was derived from the multiple regression equation, to represent the probability of a correlation between a specific morphological characteristic of the plaque and a symptomatic lesion. The results are reported as P values, odds ratios (OR) and 95% confidence intervals (CI). Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), diagnostic accuracy and optimal cutoff values were calculated from receiver-operating characteristic curves. Values with the highest Youden-index (sensitivity+specificity−1) were identified as optimal cutoff values. A P-value <0.05 was considered to indicate statistical significance. The data were analyzed with the SPSS 22.0 statistical system software (IBM Corp., Armonk, NY, USA).

**Results**

Clinical and Pathological Characteristics of Symptomatic and Asymptomatic Patients Presenting With Unstable
Table 1. Clinical and Pathological Characteristics of Symptomatic and Asymptomatic Study Groups of Patients Undergoing Carotid Endarterectomy

| Clinical characteristics | Symptomatic (n=67) | Asymptomatic (n=15) | OR (95% CI) | P value |
|--------------------------|--------------------|---------------------|-------------|---------|
| Age, years               | 73.2±6.9           | 71.3±7.6            | 1.88 (0.38–9.25) | 0.667   |
| Men                      | 61 (91)            | 13 (87)             | 1.56 (0.28–8.64) | 0.972   |
| Diabetes mellitus        | 25 (37)            | 5 (33)              | 1.19 (0.37–3.88) | 0.994   |
| Hypertension             | 53 (79)            | 12 (80)             | 0.95 (0.23–3.82) | 0.783   |
| Dyslipidemia             | 57 (85)            | 11 (73)             | 2.07 (0.55–7.81) | 0.476   |
| Chronic kidney disease   | 17 (25)            | 8 (53)              | 0.30 (0.09–0.94) | 0.069   |
| Current smoker           | 22 (33)            | 5 (33)              | 0.98 (0.30–3.21) | 0.790   |
| History                  |                    |                     |             |         |
| TIA or cerebral infarction | 15 (22)         | 3 (20)              | 1.15 (0.29–4.63) | 0.886   |
| Coronary artery disease  | 9 (13)             | 3 (20)              | 0.62 (0.15–2.64) | 0.805   |
| Peripheral artery disease| 4 (6)              | 2 (13)              | 0.41 (0.07–2.50) | 0.659   |
| Prior drug therapy       |                    |                     |             |         |
| Statin                   | 9 (13)             | 5 (33)              | 0.31 (0.09–1.12) | 0.141   |
| Aspirin                  | 6 (9)              | 4 (27)              | 0.27 (0.07–1.12) | 0.145   |
| Clopidogrel              | 3 (4)              | 2 (13)              | 0.30 (0.05–2.01) | 0.485   |
| Cilostazol               | 1 (1)              | 1 (7)               | 0.22 (0.01–3.60) | 0.804   |
| Days between onset of symptoms and operation | 52±50 | – | – |         |
| Admission laboratory data|                    |                     |             |         |
| Glycemia, mg/dL          | 136±51             | 128±55              | 2.06 (0.66–6.44) | 0.343   |
| Cholesterol, mg/dL       | 118±32             | 101±25              | 4.33 (1.34–14.0) | 0.025   |
| Low-density              | 52±17              | 54±17               | 0.18 (0.05–0.64) | 0.015   |
| High-density             | 2.4±0.8            | 2.0±0.8             | 4.99 (1.48–16.8) | 0.017   |
| LDL-C to HDL-C ratio     | 156±76             | 191±113             | 0.31 (0.09–1.12) | 0.141   |
| Pathological characteristics|                    |                     |             |         |
| Plaque area, mm²         | 41.3±19.4          | 34.9±20.4           | 3.07 (0.97–9.71) | 0.096   |
| Cross-sectional area luminal narrowing, % | 84.1±11.0 | 83.6±11.2 | 4.39 (0.54–36.1) | 0.257   |
| Plaque rupture            | 44 (66)            | 2 (13)              | 12.4 (2.58–59.9) | <0.001  |
| Plaque erosion            | 9 (13)             | 4 (27)              | 0.43 (0.11–1.63) | 0.380   |
| Calcified nodule         | 4 (6)              | 0 (0)               | 0.759      |         |
| Luminal thrombi          | 57 (85)            | 6 (40)              | 8.55 (2.49–29.3) | <0.001  |
| Thinnest fibrous cap, μm | 50.4±19.2          | 78.3±33.6           | 0.10 (0.03–0.36) | <0.001  |
| Calcification            | 62 (93)            | 14 (93)             | 0.89 (0.10–8.19) | 0.659   |
| Extent of calcification  | 1.5±0.9            | 1.7±0.9             | 0.57 (0.18–1.79) | 0.499   |
| <15μm microcalcification in the fibrous cap | 53 (79) | 7 (47) | 4.33 (1.34–14.0) | 0.025   |
| Nodular calcification    | 26 (39)            | 2 (13)              | 4.12 (0.86–19.8) | 0.114   |
| Fragmented or sheet calcification | 62 (93) | 14 (93) | 0.89 (0.10–8.19) | 0.659   |
| Maximum thickness of calcification, μm | 679±552 | 799±746 | 0.35 (0.09–1.35) | 0.201   |
| Infraplaque hemorrhage   | 63 (94)            | 12 (80)             | 3.94 (1.04–19.5) | 0.213   |
| Infraplaque hemorrhage, sections | 1.7±0.6 | 1.2±0.9 | 4.33 (1.34–14.0) | 0.025   |
| Infraplaque microvessels, /mm² | 2.5±1.9 | 1.6±1.1 | 5.94 (1.24–28.4) | 0.031   |
| Inflammatory cells within cap | 1.8±0.5 | 1.3±0.9 | 3.80 (1.11–13.0) | 0.064   |
| Foamy macrophages within cap | 1.6±0.6 | 1.1±0.8 | 3.07 (0.97–9.71) | 0.096   |
| Hemosiderin-laden macrophages within cap | 1.5±0.6 | 0.9±0.7 | 4.38 (1.13–16.9) | 0.048   |
| Immature fibrillization  | 66 (99)            | 13 (87)             | 10.2 (0.86–120) | 0.148   |
| Myxoid change in media   | 61 (91)            | 13 (87)             | 1.56 (0.28–8.64) | 0.972   |
| Necrotic core            | 63 (94)            | 13 (87)             | 2.42 (0.40–14.6) | 0.659   |
| >120° NC                 | 58 (87)            | 9 (60)              | 4.30 (1.23–15.0) | 0.042   |
| Eccentric shape          | 58 (87)            | 13 (87)             | 0.99 (0.19–5.14) | 0.683   |

Values are mean±SD or number (%) of observations. CI, confidence interval; OR, odds ratio; TIA, transient ischemic attack.
Plaques.
The 82 patients enrolled in the study comprised 67 symptomatic and 15 asymptomatic patients. Plaques were resected from all patients and examined histopathologically. The clinical characteristics of the 67 symptomatic and 15 asymptomatic patients and the pathological characteristics of the lesions analyzed in each group are compared in Table 1. By single-variable analysis, several pathological characteristics were significantly associated with symptomatic manifestations: plaque rupture, minimum TFC overlying a NC, microcalcifications in the fibrous cap, intraplaque hemorrhage, intraplaque microvessels, hemosiderin-laden macrophages within the cap and an extensive NC. Figure 1 illustrates the main characteristics of plaque instability. Table 2 shows the prevalence of plaque rupture, erosion and a calcified nodule among patients presenting with stroke, vs. TIA, vs. no symptoms. The prevalence of plaque rupture was highest (73%) in patients presenting with stroke, significantly higher than in patients presenting with TIA (33%) or no symptoms (13%). In contrast, the prevalence of a calcified nodule was significantly higher in patients presenting with a TIA (25%) than in patients presenting with stroke (2%) or no symptom (0%).

By multiple-variable logistic regression analysis, the presence of plaque rupture, minimum TFC, the presence of microcalcifications in the fibrous cap and intraplaque microvessels were independent correlates of symptomatic plaques (Table 3).

**Diagnostic Performance of Predictors of Plaque Instability**
We used receiver-operating characteristic statistics to examine the diagnostic performance of these indices of plaque instability (Figure 2, Table 4). Among all correlates of symptomatic cerebral ischemic event, minimum TFC had the highest diagnostic performance (area under the curve [AUC] 0.78, optimal cutoff value 70.2 μm, sensitivity 90%, specificity 60%, PPV 91%, NPV 56%, diagnostic accuracy 84%). However, combining the 4 independent correlates into a single score, using the equation derived from the multiple-variable logistic regression model (Logit(Score)=0.179+2.277* (insert 1 if plaque rupture present; otherwise 0)−0.355* (insert minimum TFC in mul-

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**Table 2. Rates of Plaque Rupture, Erosion and a Calcified Nodule Among the Subgroups of Patients Undergoing Carotid Endarterectomy**

| No. of plaques (%) | Major ipsilateral stroke (n=55) | TIA (n=12) | Asymptomatic (n=15) | Stroke vs. TIA P value | TIA vs. asymptomatic P value |
|--------------------|-------------------------------|------------|---------------------|-----------------------|-----------------------------|
| Plaque rupture     | 40 (73)                       | 4 (33)     | 2 (13)              | 0.023                 | <0.001                      |
| Plaque erosion     | 7 (13)                        | 2 (17)     | 4 (27)              | 0.917                 | 0.360                       | 0.535                        |
| Calcified nodule   | 1 (2)                         | 3 (25)     | 0                   | 0.002                 | 0.599                       | 0.040                        |

Values are mean±SD or numbers (%) of observations. TIA, transient ischemic attack.
Pathological Quantification of Carotid Instability

These 4 independent correlates in the diagnosis of pathologically unstable plaques in patients with carotid artery stenoses was very high. To the best of our knowledge, this study is the first to compare the pathological indices of unstable plaque by multiple-variable analysis, and then quantify the instability of carotid artery plaques in patients undergoing CEA.

Comparison With Previous Studies

Our pathological observations are concordant with those reported previously. In this study, the proportion of symptomatic carotid plaques associated with plaque rupture was higher than that of asymptomatic plaques, and the prevalence of plaque rupture in patients presenting with stroke was higher than that of patients with presenting with a TIA or of asymptomatic patients. Previous studies found that symptomatic carotid artery disease is more

Discussion

The main observations made in this study were (1) that although several pathological characteristics were associated with symptomatic plaques, plaque rupture, minimum TFC, microcalcifications in the fibrous cap and intraplaque microvessels were independent correlates, and (2) when combined into a single score, the performance of

Table 3. Pathological Lesion Characteristics Independently Associated With Symptomatic Ischemic Cerebrovascular Events

| Variable                                      | OR (95% CI)   | P value |
|-----------------------------------------------|---------------|---------|
| Presence of plaque rupture                    | 9.75 (1.62–58.6) | 0.013   |
| Minimum thin fibrous cap (10μm)              | 0.70 (0.51–0.96) | 0.025   |
| Presence of microcalcification in the fibrous cap | 7.82 (1.35–45.4) | 0.022   |
| Intraplaque microvessels (/mm²)               | 1.91 (1.02–3.57) | 0.043   |

CI, confidence interval; OR, odds ratio.

Figure 2. Pathological characteristics that identify symptomatic plaques individually as well as when combined in a score. Receiver-operating characteristic curves for (A) minimum fibrous cap thickness, (B) intraplaque microvessels to identify symptomatic plaque individually and (C) combined into a score. The score, which includes the presence of plaque rupture and microcalcifications in the fibrous cap, was calculated as described in the text.

Table 4. Outcome of Receiver-Operator Characteristics Analysis of Pathological Variables of Unstable Plaque

| Variable                                      | AUC (95% CI) | Cutoff | Sensitivity | Specificity | Predictive value Positive | Predictive value Negative | Diagnostic accuracy |
|-----------------------------------------------|--------------|--------|-------------|-------------|---------------------------|--------------------------|---------------------|
| Plaque rupture                                | Present      |        | 65.7        | 86.7        | 95.7                      | 36.1                     | 69.5                |
| Minimum thin fibrous cap (10μm)              | 0.78 (0.62–0.94) | 70.2μm | 89.6        | 60.0        | 90.9                      | 56.3                     | 84.1                |
| Fibrous cap microcalcifications               | Present      |        | 79.1        | 53.3        | 88.3                      | 36.4                     | 74.4                |
| Intraplaque microvessels (2.20/mm²)          | Present      |        | 47.8        | 86.7        | 94.1                      | 27.1                     | 54.9                |
| Score                                         | 0.92 (0.85–0.99) | 0.814  | 89.6        | 86.7        | 96.8                      | 65.0                     | 89.0                |

AUC, area under the curve; CI, confidence interval.

tiples of 10μm)+2.057* (insert 1 if microcalcification in the fibrous cap present; otherwise 0)+0.646* (insert intraplaque microvessels/mm²), increased the diagnostic performance to an AUC of 0.92 (95% CI 0.85–0.99; optimal cutoff value 0.814; sensitivity 90%; specificity 87%; PPV 97%; NPV 65%; diagnostic accuracy 89%). The application of this score to grade the instability of 2 separate lesions is illustrated in Figure 3.
Measuring Plaque Vulnerability

The individual and combined power of these plaque characteristics to measure plaque instability is incompletely understood. In this study, we combined these previously described individual characteristics of plaque instability into a single score. From our comprehensive pathological study and multiple-variable analysis, we identified the factors correlating with plaque instability. Among all the pathological indices, plaque rupture, minimum TFC, presence of microcalcifications and intraplaque microvessels were the only independent correlates of symptomatic plaque in this sample of patients with carotid artery stenosis.

Among all the pathological factors we examined, minimum TFC was the most accurate to quantify plaque instability. Furthermore, when combined into a score, these risk factors were highly accurate to quantify plaque instability and identify unstable plaques in patients with carotid artery stenoses. Although plaque rupture has been described as one of the major features of unstable plaque in histological studies,1,4,30 plaque rupture is not the only characteristic of an unstable atherosclerotic lesion. A representative example of unstable plaque without plaque rupture is shown in Figure S1.

Insignificant Variables by Multiple-Variable Analysis

Intraplaque hemorrhages, hemosiderin-laden macrophages and extensive NC, although related to unstable plaques by single-variable analysis, were no longer statistically significant by multiple-variable analysis. Although plaques removed ≤60 days after the most recent event are more unstable after a stroke than after a TIA, the instability persists after a TIA, and plaques removed >180 days after a recent event are less unstable after a stroke than after a TIA.40 Because >80% of symptomatic patients presented with major ipsilateral stroke in this study, and the average timing of CEA was 52±50 days after the onset of symptoms, intraplaque hemorrhages, inflammatory cells and...
extensive NC might no longer be significant factors because of the long interval between the onset of symptoms and CEA. In contrast, because the statistical significance between plaque instability and intraplaque microvessels is not related to the time since onset of stroke, it might remain an independent predictor of unstable plaque by multiple-variable analysis. Furthermore, intraplaque hemorrhages have been correlated with plaque rupture in symptomatic carotid artery stenosis. It is noteworthy that plaque rupture and intraplaque hemorrhages were correlated in this study \((r=0.362; \, r^2=0.131; \, P<0.001)\). Therefore, it might be a confounding factor in the multiple-variable analysis.

### Higher Prevalence of Calcified Nodules in Cases of TIA Than of Stroke

Calcified nodules might be associated with more stable atherosclerotic lesions than plaque rupture in symptomatic patients. Several matrix metalloproteinases produced by activated macrophages digest fibrillar collagen, thus reducing the plaque’s mechanical stability. Our comparisons of plaques with rupture and with calcified nodules showed significantly less severe infiltration of inflammatory cells \((1.9\pm0.4 \, \text{vs.} \, 1.3\pm0.9, \, P=0.015)\) and foamy macrophages \((1.7\pm0.5 \, \text{vs.} \, 1.0\pm0.6, \, P=0.037)\) in those with a calcified nodule \((\text{Table S1})\). Therefore, calcified nodules might be more frequently observed in patients with TIA, compared with those with stroke. As for coronary artery disease, Jia et al showed in an optical coherence tomography (OCT) analysis that the prevalence of a calcified nodule was higher in patients with non-ST-segment elevation myocardial infarction than in those with ST-segment elevation myocardial infarction \((15\% \, \text{vs.} \, 0\%, \, P=0.004)\).

### Clinical Perspectives

First, applying the indices of plaque instability derived in this study using techniques such as 3-dimensional or contrast-enhanced ultrasonography, magnetic resonance imaging (MRI), positron-emission tomography (PET), which can be used to measure these indices, may supplement conventional plaque imaging and improve our prediction of adverse CVE. High-resolution MRI is the best means of imaging carotid plaques and can effectively visualize plaque rupture, intraplaque hemorrhages and TFC: 7-tesla MRI might enable further detailed visualization of the structure of carotid plaques. Contrast-enhanced ultrasound allows quantification of intraplaque neovascularization as a feature of instability of carotid plaque, and has been closely correlated with histopathological observations. The 1-mm resolution of OCT is capable of revealing microcalcifications as the accumulation of small, punctate high-density signals within the fibrous cap. These microcalcifications in the atherosclerotic plaque can also be identified with sodium fluoride PET or sodium fluoride PET/CT. However, further technological developments are needed, including of spatial resolution, to detect unstable plaques more precisely. Second, we can verify the consistency between a preoperative diagnosis made with these imaging techniques and the plaque instability quantified by our novel pathological score. In our study, the plaque instability diagnosed by preoperative examinations including ultrasonography, MRI and CT was consistent with the pathological analysis of plaque instability in 72 of 82 patients \((88\%)\). Third, the plaque instability score might be a predictor of CVE. During the 6-month follow-up of, CVE occurred in 2 of 55 patients presenting with major ipsilateral stroke. The mean instability score was 97.8±2.3\% in patients with CVE compared with 90.2±17.1\% in those without CVE \((P=0.329, \text{Table S2})\). Fourth, carotid artery plaques with certain morphological features might be better targets for carotid artery stenting (CAS). Previous studies have shown that some morphological plaque features are associated with cerebrovascular complications pertaining to CAS. Tsutsumi et al showed that severe calcification of the carotid artery might be a cause of incomplete stent expansion despite aggressive dilatation. Bicknell and Cheshire reported that lipid-rich plaque was an independent predictor of distal embolization during the procedure. Furthermore, Kologidj et al described extensive inflammation as an important risk factor of restenosis after CAS. In our study, age and the thickness of calcification were positively correlated \((\text{Figure S2})\). Infiltration of inflammatory cells, as well as foamy and hemosiderin-laden macrophages, was less observed in patients with NC <120\% than in those with NC ≥120\% \((\text{Table S3})\). These observations suggest that non-elderly patients without severe calcification or extensive NC in plaque would be likely to preferentially benefit from CAS. Fifth, several studies have shown that the mechanisms of carotid plaque instability are similar to those of plaque found in the coronary circulation. Although, unlike the myocardial circulation, the carotid vascular bed is exposed to a high blood flow. Therefore, this study should provide useful information, including the identification of unstable plaques, about patients presenting with coronary artery disease.

### Study Limitations

The sample size of this retrospective, observational study, conducted at 3 medical centers, was small. Our results need to be confirmed by a study including a larger number of patients. Second, in our population, the median time between the last adverse clinical event and CEA was 52±50 days, whereas the current professional practice guidelines recommend that patients presenting with symptomatic carotid stenosis undergo CEA within 14 days of the last event. Therefore, in further histopathological analyses, factors such as infiltration by inflammatory cells could turn out to be strong predictors of unstable plaques in patients undergoing CEA. Third, instead of being functional, this study was mainly a quantitative, morphological analysis of excised specimens of carotid plaques, which did not include measurements of enzymatic concentrations derived from foamy macrophages, such as that of matrix metalloproteinase. Fourth, although observations that are not derived from atherosclerotic plaques (e.g., wall shear stress) have also been found to predict plaque instability, this study was limited to pathological indices to quantify plaque instability.

### Conclusions

This pathological analysis of carotid artery plaques suggested that our diagnostic scoring may facilitate understanding of plaque instability. Pathological quantification by this scoring simplified the assessment of carotid artery plaques, instead of requiring the wide variety of pathological characteristics reflecting plaque instability.

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Supplementary File 1

Appendix S1. Potential Characteristics of Unstable Plaque

Figure S1. Representative example of unstable plaque without plaque rupture.

Figure S2. Positive correlation between age and maximum thickness of calcification (r=0.378, r^2 =0.143, P<0.001) in patients undergoing carotid endarterectomy.

Table S1. Comparison of plaque rupture and calcified nodule groups of patients undergoing carotid endarterectomy

Table S2. Comparison of CVE and no CVE groups of patients undergoing carotid endarterectomy

Table S3. Comparisons of NC ≤120° and NC >120° groups of patients undergoing carotid endarterectomy

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