4-Hydroxyl-2-Nonenal Localized Expression Pattern in Retrieved Clots is Associated with Large Artery Atherosclerosis in Stroke Patients

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Running title: 4-HNE Localized Expression in Ischemic Stroke Clot

Keywords: 4HNE, large artery atherosclerosis, acute ischemic stroke, clot, immunohistochemistry

Abbreviations

BA, basilar artery; CE, cardioembolic; CML, carboxymethyl lysine; HE, hematoxylin & eosin; HIF-1α, hypoxia inducible factor-1α; NIHSS, National Institutes of Health Stroke Scores; ICA, internal carotid artery; LAA, large artery atherosclerosis; MCA, middle cerebral artery; MMP9, matrix metalloproteinase 9; NLRP3, NOD-like receptor family pyrin domain containing 3; ox-LDL, oxidized low-density lipoprotein cholesterol; RBCs, red blood cells; rt-PA, recombinant tissue-type plasminogen activator; TICI score, thrombolysis in cerebral infarction score; WBCs, white blood cells; 4-HNE, 4-hydroxyl-2-nonenal; 8-OHdG, 8-hydroxy-2'-deoxyguanosine

Abstract

Objectives: The relationship between stroke etiology and clot pathology remains controversial.

Materials and Methods: We performed histological analysis of clots retrieved from 52 acute ischemic stroke patients using hematoxylin & eosin staining and immunohistochemistry (CD42b and oxidative/ hypoxic stress markers). The correlations between clot composition and the stroke etiological group (i.e., cardioembolic, cryptogenic, or large artery atherosclerosis) were assessed.

Results: Of the 52 clots analyzed, there were no significant differences in histopathologic composition (e.g., white blood cells, red blood cells, fibrin, and platelets) between the 3 etiological groups ($P = .92$). By contrast, all large artery atherosclerosis clots showed a localized pattern with the oxidative stress marker 4-hydroxyl-2-nonenal ($P < .01$). From all 52 clots, 4-hydroxyl-2-nonenal expression patterns were localized in 28.8% of clots, diffuse in 57.7% of clots, and no signal in 13.5% of clots.

Conclusions: A localized pattern of 4-hydroxyl-2-nonenal staining may be a novel and effective marker for large artery atherosclerosis (sensitivity 100%, specificity 82%).

Introduction

Embolic clot material for histological analysis can be obtained by mechanical thrombectomy. Although the properties of clots obtained from the coronary artery were reported to be associated with atherosclerosis lesions,[1,2,3] clots from an acute ischemic stroke may originate from various sources including a mural cardiac clot, intra or extracranial atherosclerosis lesions, venous 'paradoxical' sites,[4] or an unknown source.[5] Because the risk of stroke recurrence is as high as 26% over 5 years,[6] histological clot analysis is important to improve diagnostic accuracy and to reduce the recurrence rate.

Previous studies assessing histological clot composition have largely focused on basic clot morphology using hematoxylin & eosin (HE) staining for white blood cells (WBCs), red blood cells (RBCs), and fibrin/platelet.[7,8,9,10,11] Immunohistochemical analyses for von-Willebrand factor[12] and inflammation markers (e.g., CD3, CD20, and CD68)[9] have also been reported in human clots. However, the relationship between stroke etiology and clot pathology remains controversial. Furthermore, the expression of oxidative stress markers, which largely correlates with the pathogenesis of ischemic stroke, has not been examined in clots.

In the present study, we performed a histological composition analysis on clots retrieved by mechanical thrombectomy in acute stroke patients.

Materials and Methods

Patient Samples

Between June 2019 and April 2020, a total of 82 patients received thrombectomy procedures for acute ischemic stroke at Okayama City Hospital, Ohnishi Neurological Center or Tsuyama Chuo Hospital. Of these patients, we recruited 52

patients from who analyzable-sized clots were retrieved. All subjects were ethnic Japanese >20 years of age. Approval was obtained from the medical ethics committee of each hospital. Written informed consent was obtained from the patients or their nearest relatives. Clinical data collected from the patients included demographic features, cerebrovascular risk factors (diabetes, hypertension, hyperlipidemia), National Institutes of Health Stroke Scores at baseline, use of tissue-type plasminogen activator, the device used, and the thrombolysis in cerebral infarction score. A classification of ischemic stroke was determined using the modified TOAST stroke subtype algorithm.[13]

Histochemistry

The retrieved clot material was immediately fixed overnight with 4% paraformaldehyde, followed by paraffin embedding and microtome sectioning (4-μm thick). All clots were stained with HE.

For immunohistochemical analysis, the clots were stained with the following primary antibodies: rabbit anti-CD42b (1:100; ab183345; Abcam), mouse anti-8-hydroxy-2'-deoxyguanosine (1:100; N45.1; JaICA), mouse anti-4-hydroxyl-2-nonenal (4-HNE; 1:40; HNEJ-2; JaICA), mouse anti-3-nitrotyrosine (1:2000; ab61392; Abcam), rabbit anti-carboxymethyl lysine (1:1000; ab27684; Abcam), mouse anti-hypoxia inducible factor-1α (1:200; AF1935; R&D systems), goat anti-NOD-like receptor family pyrin domain containing 3 (1:100; ab4207; Abcam), and rabbit anti-matrix metalloproteinase 9 (MMP9; 1:100; ab38898; Abcam). Each primary antibody was omitted for negative control staining. The sections were incubated with each primary antibody at 4°C overnight, followed by an appropriate biotin-labeled secondary antibody (1:500; Vector Laboratories, Burlingame, CA, USA). The sections were then incubated with the avidin-

biotin-peroxidase complex (Vectastain ABC Kit; Vector Laboratories) and the signal was visualized with diaminobenzidine tetrahydrochloride.

Detection and Analyses

The histochemistry sections were imaged at ×100 magnification using a digital microscope camera (Olympus BX-51; Olympus Optical Co., Tokyo, Japan). For HE- and CD42b-staining, the areas with WBCs, RBCs, fibrin, and platelets were evaluated using color-based segmentation (ImageJ software; National Institutes of Health, Bethesda, MD, USA).

Statistics

All data are expressed as mean ± standard deviation. Variables were analyzed using the m × n χ^2 test and the Fisher's test for categorical variables, or the Kruskal–Wallis test for continuous variables. All statistical analyses were performed using statistical software (ystat 2018; IgakuTosho Shuppan). $P < .05$ was considered statistically significant.

Results

Clinical Characteristics of the Patients

A total of 52 clots were retrieved from 52 patients. Stroke etiologies were cardioembolic (CE) in 61.5% of clots, cryptogenic in 25.0% of clots, and large artery atherosclerosis (LAA) in 13.5% of clots. The median age was 76.8 ± 12.3 years (range, 45–94 years). The median baseline National Institutes of Health Stroke Score was 18.6 ± 9.7 points. Intravenous tissue-type plasminogen activator was used in 46.2% of patients. The location of the vessel occlusion was the internal carotid artery in 38.5% of

patients, the middle cerebral artery in 55.8% of patients, and the basilar artery in 5.8% of patients. A stent retriever was used in 53.8% of patients, while a suction catheter was used for collecting clots in 46.2% of patients. The thrombolysis in cerebral infarction score 2b/3 rate was 92.3%. There were no significant differences in the clinical characteristics between the 3 groups (Table 1).

Histological Analysis

The quantified histological composition of the 52 clots is shown in Figure 1 and Table 2. The average WBCs, RBCs, fibrin, and platelet contents were 3.5%, 41.1%, 49.1%, and 6.4%, respectively. There were no significant differences in the histopathologic composition between the 3 groups ($P = .92$). However, CE patients (52.1%) showed a trend toward a higher average fibrin composition than cryptogenic (48.6%) and LAA (42.2%) patients. Furthermore, LAA patients (4.6%) showed a trend toward a higher average WBC and RBC composition than for CE (3.3%) and cryptogenic (3.3%) patients.

Even when the clots (14 cases) were divided into the proximal, median, and distal portions, there were no significant differences in the average clot compositions between the different portions (Fig. 2; $P = .68$); the percentage of WBCs, RBCs, and fibrin/platelets was 3.0%, 43.2%, and 53.8%, respectively, for the proximal portion, 2.5%, 40.5%, and 57.0%, respectively, for the median portion, and 2.4%, 43.5%, and 54.1%, respectively, for the distal portion.

Immunohistochemical Analysis of Oxidative/Hypoxic Stress Markers

The proportion of clots that expressed 8-hydroxy-2’-deoxyguanosine, 4-HNE, NOD-

like receptor family pyrin domain containing 3, or MMP9 were 11.5%, 86.5%, 25.0%, and 53.8%, respectively. MMP9 expression was significantly higher in CE patients and significantly lower in cryptogenic patients ($P < .05$). However, no clots were positive for 3-nitrotyrosine, carboxymethyl lysine, or hypoxia inducible factor-1α (Fig. 3). When 4-HNE expression patterns from all clots were categorized into a localized pattern (28.8%), a diffuse pattern (57.7%), or no signal (13.5%), all LAA clots showed the localized pattern (Fig. 4), The rate of the localized pattern was significantly higher in the LAA group compared with the other 2 groups ($P < .01$).

Discussion

The present study provided a histological and immunohistochemical analysis of clots retrieved from 52 patients with acute ischemic stroke. There were no correlations between clot composition and stroke pathogenesis using histological analysis. However, immunohistochemical analysis of oxidative stress markers showed localized 4-HNE expression in all LAA patients. Thus, 4-HNE may be a novel and effective marker for LAA (sensitivity 100%, specificity 82%).

The widespread use of mechanical thrombectomy in acute ischemic stroke allowed us to analyze the correlations between clot histological composition (e.g., WBCs, RBCs, fibrin, and platelets) and stroke pathogenesis. Classically, clots in the CE were classified as ‘red clots’ that contained mixtures of RBCs and fibrin and that originated from regions of slow blood flow such as the left atrial appendage. Furthermore, clots in the LAA were regarded as ‘white clots‘ that contained platelets and that originated from regions of rapid blood flow.[14] Although CE clots were reported to have a higher RBC composition,[8] there are contrasting findings of a higher RBC composition in LAA

compared with CE.[7,9,10] Furthermore, in a study of 105 cases, LAA had the highest platelet composition.[11] Thus, the relationship between stroke pathogenesis and clot composition remains controversial. In the present study, LAA clots showed a trend toward having a higher RBC composition (48.2%; Table 2). Nevertheless, there were no significant differences in clot composition between the different stroke types (Fig. 1), suggesting that it is difficult to predict stroke etiology using classical clot histological composition analyses.

Many studies have reported a relationship between oxidative stress and thrombosis. For example, oxidative stress and inflammation correlate with the lysis resistance of fibrin in the clot.[15] 4-HNE is a marker of lipid peroxidation via free radicals[16] and contributes to angiogenesis and platelet aggregation in atherosclerotic lesions in the presence of oxidized low-density lipoprotein cholesterol.[17,18] Blood levels of 4-HNE were also reported to increase in acute ischemic stroke patients.[19] To our knowledge, the present study provides the first analysis of oxidative stress immunohistochemistry in clots of stroke patients (Figs. 3 and 4). Specifically, we found a localized expression of 4-HNE in LAA clots (Fig. 4), which formed the core structure of the atherothrombosis. Because the fibrous cap and lipid core frequently express 4-HNE in atherosclerotic plaques,[20,21] the retrieved LAA clot still contained the core structure.

In summary, we found that localized expression of 4-HNE in the clot was associated with an LAA etiology. Thus, stroke patients with the 4-HNE localized pattern clot may respond better to free radical scavenger therapy, which suppresses oxidative stress and stabilizes atherosclerosis.[22] Further studies are required to validate this hypothesis.[23,24]

Conflict of interest

The authors declare that they have no conflicts of interest.

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Figure Legends

Figure 1. *Clot composition of the patient cohort. (a) A gross photo of a retrieved clot. (b) A hematoxylin & eosin-stained section of a retrieved clot. (c–e) Histopathological analysis of a retrieved clot using ImageJ software. Expression of (c) white blood cells (WBCs), (d) red blood cells (RBC), and (e) fibrin. (f) Immunohistochemical staining for platelets (cluster of differentiation 42b [CD42b]; brown). (g) Graphical representation of the clot composition of each patient. Scale bars in (b–f) = 100 μm.*

Figure 2. *Average clot composition for each clot region. (a, b) When the distal and proximal side (arrow) of the retrieved clot was identified, the clots were divided into 3 parts (proximal, median, and distal) and the histological composition was assessed (n = 14). There were no significant differences in the average clot composition between the different regions. Scale bar = 10 mm.*

Figure 3. *Immunohistochemical analysis of oxidative/hypoxic stress markers. Immunohistochemical staining for (a) 8-hydroxy-2'-deoxyguanosine (8-OHdG), (b) 4-hydroxyl-2-nonenal (4-HNE), (c) 3-nitrotyrosine (3-NT), (d) carboxymethyl lysine (CML), (e) hypoxia inducible factor-1α (HIF-1α), (f) NOD-like receptor family pyrin domain containing (NLRP3), and (g) matrix metalloproteinase 9 (MMP9). Some clots expressed 8-OHdG, 4-HNE, NLRP3, and MMP9. MMP9 expression was significantly higher in clots from cardioembolic (CE) patients and significantly lower in clots from cryptogenic patients (*P < .05). All scale bars = 100 μm.*

Figure 4. *Expression pattern analysis of 4-hydroxyl-2-nonenal (4-HNE). The top panels (a–c) show the expression pattern of 4-HNE categorized into (a) a localized pattern, (b) a diffuse pattern, and (c) no signal. The middle panels (d–f) show a schematic of 4-HNE expression (red point; 4-HNE positive cells). The lower panels (g) show that all clots with a large artery atherosclerosis (LAA) etiology exhibited a localized pattern (significantly higher incidence than that for the other 2 groups,**P < .01). Scale bar = 100 μm.*

Figure 1. *Clot composition of the patient cohort.*

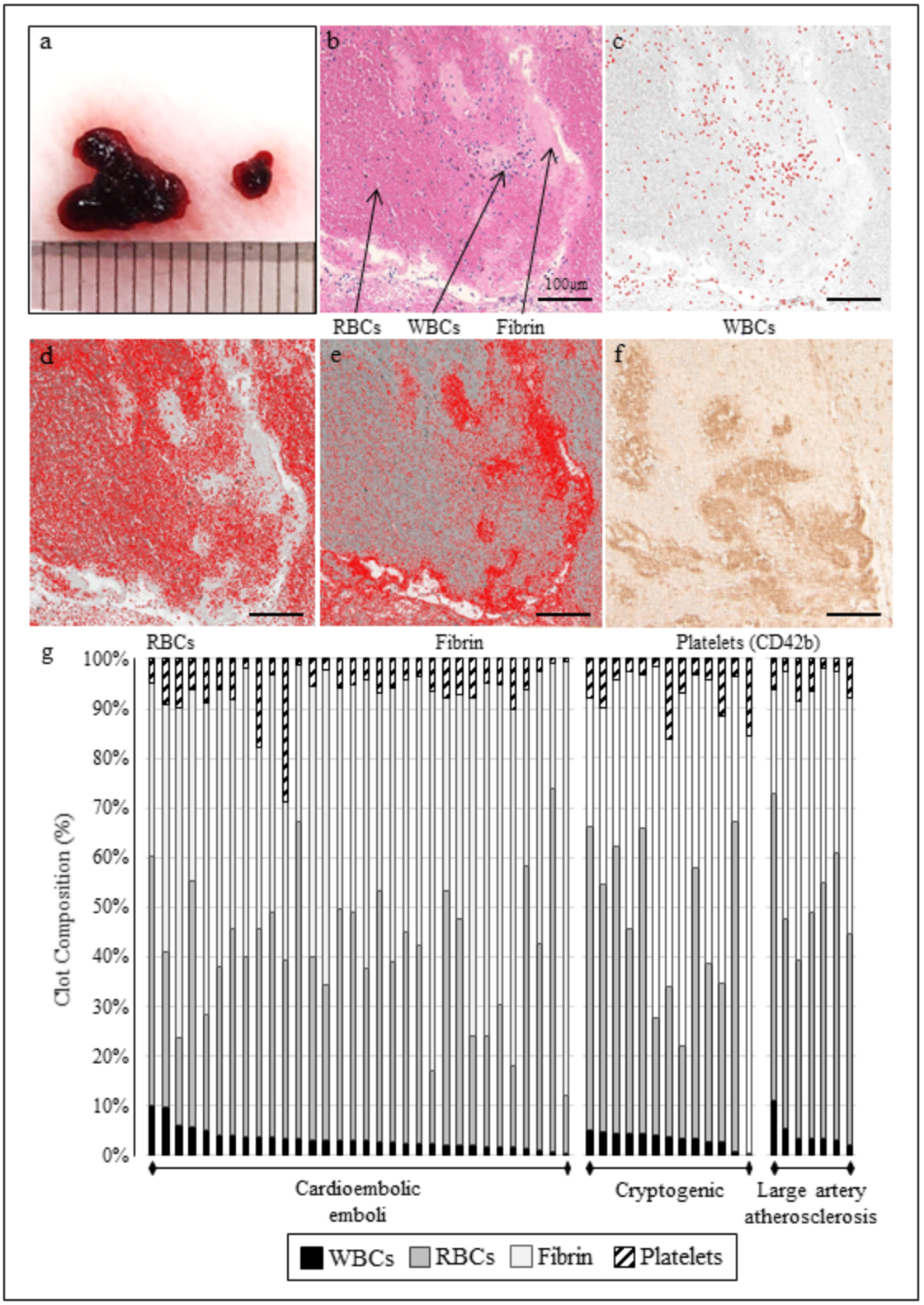
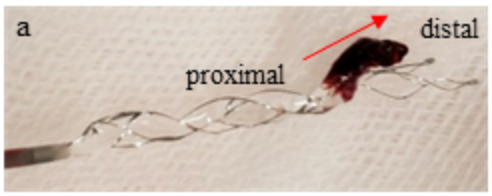
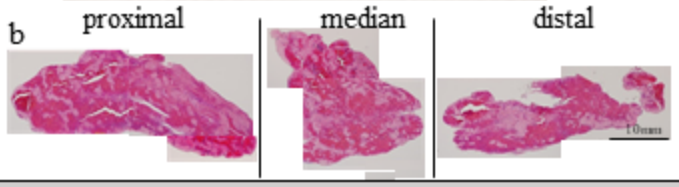


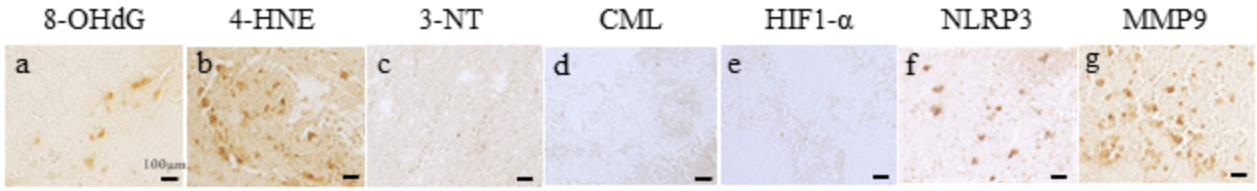
Figure 2. *Average clot composition for each clot region.*





n = 14	WBCs	RBCs	Fibrin/platelets	*p*
proximal	3.0%	43.2%	53.8%	
median	2.5%	40.5%	57.0%	0.68
distal	2.4%	43.5%	54.1%	

Figure 3. *Immunohistochemical analysis of oxidative/hypoxic stress markers.*



	8-OHdG	4-HNE	3-NT	CML	HIF1-α	NLRP3	MMP9
All cases	6/52 (11.5%)	45/52 (86.5%)	0/52 (0%)	0/52 (0%)	0/52 (0%)	13/52 (25.0%)	28/52 (53.8%)
Cardioembolic emboli	4/32 (12.5%)	29/32 (90.6%)	0/32 (0%)	0/32 (0%)	0/32 (0%)	9/32 (28.1%)	22/32 (68.8%)
Cryptogenic	0/13 (0%)	9/13 (69.2%)	0/13 (0%)	0/13 (0%)	0/13 (0%)	1/13 (7.7%)	2/13 (15.4%)
Large artery atherosclerosis	2/7 (28.6%)	7/7 (100%)	0/7 (0%)	0/7 (0%)	0/7 (0%)	3/7 (42.9%)	4/7 (57.1%)
p	0.49	0.29	-	-	-	0.42	<0.05

Figure 4. *Expression pattern analysis of 4-HNE.*

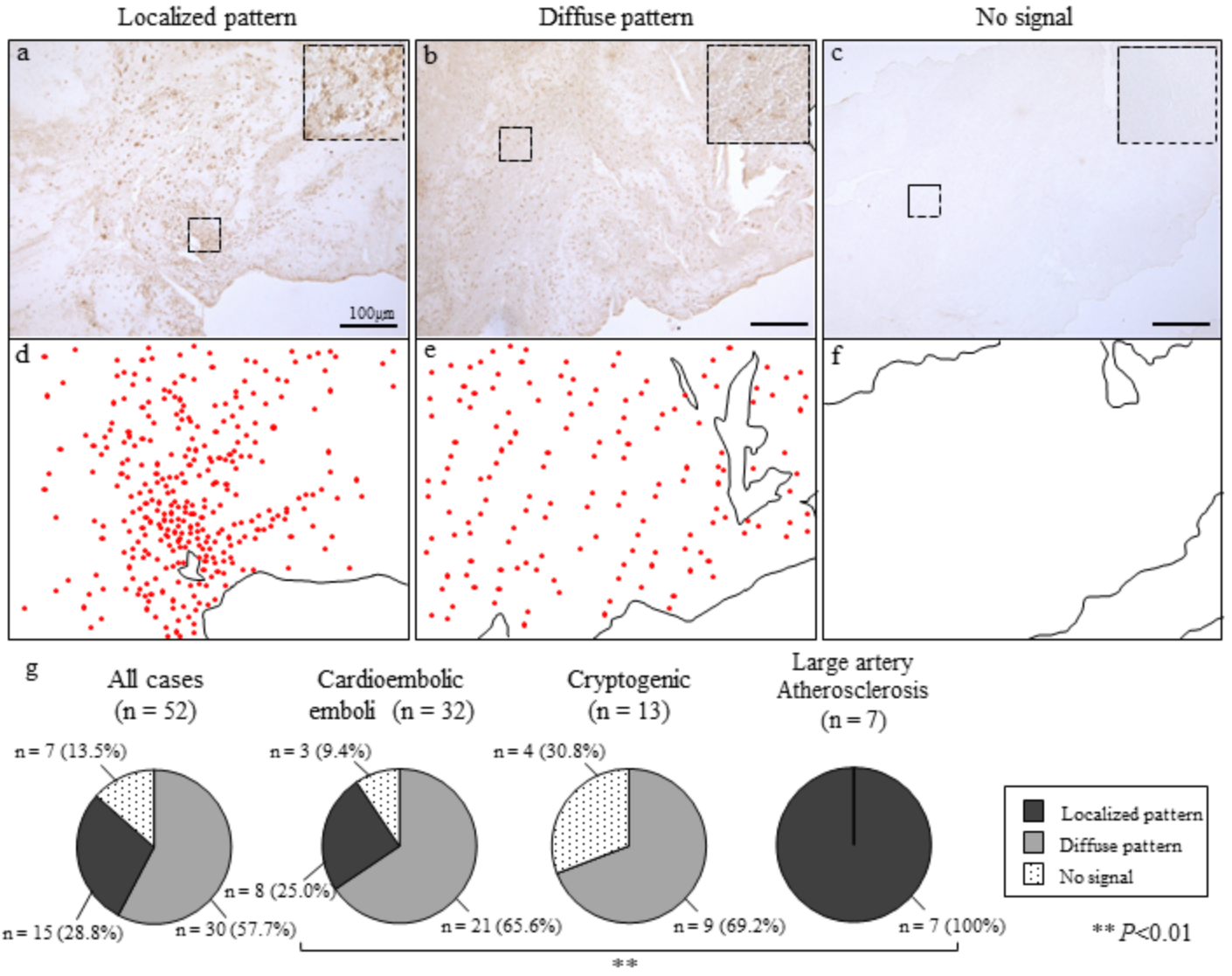


Table 1. *Clinical characteristics*

Clinical characteristic	All cases n = 52	Cardioembolic emboli n = 32 (61.5%)	Cryptogenic n = 13 (25%)	Large artery atherosclerosis n = 7 (13.5%)	*p*
Age (years)	76.8 ± 12.3	78.9 ± 11.0	75.2 ±12.4	70.0 ±15.0	0.39
Male	27 (51.9%)	17/32 (53.1%)	4/13 (30.8%)	6/7 (85.7%)	0.17
Diabetes	12 (23.1%)	9/32 (28.1%)	2/13 (15.4%)	1/7 (14.3%)	0.95
Hypertension	28 (53.8%)	19/32 (59.4%)	5/13 (38.5%)	4/7 (57.1%)	0.62
Hyperlipidemia	17 (32.7%)	11/32 (34.4%)	5/13 (38.5%)	1/7 (14.3%)	0.81
Platelet count ($\times 10^4/\mu L$)	20.6 ± 7.0	20.1 ± 7.0	18.9 ± 5.1	25.7 ± 8.5	0.25
Baseline NIHSS	18.6 ± 9.7	20.6 ± 9.8	16.5 ± 9.8	15.0 ± 6.0	0.28
Intravenous rt-PA	24 (46.2%)	12/32 (37.5%)	8/13 (61.5%)	4/7 (57.1%)	0.5
Location of occlusion					
- ICA	20 (38.5%)	11/32 (34.3%)	3/13 (23.1%)	6/7 (85.7%)	0.06
- MCA	29 (55.8%)	19/32 (59.4%)	9/13 (69.2%)	1/7 (14.3%)	0.14
- Basilar artery	3 (5.8%)	2/32 (6.3%)	1/13 (7.7%)	0/7 (0%)	0.91
Device used					
- Stent retriever	28 (53.8%)	19/32 (59.4%)	5/13 (38.5%)	4/7 (57.1%)	0.91
- Suction catheter	24 (46.2%)	13/32 (40.6%)	8/13 (61.5%)	3/7 (42.9%)	
TICI ≧ 2b	48 (92.3%)	30/32 (93.8%)	11/13 (84.6%)	7/7 (100.0%)	0.87

Table 2. *Histopathologic composition of the clots related to stroke pathogenesis*

	WBCs	RBCs	Fibrin	Platelets	*p*
All cases (n = 52)	3.5%	41.1%	49.1%	6.4%	
Cardioembolic emboli	3.3%	38.1%	**52.1%**	6.6%	0.92
Cryptogenic	3.3%	41.0%	48.6%	**7.0%**	
Large artery atherosclerosis	**4.6%**	**48.2%**	42.2%	5.1%	