ORIGINAL ARTICLE

Atherosclerosis-Related Circulating MicroRNAs as a Predictor of Stroke Recurrence

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Abstract MicroRNAs (miRNAs) are short sequenced non-coding RNAs that posttranscriptionally regulate gene expression. We investigated circulating miRNA expression levels in acute stroke patients and its relationship with future vascular event. We included acute ischemic stroke patients who admitted to a university hospital between May 1, 2011, and July 31, 2012, and the patients with vascular risk factors but not incident stroke as controls. We collected 5 ml of venous blood, and circulating miRNA levels were evaluated by quantitative real-time polymerase chain reaction. Five miRNAs (miR-17, miR-21, miR-106a, miR-126, and miR-200b), which had been reported to be related to atherosclerosis, were measured. The levels of miRNAs were compared with the presence of acute stroke, vascular risk factors, stroke subtypes, and stroke recurrence after index stroke. A total of 120 patients were included in the study, with 83 acute stroke patients. The levels of miR-17 were significantly increased in acute stroke patients, and the levels of miR-126 had positive correlation with cerebral atherosclerosis ($r=0.254$, $p=0.021$). Among the 83 stroke patients, eight experienced stroke recurrence during follow-up and higher level of miR-17 was associated with shorter event-free survival ($p=0.047$). This study shows that the miR-17 level was elevated in acute ischemic stroke and associated with future stroke recurrence.

Keywords MicroRNA · Atherosclerosis · Cerebral infarction · Biomarker · Prognosis

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Introduction

The incidence of stroke is increasing rapidly in concert with societal aging and is related to high mortality and disability. Although diagnostic accuracy has been aided by the advents of new brain imaging modalities, little progress has been achieved with respect to the development of blood biomarkers that predict stroke outcome or the burden of underlying atherosclerosis [1]. The introduction of various blood biomarkers of ischemic heart disease has enormously improved treatment strategy and clinical study performance on myocardial infarction, and thus, efficient blood biomarkers of ischemic stroke and cerebral artery atherosclerosis based on disease pathophysiology are urgently required [2]. MicroRNAs (miRNAs) are small non-coding RNAs with 20–25 nucleotides that posttranscriptionally regulate gene expression. Recently, circulating miRNAs have been studied in several disease backgrounds, and their expression patterns have been found to reflect disease subtype and prognosis. These findings extend

the scope of miRNA from being representative of tissue-specific local machinery to being systemic representative of disease burden [3].

Several miRNAs have been studied in stroke using experimental models and small groups of patients [4–6]. Studies on animal models showed dynamic changes in the miRNA expression, and in particular, miR-200b and miR-298 levels were increased both in ischemic and hemorrhagic stroke models [4]. Another study on young stroke patients reported that miRNA expression patterns vary according to stroke mechanism [5]. In a recent study, symptomatic and asymptomatic carotid plaques harvested after carotid endarterectomy were found to exhibit different miRNA expression profiles [7]. However, little available data supports a relation between circulating miRNA levels in acute stroke and its prognosis.

We hypothesized that miRNAs related to atherosclerosis or hypoxia would be altered in the blood of acute ischemic stroke patients and their initial expression levels will be able to reflect atherosclerosis activity and to predict future vascular event. In this regard, we selected five miRNAs related with atherosclerosis by literature review and evaluated their expression patterns in acute stroke patients and control subjects with vascular risk factors.

Methods

Patient Inclusion and Clinical Variables

Between May 1, 2011, and July 31, 2012, acute ischemic stroke patients who admitted to Seoul National University Hospital were enrolled, and those patients who were admitted to control vascular risk factors or other neurological problems but without incident stroke during the same time period served as a control group. We obtained basic demographic data, clinical history including vascular risk factors, stroke subtypes, laboratory data and brain MR imaging (MRI)/MR angiography (MRA). Vascular risk factors included hypertension (systolic blood pressure >140 mmHg or diastolic blood pressure >90 mmHg or on antihypertensive medication), diabetes mellitus (fasting blood sugar >7.0 mmol/L or hemoglobin A1c >6.5 % or on glucose-lowering medication), heart disease with potential embolic source, and smoking status. Stroke subtype was determined by Trial of Org 10172 in Acute Stroke Treatment classification as (1) large artery atherosclerosis (LAA) with significant stenosis in a relevant artery, (2) cardioembolic infarction (CE) with a documented potential cardioembolic source without relevant artery stenosis, and (3) small vessel occlusion (SVO), when an infarction had a typical location and size of lacunar stroke without large artery stenosis or a potential embolic source [8]. Patients with other stroke etiology, such as a genetic cause, a hypercoagulable state, combined malignancy, vasculitis, or an infection-related condition were excluded. Written informed consent was obtained from each patient before inclusion, and this study was approved by the institutional review board of Seoul National University Hospital (0906-028-282) and performed conform the declaration of Helsinki.

Atherosclerotic Burden and Clinical Outcome Assessment

Brain MRI was performed using a 3.0-T unit (Signa, GE medical systems, Milwaukee, WI, USA) and an eight-channel head coil including T1- and T2-weighted, diffusion weighted, fluid-attenuated inversion recovery, gradient echo images, and time-of-flight MRA. The degree of cerebral atherosclerosis was determined using brain MRA findings with previously reported scoring system [9]. In brief, stenosis was graded into three levels: 0 indicating <50 % stenosis, 1 indicating 50 to 99 % stenosis, and 2 indicating occlusion, which was assessed separately from anterior cerebral, middle cerebral, posterior cerebral, internal carotid, vertebral, and basilar arteries [9]. The atherosclerosis score was defined as the sum of the scores of these arteries on three-dimensional MRA [9]. High-resolution vessel wall MRI was additionally performed among the 12 stroke patients with intracranial atherosclerosis by previously described protocol, and enhanced atheroma from T1 enhancement protocol was considered as vulnerable plaque [10]. The target atherosclerotic vessel for high-resolution MRI evaluation was determined from initial MRA image review. The black blood technique with fat pre-regional saturation pulses of 80-mm thickness to saturate incoming arterial flow was used, and high-resolution MR sequences using a 3.0-T unit (Verio, Siemens Medical Solution, Erlangen, Germany) included T1-weighted images acquired with repetition time (TR)/echo time (TE)=600/12 ms, T2-weighted images acquired with TR/TE=2910/70 ms, proton density (PD) images acquired with TR/TE=2500/30 ms, and T1-weighted images with gadolinium enhancement. All images were taken with a field of view=120×120 mm, slice thickness 2 mm, matrix size=384×269, and number of average=4. Patient functional status was measured by National Institutes of Health Stroke Scale on admission and by modified Rankin's score at discharge, and further dichotomized as good outcome when discharge modified Rankin's score was 0 or 1. All stroke patients were monitored regularly after discharge every 2 or 3 months to detect recurrence of cerebral infarction.

MicroRNA Sample and Expression Levels

A peripheral blood sample of 5 ml was harvested in an EDTA bottle from each patient. For acute stroke patients, blood sample was harvested within 7 days after symptom onset. Plasma samples were immediately extracted after centrifugation at 1500g for 15 min at 4 °C and stored at −70 °C until required for analysis. miRNAs were extracted from 200 µl of plasma aliquot

using a miRNeasy kit (Qiagen, GmbH, Germany). About 150 ng of RNA mixture was collected from each plasma sample, and miRNA quantitative real-time polymerase chain reaction (qRT-PCR) was performed using the SYBR GreenER™ miRNA qRT-PCR kits (Invitrogen, Carlsbad, CA) and the respective primers (Applied Biosystems, Foster City, CA). We determined to evaluate five different miRNAs: miR-17 [11, 12], miR-21 [12, 13], miR-106a [14], miR-126 [15, 16], and miR-200b [6, 17], which had been associated with vascular pathologies and atherosclerosis as illustrated in Supplementary Table 1. We used miR-16 as a standard because several studies have shown its stable expression in human blood [3, 5, 18]. Each sample was tested three times, and relative miRNA expression levels were determined using the formula $2^{-\Delta CT}$ (ΔCT = mean Ct [miRNA] − mean Ct [miRNA-16]).

Statistical Analysis

Continuous values are expressed as means±standard deviations and categorical values as numbers of the patients. Continuous values were tested for normality using the Shapiro-Wilk test and converted to natural logs when appropriate. The Student's t test or Mann-Whitney U test was used to analyze continuous variables and chi-squared test to analyze categorical values. Clinical and laboratory variables including miRNA levels were compared between acute stroke patients and control group. Multivariate logistic regression analysis was performed to evaluate independent miRNA change after acute infarction and included age, gender, and those variables with p value <0.05 from bivariate analysis. Among the stroke patients, miRNA levels were compared in terms of stroke subtype, neurological outcome, and the presence of atherosclerosis. Receiver operating characteristic curves (ROC) were generated to determine sensitivity and specificity values of acute ischemic stroke diagnosis by miRNA level. Pearson's correlation analysis was used to examine relationship between clinical variables including atherosclerosis score and miRNA levels. The levels of miRNA were compared between the patients with plaque enhancement and those with non-enhanced plaque from high-resolution vessel wall MRI. Finally, clinical and laboratory variables were compared between the patients who experienced stroke recurrence and those without recurrence. Kaplan-Meier survival curves with log-rank test were applied to compare stroke recurrence-free survival between the patients with higher miR-17 level and those with lower levels by GraphPad Prism ver. 5 (GraphPad, La Jolla, CA, USA). All the statistical

Table 1 Clinical characteristics and microRNA profiles between stroke patients and controls

	Control	Acute stroke	P value
Total patients	37	83	
Age	65.3±11.2	66.2±14.0	0.743
Female patients, no. (%)	22 (59.5)	28 (33.7)	0.008
Hypertension, no. (%)	22 (59.5)	54 (65.1)	0.682
Diabetes mellitus, no. (%)	8 (21.6)	34 (41.0)	0.040
Heart disease, no. (%)	6 (16.2)	27 (32.5)	0.059
Systolic blood pressure (mmHg)	129.9±16.5	142.0±26.1	0.003
Diastolic blood pressure (mmHg)	76.4±9.1	81.1±14.9	0.039
White blood cell (/μl^3)	6343.5±2574.5	7623.5±2823.8	0.020
hsCRP, median (IQR, mg/dl)	0.08 (0.03–034)	0.13 (0.04–0.42)	0.536
Fasting blood sugar (mg/dl)	103.6±34.9	117.3±44.9	0.057
Hemoglobin A1c (%)	6.1±0.8	6.6±1.4	0.032
Total Cholesterol (mg/dl)	178.9±40.8	173.9±41.3	0.995
High density lipoprotein (mg/dl)	50.0±11.7	45.8±11.2	0.264
Triacyl glycerol (mg/dl)	116.9±39.3	119.2±64.3	0.515
Low density lipoprotein (mg/dl)	110.0±31.5	111.6±37.0	0.603
Fibrinogen (mg/dl)	297.7±58.4	321.6±87.7	0.063
Homocystein (μmol/l)	10.0±2.1	10.3±3.3	0.899
miR-17, median (IQR)	0.0805 (0.0375–0.1049)	0.1031 (0.0625–0.1402)	0.013
miR-21, median (IQR)	0.0886 (0.0706–0.1297)	0.1116 (0.0529–0.2017)	0.386
miR-106a, median (IQR)	0.0530 (0.0389–0.0878)	0.0794 (0.0486–0.1257)	0.022
miR-126, median (IQR)	0.1020 (0.0555–0.1788)	0.1543 (0.0506–0.2467)	0.148
miR-200b, median (IQR)	0.1190×10^{-3} (0.0626×10^{-3}–0.1623×10^{-3})	0.1392×10^{-3} (0.0862×10^{-3}–0.2518×10^{-3})	0.186

hsCRP highly sensitive C-reactive protein, *IQR* interquartile range, *miR* microRNA

Table 2 Multivariate logistic regression analysis predicting acute cerebral infarction

	P value	Odds ratio	Confidence interval
Age	0.941	1.001	0.968–1.035
Gender (female)	0.171	1.912	0.757–4.833
Systolic blood pressure	0.025	1.024	1.003–1.045
Diabetes mellitus	0.417	0.656	0.237–1.816
Ln(WBC)[a]	0.006	8.572	1.842–39.900
Ln(miR-17)[a]	0.019	1.895	1.111–3.234

[a] The values of white blood cell count and microRNA-17 (miR-17) were converted by natural logarithm because they were not normally distributed
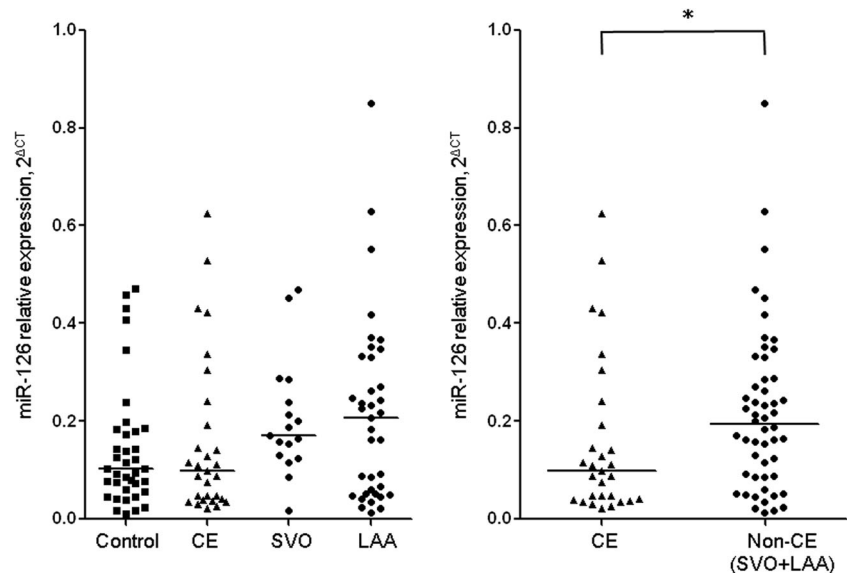
analyses were performed using SPSS ver. 19 (SPSS, Chicago, IL) unless otherwise indicated, and statistical significance was accepted for *p* value <0.05.

Results

Plasma Levels of miR-17 were Significantly Higher in Acute Cerebral Infarction Patients

A total of 120 patients were enrolled in this study, including 83 acute ischemic stroke patients and 37 controls. Stroke subtypes included 35 LAA, 17 SVO, and 31 CE. The levels of miR-17 (*p*=0.013) and miR-106a (*p*=0.022) were elevated in acute ischemic stroke patient (Table 1), and the increase in miR-17 remained significant after multivariate logistic regression analysis adjusted for age, gender, diabetes mellitus, white blood cell count, and systolic blood pressure (Table 2). The area under the ROC of miRNA-17 was 0.642 (37.4 % sensitivity and 89.2 % specificity at a cutoff of 0.1265, *p*=0.013,

Supplementary Fig. 1). miRNA expression levels were not significantly different regardless of gender, vascular risk factors, or neurological outcome (Supplementary Table 2), but miR-126 level was higher among the patients with atherosclerosis than those without atherosclerotic stenosis (*p*=0.044, Supplementary Table 2).

miRNA Expressions Reflected Stroke Subtypes and Atherosclerosis Burden

When stroke subtypes were dichotomized into CE and non-CE stroke groups including LAA and SVO, miR-126 (*p*=0.018) and miR-200b (*p*=0.024) levels were found to be higher in non-CE patients (Fig. 1, Supplementary Table 3). Atherosclerosis scores from MRA positively correlated with miR-21 (*r*=0.223, *p*=0.043) and with miR-126 levels (*r*=0.254, *p*=0.021, Supplementary Table 4), whereas stroke onset, infarction volume, and stroke severity were not. The levels of miR-126 also positively correlated with white blood cell count, but negatively correlated with age (Supplementary Table 4). Plaque enhancement from vessel wall MRI was associated with elevated levels of miR-17 (*p*=0.048), miR-126 (*p*=0.030), and miR-200b (*p*=0.030, Fig. 2, Supplementary Table 5).

The Higher miR-17 Level was Associated with Future Stroke Recurrence

Among 83 stroke patients, eight patients experienced recurrent cerebral infarction during median follow-up period of 24 months. Stroke patients who experienced recurrent stroke during follow-up period exhibited higher levels of miR-17 (*p*=0.013) and miR-106a (*p*=0.015, Table 3). Other clinical variables and laboratory data were not different between the

Fig. 1 The microRNA levels by stroke subtype. miR-126 levels were elevated in non-cardioembolic stroke patients (including large artery atherosclerosis patients and patients with small vessel occlusion) than in cardioembolic stroke patients (*n*=83, Mann-Whitney *U* test, *p*=0.018). *Bars* represent median values (**P*<0.05). *CE* cardioembolism, *SVO* small vessel occlusion, *LAA* large artery atherosclerosis

Fig. 2 The microRNA levels according to plaque enhancement from high resolution vessel wall MR imaging. Representative high-resolution vessel wall MR imaging from two different pontine infarction patients due to basilar artery atherosclerosis show non-enhancing plaque (**a**, *arrow*) and enhancing plaque (**b**, *arrow*) in basilar artery. The levels of miR-17 ($p=0.048$), miR-126 ($p=0.030$), and miR-200b ($p=0.030$) were significantly elevated in stroke patients with enhanced plaque in patients with non-enhanced plaque ($n=12$, Mann-Whitney U test). *$P<0.05$
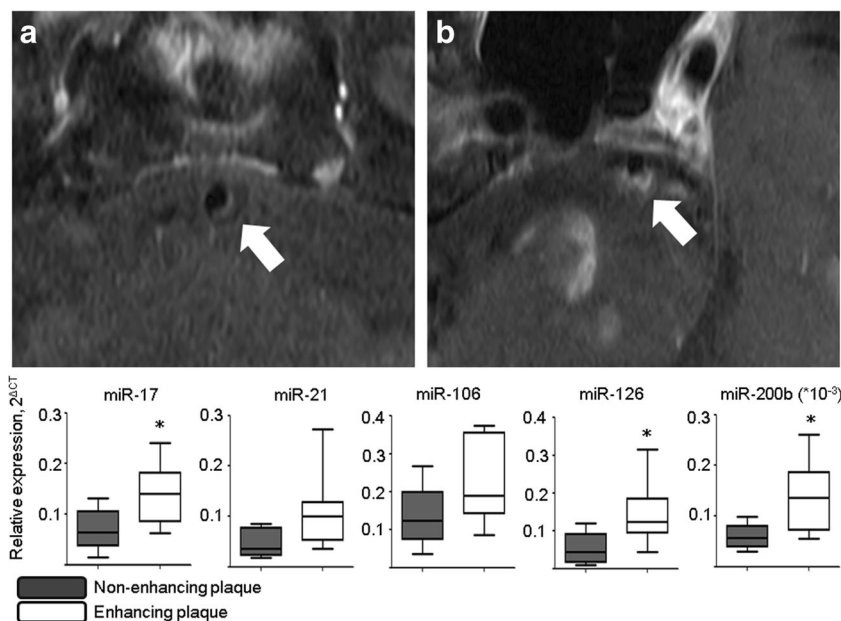
two groups. When stroke patients were dichotomized by the median value of miR-17 level (cutoff value=0.1031), higher level of miR-17 was associated with shorter event-free survival ($p=0.047$, Fig. 3).

Discussion

This study shows acute ischemic stroke was associated with elevated plasma levels of miR-17, and its higher level was

related to future stroke recurrence. The miRNA expression patterns of stroke subtypes differed; miR-126 levels correlated with the degrees of cerebral atherosclerosis from brain MRA and were significantly lower in CE stroke patients than in atherosclerotic stroke patients, emphasizing its role in vascular dysfunction. This is the first study suggesting prognostic value of miRNA in cerebral infarction.

Two hypotheses could be proposed to explain the origins of circulating microRNAs in stroke patients. First, apoptotic microparticles from brain endothelial cell, vulnerable

Table 3 Comparison between stroke patients who experienced stroke recurrence and stroke patients without recurrence

	With recurrence	Without recurrence	P value
Total patients	8	75	
Age	61.5±22.7	66.7±12.9	0.326
Female patients, no. (%)	3 (37.5)	25 (33.3)	0.999
Hypertension, no. (%)	4 (50.0)	50 (66.7)	0.441
Diabetes mellitus, no. (%)	3 (37.5)	31 (41.3)	0.999
Heart disease, no. (%)	5 (62.5)	22 (29.3)	0.107
Systolic blood pressure (mmHg)	136.6±26.6	142.6±26.1	0.544
White blood cell (/μl^3)	9030.0±3927.3	7473.5±2627.1	0.307
hsCRP >0.20 mg/dl, no. (%)	4 (50.0)	27 (36.0)	0.464
Fasting blood sugar (mg/dl)	104.5±39.0	116.1±40.8	0.447
Low density lipoprotein (mg/dl)	92.1±24.9	110.0±39.6	0.216
Fibrinogen (mg/dl)	360.4±118.7	319.3±78.9	0.187
miR-17, median (IQR)	0.1635 (0.1068–0.2154)	0.0885 (0.0621–0.1512)	0.013
miR-21, median (IQR)	0.2324 (0.1041–0.3051)	0.1093 (0.0508–0.2094)	0.075
miR-106a, median (IQR)	0.1155 (0.0822–0.1934)	0.0743 (0.0445–0.1341)	0.015
miR-126, median (IQR)	0.2651 (0.0865–0.3466)	0.1449 (0.0476–0.2702)	0.395
miR-200b, median (IQR)	0.1715×10^{-3} (0.0862×10^{-3}–0.3152×10^{-3})	0.1389×10^{-3} (0.0751×10^{-3}–0.3015×10^{-3})	0.054

hsCRP highly sensitive C-reactive protein, *IQR* interquartile range, *miR* microRNA
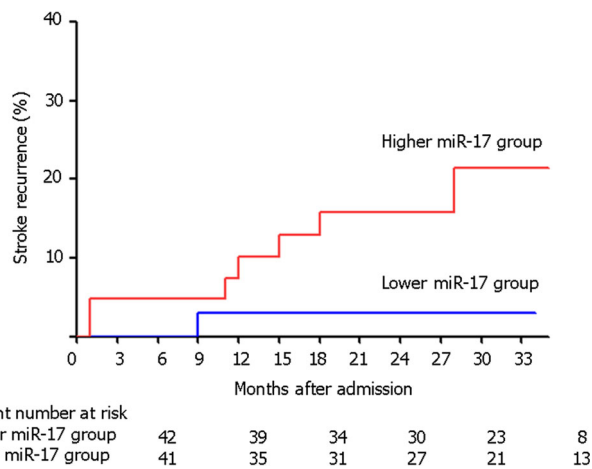
Fig. 3 Survival curve analysis predicting event free survival between the groups with higher and lower miR-17 level. When stroke patients were dichotomized by the median value of miR-17 levels (cutoff value= 0.1031), the patients group with higher miR-17 showed shorter stroke recurrence-free survival than those patients with lower miR-17 levels (p=0.047, log-rank test)

atheromatous plaque, or injured nervous tissue might be a potential source, as they have stable microenvironment protected by hydrophobic lipid membrane [19]. Second, miRNAs might be produced after acute ischemia because they have tissue protective function [20, 21]. This study shows that miR-17 was elevated among acute cerebral infarction patients: around 25 % of risk factor combined controls. Angiogenesis with increased vascular endothelial growth factor have been observed after acute cerebral ischemia, and the miRNA 17-92 cluster is known to augment angiogenesis by suppression of antiangiogenic thrombospondin-1 and connective tissue growth factor [11, 20]. Reactive angiogenesis after acute ischemic stroke may underlie miR-17 elevation after acute cerebral infarction [22]. It remains to be determined whether circulating microRNAs are actively secreted protective material or by-products from vulnerable atheroma.

This study suggested prognostic implication of miR-17 in stroke patients, although the exact pathomechanism is yet illusive. The level of circulating miR-17 may reflect systemic atherosclerosis activity in stroke patients, which is related to future vascular event. Many studies have shown that miR-17 harbors oncogenic potential and elevated miR-17 level is associated with poor prognosis in cancer patients by increased angiogenesis [23, 24]. The progression of atherosclerosis is a complex pathological process that involves inflammatory cell infiltration, smooth muscle cell hyperplasia, and reactive angiogenesis [25]. Vulnerable plaque from high-resolution vessel wall MRI was associated with elevated miR-17, miR-126, and miR-200b levels compared to non-enhanced plaque,

suggesting their role in atheroma neoangiogenesis and inflammation.

The miR-126 levels were increased in patients with atherosclerosis and significantly higher in non-CE stroke group. Endothelial cells are known to express abundant miR-126 to protect vascular integrity, which decreases inflammatory cell adhesion by inhibiting the expression of vascular cell adhesion molecule-1.[16] Therefore, the elevation of miR-126 levels in patients with advanced atherosclerosis could be counter-reaction against atheroma progression. However, increased baseline miR-126 levels were also reported to be associated with incident myocardial infarction from a cohort study, suggesting bidirectional role of miR-126 in atherosclerosis pathophysiology [26].

This study has several limitations. First, all the patients were Korean and the number of included patients was small because we did not include every stroke patient within the study period. We excluded stroke etiologies other than LAA, CE, or SVO, and patients with an undetermined stroke subtype, which might result in selection bias. Second, the miRNAs studied were selected from previous studies performed on various vascular diseases, which might not be an ideal way of deriving sensitive and specific biomarkers assessing brain neuronal damage. However, selected miRNAs reflected atherosclerosis burden and activity among stroke patients, which will help to predict future vascular event. Third, although atherosclerosis is a systemic disease, we only evaluated cerebral vessels to determine atherosclerosis score, which could have underestimated overall atherosclerosis burdens.

Summarizing, we studied five atherosclerosis-related miRNAs in acute cerebral infarction patients, and found that plasma miR-17 levels were increased in acute ischemic stroke and its higher level was associated with future vascular event after index stroke. The levels of miR-126 were increased in patients with atherosclerosis and correlated with atherosclerosis burden. Future studies are warranted to determine the pathomechanism responsible for these increases and to evaluate biomarker potentials.

Ethical Approval All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional: Seoul National University Hospital, 0906-028-282) and with the Helsinki Declaration of 1975, as revised in 2008. Informed consent was obtained from all patients for being included in the study.

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