

Role of MicroRNAs in Thrombus and Future Vascular Events Among Patients With Stroke

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Dear Sir:

Despite the application of endovascular thrombectomy, approximately 30% of patients with stroke encounter neurological deterioration following thrombectomy, and a significant proportion of patients face vascular events, including stroke recurrence, even with optimal antithrombotic management.¹ Recent strides in advanced histological analysis, incorporating transcriptomic analysis and immunohistochemistry, offer a promising avenue for obtaining new insights.² Consequently, our efforts have been directed towards identifying a set of microRNAs with prognostic significance from the thrombi of patients with stroke and evaluating their therapeutic potential through *in vitro* disease models.

Patients with ischemic stroke who underwent intra-arterial thrombectomy at a university hospital between February 2016 and March 2020 were included in the first cohort. MicroRNA expression was compared between patients with and without vascular events. The microRNA profiles from thrombus analysis were validated using blood samples from another cohort of patients with stroke who underwent 18F-fluorodeoxyglucose positron emission tomography to predict stroke recurrence. The therapeutic effects of the identified microRNAs were examined using an endothelial ischemic injury model. More detailed study methods are provided in Supplementary Methods.

A total of 79 thrombi from 103 patients within the thrombus analysis cohort were included (mean age=70.7±11.9 years, 33 female patients) and 21 vascular events (26.6%) were identified

(Supplementary Figure 1). Microarray analysis revealed significant upregulation of 19 microRNAs, whereas 11 were downregulated in patients experiencing vascular events (Figure 1A). Principal component analysis of the microarray data revealed distinct but intermixed expression signatures between the two groups (Figure 1B). Subsequently, six microRNAs were selected for validation based on microarray analysis results and a literature review (Supplementary Table 1). Quantitative real-time polymerase chain reaction from thrombus analysis revealed significantly elevated expression of miR-93-5p among patients with vascular events (3.82 ± 5.42 vs. 11.2 ± 14.5 , $P < 0.01$), whereas miR-20b-5p demonstrated a tendency towards elevation (11.7 ± 13.7 vs. 29.1 ± 29.3 , $P = 0.07$) (Figure 1C). A detailed comparison of the clinical information and microRNA expression levels between patients with and without vascular events is presented in Supplementary Table 2. The Cox proportional hazards model did not reveal an independent relationship between miR-93-5p expression and vascular events (Supplementary Table 3).

Receiver operating characteristic curve analysis that predicted vascular events using miR-93-5p yielded insignificant results (area under the curve=0.608, 95% confidence interval=0.416–0.799; $P = 0.25$) (Supplementary Figure 2A). After dichotomizing patients based on miR-93-5p expression levels from thrombus, Kaplan-Meier survival curve analysis demonstrated significantly shorter event-free survival time among patients with higher miR-93-5p expression (log-rank test, $P = 0.03$) (Supplementary Figure 2B). A waterfall plot based on miR-93-5p expression high-

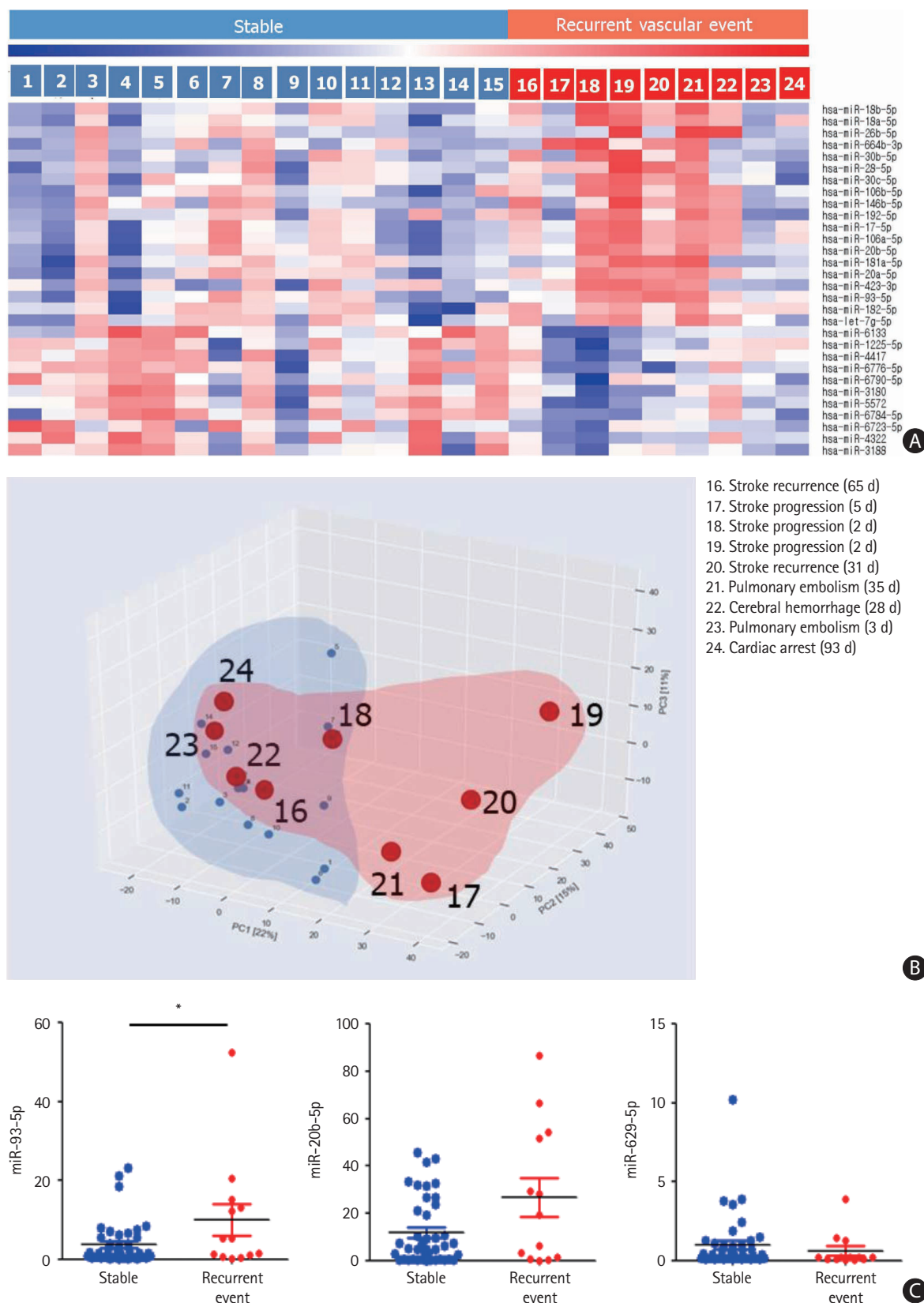


Figure 1. MicroRNA expression level analysis from the thrombus. (A) Microarray analysis demonstrated that 19 microRNAs were significantly upregulated and 11 were downregulated in patients with vascular events. (B) Principal component analysis showed a distinctive pattern of microRNA expression signature in patients with vascular events (red dots), although there was considerable overlap with those with stable prognosis (blue dots). (C) Validation by quantitative real-time polymerase chain reaction showed that the expression levels of miR-93-5p were significantly elevated in patients with vascular events. * $P < 0.05$.

lighted the number of patients with vascular events (red arrow) or death (orange arrow) on the side with higher miR-93-5p expression (Supplementary Figure 2C). Although patients with vascular events tended to exhibit elevated miR-93-5p expression in venous blood samples obtained at the end of thrombectomy ($P=0.058$) (Supplementary Figure 2D), the expression levels of miR-93-5p in the blood and thrombus were not correlated ($r=0.0275$, $P=0.90$) (Supplementary Figure 2E).

Correlation analysis between microRNAs and laboratory variables indicated that miR-93-5p expression levels were positively correlated with the National Institutes of Health Stroke Scale score at hospital discharge ($r=0.331$, $P=0.02$) (Supplementary Figure 3A), e/e' from echocardiography ($r=0.321$, $P=0.02$) (Supplementary Figure 3B), and serum parathyroid hormone levels ($r=0.451$, $P<0.01$) (Supplementary Figure 3C). Whereas, miR-93-5p expression levels were negatively correlated with serum vitamin D levels ($r=-0.375$, $P<0.01$) (Supplementary Figure 3D). Immunohistochemistry for miR-93-5p in the thrombus revealed abundant positive signals throughout the thrombus sections (Supplementary Figure 3E and F). Collectively, these data suggest that miR-93-5p is closely associated with thrombus formation.

Following the outcomes from the initial cohort, in the second cohort, we validated the expression patterns of miR-93-5p and

miR-17-5p; the latter shares the same seed sequence as miR-93-5p and is a crucial member of the miR-17-92 cluster. Patients experiencing recurrent stroke exhibited elevated levels of both miR-93-5p (2.33 ± 1.42 vs. 3.60 ± 1.72 , $P=0.04$) (Figure 2A) and miR-17-5p (2.21 ± 1.19 vs. 3.24 ± 1.68 , $P<0.05$) (Figure 2D) than those with a stable prognosis (Supplementary Table 4). Interestingly, circulating miR-93-5p levels were positively associated with glucose uptake in the amygdala ($r=0.326$, $P=0.01$) (Figure 2B and C), whereas miR-17-5p levels were correlated with glucose uptake in the carotid artery ($r=0.270$, $P<0.05$) (Figure 2E and F).

Endothelial injury following oxygen glucose deprivation (OGD) correlated with augmented expression of miR-17-5p ($P=0.03$) (Figure 3A) and miR-93-5p ($P=0.03$) (Figure 3B) in human umbilical vein endothelial cells and human brain microvascular endothelial cells. The application of mimic or antagonistic sequences effectively altered the expression of the target microRNAs (Supplementary Figure 4). Notably, the application of their antagonistic sequences resulted in a pronounced enhancement in cell survival (OGD vs. miR-17-5p antagonist, $P<0.01$; OGD vs. miR-93-5p antagonist, $P=0.10$) (Figure 3C) along with a concomitant reduction in cytotoxic lactate dehydrogenase (OGD vs. miR-17-5p antagonist, $P<0.01$; OGD vs. miR-93-5p antagonist, $P<0.01$)

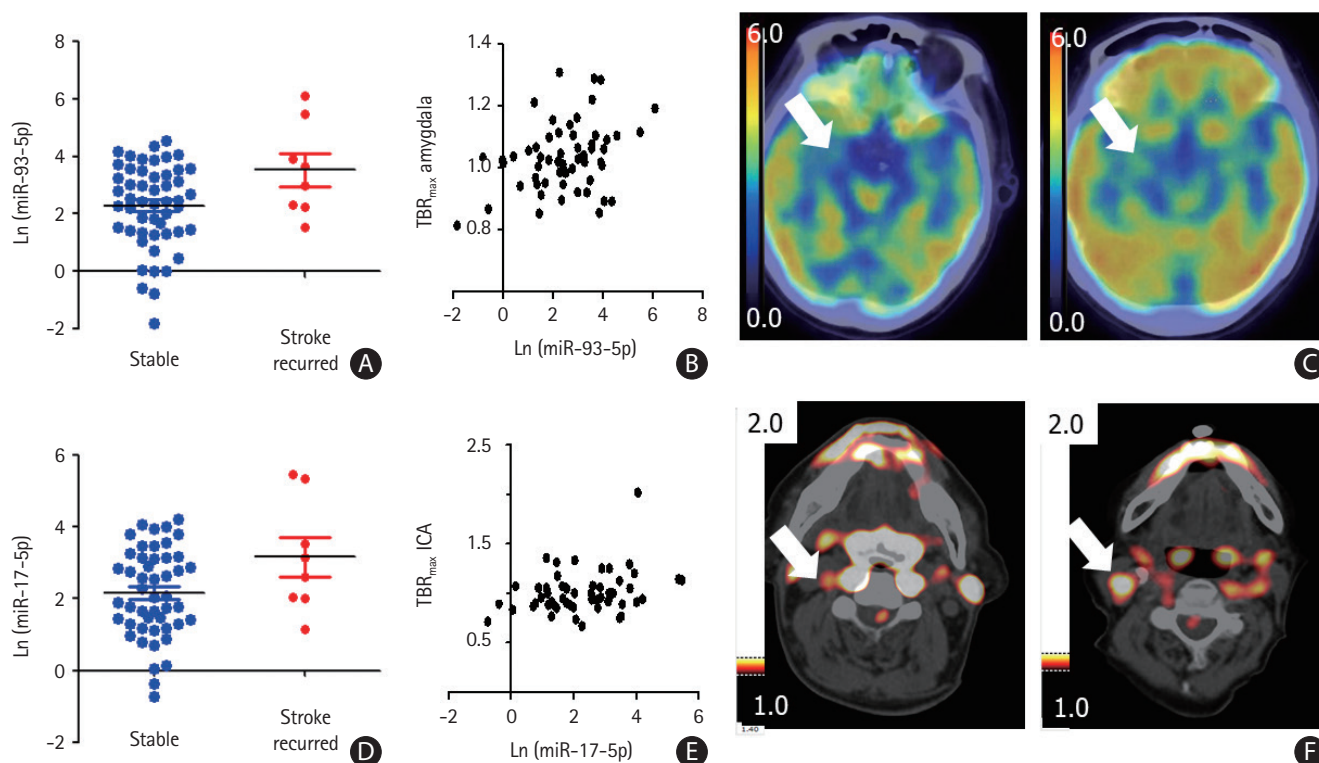


Figure 2. Impact of miR-17-5p/miR-93-5p among patients with stroke in the Cerebral Atherosclerosis Research with Positron Emission Tomography (CARPET) cohort. (A) Expression levels of miR-93-5p were elevated in patients with vascular events in the CARPET cohort. (B and C) miR-93-5p expression was correlated with the metabolic activity of the amygdala. (D) Expression levels of miR-17-5p were elevated in patients with recurrent stroke. (E and F) miR-17-5p expression was correlated with glucose uptake in the carotid artery. TBR_{max}, target to background ratio, maximal; ICA, internal carotid artery.

(Figure 3D). Western blot analysis revealed that the antagonistic sequence of miR-17-5p significantly increased the expression of programmed death ligand 1 (PD-L1) (OGD vs. miR-17-5p antagonist, $P=0.03$) (Figure 3E). Additionally, the expression of phosphorylated serine/threonine protein kinases was significantly increased by the antagonistic sequences of both miR-17-5p and miR-93-5p (OGD vs. miR-17-5p antagonist, $P=0.03$; OGD vs. miR-93-5p antagonist, $P=0.03$) (Figure 3E).

This study demonstrates that the expression signature of microRNAs from thrombi may be a potential predictor of future vascular events after stroke. The elevation of miR-93-5p was found to be associated with future vascular events among survivors of stroke by investigating two different specimens, thrombus and blood, from two distinctive prospective cohorts. Using *in vitro* models, the antagonistic sequences of miR-93-5p and miR-17-5p were found to mitigate endothelial cell death, highlighting their potential roles in the intricate processes of thrombus formation.

The impact of miR-93-5p on future vascular events appears to

be multifaceted given its positive association with brain activity in the amygdala, a hub for emotional, hormonal, and circadian homeostasis control. Recent studies revealed that decreased PD-L1 in thrombi or atheromas is associated with future vascular events.^{3,4} This study underscores that elevated amygdala activity correlates with increased systemic expression of the miR-17/92 family, potentially attenuating a key modulator of the adaptive immune system and promoting thromboembolic tendencies among survivors of stroke.⁵ However, the results of this study should be interpreted with caution because the relationship between microRNAs and vascular events was not independent after adjusting for clinical variables in the first cohort, and the correlation between miR-93-5p and amygdala activity was weak in the second cohort. This suggests that miR-93-5p may act as a confounding factor indirectly related to vascular events.

This study has several limitations. First, the sample size was relatively small and microRNA analysis proved unfeasible in approximately 20% of the harvested thrombi. Second, while the modulatory effects of the identified microRNAs were explored

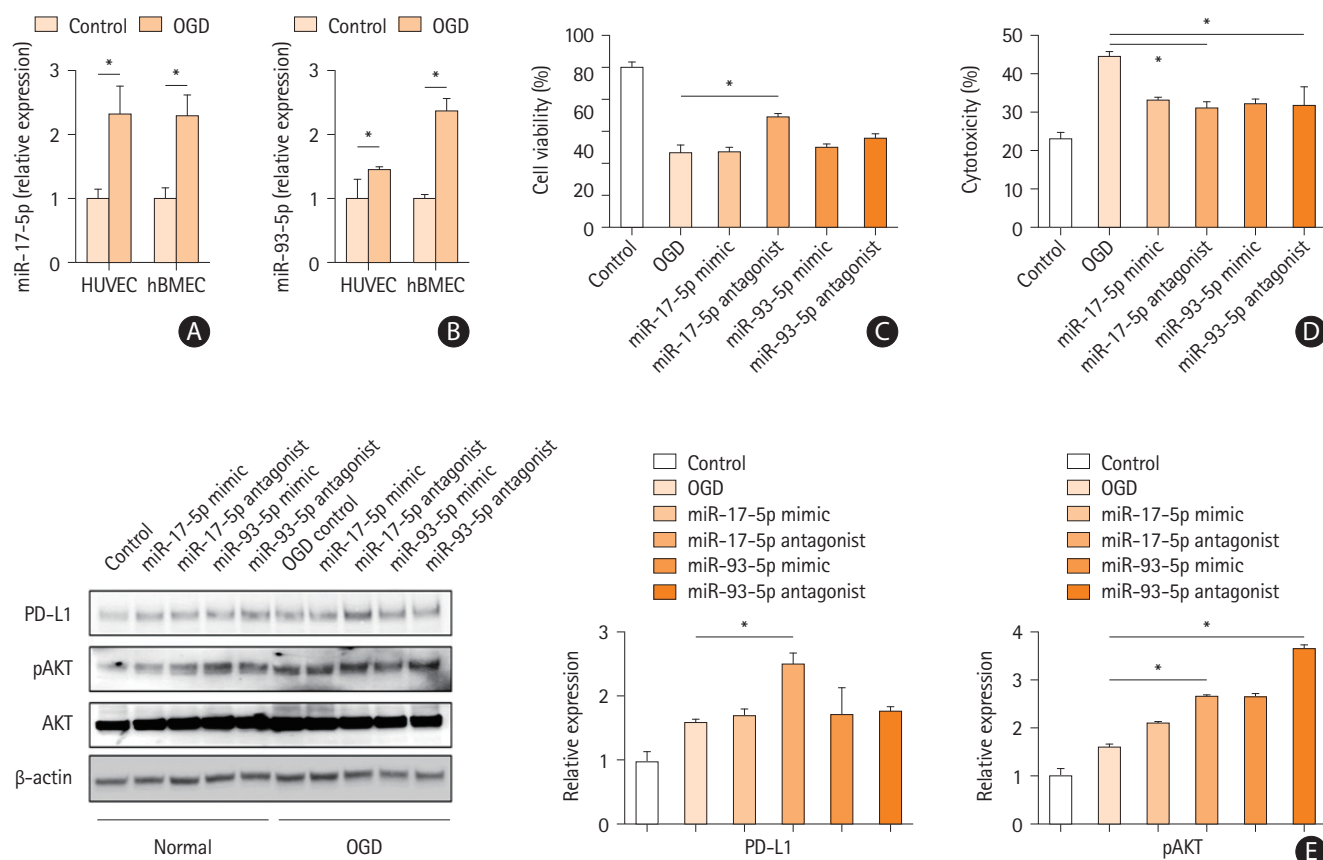


Figure 3. Effect of miR-17-5p/miR-93-5p antagonistic sequence in the *in vitro* endothelial injury model. Oxygen glucose deprivation (OGD) resulted in increased expression of miR-17-5p (A) and miR-93-5p (B) in human umbilical vein endothelial cells (HUVEC) and human brain microvascular endothelial cells (hBMEC). The antagonistic sequence of miR-17-5p increased cell viability following OGD (C), and the antagonistic sequences of both miR-17-5p and miR-93-5p reduced the cytotoxic response (D). (E) Programmed death ligand 1 (PD-L1) protein expression was increased by miR-17-5p antagonist treatment, and phosphorylated serine/threonine kinase (pAKT) levels were elevated by both miR-17-5p and miR-93-5p antagonists. * $P<0.05$.

using *in vitro* models, the absence of *in vivo* investigations hindered a comprehensive understanding of the pathophysiological role and systemic impact of miR-93-5p in the link between thrombosis and immune reactions. Whether a therapeutic strategy to mitigate microRNA expression or its downstream pathways can prevent future vascular events warrants further study.

Supplementary materials

Supplementary materials related to this article can be found online at <https://doi.org/10.5853/jos.2025.00150>.

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Conflicts of interest

The authors have no financial conflicts of interest.

Author contribution

Conceptualization: JMK, KHJ, KYP. Study design: JMK. Methodology: HBJ, YM, SH, TKN, HHC, RL, JWS, HRK. Data collection: HBJ, YM, SH, TKN, HHC, RL, JWS, HRK. Investigation: HBJ, YM, SH, TKN, HHC, RL, JWS, HRK. Statistical analysis: JMK. Writing—original draft: JMK, YM, SH. Writing—review & editing: all authors. Funding acquisition: JMK. Approval of final manuscript: all authors.

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Supplementary Methods

Experimental models and subject details

Patient inclusion in this study involved two prospective cohorts, as illustrated in Supplementary Figure 1. The first cohort, Thrombus Analysis of Ischemic Stroke (TAOIST), focused on investigating the expression signature of microRNAs derived from thrombi obtained through endovascular thrombectomy (EVT) in stroke patients.¹ This cohort spanned the period between February 2017 and March 2020 (Supplementary Figure 1A). The study protocol was reviewed and approved by the Institutional Review Board of Chung-Ang University Hospital, with informed consent obtained from the patient's caregiver or guardian (IRB Number: 1621-003-264). The enrolled patients underwent a comprehensive stroke etiology evaluation, including brain magnetic resonance imaging, Doppler echocardiography, 24-hour Holter monitoring, and a lipid panel. Additional information concerning bone mineral status, assessed via dual-energy X-ray absorptiometry and serum bone metabolism markers, including parathyroid hormone (PTH), vitamin D, and osteocalcin, was also collected.

The second cohort, named Cerebral Atherosclerosis Research with Positron Emission Tomography (CARPET), utilized 18F-fluorodeoxyglucose positron emission tomography (FDG PET) to predict future vascular events after stroke.² This cohort's study protocol was also reviewed and approved by the Institutional Review Board of Chung-Ang University Hospital, with informed consent obtained from the patient's caregiver or guardian (IRB Number: C2015061). All patients in this cohort underwent FDG PET once stabilized, and a 5 mL venous blood sample was obtained in an ethylenediaminetetra-acetic acid (EDTA) tube. MicroRNA expression analysis was available for 70 out of the 110 patients in this cohort (Supplementary Figure 1B).³

Patients enrolled in both cohorts received medical treatment, including antiplatelet or anticoagulant therapy, along with the control of vascular risk factors following the most recent stroke management guidelines. Information on sex, age, and race was self-reported by participants. Initial and discharge neurological severity were assessed using the National Institutes of Health Stroke Scale (NIHSS) by experienced neurologists. Subsequent follow-ups occurred post-discharge from the outpatient clinic, monitoring for vascular event after stroke which was defined as the occurrence of objectively proven thromboembolic events or vascular death defined as follows⁴:

Cardiovascular mortality

Cardiovascular mortality refers to death that is primarily from cardiovascular causes, including:

- Heart failure, cardiogenic shock, native, mechanical or bio-

prosthetic valve dysfunction, myocardial infarction, stroke, thromboembolism, bleeding, tamponade, vascular complication, arrhythmia or conduction system disturbances, cardiovascular infection (e.g., mediastinitis, endocarditis), or other clear cardiovascular cause.

- Intraprocedural death—caused by immediate/latent complications of a cardiovascular procedure.
- Sudden death—sudden natural death presumed to be of cardiac cause that occurs within 1 hour of onset of symptoms in witnessed cases, and within 24 hours of last being seen alive when it is unwitnessed. In autopsied cases, it is defined as the natural unexpected death of unknown or cardiac cause.
- Death of unknown causes.⁴

Myocardial infarction

Myocardial infarction, as defined according to the latest universal definition of myocardial infarction, currently: a rise and/or fall of cardiac troponin with at least one value above the 99th percentile and/or symptoms suggestive of ischemia, new significant electrocardiogram (ECG) changes, imaging evidence of new loss of viable myocardium or new regional wall motion abnormality in a pattern consistent with an ischemic etiology or identification of a coronary thrombus by angiography/intracoronary imaging or by autopsy. History of myocardial infarction also includes episodes of symptoms suggestive of myocardial ischemia, which are accompanied by presumed new ischemic ECG changes or ventricular fibrillation; coronary intervention-related myocardial infarction; and CABG-related myocardial infarction.⁴

Stroke

- An acute episode of focal or global neurological dysfunction (lasting for ≥ 24 h or until death) caused by an infarction or hemorrhage in the brain, spinal cord, or retina resulting in cell damage based on pathological, imaging, or other objective evidence. Stroke does not include non-vascular neurological deficits.
- Ischemic stroke is defined as an acute episode of focal, cerebral, spinal, or retinal dysfunction that is caused by central nervous system infarction, where the neurological dysfunction lasts for ≥ 24 hours. Ischemic stroke may result in hemorrhage (hemorrhagic transformation).
- Hemorrhagic stroke is defined as an acute episode of focal or global neurological dysfunction of the brain, spinal cord or retina that is caused by a spontaneous (not traumatic) collection of intraparenchymal, intraventricular, and/or sub-arachnoid blood, where the neurological dysfunction lasts for ≥ 24 hours. Hemorrhagic stroke does not include sub-dural hematomas.
- Unspecified stroke is defined as an acute episode of focal or global neurological dysfunction that is caused by a presumed

infarction or hemorrhage to the central nervous system, where the neurological dysfunction lasts for ≥ 24 hours but with insufficient information to allow categorisation as either ischemic or hemorrhagic stroke.⁴

Pulmonary embolism

A condition in which one or more emboli, usually arising from a thrombus formed in the veins, are lodged in and obstruct the pulmonary arterial system.⁴

Systemic embolism

Systemic embolism is defined as a hospital encounter with a principal diagnosis of an arterial embolism and thrombosis, excluding stroke or transient ischemic attack.⁴

Ischemia progression

Ischemia progression is neurological deterioration due to ischemic stroke progression defined as two or more increase of NIHSS score compared to the best neurological status after stroke within 7 days.⁵

In cases where a patient could not visit the clinic, information was obtained either from a caregiver or through telephone communication.

MicroRNA expression analysis

In the TAOIST cohort, the harvested thrombus underwent meticulous preservation in a nitrogen tank, maintaining a temperature below -70°C . Simultaneously, a venous blood sample of 5–10 mL was drawn from each patient upon completion of the thrombectomy procedure. To facilitate comprehensive analyses, each thrombus was dissected into two parts, one allocated for microRNA extraction and the other for immunohistochemistry analysis to elucidate the expression pattern of potential target messenger RNAs for microRNAs.

For microRNA extraction, the thawed thrombus underwent dissolution, and the miRNeasy kit (Qiagen, Hilden, Germany) was employed following the manufacturer's protocol. We first utilized previous microarray analysis information derived with the Affymetrix miRNA 4.0 array (Thermo Fisher Scientific, Waltham, MA, USA) which contains 2,578 human mature microRNAs from 24 patients to derive microRNAs related to stroke etiology.¹ The original study investigated the differential expression of microRNA profile according to stroke etiology by including 8 patients with large artery atherosclerosis, 8 patients with cardioembolic stroke due to atrial fibrillation, and 8 patients with cardioembolic stroke with valvular heart disease. In this study, we categorized previous data from the same patients into two groups: 9 subjects with

and 15 subjects without vascular events after index stroke.

MicroRNA expression normalization utilized the Robust Multi-Average algorithm, and a heat map was constructed highlighting microRNAs with significant alterations of more than two folds ($P < 0.05$) between the two groups. Principal component analysis involved deriving eigenvalues and eigenvectors from the microRNA expression signature of each thrombus. Significantly altered microRNAs from the microarray analysis were investigated by quantitative real-time polymerase chain reaction (qRT-PCR) in another 55 patients within the same cohort. All samples underwent three examinations, and the relative amount of the target microRNA was determined using $2^{-\Delta\text{Ct}}$ with the ratio between the microRNA and miR-652 as a reference, derived from the standard curve. Correlation analysis explored relationships between microRNA expression levels and clinical/laboratory variables.

Validation of the derived microRNA from the first cohort was carried out in the second cohort. MicroRNA extraction from the venous sample employed a commercial kit (Qiagen) after centrifuging serum at 1,000 g for 10 minutes, as described previously.³ Correlation analysis between microRNA expression levels and the metabolic activity of each organ, measured from FDG PET images, was conducted. Like the first cohort, all samples underwent three examinations, and relative expression levels of the microRNA were determined using $2^{-\Delta\text{Ct}}$ with the ratio between the microRNA and RNA, U6 small nuclear 2, as a reference. Supplementary Table 1 provides comprehensive details regarding the location, seed sequence, and pertinent literature related to the selected microRNA.

Immunohistochemistry for microRNA expression in the thrombus

MicroRNA *in situ* hybridization was conducted on representative paraffin thrombus sections following a meticulous protocol. The paraffin-fixed thrombus underwent deparaffinization using xylene and a 99% ethanol solution at room temperature, followed by rehydration with phosphate-buffered solution. Subsequently, each slide received approximately 200 μL of proteinase K solution, incubated for 10 minutes at 37°C in a humidifying incubator, and underwent dehydration. The hybridization mixture, consisting of 1 μL of a 100 μM locked nucleic acid probe and 9 μL of transfer buffer, was denatured at 90°C for 4 minutes. To achieve a probe concentration of 100 nM, 990 μL of hybridization buffer was added. The hydration mixture was applied to each slide, followed by incubation at 55°C for 2 hours. A positive control, utilizing a 10 nM locked nucleic acid probe of U6 small nuclear RNA, and a 40 nM double digoxigenin locked nucleic acid microRNA probe labeled the microRNA of interest.

After multiple rounds of stringent washing, visualization of the microRNA signal was achieved by applying a 1:800 dilution of alkaline phosphatase-conjugated anti-Digoxigenin antibody. This rigorous *in situ* hybridization procedure allowed for precise localization and visualization of the targeted microRNA within the thrombus sections.

In vitro endothelial injury model

To simulate vascular injury under conditions of ischemic stroke, we established a oxygen glucose deprivation (OGD) model using commercially available human umbilical vein endothelial cells (HUVEC; CEFO, Seoul, Korea) and human brain microvascular endothelial cells (hBMEC) obtained from a deceased donor following Institutional Ethical Review of Seoul National University Hospital (E-2201-004-1285). Both endothelial cell types were cultured in endothelial cell growth medium (Clonetics, San Diego, CA, USA) at 37°C under an atmosphere containing 95% O₂ and 5% CO₂. OGD was induced by incubating the cells in a hypoxia chamber with a gas mixture containing 5% CO₂, 94% N₂, and 1% O₂ for 6 hours using glucose-free Dulbecco's modified eagle medium (Sigma, St. Louis, MO, USA). We compared the expression levels of miR-17-5p and miR-93-5p between pre- and post-OGD conditions to compare the impact of OGD. Total RNA was extracted from cells using the RNeasy mini kit (Qiagen), followed by cDNA synthesis using the miRCURY LNA RT kit (Qiagen). UniSp6 RNA spike-in controls were incorporated to assess the reverse transcription reaction. For quantifying miRNA expression levels, the miRCURY SYBR Green qPCR kit (Qiagen) was utilized with miRCURY LNA miRNA PCR assay (Qiagen).

To further elucidate the cellular response, we compared cellular viability and cytotoxicity following OGD conditions with the application of miR-17-5p and miR-93-5p mimics and antagonistic sequences. HUVEC cells were transfected with the miRNA-17-5p and miRNA-93-5p mimic or antagonist using Lipofectamine 3000 (Thermo Fisher Scientific) according to the provided protocol. Cell viability was assessed using the water-soluble tetrazolium salt 1 assay (Dynebio, Seongnam, Korea). For the lactate dehydrogenase (LDH) assay, cells were incubated with LDH solution, and the absorbance was measured at 490 nm wavelength. Analysis of cell survival-related proteins and potential target proteins of miR-17-5p/miR-93-5p was conducted through Western blot analysis. Primary antibodies used included anti-programmed death ligand 1 (PD-L1; Cell Signaling Technology, Danvers, MA, USA; 1:1,000), anti-phosphorylated serine/threonine kinase (pAKT; Cell Signaling Technology, 1:1,000), anti-AKT (Cell Signaling Technology, 1:1,000), and anti-β-actin (Cell Signaling Technology, 1:2,000). Band intensity was quantified using ImageJ Gel Analysis (National Institutes of Health, Bethes-

da, MD, USA), normalized to internal β-actin expression levels.

Statistical analysis

Categorical variables underwent comparison through the chi-square test or Fischer's exact test, while continuous variables were assessed using Student's t-test or the Mann-Whitney U test, as appropriate. Spearman's correlation analysis was employed to explore associations between microRNA expression levels and laboratory variables. All statistical analyses were executed using SPSS version 23.0 (IBM Corp., Armonk, NY, USA). A significance level of $P < 0.05$ was deemed as statistically significant.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supplementary Table 1. Sequences and potential role of selected microRNAs for quantitative real-time polymerase chain reaction

Name	Location in chromosome	Sequence	Potential role in vascular disease
miR-17-5p	Chr13: 92003326-92003348	CAAAGUGCUUACAGUGCAGGUAG	Elevated among the patients with vulnerable cerebral atherosclerosis ⁶
miR-20b-5p	ChrX: 134169809-134169877	CAAAGUGCUCAUAGUGCAGGUAG	Promotes cardiac hypertrophy by the inhibition of Mitofusin 2 mediated calcium signaling ⁷
miR-93-5p	Chr7:99691438-99691460	CAAAGUGCUGUUCGUGCAGGUAG	Elevated in the thrombi of patients with stroke and neurological deterioration ¹
miR-629-5p	Chr15:70371766-70371786	UGGGUUUACGUUGGGAGAACU	Decreased in the thrombi of patients with stroke and neurological deterioration ¹
miR-450b-5p	ChrX:133674261-133674282	UUUUGCAAUAUGUUCUGAAUA	Elevated in animal model of transient ischemic attack ⁸
let7i-5p	Chr12:62997471-62997492	UGAGGUAGUAGUUUGUGCUGUU	Decreased among stroke patients and predicted to regulate leukocyte response ⁹

Supplementary Table 2. Comparison of clinical and laboratory variables between the patients with and without vascular events from thrombus analysis of ischemic stroke cohort

	Patient without event (n=43)	Patient with event (n=12)	P
Age (yr)	71.8 (13.0)	69.4 (11.1)	0.56
Female sex	17	3	0.36
Hypertension	22	7	0.66
Diabetes	12	4	0.71
Smoking	7	4	0.19
Atrial fibrillation	21	5	0.66
Previous stroke	11	1	0.20
Initial NIHSS	14.0 (6.7)	20.2 (5.8)	<0.01
Discharge NIHSS	5.3 (5.2)	20.8 (12.9)	<0.01
WBC ($\times 10^9/L$)	9.10 (4.92)	7.81 (3.15)	0.40
Hematocrit (%)	37.6 (5.8)	37.9 (7.3)	0.90
Platelet ($\times 10^9/L$)	213.0 (62.1)	204.8 (112.9)	0.74
eGFR (mL/min/1.73 m ²)	80.7 (31.6)	87.3 (25.1)	0.50
Vitamin D (nmol/L)	19.4 (9.6)	12.6 (5.7)	<0.01
PTH (pg/mL)	93.8 (52.7)	125.3 (104.1)	0.17
e/e'	14.7 (6.5)	14.0 (7.7)	0.78
miR-20b-5p (relative expression)	11.7 (13.7)	29.1 (29.3)	0.07
miR-93-5p (relative expression)	3.82 (5.42)	11.2 (14.5)	<0.01
miR-629-5p (relative expression)	1.82 (2.83)	2.54 (3.37)	0.46
miR-450b-5p (relative expression)	2.15 (2.97)	1.89 (3.25)	0.82
let7i-5p (relative expression)	9.81 (11.9)	12.2 (12.7)	0.54

Data are presented as mean (SD) for continuous variables and number for categorical variables.

SD, standard deviation; NIHSS, National Institutes of Health Stroke Scale; WBC, white blood cell; eGFR, estimated glomerular filtration rate; PTH, parathyroid hormone.

Supplementary Table 3. Cox proportional hazard model predicting future vascular event

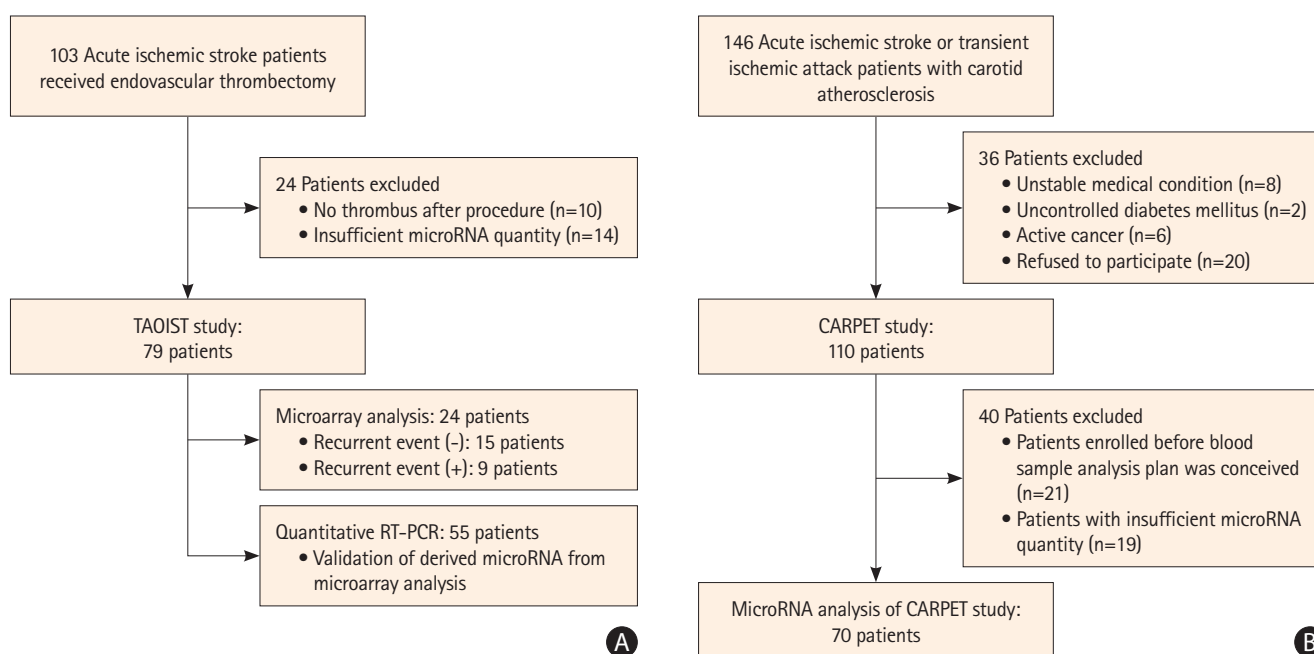
	Hazard ratio	95% confidence interval	P
Discharge NIHSS	1.08	1.04–1.12	<0.01
Vitamin D	0.00	0.00–0.00	0.98
miR-93-5p	1.27	0.27–5.95	0.76

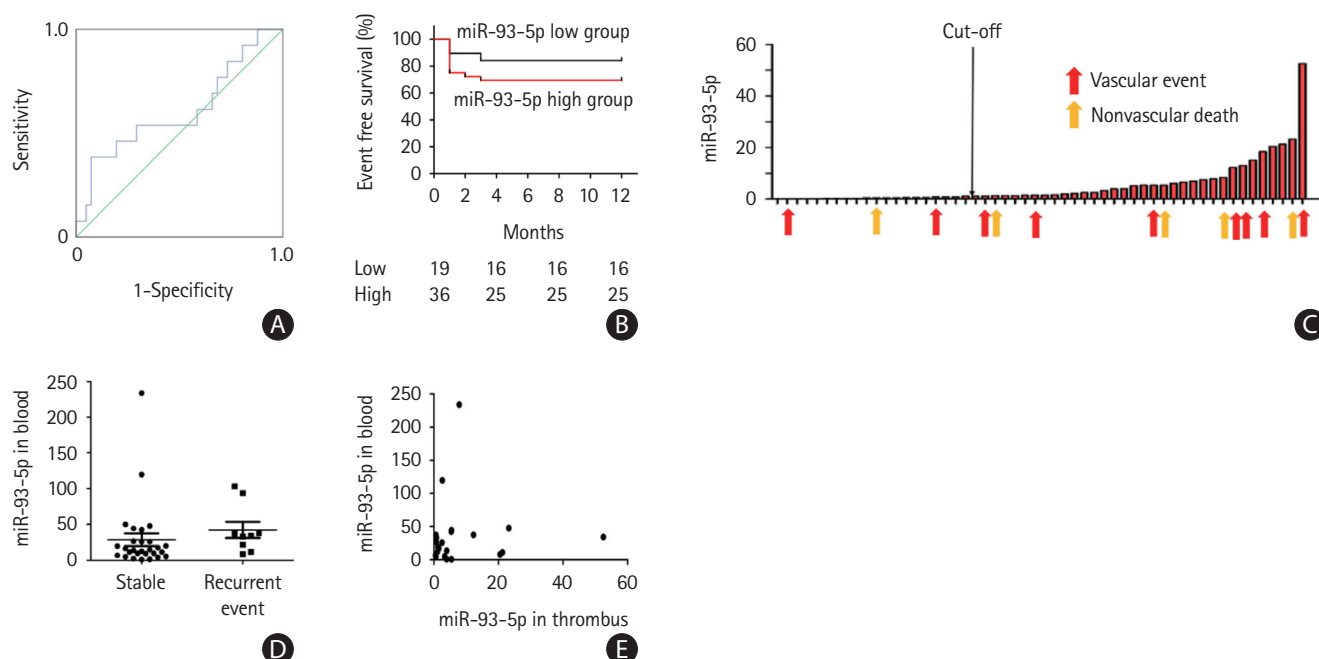
NIHSS, National Institutes of Health Stroke Scale.

Supplementary Table 4. Comparison of clinical and laboratory variables between the patients with and without vascular events from Cerebral Atherosclerosis Research with Positron Emission Tomography (CARPET) cohort

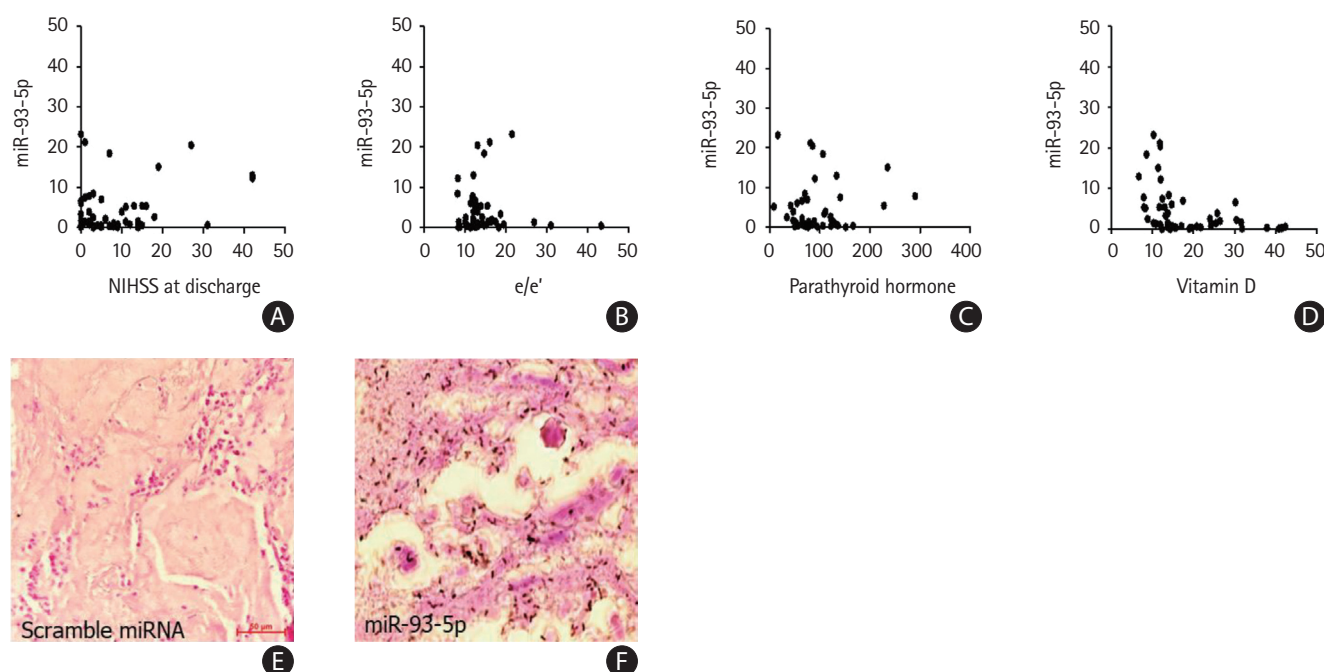
	Patients without recurrence (n=63)	Patients with recurrence (n=7)	P
Age (yr)	71.6 (10.0)	66.1 (12.8)	0.19
Female sex, n (%)	28 (44.4)	1 (14.3)	0.23
Ln (miR-17-5p) (relative expression)	2.21 (1.19)	3.24 (1.68)	<0.05
Ln (miR-93-5p) (relative expression)	2.33 (1.42)	3.60 (1.72)	0.04
Ln (let7i-5p) (relative expression)	3.88 (1.20)	4.43 (1.11)	0.25
Ln (miR-450b-5p) (relative expression)	3.67 (5.33)	4.42 (2.36)	0.14
Ln (miR-629-5p) (relative expression)	2.26 (1.81)	3.38 (0.76)	0.11

Values are presented as mean (standard deviation) unless otherwise indicated. Ln (miR) stands for the natural logarithmic transformation of the relative expression level of each microRNA, which is derived by calculating $2^{-\Delta Ct}$ with the ratio between the microRNA and RNA, U6 small nuclear 2 as a reference from the standard curve.

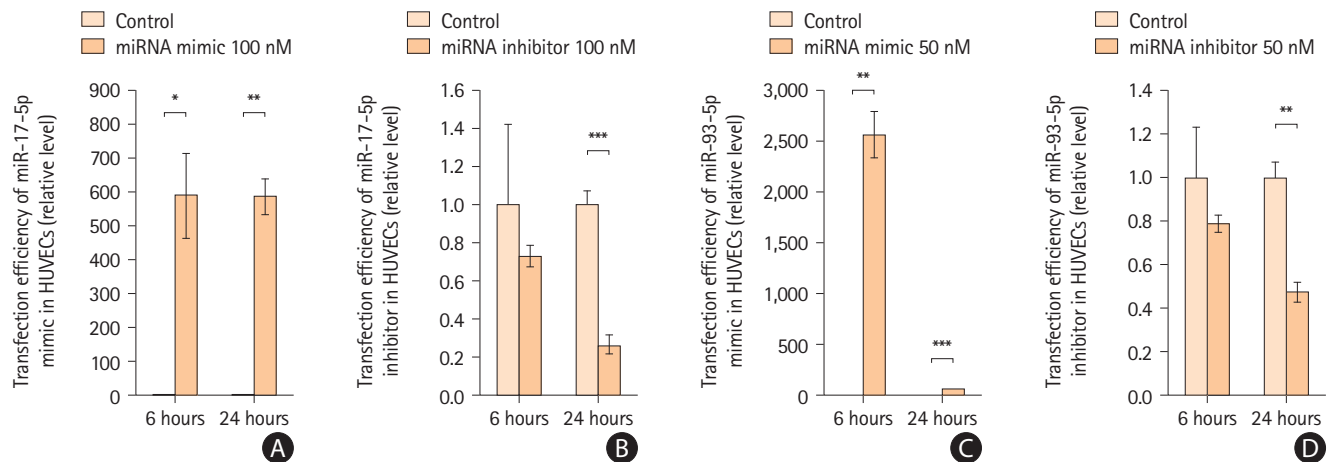
**Supplementary Figure 1.** Schematic diagram of the study. (A) The patients who received endovascular thrombectomy were eligible for inclusion in the Thrombus Analysis of Ischemic Stroke (TAOIST) cohort. A total of 79 patients with thrombus were included after excluding 24 patients without sufficient thrombus or microRNA. (B) The prognostic significance of derived thrombus from the TAOIST cohort was validated from 70 blood samples from another cohort—Cerebral Atherosclerosis Research with Positron Emission Tomography (CARPET)—which applied 18F-fluorodeoxyglucose positron emission tomography to understand atherosclerosis pathophysiology after stroke by including 110 acute ischemic stroke patients. RT-PCR, real-time polymerase chain reaction.



Supplementary Figure 2. Prognostic significance of miR-93-5p expression from thrombus. (A) The receiver operating characteristic curve analysis predicting vascular event after stroke with miR-93-5p derived an insignificant result. (B) The Kaplan-Meier survival curve analysis demonstrated a significant difference between the patients with lower (black line) and higher miR-93-5p expression (red line) from thrombus. (C) The waterfall plot shows that the vascular event and death are prevalent among the patients with higher miR-93-5p levels than the cut-off point. (D) The blood sample at the time of endovascular thrombectomy analysis shows elevated tendency of miR-93-5p expression among the patients with vascular event than those with stable prognosis. (E) However, there was no correlation between the miR-93-5p levels in the blood and those in the thrombus.



Supplementary Figure 3. Correlation analysis of altered microRNAs and laboratory variables, and immunohistochemistry of miR-93-5p. Correlation analysis between microRNAs and laboratory variables disclosed that miR-93-5p showed significant correlation with neurological severity as NIHSS at discharge (A), e/e' (B), and serum parathyroid hormone (C), and negative association with vitamin D levels (D). The immunohistochemistry showed that, in comparison with scrambled RNA (E), miR-93-5p is abundantly expressed in thrombi (F). NIHSS, National Institutes of Health Stroke Scale.



Supplementary Figure 4. MicroRNA modulation effect following the application of microRNA mimics and antagonistic sequence. The application of mimics (A) or antagonistic sequence (B) of miR-17-5p significantly altered the miR-17-5p expression in HUVEC. The application of mimics (C) or antagonistic sequence (D) of miR-93-5p also efficiently changed the expression of miR-93-5p in HUVEC. HUVEC, human umbilical venous endothelial cells. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.