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Relationship Between MicroRNA Signature and Arterial Stiffness in Patients With Ischemic Stroke

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Background and Purpose We investigated whether circulating microRNAs (miRNAs) is associated with arterial stiffness in patients with acute ischemic stroke.

Methods We recruited patients with acute ischemic stroke who were admitted to a university hospital stroke center and underwent carotid-femoral pulse wave velocity (cfPWV) measurement using SphygmoCor (AtCor Medical, Sydney, Australia) and brachial-ankle PWV using a volume-plethysmography device (VP-1000, Omron Colin, Komaki, Japan). Circulating miRNAs were measured in venous blood samples stored in EDTA. We selected five miRNAs (miR-17, miR-93, miR-450, miR-629, and let-7i) related to atherosclerosis based on a literature review. Pearson's correlation analysis was applied to the correlations between miRNAs and arterial stiffness parameters. Finally, multivariable linear regression analysis was performed to identify the independent factors for cfPWV.

Results This study included 70 patients (age=71.1±10.3 years [mean±SD], 29 females). The expression levels of miR-93 (r=-0.27, p=0.049) and let-7i (r=-0.27, p=0.039) were inversely correlated with cfPWV. Multivariable linear regression analysis including age, hypertension, and estimated glomerular filtration rate showed that let-7i was independently related with cfPWV (standardized coefficient=-0.262, p=0.036). Correlation analysis indicated that let-7i was positively associated with visceral muscle Hounsfield units on computed tomography (r=0.264, p=0.043).

Conclusions The expression level of let-7i was independently related to arterial stiffness in patients with cerebral infarction, suggesting that it plays a pathophysiological role in atherosclerosis.

Keywords microRNAs; arterial stiffness; acute ischemic stroke.

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INTRODUCTION

The aging society has resulted in stroke becoming one of the most common causes of death and long-term neurological disability in adults.[1] Atherosclerosis is a principle mechanism of stroke, and various studies have been conducted to develop blood biomarkers of ischemic stroke based on atherosclerosis pathophysiology.[2] Arterial stiffness is the hallmark of the aging process involving decreased elasticity, and is the consequence of many diseases such as atherosclerosis, diabetes mellitus (DM), and chronic renal disorder.[3] Arterial stiffness is correlated with conditions associated with an increased cardiovascular disease risk, including hypertension,[4] heart failure,[5] coronary artery disease,[6] and atrial fibrillation.[7] Several recent studies have found an association between arterial stiffness and stroke.[8-10]

MicroRNAs are small noncoding RNAs consisting of approximately 22 nucleotides that interact with multiple target messenger RNAs, and they play an important role in posttranscriptional gene regulation and function.[11] There is now considerable evidence for the role of circulating microRNAs in arteriosclerosis.[12-14] Studies of patients with ischemic stroke

found that several microRNAs were elevated among the patients with unstable atherosclerosis,[14] and that let-7i was related to the blood markers of systemic inflammation.[15] Another study found that miR-93 regulated the expression of vascular endothelial growth factor (VEGF), which is related to endothelial integrity and renal microvascular complication following hyperglycemia.[16]

We hypothesized that circulating microRNAs are associated with arterial stiffness in patients with acute ischemic stroke and that their expression levels reflect the degree of atherosclerosis. We selected five microRNAs related to atherosclerosis through a literature review and evaluated expression levels in patients with acute ischemic stroke.

METHODS

Patient inclusion

The present study was a post-hoc analysis of the Cerebral Atherosclerosis Research with Positron Emission Tomography (CARPET) study that applied whole-body 18F-fluorodeoxyglucose (FDG) PET to patients with cerebral infarction or transient ischemic attack with the aim of understanding the pathophysiology of cerebral atherosclerosis. The study was reviewed and approved by the Institution Review Board of Chung-Ang University Hospital (IRB No. C2015061), and written informed consent was obtained from each patient. We prospectively included patients with stroke and carotid atherosclerosis of ≥50% on brain computed tomography (CT) angiography, but excluded patients with overt cancer or autoimmune diseases, advanced renal impairment with an estimated glomerular filtration rate (eGFR) <30 mL/min/1.73 m^2, uncontrolled DM, or other unstable medical conditions (Fig. 1). Among the 110 included participants, blood sample analysis was finally performed on 70 patients. We obtained basic demographic data and the clinical history including vascular risk factors such as hypertension, DM, and smoking, and laboratory test results. When a patient had been stabilized after their index event, FDG PET, blood test for microRNA expression analysis, and arterial stiffness evaluation by measuring brachial-ankle and carotid-femoral pulse wave velocity (baPWV and cfPWV, respectively) were performed. Brain imaging and whole-body CT data were analyzed to understand the relationships between arterial stiffness and microRNA expression levels.

Recent studies found that the functional role of microRNAs was related to metabolic homeostasis in muscle and fat tissue.[17,18] To understand the impact of muscle and fat tissue on circulating microRNAs, we measured their quantitative and qualitative indexes at the third lumbar vertebra level as described previously.[19,20] Each area of muscle and fat was measured after drawing the region of interest by tracing along the anatomical border, and muscle and fat indexes were calculated by dividing the area by the height in meters squared for each patient. Muscle and fat Hounsfield units (HU) were also derived as the mean attenuation in each region of interest.

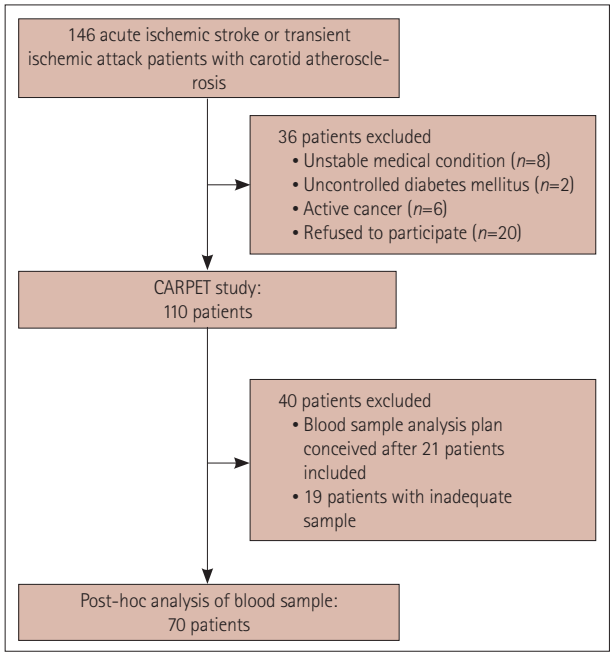

Fig. 1. Flowchart of the included patients. CARPET, cerebral atherosclerosis research with positron emission tomography.

Arterial stiffness analysis

When a patient had been stabilized after their stroke, arterial stiffness was assessed using a cfPWV measurement (SphygmoCor, AtCor Medical, Sydney, Australia). The measurement was performed with the patient in a supine position after 10 min of rest. The tonometer was applied to the right common carotid and femoral arteries to measure the pulse-wave travel time (t). The direct straight distance between two measurements sites was measured using a tape measure (d), and cfPWV was then calculated using PWV=0.8 d/t (m/s) as proposed recently.[21] For the sensitivity analyses, baPWV was also obtained using a volume-plethysmography device (VP-1000, Omron Colin, Komaki, Japan) with the patient in a supine position as reported previously.[22] The pulse pressure was derived as the difference between the systolic and diastolic blood pressures. Patients also underwent transcranial Doppler (TCD) sonography to measure the pulsatility index (PI, calculated as [peak systolic flow velocity minus peak diastolic flow velocity]/[mean flow velocity]) of the basilar artery (BA). We measured PI in the BA because the middle cerebral artery (MCA) was not detected in 21 patients with poor temporal windows. The TCD sonography was performed using a 2-MHz pulsed-wave

and range-gated TCD probe (Digital PMD100 or ST3 Digital PMD150, Spencer Technologies, Redmond, WA, USA) with a 9-mm sample volume and 100-mW/cm^2 transmit power in the supine position.

MicroRNA sample end expression levels

A 5-mL venous blood sample was obtained in an EDTA bottle from each patient when performing whole-body FDG PET. Plasma samples were immediately extracted after centrifugation at 1,500×g for 15 min at 4°C and stored at -70°C until they were analyzed. Five microRNAs related to atherosclerosis pathophysiology were selected based on an extensive literature review. The primer sequence, potential targets, and previous literature of the selected microRNAs are listed in Table 1. The microRNAs were extracted using a commercial kit (QIAGEN GmbH, Hilden, Germany) after centrifuging the serum at 1,000×g for 10 min as reported previously.[14] All samples were examined three times, and the relative amount of the target microRNA was determined by calculating $2^{-\Delta Ct}$ with the ratio between the microRNA and RNA, with U6 small nuclear RNA as a reference concentration derived from the standard curve.

Statistical analysis

Continuous values are expressed as mean±SD values, and categorical values are expressed as numbers of the patients with percentages. Continuous values were tested for normality using the Shapiro-Wilk test. We first analyzed the correlations between circulating microRNA levels and laboratory variables including arterial stiffness using Pearson's correlation analysis. Simple linear regression analysis was performed to assess the associations between cfPWV and microRNA levels as well as clinical and laboratory variables including eGFR using the Modification of Diet in Renal Disease equation. Multiple linear regression analysis was performed to determine whether circulating microRNAs were independently related to arterial stiffness by including biologically plausible variables for which $p<0.10$ in simple linear regression.

We also performed mediation analyses to further understand the interactions between circulating microRNAs and muscle and arterial stiffnesses using the Hayes macro-application "PROCESS" for SPSS.[23] We applied model 4 of the Hayes macro function to examine the total, direct, and indirect effects of microRNAs on cfPWV with muscle HU as a mediator (model A). The path coefficients were derived from the beta coefficients (β values) of the multivariable regression models and represented the magnitude of the associations among variables. The total β effect represents the effect of microRNA on cfPWV when no other mediator was included in the model, while the direct β effect represents the effect of microRNA on cfPWV when muscle HU was included as a mediator. Indirect effects represent the effect of microRNA on PWV through muscle HU. If an indirect effect was significant, it can be concluded that mediation occurred, with the significance tested using bootstrapped 95% confidence intervals (CIs). We also performed a mediation analysis to examine the total, direct, and indirect effects of muscle HU on cfPWV with microRNA as a mediator (model B).

All of the statistical analyses were performed using SPSS (version 22.0, IBM Corp., Armonk, NY, USA), and significance was accepted at $p<0.05$.

RESULTS

This study included 70 patients who experienced an acute ischemic stroke or transient ischemic attack between November 1, 2016 and May 31, 2019 (age=71.1±10.3 years, 29 females). Baseline demographic and clinical characteristics are listed in Table 2. We found that age, hypertension, DM, and eGFR were significantly related to cfPWV (Table 3). We also found that let-7i-5p (r=-0.302, p=0.025) (Fig. 2A) and miR-93-5p (r=-0.266, p=0.049) (Fig. 2B) were negatively correlated with cfPWV among the five analyzed microRNAs (Table 3). The sensitivity analysis demonstrated that let-7i was negatively correlated with baPWV (r=-0.302, p=0.025) (Fig. 2C), but there was no significant relationship between miR-93-5p and

Table 1. The sequences and potential roles of selected microRNAs

Name	Location in chromosome	Sequence	Potential target gene
Let-7i-5p	Chr12: 62997471–62997492	UGAGGUAGUAGUUUGUGCUGUU	Decreased in patients with stroke and predicted to regulate leukocyte responses[15]
miR-17-5p	Chr13: 91350605-91350688	CAAAGUGCUUACAGUGCAGGUAG	miR-17-5p elevated in patients with stroke and unstable plaque, and was related to stroke recurrence[14]
miR-93-5p	Chr7: 99691438–99691460	CAAAGUGCUGUUCGUGCAGGUAG	Elevated in the thrombi of patients with stroke and neurological deterioration[42]
miR-450-5p	ChrX: 133674261–133674282	UUUUGCAAUAUGUUCCUGAAUA	Elevated in an animal model of transient ischemic attack[43]
miR-629-5p	Chr15: 70371766–70371786	UGGGUUUACGUUGGGAGAACU	Promoted the invasion of lung cancer by increasing cell invasion and endothelial cell permeability[44]

Table 2. Demographic and clinical characteristics of the included patients (*n*=70)

Characteristic	Value
Age, years	71.1±10.3
Sex, female	29 (41.4)
Body mass index, kg/m^2	23.1±3.3
Stroke subtypes	
Large-artery atherosclerosis	28 (40.0)
Cardioembolism	15 (21.4)
Small-vessel occlusion	3 (4.3)
Others or unknown	16 (22.9)
Transient ischemic attack	8 (11.4)
Hypertension	56 (80.0)
Diabetes mellitus	32 (45.7)
Current smoker	17 (24.3)
Initial NIHSS score	6.7±7.5
Pulse pressure, mm Hg	68.5±20.0
White blood cell count, ×10^9/L	7.6±2.5
Hemoglobin, g/dL	13.2±1.8
Platelets, ×10^9/L	230.7±58.2
LDL cholesterol, mg/dL	90.0±29.5
HbA1c, %	6.4±1.2
High-sensitivity CRP, mg/L	6.8±18.1
eGFR, mg/min/1.73m^2	89.4±27.2
Carotid-femoral PWV, m/s	13.0±2.1
Brachial-ankle PWV, m/s	2.1±0.6
PI in the BA	1.1±0.2
Ejection fraction, %	62.6±6.5
E/e' ratio	12.7±3.1

Data are mean±SD or *n* (%) values.
BA, basilar artery; CRP, C-reative protein; E, early diastolic mitral inflow velocity; e', early diastolic mitral annulus velocity; eGFR, estimated glomerular filtration rate; HbA1c, glycated hemoglobin; LDL, low-density lipoprotein; NIHSS, National Institude of Health Stroke Scale; PI, pulsatility index; PWV, pulse wave velocity.

Table 3. Results of bivariable linear regression analysis of factors related to the carotid-femoral pulse wave velocity

	Coefficient (β)	Standard error	*p*
Age	0.076	0.023	0.002
Sex, female	0.435	0.526	0.411
Hypertension	1.327	0.626	0.038
Diabetes mellitus	0.697	0.520	0.184
eGFR	-0.021	0.009	0.025
Body mass index	-0.080	0.081	0.327
Let-7i	-0.005	0.003	0.039
miR-93-5p	-0.005	0.004	0.049
miR-17-5p	-0.010	0.007	0.161
miR-450b	0.022	0.063	0.728
miR-629-5p	-0.026	0.014	0.066

eGFR, estimated glomerular filtration rate.

baPWV (*r*=-0.155, *p*=0.248) (Fig. 2D). Multivariable linear regression analysis adjusted for age, hypertension, and eGFR revealed an independent relationship between let-7i and cfPWV (β=-0.262, *p*=0.036) (Table 4). The relationship between miR-93-5p and cfPWV did not remain independent after the multivariable linear regression analysis was adjusted for age, hypertension, and eGFR (β=-0.088, *p*=0.512).

We also analyzed whether let-7i was related to cardiac dysfunction or intracranial arterial stiffness. Let-7i was not significantly associated with the cardiac ejection fraction on echocardiography (*r*=0.056, *p*=0.673) (Fig. 3A) or the PI in the BA (*r*=0.037, *p*=0.782) (Fig. 3B). Circulating let-7i levels were positively correlated with abdominal muscle HU (*r*=0.264, *p*=0.043) (Fig. 3C), but they were not significantly associated with muscle index (*r*=0.165, *p*=0.212) (Fig. 3D), subcutaneous fat index (*r*=0.162, *p*=0.222) (Fig. 3E), visceral fat index (*r*=0.012, *p*=0.929) (Fig. 3F), or visceral HU (*r*=-0.064, *p*=0.627).

We performed mediation analyses to further explore the relationships among microRNA, muscle HU, and cfPWV (Fig. 4). Model A indicated that the total effect of let-7i on PWV was not mediated by muscle HU, although a positive association was found between let-7i and muscle HU (β=0.1250, *p*=0.031) (Fig. 4A). Bootstrapping analysis indicated a nonsignificant indirect effect of let-7i on cfPWV via muscle HU (β=0.0004, 95% CI=-0.0015 to 0.0020). Model A indicated a significant direct effect of let-7i on cfPWV (β=-0.0055, *p*=0.034). Model B indicated nonsignificant total and direct effects of muscle HU on cfPWV (β=-0.0094 and *p*=0.874, and β=0.0029 and *p*=0.630, respectively), but a significant indirect effect of muscle HU on cfPWV via let-7i (β=-0.0382, 95% CI=-0.0919 to -0.0013) (Fig. 4B).

DISCUSSION

Circulating microRNAs were associated with systemic arterial stiffness in patients with stroke. The negative correlation between let-7i and cfPWV remained significant after adjusting for clinical variables. These findings suggest that there is a pathophysiological link between let-7i and arterial stiffness in patients with ischemic stroke, possibly mediated by the muscular system.

This study found that the level of circulating let-7i was negatively correlated with PWV using two different measurement techniques in patients with cerebrovascular disease. Arterial walls thicken and stiffen due to the loss of elastic tissue and fibrosis, which are caused by age, hypertension, and atherosclerosis.[3,24,25] The most widely used modality for arterial stiffness assessment is PWV, which reflects arterial elasticity. Both cfPWV and baPWV have been found to be significantly associated with various vascular risk factors and are frequently

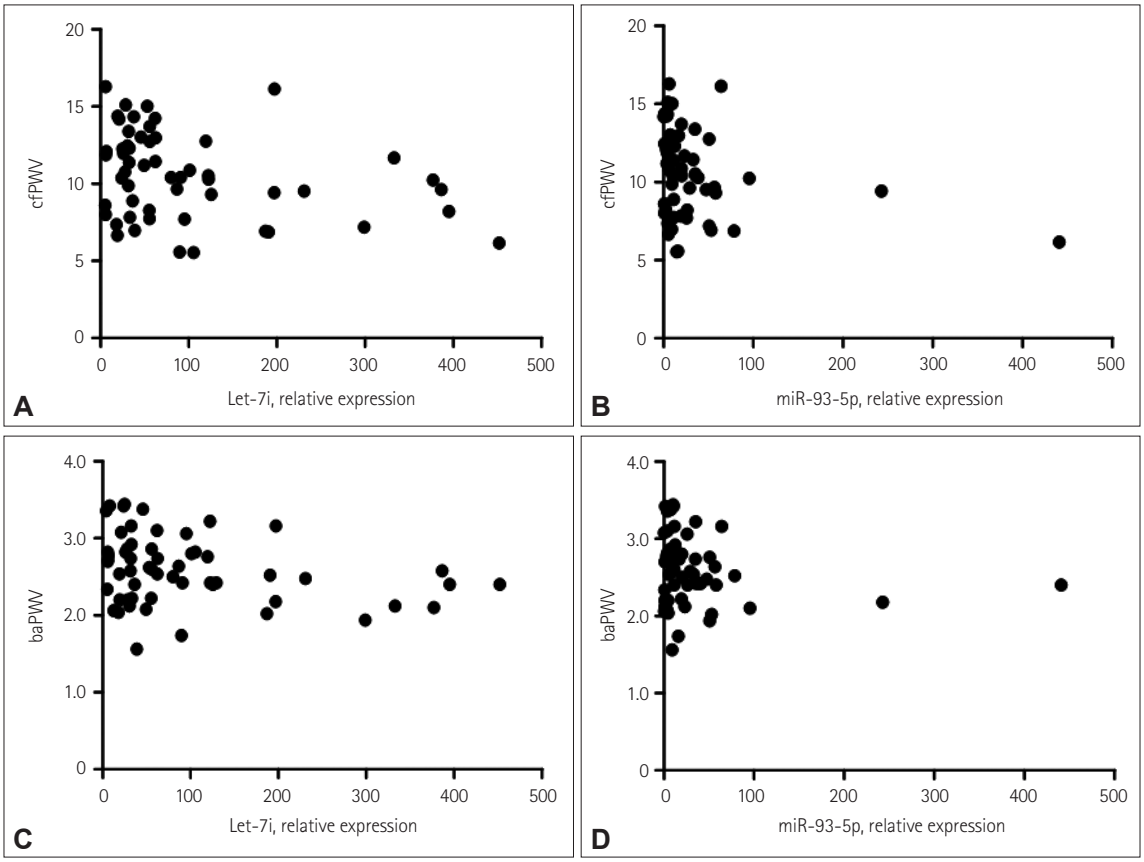

Fig. 2. Correlation between microRNAs and pulse wave velocities. A-D: The correlation analysis demonstrated that the cfPWV was negatively associated with let-7i (r=-0.302, p=0.025; A) and miR-93-5p (r=-0.266, p=0.049; B). The association between the baPWV and let-7i was also significant (r=-0.302, p=0.025; C). There was no significant relationship between miR-93-5p and baPWV (r=-0.155, p=0.248; D). baPWV, brachial-ankle PWV; cfPWV, carotid-femoral PWV; PWV, pulse wave velocity.

Table 4. Results of multivariable linear regression analysis with carotid-femoral pulse wave velocity as a dependent variable

	Coefficient (β)	Standard error	*p*
Age	0.065	0.030	0.032
Hypertension	0.530	0.708	0.458
eGFR	-0.013	2.647	0.360
Let-7i	-1.447	0.621	0.036

eGFR, estimated glomerular filtration rate.

used in clinical practice.[22] We chose cfPWV as the standard for arterial stiffness in our study because baPWV includes arterial stiffness of the distal lower extremities, which is not related to the cerebral vasculature and might greatly underestimate the arterial compliance of patients with stroke.[26] Elevated arterial stiffness induces an increase in systolic blood pressure and decrease in diastolic blood pressure, which results in structural changes in the arterial wall, such as increased collagen deposition and vessel wall calcification.[27,28] Increased arterial pulse pressure induces blood–brain barrier disruption and endothelial dysfunction, leading to cerebral arterial atherosclerosis and small arteriolar degeneration.[28,29] The let-7 family, discovered in *Caenorhabditis elegans*, has been known to regulate developmental milestones and cancer formation.[30,31] The human let-7 family contains 13 members among 9 chromosomes, including let-7a (encoded by 3 genes), let-7b, let-7c, let-7d, let-7e, let-7f (encoded by 2 genes), let-7g, let-7i, miR-98, and miR-202.[32] *In vivo* and *in vitro* studies found that decreased let-7 expression affected the expressions of multiple proinflammatory genes associated with plaque formation.[33] Among patients with coronary artery disease, let-7i-5p expression levels were negatively correlated with TLR4 expression, which leads to heart failure.[34]

Muscle HU is a marker of overall muscle density, and aging, obesity, and DM are associated with decreased muscle HU, whereas endurance training is related to increased muscle HU.[35] Regular exercise training is associated with antiatherogenic adaptation in vascular function and structure, irrespective of traditional vascular risk factors.[36] Short-term aerobic exercise can reduce arterial stiffness in older adults with DM.[37] Circulating factors such as myokines or microRNAs may orchestrate or reflect the interaction between the vascular and muscular systems.[36] The positive association between let-7i

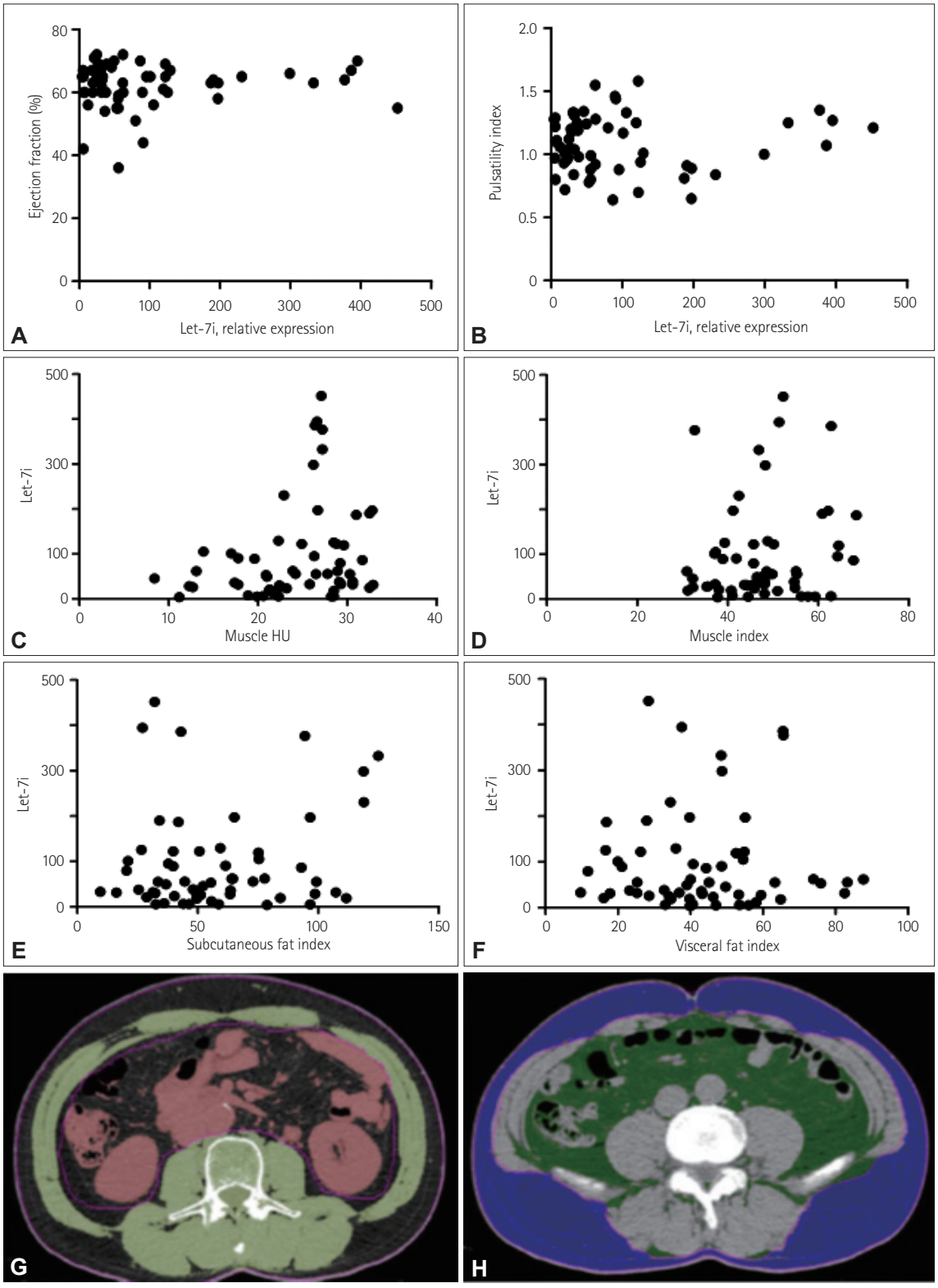

Fig. 3. Correlation between let-7i and potential factors related to arterial stiffness. A-H: The let-7i expression levels were not significantly associated with the cardiac ejection fraction (r=0.0562, p=0.673; A) or the pulsatility index in the basilar artery (r=0.0374, p=0.782; B). Representative CT images for the measurements of muscle and fat parameters showed that the abdominal muscle index and HU were derived at the third lumbar level (yellowish green, G), and subcutaneous (blue, H) and visceral (green, H) fat indexes were derived separately. The circulating let-7i levels were significantly associated with the abdominal muscle HU (r=0.264, p=0.043; C), but not with the muscle index (r=0.165, p=0.212; D) or the subcutaneous (r=0.162, p=0.222; E) or visceral (r= 0.012, p=0.929; F) fat indexes. HU, Hounsfield units.

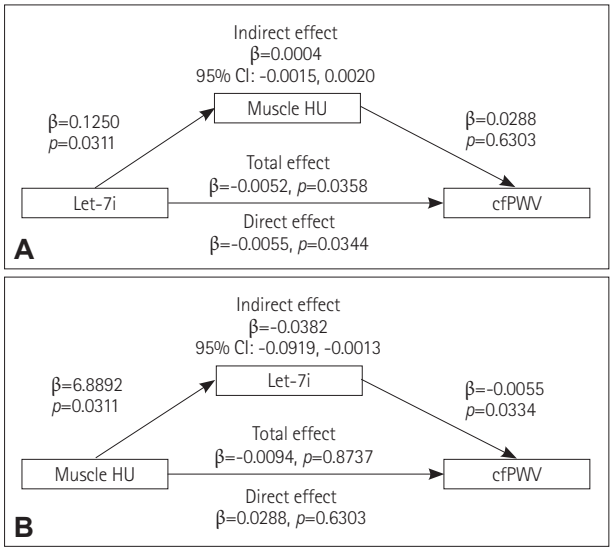

Fig. 4. Mediation analyses of the associations among let-7i, muscle HU, and cfPWV. A: Model A with muscle HU as a mediator (r^2=0.240, p=0.013) indicated that the direct effect of let-7i on cfPWV was significant (β=-0.0055, p=0.034) and the indirect effect via muscle HU was not significant (β=0.0004, 95% CI=-0.0015–0.0020). B: Model B with let-7i as a mediator (r^2=0.436, p<0.001) indicated that the direct effect of muscle HU on cfPWV was not significant (β=0.0288, p=0.630) and that the indirect effect via let-7i was significant (β=-0.0382, 95% CI=-0.0919–-0.0013). Each model was adjusted for age, hypertension, and eGFR. cfPWV, carotid-femoral pulse wave velocity; CI, confidence interval; eGFR, estimated glomerular filtration rate; HU, Hounsfield units.

and muscle HU suggested that muscle quality or its metabolic system are closely related to arterial elasticity. Previous studies found that the let-7 family were potent regulators of glucose metabolism and were associated with insulin resistance.[38] Our mediation analysis indicated that let-7i is an important factor in arterial stiffness, independent of a muscle integrity image marker. Future studies are warranted to understand the mechanistic role of let-7i on muscular integrity and arterial elasticity.

The miR-93-5p level was also negatively correlated with PWV in patients with stroke. Studies on various cancer cells indicated that miR-93 expression was associated with inflammation and oxidative stress.[39,40] Those studies found that miR-93-5p was associated with elevation of inflammatory cytokine levels including IL-8 and VEGF, and with inhibition of the NRF2 pathway, and is an important factor in the mechanism of cellular defense against oxidative stress.[40] Another study of patients with coronary artery disease found that miR-93-5p was correlated with serum cholesterol level, but negatively correlated with ABCA1, suggesting that miR-93-5p regulates ABCA1 expression by targeting 3'UTR.[41] A recent study that focused on the microRNA expression patterns in the thrombi of patients with stroke found that elevated miR-93-5p in the thrombus was related to neurological deterioration after a stroke.[42] However, differences in the sources, sampling times, and quantification references for microRNA may affect the relationship between the expression signature of microRNAs and laboratory data.

This study had several limitations. First, all of the included patients were recruited from a single university hospital and the sample was small. Second, we selected five microRNAs through a literature review, rather than through an unbiased analysis. Third, the multiple-comparisons problem may have occurred in the study results because a post-hoc analysis was performed with multiple variables. However, we applied two different measurement techniques for arterial stiffness (cfPWV and baPWV), and the derived results seem to be biologically plausible considering previous findings.

Circulating let-7i was negatively associated with arterial stiffness and positively associated with muscle density in patients with stroke. Whether its expression levels could be a biomarker or therapeutic target of arterial stiffness requires future investigations.

Availability of Data and Material

The datasets generated or analyzed during the study are available from the corresponding author on reasonable request.

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Conflicts of Interest

The authors have no potential conflicts of interest to disclose.

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