Plasma miR-126 and miR-143 as Potential Novel Biomarkers for Cerebral Atherosclerosis

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Background: Cerebral atherosclerosis is the most important mechanism for ischemic stroke. However, specific plasma biomarkers to assess atherosclerosis susceptibility are still lacking. Circulating miRNAs have been shown to be promising biomarkers for various pathologic conditions. We investigated whether plasma miR-126 and miR-143 could be used as biomarkers for identifying and evaluating cerebral atherosclerosis. Results showed that miR-143 and miR-126 might participate in the process of atherosclerosis and were minimally affected by cerebral infarction. Using Pearson correlation analysis, we showed that miR-126 and miR-143 were correlated with the presence and severity of cerebral atherosclerosis. The ability of miR-126 and miR-143 to differentiate atherosclerosis patients from healthy controls was demonstrated via a receiving operating characteristic curve with high specificity and sensitivity. Our data thus indicate that miR-126 and miR-143 may be potential specific biomarkers for atherosclerosis.

Keywords: Circulating miRNA—atherosclerosis—LAA stroke—biomarker

Introduction

MicroRNAs (miRNAs) are endogenous, noncoding single-stranded RNAs that regulate gene expression by recognizing binding sites located in the 3′ untranslated region (3′ U TR) of mRNA targets.[1-3] MiRNAs participate in a variety of essential biological processes, including proliferation, differentiation, apoptosis, necrosis, autophagy, development, and aging.[4,5] Circulating miRNAs have recently emerged as potential biomarkers for various diseases, such as colorectal cancer,[6] myocardial infarction,[7,8] and acute myeloblastic leukemia.[9]

Evidence increasingly points to miRNAs participating in the physiological and pathological processes of atherosclerosis and atherosclerotic thrombotic cerebral infarction.[10-12] MiR-126 is among the most studied miRNAs in vascular biology. It negatively regulates the vascular endothelial growth factor pathway and plays an important role in maintaining vascular integrity, angiogenesis, and wound repair. Recent studies have shown that endothelial miR-126-5p maintained a proliferative reserve in endothelial cells through suppression of the Notch1 inhibitor delta-like 1 homolog (Dlk1), thereby preventing atherosclerotic lesion formation. MiR-143, meanwhile, targets a network of transcription factors, including Klf4, myocardin, and Elk-1 to promote differentiation and repress proliferation of smooth muscle cells.[13]

Abbreviations: AML, acute myeloblastic leukemia; AUC, area under the ROC curve; EC, endothelial cells; miRNA, MicroRNA; MVs, micro vesicles; ROC, receiving operating characteristic; TNF-α, tumor necrosis factor-α; VCAM-1, vascular cell adhesion molecule-1; VEGF, vascular endothelial growth factor

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Ischemic stroke (IS) is characterized by high mortality and morbidity. Eighty percent of IS caused by arterial occlusion or stenosis initiated by atherosclerosis.[14] Atherosclerosis is a condition caused by hyperlipidemia-induced chronic inflammation of the vessel wall orchestrated by a complex interplay of various cell types, such as endothelial cells, smooth muscle cells, and macrophages which miR-126 and miR-143 are involved in as mentioned above. However, the diagnosis of cerebral atherosclerosis depends mainly on neuroimaging techniques and lack of circulating biomarkers. To date, no studies have addressed the relationship between cerebral atherosclerosis and miRNAs. The aims of this study are hence to assess whether plasma miR-126 and miR-143 can be used as biomarkers for identifying and evaluating cerebral atherosclerosis.

Materials and Methods

Participants

(1) *Large-artery atherosclerosis group (LAA group).* LAA stroke patients from the Trial of Org 10172 in Acute Stroke Treatment (TOAST)[15] classification admitted to the Department of Neurology at Medical College of Qingdao University from January 2013 to October 2013 were enrolled in the study (n = 57; 37 men and 20 women; mean age = 60.5 ± 10.1 years). The presence of cerebral atherosclerosis was defined as when stenosis was significant (≥50%). The severity of cerebral atherosclerosis was determined based on the number of arteries with cerebral atherosclerosis. All patients underwent CT and/or MRI, TCD or brain ultrasound, brain MRA or CT angiography (CTA) or DSA, cardiac ultrasound, and other tests to identify cerebrovascular and heart disease conditions. Those suffering from cardioembolism stroke with either determined or undetermined etiology, severe heart disease and recent myocardial infarction or angina pectoris disorders, severe infections, severe nephrosis or liver disease, thrombotic diseases or tumors were excluded from the study.

(2) *Atherosclerosis (AS) group and control group.* AS group (n = 41; 18 men and 23 women; mean age = 59.2 ± 11.1 years) and control group (n = 50; 26 men and 24 women; mean age = 61.3 ± 12.4 years) patients were given a health check at the same time and in the same place as the LAA group. Brain CT or MRI was used to confirm that the patients were free from any previous history of stroke. Atherosclerosis and angiostenosis were assessed through examination of cerebrovascular TCD/MRA/CTA. Patients with cerebral atherosclerosis and vascular stenosis greater than or equal to 50% were included in the AS group; otherwise, they were included in the control group. Exclusion criteria were the same as for the LAA group.

The severity of cerebral atherosclerosis was determined based on the number of arteries with atherosclerotic stenosis (≥50%) or occlusion. Both the LAA and AS groups were hence further divided into 3 subgroups (single, double, and multiple arteries with cerebral atherosclerosis).

The study was approved by the hospital's Ethics Committee, and informed consent was obtained from all participants.

Blood Samples

Blood samples for the 3 groups were collected via venipuncture in the early morning following admission. The samples were stored in tubes containing sodium EDTA, centrifuged at 3000 r/min for 10 minutes at room temperature. The plasma was carefully transferred into Eppendorf tubes and stored at −80°C until further processing.

RNA Purification, MiRNA qRT-PCR and Data Processing

Total RNA was extracted from 400 ul plasma using TRIzol LS Reagent (TAKARA, Dalian, China), as described previously.[7] Quantitative real-time PCR (qRT-PCR) was conducted using RotorGENE-3000 and a RotorGene6 real-time PCR detection system (CorbettResearch America) following the reverse transcription into cDNA, which was performed in triplicate using the One Step PrimeScript miRNA cDNA Synthesis Kit (Takara, Japan). All miRNA primers were purchased from Takara. MiRNA expression was normalized to small nucleolar RNA U6.

Relative expression of miRNAs was calculated using $2^{-\triangle\triangle Ct}$ method in duplicate experiments (change fold = $2^{-((\text{Mean } \triangle Ct \text{ Target}] - (\text{Mean } \triangle Ct \text{ Calibrator}),}$ $\triangle Ct = Ct^{Target} - Ct^{Calibtator}$).[16] A composited score (denoted as miRNA-score) was defined to represent the cumulative levels of the miRNA (miR-AS, miR-LAA) in the patients compared with the control group as described previously.[17] The miRNA-score of each sample was calculated as the sum of the inverted-normalized signals of the miRNA and adjusted by subtracting a constant (the minimal score) so that the range of scores starts at 0.The inverted-normalized signals were calculated for each miRNA by subtracting the normalized Ct from 50, so that high values represent high levels.

Statistical Analysis

Statistical analysis was performed using SPSS software for Windows (version 17.0). The relative expression of miRNAs was calculated using the $2^{-\triangle\triangle Ct}$ method in

Table 1. *Basic data of the participants in the study*

Characteristics	Total patients N = 148	Control group N = 50	AS group N = 41	LAA group N = 57	*P*
Age (years)	61.77 ± 9.94	59.86 ± 10.77	62.68 ± 8.92	62.79 ± 9.80	.249
Male/female (n/n)	108/40	36/14	29/12	43/14	.859
Smoking (n, %)	54 (36%)	12 (24%)	11 (27%)	31 (54%)	.003
Drinking (n,%)	52 (35%)	12 (24%)	10 (24%)	30 (53%)	.014
HBP (n,%)	89 (60%)	20 (40%)	26 (63%)	43 (75%)	.001
DM (n,%)	35 (24%)	5 (10%)	10 (24%)	20 (35%)	.010
CAD (n,%)	38 (26%)	11 (22%)	12 (29%)	15 (26%)	.725
HDL (mmol/L, $x\pm$ s)	1.16 ± .27	1.22 ± .28	1.22 ± .25	1.06 ± .24	.001
LDL (mmol/L, $x\pm$ s)	2.45 ± .73	2.50 ± .74	2.54 ± .70	2.35 ± .74	.381
TC (mmol/L, $x\pm$ s)	4.40 ± .99	4.15 ± .92	4.43 ± 1.08	4.67 ± .92	.022
TG (mmol/L, $x\pm$ s)	1.71 ± 1.19	2.00 ± 1.73	1.49 ± .77	1.63 ± .76	.117
hs-CRP (mg/L, $x\pm$ s)	9.5 ± 21.53	7.02 ± 23.73	4.42 ± 5.43	15.34 ± 25.38	.027

Abbreviation: CAD, coronary artery atherosclerosis disease; DM, diabetes mellitus; HBP, hypertension; HDL, high-density lipoprotein; hs-CRP, hypersensitivity C response protein; LDL, low-density lipoprotein; TC, total cholesterol; TG, total triglyceride.

duplicate experiments (change fold = $2^{-((\text{Mean } \triangle\text{Ct Target}) - (\text{Mean } \triangle\text{Ct Calibrator}),}$ $\triangle Ct = Ct^{Target} - Ct^{Calibtator}$).[16] A composited score (denoted as miRNA-score) was defined to represent the cumulative levels of miRNA (miR-AS, miR-LAA) compared with the control group, as described previously.[17] The miRNA-score of each sample was calculated as the sum of the inverted-normalized signals of the miRNA and adjusted by subtracting a constant (the minimal score) so that the range of scores started at 0. The ability to distinguish the cerebral atherosclerosis and control groups was characterized by the receiver operating characteristic (ROC) curve, and the area under the ROC curve (AUC) was calculated.

All miRNA values are expressed as mean ± SD. Comparisons of parameters among greater than or equal to 3 groups were analyzed by repeated measures ANOVA. Independent-sample *t* test was used for 2-group comparisons, and Chi-square test was used for categorical variables. A value of $P < .05$ was considered statistically significant.

Results

Basic Data

No significant differences ($P > .05$) were found for average age, gender composition, coronary disease, TG level, and LDL level among the 3 groups. The percentages of the LAA group and the AS group were significantly higher than those of the control group when hypertension, diabetes, smoking, and alcohol consumption were taken into account ($P < .05$). TC level and hs-CRP level were significantly higher in the LAA group than in the atherosclerosis and control groups ($P < .05$). The HDL level in the LAA group, meanwhile, was significantly lower than in the other 2 groups ($P < .05$; Table 1).

MiRNA Expression in Plasma Samples

After all the risk factors listed in the Table 1 (hypertension, smoking, alcohol consumption, diabetes, TC level, HDL level, and hs-CRP level) were adjusted using binary logistic regression analysis, the levels of miR-126-5p and miR-143-3p were significantly lower in the AS and LAA groups ($P < .05$) compared with the control group. The plasma levels of miR-126-5p and miR-143-3p, meanwhile, were higher in the LAA group than in the AS group, but the difference was not statistically significant ($P > .05$; Table 2).

Correlation of Plasma Levels of miR-126 and miR-143 with the Presence and Severity of Cerebral Atherosclerosis

According to the presence and severity of cerebral atherosclerosis, the LAA group and AS group were divided into 3 subgroups (single, double, and multiple arteries).

Table 2. *Plasma levels of miRNAs in 3 groups*

	Control group	AS group	LAA group	*P* value		
				Control-AS	Control-LAA	AS-LAA
miR-126 (△Ct ± SD)	3.88 ± .21	6.32 ± .27	6.04 ± .21	<.001*	.024†	.083
miR-143 (△Ct ± SD)	4.53 ± .19	6.79 ± .24	6.44 ± .22	<.001*	.034†	.076

Relative expression level of miRNAs is presented as △Ct ± SD. *P* values were calculated using independent-samples *t* test.
*$P < .001$.
†$P < .05$.

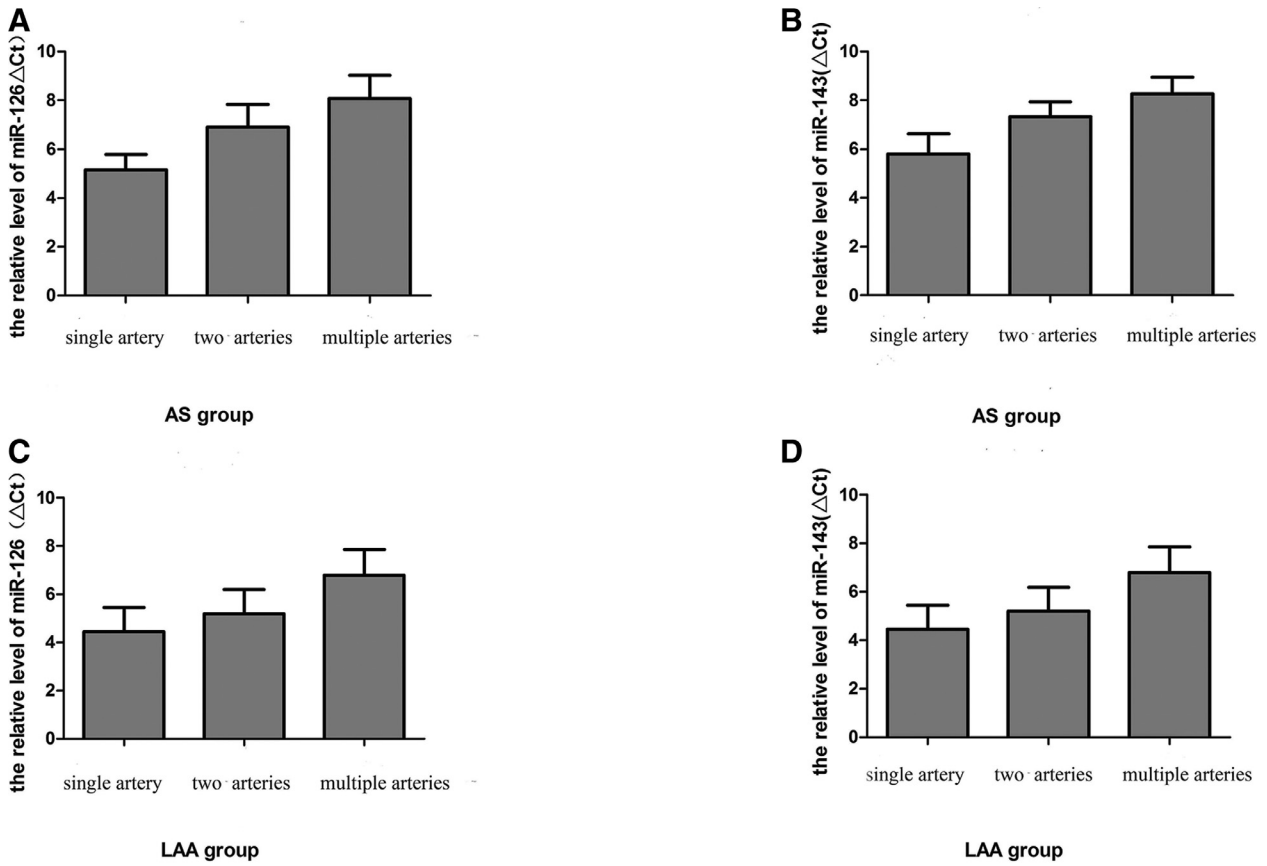

Figure 1. *Plasma levels of miR-126 and miR-143 in 3 subgroups of AS and LAA (△Ct). (A) Plasma levels of miR-126 in 3 subgroups of AS; (B) Plasma levels of miR-143 in 3 subgroups of AS; (C) Plasma levels of miR-126 in 3 subgroups of LAA; (D) Plasma levels of miR-143 in 3 subgroups of LAA.*

Plasma levels of miR-126 and miR-143 from these 3 subgroups were analyzed by ordinal logistic regression. Results showed that the levels of miR-126 and miR-143 were correlated with the severity of cerebral atherosclerosis (Fig 1 and Table 3).

Specificity and Sensitivity of miR-126 and miR-143 for Determining Cerebral Atherosclerosis

As the plasma levels of miR-126 and miR-143 might be influenced by both technical and biological variations, we combined the levels of miR-126 and miR-143 (including the LAA and AS groups) into a single score to provide an improved signal-to-noise ratio. The miR-126 and miR-143 scores represented the plasma levels of each. The ability of miR-126 and miR-143 to differentiate AS patients from the healthy controls was shown by the ROC curve, with AUCs of .859 (95% CI .792-.925, $P < .001$) and .866 (95% CI .805-.928, $P < .001$), respectively. By using threshold scores of 4.675 and 5.045, above which patients were placed in the AS group, we achieved sensitivity (85.6% and 86.7%) and specificity (73.3% and 68.9%) percentages for miR-126 and miR-143, respectively (Figs 2 and 3).

Discussion

Cerebral atherosclerosis is the most important mechanism of IS. However, specific plasma biomarkers to assess AS susceptibility are lacking. The discovery of new biomarkers for AS could be used to identify individuals at risk of IS, allowing for early intervention that may delay the development and progression of atherosclerosis.

Table 3. *Pearson correlation analysis of miR-126, miR-143, and cerebral atherosclerosis in LAA and AS group*

	miRNA	r	*P*
AS group	miR-126	−.841	<.001
	miR-143	−.826	<.001
LAA group	miR-126	−.681	<.001
	miR-143	−.723	<.001

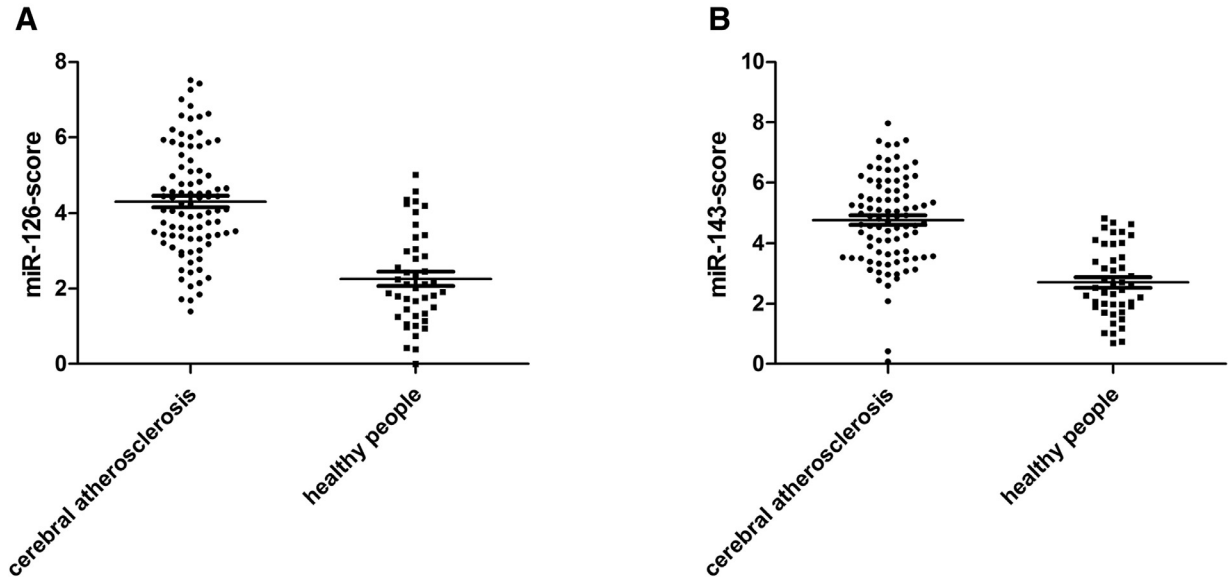

Figure 2. *miRNA-score in the AS patients (including LAA group and AS group) and healthy people. (A) miR-126 (B) miR-143.*

MiRNAs are present in circulation in a highly stable form. They are resistant to ribonucleases, freeze-thaw cycles, and other drastic experimental conditions.[18] Consequently, plasma samples can be stored at −20°C or −80°C for several months, with no notable degradation of miRNAs, which may thus be considered ideal candidate biomarkers.[19]

Our significant findings were that miR-143 and miR-126 might participate in the atherosclerosis process and were minimally affected by cerebral infarction. Pearson correlation analysis revealed that miR-126 and miR-143 were correlated with the presence and severity of cerebral atherosclerosis. Interestingly, the plasma levels of miR-126 and miR-143 were slightly higher in the LAA group than that in the AS group, although this had no statistical significance. These results suggest that plasma miRNAs increased due to release from damaged or circulating brain cells following IS, in line with earlier research by Mayr.[20] Our observations support the assertion that miR-126 and miR-143 may be useful biomarkers for cerebral atherosclerosis. However, the reasons behind the decrease in miR-126 and miR-143 levels in the LAA and AS groups remain unclear. We suspect that circulating miRNAs are mainly released by normal cells, and thus decrease when the cells are injured or degenerated, which indirectly supports the idea that miR-126 and miR-143 play important roles in the normal development of the cerebrovascular system.

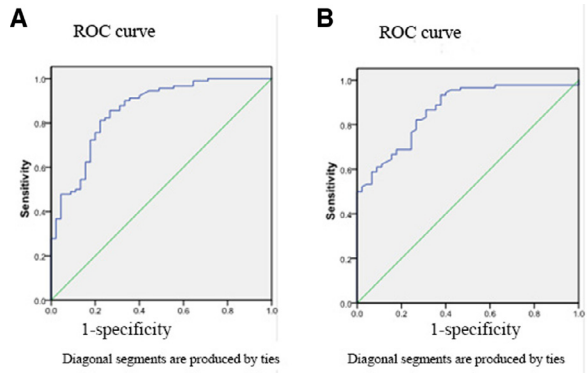

Figure 3. *ROC curve of miR-126 (A) and miR-143 (B) to distinguish AS patients from controls. The AUC are .859 (95% CI .792-.925, P < .001) and .866 (95% CI .805-.928, P < .001) for miR-126 and miR-143, respectively.*

It is well known that inflammatory and immune responses play key roles in occurrence and development of atherosclerosis.[15] Notably, the up-regulated miR-126 inhibited vascular cell adhesion molecule-1 and tumor necrosis factor-α expression, limited the leukocyte adherence to endothelial cells,[15] reducing the inflammatory response involved in the atherosclerosis process. Hergenreider et al[21] reported that microvesicles containing miR-143 injected into mice with AS could reduce the formation of AS in mice. Our findings were also supported by previous observations from plasma specimens of IS patients in which circulating miR-126 was lower in IS patients than healthy controls, indicating that miR-126 may be a useful biomarker for IS.[22] Furthermore, the inhibition expression of miR-143 caused a doubling of the proliferative rate of vascular smooth muscle cells, demonstrating miR-143's role in negatively regulating vascular smooth muscle cell proliferation.[13] Overall, and in line with existing research, our results demonstrated that miR-143 and miR-126 may be of great value in predicting the process of AS.

We also considered whether circulating levels of miR-126 and miR-143 have any clinical significance and whether miRNAs might be valuable biomarkers in the diagnosis of atherosclerotic patients. Taking the

respective levels of the 2 miRNAs, we defined a score to distinguish cerebral atherosclerotic patients from the control group. The resultant ROC curve displayed high specificity and sensitivity, indicating that miR-126 and miR-143 may potentially be specific biomarkers for atherosclerosis.

In summary, in this case-control study, we found that plasma level of miR-126 and miR-143 might be of great worth in predicting cerebral atherosclerosis, and less be affected by cerebral infarction. These conclusions shed light on current scientific knowledge of cerebral atherosclerosis and may provide novel treatment options for cerebrovascular diseases. It should be noted, however, that the number of cases involved in this study was limited. Further multicenter research with an expanded number of cases is required to reliably evaluate these data in a clinical setting.

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