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Original Paper

Upregulated Serum MiR-146b Serves as a Biomarker for Acute Ischemic Stroke

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Key Words

Serum miRNA • miR-146b • Acute Ischemic Stroke • AIS • Biomarker

Abstract

Background/Aims: Stroke is a major cerebrovascular disease threatening human health and life with high morbidity, disability and mortality. It is aimed to find effective biomarkers for the early diagnosis on stroke. ***Methods:*** The expressions of 17 previously reported stroke-associated miRNAs were measured using quantitative RT-PCR and the expressions of plasma high-sensitivity C reactive protein (hs-CRP) and serum interleukin 6 (IL-6), the pro-inflammation markers in brain injury, were examined using enzyme-linked immunosorbent assay in 128 acute ischemic stroke (AIS) patients and control group. ***Results:*** Serum miR-146b expression was significantly increased within 24 hours after stroke onset in patients compared with control group. In addition, the upregulation of serum miR-146b was strong positively correlated with plasma hs-CRP, infarct volume and National Institutes of Health Stroke Scale (NIHSS) score, and moderate positively correlated with serum IL-6 of patients. Importantly, the combination of plasma hs-CRP and serum miR-146b gained a better sensitivity/specificity for prediction of AIS (AUC from 0.782 to 0.863). ***Conclusion:*** Our preliminary findings suggested that upregulated serum miR-146b in acute ischemic stroke might be a potential biomarker for AIS evaluation.

Introduction

Stroke is a major cerebrovascular disease threatening human health and life with high morbidity, disability and mortality [1]. At present, the diagnosis of stroke depends on clinical examination and neuro-imaging techniques [2]. However, many patients cannot afford to undergo MRI, and many poor areas of China cannot accommodate such devices. Thus, it is critically important to find effective biomarkers for the early diagnosis on acute stroke and

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to establish biological early diagnosis. The use of serum biomarkers is considered to be the most valuable adjunct to routine clinical examination and imaging data. Cerebral ischemia and reperfusion injury activate a robust inflammatory response exacerbating cell death and eventually leading to dysfunction of the blood–brain barrier [3]. Accumulating evidence suggests that inflammation plays an important role in the development of cardiovascular and cerebrovascular diseases [4, 5]. Markers of inflammation, such as plasma high sensitivity C reactive protein (hs-CRP) and interleukin-6 (IL-6) are associated with stroke [6, 7].

MiRNAs are a class of naturally occurring short non-coding RNAs that regulate the expression of a wide range of genes and play an important role in various biological functions including cell differentiation, development, immune responses, metabolism, and carcinogenesis [8-10]. Prior studies indicate miRNA are dysregulated in the blood and brain of rodent ischemic stroke [11-14]. In addition, several miRNAs including miR-21, miR-210 and miR-223, et al. were reported as the biomarkers for ischemia stroke [15-19]. Recently, serum exosomal MiR-9 and miR-124 are two brain-specific miRNAs and are promising biomarkers for diagnosing AIS and evaluating the degree of damage caused by ischemic injury [20] and that elevated serum expression of miR-15a, miR-16, and miR-17-5p is strongly associated with AIS [21]. Serum circulating miRNA-221-3p and miRNA-382-5p might be used as potential non-invasive biomarkers for the diagnosis of ischemic stroke [22]. However, whether these deregulated circulating miRNAs are reliable for acute ischemia stroke (AIS) risk prediction, diagnosis or outcome prediction in Chinese patients, additional study is required.

In this study, we demonstrated that miR-146b was remarkably deregulated in the serum of patients with AIS and this upregulation of miR-146b was strong or moderate correlated with plasma hs-CRP, infarct volume and NIHSS score of AIS patients. Moreover, the combination of hs-CRP and miR-146b won 10.4% increase of sensitivity/specificity compared with that of plasma hs-CRP alone for AIS diagnosis. Thus, serum miR-146b might be a potential candidate for AIS prediction.

Materials and Methods

Patients and blood collection

One hundred and twenty-eight consecutive patients with acute ischemic stroke within 24 hours after symptomon set admitted to the department of neurology from July 2014 to July 2016 were prospectively enrolled. One hundred and two control participants without prior history of stroke, myocardial infarction or peripheral vascular disease were also recruited from a large pool of individuals seeking routine health checkups, whose age and gender matched that of the stroke patients. Written consent for tissue donation (for research purposes) was obtained from the patients or their close relatives before serum collection and the protocol was approved by the medical ethics committee of RuiKang Hospital Affiliated to Guangxi University of Chinese Medicine. A complete description of the sample characteristics is provided in Table 1. Exclusion criteria were being under 18 years old, being on thrombolytic or anticoagulant therapies, intracerebral hemorrhage or hemorrhagic transformation, other complicating neurological or neuropsychological diseases, cancer, comorbidity with proinflammatory conditions, and clinical signs of infection at any time during the study.

Neurological deficit was assessed by at least two experienced stroke neurologists, using the National Institutes of Health Stroke Scale (NIHSS) at admission and 24 hours after stroke onset. The MRI was performed with a 3.0 Tesla whole-body imaging system. Diffusion weighted imaging (DWI) lesion volumes were measured using a manual tracing technique by researchers blinded to clinical and laboratory data. The perimeter of the area of abnormal high-signal intensity was traced on each DWI map. The total lesion volume was calculated as the sum of the infarct area on each DWI slice (slice thickness + interslice gap).

Five milliliters of venous blood was collected into serum separator tubes in the absence of any coagulant from each patient within 24 hours of stroke onset or control group. Blood was allowed to spontaneously clot and was centrifuged at 1600 revolutions per minute for 5 minutes. The supernatant was recovered and centrifuged at 12, 000 g for 10 minutes at 4°C to completely remove cell debris. Aliquots of serum were collected and immediately frozen, and stored at -80°C until analysis.

RNA isolation and quantitative real time PCR

Total RNA was isolated using Trizol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturers' recommendations. An A260 nm/A280 nm ratio of 1.9 and a 28S/18S ratio of 1.8 were minimum requirements for the following PCR. Total RNA (2μg) was reverse transcribed with the SuperScript III First-Strand Synthesis System (Invitrogen). The Taqman quantitative RT-PCR was used for amplification with miRNA specific primers (cat#4427975; Applied Biosystems, Foster City, CA, USA). Reactions were incubated in a 96-well optical plate at 95°C for 5 min, followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min. PCR reactions were run on a StepOne Plus real-time PCR machine (Applied Biosystems) and the relative expression was calculated using the $2^{-\Delta\Delta}$CT method. U6 was used as the control.

Table 1. Baseline characteristics of AIS patients. * NIHSS = National Institutes of Health Stroke Scale

	Control (n=102)	AIS (n=128)
Mean age	65.36±16.32	68.42±17.26
Male	74(72.5%)	109 (85.2%)
Hypertension	68(66.7%)	113 (88.3%)
Smoker	53(51.9%)	63 (49.2%)
Diabetics	38(37.3%)	55 (42.9%)
Coronary disease	38(37.3%)	49 (38.3%)
Dyslipidemia	34(33.3%)	98 (76.6%)
NIHSS score	0	6.2 (±7.4)

Concentrations of serum hsCRP and serum IL-6

Plasma high-sensitivity C-reactive protein (hs-CRP) levels within 24 hours after stroke onset were assayed by the clinical laboratory in our hospital. Serum IL-6 level were analyzed using a sandwich enzyme linked immunosorbent assay (ExCell, Shanghai, China), and all assays were performed in duplicate.

Statistical analysis

The NCSS PASS 2008 v8.0.8 (UT, USA) were applied for statistical analyses. Descriptive statistics (mean, standard deviation) were used for assessment of the study data. Besides, the analysis of continuous variables was performed using t-test for parametric variables (serum IL-6 and plasma hs-CRP) and using Kruscal-Wallis and Mann-Whitney U test or Wilcoxon Signed Rank test for non-parametric variables (serum miRNA, infarct volume and NIHSS score). The added effects of serum miRNA were tested by receiver operating characteristic (ROC) curve analysis. Correlation analysis was calculated with Spearman test. A two-sided p value <0.05 was considered statistically significant.

Results

Serum miRNAs and serum hsCRP, IL-6 levels levels in AIS patients

At first, plasma hs-CRP and serum IL-6 were also measured in these AIS patients and control participants. As expected, plasma hs-CRP (Fig. 1A) and serum IL-6 (Fig. 1B) were significantly upregualted in AIS patients (n=128) compared with control group (n=102).

Increasing evidences suggest that circulating miRNAs may serve as biomarker for disease. To identify candidate miRNAs with the capability to discriminate ischemic stroke and normal controls, 17 previously reported stroke-associated miRNAs (Table 2) were initially screened by qRT-PCR in randomly selected 30 AIS patients compared with 30 control participants. Here, it was found that there is statistically significant increase in serum miR-21, miR-145, miR-29b and miR-146b but decrease in serum miR-23a and miR-221 levels in AIS patients (n=30) within 24 hours after stroke onset ($p<0.001$) compared with control group (n=30) (Table 2). No significant difference existed between AIS patients and control group for the other 11 serum miRNAs (Table 2). To further verify the deregulation of serum miR-21, miR-23a, miR-29b, miR-145, miR-146b and miR-221, the expressions of these miRNAs were measured in much more AIS patients and control participants. Consistent with the previous results, miR-146b was significantly upregulated in the serum of AIS patients (n=128) compared with control group (n=102) (Fig. 2A). Meanwhile, miR-21 and miR-221 were deregulated, but miR-145, miR-29b and miR-23a showed no significance in the serum of AIS patients (data not shown).

Correlation between serum miR-146b and proinflammatory markers

Elevated plasma hs-CRP levels were reported to be positively correlated with infarct volume and NIHSS score at admission, thus, plasma hs-CRP level can reflect the severity of AIS and might be useful as a clinical tool for predicting outcome (20-22). Interesting, a moderate positive correlation existed between the expression of serum miR-146b and that of plasma hs-CRP (r=0.5231) (Fig. 2B) or that of IL-6 (r=0.5305) (Fig. 2C). There is no significant correlation existed between the expression of serum miR-21 or miR-221 and that of plasma hs-CRP or IL-6 (data not shown).

Correlation between serum miR-146b and infarct volume and NIHSS score

Elevated plasma hs-CRP levels were reported to be positively correlated with infarct volume and NIHSS score (16-18), thus, we aimed to verify that the expression of serum miR-146b can reflect the severity of acute ischemic stroke and might be useful as a clinical tool for predicting outcome. It was found that the expression of serum miR-146b was positive correlated with both infarct volume (r=0.5199) (Fig. 3A) and NIHSS score (r=0.6460) (Fig. 3C). In addition, we divided our patients into two groups according to infarct volume (>3 cm^3) or NIHSS score (>5), and found a significant increase of serum miR-146b levels in patients with lesion volume >3 cm^3 (p<0.001) (Fig. 3B) or with NIHSS score >5 (p<0.001) (Fig. 3D), which suggested miR-146b level might represent the severity of AIS.

The combination of plasma hs-CRP and serum miR-146b for prediction of AIS

Area under the ROC curve (AUC) for prediction of AIS by serum miR-146b was 0.776 (95% confidence interval 0.628–0.813, p<0.001). To further evaluate the prediction

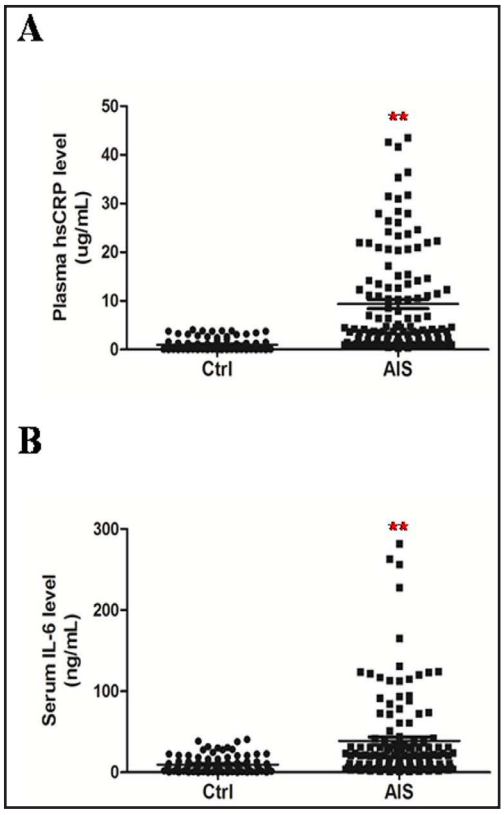

Fig. 1. Increased plasma hs-CRP and IL-6 levels in AIS patients. A. Increased serum hs-CRP level in AIS patients (n=128) compared with healthy controls (n=102). B. Increased serum IL-6 level in AIS patients (n=128) compared with healthy controls (n=102). t-test was used for Statistical analysis; **: p<0.001.

Table 2. The expression of serum miRNAs in AIS patients and control populations. *The relative expression of miRNAs was showed with the mean of CT value.

miRNAs*	Control (n=30)	AIS (n=30)	p value
Let-7b	26.3±1.3	27.4±2.1	0.129
miR-23a	28.6±1.8	30.2±2.3	0.0083
miR-126	26.4±2.1	27.3±1.6	0.216
miR-15a	25.4±1.6	26.2±2.4	0.139
miR-16	28.2±1.8	27.8±1.8	0.314
miR-17-5p	27.5±1.4	27.6±2.6	0.232
miR-19b	32.2±0.9	31.4±1.9	0.248
miR-29b	28.2±1.9	25.6±2.5	0.0054
miR-339-5p	30.4±2.6	29.4±2.7	0.083
miR-21	24.6±1.8	21.3±1.6	0.0026
miR-221	25.8±1.5	28.6±1.5	0.0053
miR-32-3p	28.6±2.9	29.4±2.1	0.106
miR-106-5p	31.4±2.6	30.6±1.4	0.092
miR-532-5p	30.8±1.8	30.2±1.8	0.213
miR-145	28.5±1.3	26.1±1.6	0.0073
miR-146b	29.2±2.1	25.4±1.5	0.0042
miR-210	26.5±1.4	26.8±2.4	0.225

value of deregulated miR-146b, the ROC curves of combination of hs-CRP, IL-6 and miR-146b were calculated. As showed in Table 3, combination of hs-CRP and miR-146b won the higher sensitivity/specificity (AUC=0.863, 95% confidence interval 0.801–0.936, p<0.001), which was 10.4% higher than that of plasma hs-CRP alone. The combination of IL-6 and miR-146b won the higher sensitivity/specificity (AUC=0.819, 95% confidence interval 0.738–0.892, p<0.001), which was 19.7% higher than that of serum IL-6 alone. In addition, the combination of hs-CRP, IL-6 and miR-146b won the highest sensitivity/specificity (AUC=0.866, 95% confidence interval 0.802–0.925, p<0.001), which was quite similar to that of combination of hs-CRP and miR-146b (AUC=0.863, 95% confidence interval 0.801–0.936, p<0.001).

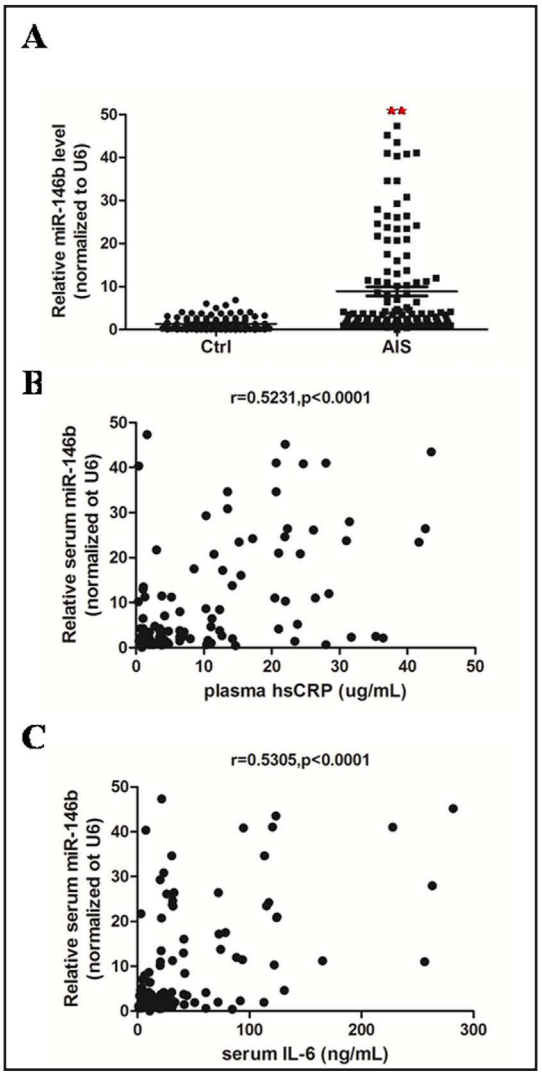

Fig. 2. The expression of serum miR-146b was correlated with plasma hs-CRP and IL-6 in AIS patients. A. Increased circulating miR-146b level strong positively correlated with plasma hs-CRP level and moderate positively correlated with serum IL-6 level in AIS patients. B. Decreased circulating miR-23a level negatively correlated with plasma hs-CRP level but not serum IL-6 level in AIS patients. C. Decreased circulating miR-221 level negatively correlated with plasma hs-CRP level but not serum IL-6 level in AIS patients. Kruscal-Wallis test was used for statistical analysis; **: p<0.001.*:P<0.01; Correlation was determined with the Spearman method.

Discussion

Recently, circulating miRNAs is found to be stable, reproducible, and have already been proposed as novel noninvasive biomarkers for the diagnosis of many neurodegenerative disorders despite of proteins as well. Studies have demonstrated that a number of circulating miRNAs associated with dysregulation of neurovascular integrity and stroke-mediated inflammation [23, 24].

Although, many circulating miRNAs have been reported to be associated with AIS, whether these deregulated miRNAs are reliable for AIS risk prediction, diagnosis or outcome prediction needs further verification by multi-researchers. In this study, nine circulating miRNAs previous reported to be associated with stroke were detected in AIS patients 24 hours after stroke onset. The levels of serum miR-21 and miR-146b were significantly increased and the level of serum miR-221 was significantly decreased in AIS patients, but no significant difference existed for other serum miRNAs. The discrepancy may due to the different subtype of stroke or time point of stroke onset. Inflammatory markers such as hs-CRP and IL-6 were used as additional diagnostic tools in AIS [6, 7], so the correlations of these deregulated circulating miRNAs and hs-CRP or IL-6 were evaluated. Excitingly, serum miR-146b was correlated with plasma hs-CRP positively,

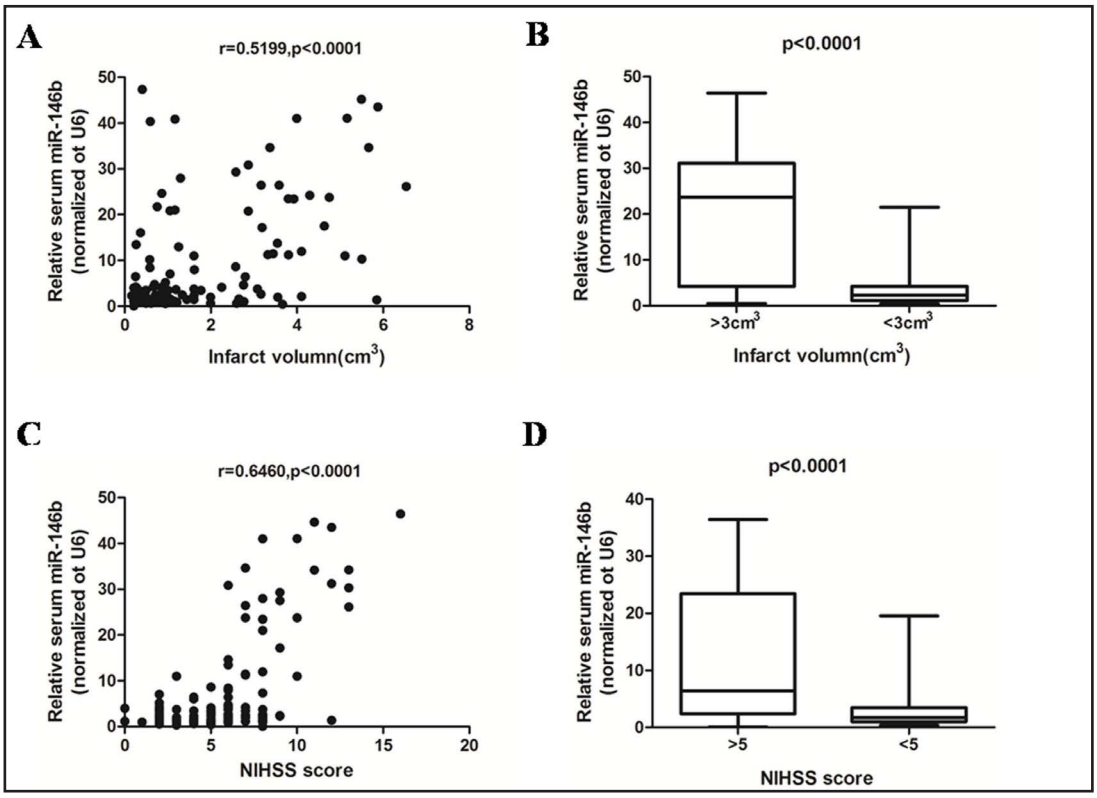

Fig. 3. The expression of serum miR-146b correlated with infarct volume and NIHSS score. A & C. Serum miR-146b levels within 24 hours after stroke onset were positively correlated with infarct volume (A) or NIHSS score (C). Correlation was determined with the Spearman method. B. Serum miR-146b levels were increased in patients with larger infarct (>3cm^3) compared to controls. Bars represent median and interquartile range. Mann-Whitney U test was used for statistical analysis. D. Serum miR-146b levels were increased in patients with higher NIHSS score (>5) compared to controls. Bars represent median and interquartile range. Wilcoxon Signed Rank test was used for statistical analysis.

suggesting serum miR-146b might be related to AIS and could serve as potential biomarkers for AIS. Furthermore, serum miR-146b was strong positive correlated with both infarct volume and NIHSS score. It has been reported that elevated plasma hs-CRP level was positively correlated with infarct volume and NIHSS score [25, 26], thus, serum miR-146b correlated with plasma hs-CRP also can reflect the severity of acute ischemic stroke and might be useful as a clinical tool for predicting outcome.

Table 3. ROC results of the miR-146b

ROC of biomarkers	Area+SD	Asymptotic Significance	Asymptotic 95% confidence interval	
			Lower bound	Upper bound
miR-146b	0.776+0.048	<0.001	0.628	0.813
CRP	0.782+0.063	<0.001	0.672	0.835
IL-6	0.684+0.046	<0.001	0.594	0.726
CRP+miR-146b	0.863+0.052	<0.001	0.801	0.936
IL-6+miR-146b	0.819+0.038	<0.001	0.738	0.892
CRP+IL-6+miR-146b	0.866+0.061	<0.001	0.802	0.925

In order to improve the sensitivity and specificity of diagnosis, the combination of multi-biomarkers are always used. The combination of hs-CRP and D-dimer improves the diagnosis accuracy of cardioembolism in the acute phase [27]. Higher hs-CRP level and lipoprotein-related phospholipase A2 (Lp-PLA2) activity are significantly associated with more severe neurologic impairment and larger infarct size in patients who have acute ischemic stroke

[28]. High-sensitivity C-reactive protein and homocysteine (HCY) are independent predictors of short-term outcome and mortality after AIS. The combined model may provide additional general prognostic information [29]. Adipocyte fatty acid-binding protein (A-FABP) and hs-CRP are associated with AIS, and may be involved in the pathogenesis of AIS [30]. In this study, the combination of hs-CRP and miR-146b won the higher sensitivity/specificity (AUC=0.863, p<0.001), which was 10.4% higher than that of plasma hs-CRP alone, while the combination of hs-CRP, miR-146b and IL-6 won a quite similar sensitivity/specificity (AUC=0.866, p<0.001). Thus, miR-146b might be the potential candidate for AIS prediction. Many of these cellular changes are mediated by changes in miRNA expression, suggesting that analysis of miRNA expression may improve understanding of stroke pathogenesis and uncover new avenues for risk assessment and therapy.

AIS patients were classified into 5 categories based on etiology, using the TOAST classification: large-artery atherosclerosis (LAA), small vessel occlusion (SVO), cardioembolism (CE), stroke of other determined etiology, and stroke of undetermined etiology [31]. There is no significance of the expression of serum miR-146b among 5 AIS groups in this study (data not shown), which may suggest that miR-146b may be a biomarker for AIS but not for separating subtypes of AIS. In fact, miR-146b is one of the most-studied miRs [32, 33], and especially in the vascular smooth muscle cell (VSMCs) proliferation, differentiation, and phenotypic switching. In cardiovascular system, miR-146b is not only important for heart and vascular development but also plays an essential role in cardiac pathological factors, such as hypertrophy and ischemia, and has been regarded as a new therapeutic target for vascular disease [34-36]. Our findings might not only provide evidences for miR-146b as a new biomarker for AIS evaluation, but also further confirm the important role of miR-146b in vascular pathology.

In this study, the sample sizes used in this study is a relatively small, further confirmation on large-scale prospective data would be advantageous. This study is performed in Chinese, whether the results are applicable to other racial groups need further verified. Furthermore, a longer follow-up study would have been preferable, which helps to evaluate the roles of miR-146b more accurately in AIS diagnosis. It is important to seek a sensitive and specific biomarker(s) for diagnosis and prognosis of AIS. Although miR-146b was found to be helpful for AIS evaluation in this study, a panel of AIS related miRNAs (including miR-9, miR-24, miR-16, miR-221 and miR-146b, et al.) even together with hs-CRP or IL-6, may be evaluated in large number of stroke patients.

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Disclosure Statement

The authors declare that they have no competing interests.

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