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MicroRNA-99a-5p in circulating immune cells as a potential biomarker for the early diagnosis of ischemic stroke

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Abstract:

OBJECTIVES: We have previously shown the neuroprotective function of microRNA-99a-5p in experimental stroke. Here, we explore its diagnostic potential for acute stroke patients.

MATERIALS AND METHODS: MiR-99a-5p levels in circulating from acute stroke patients and control were measured by real-time polymerase chain reaction. Pearson's correlation and receiver operator characteristic (ROC) curves were used to analyze clinical significance of miR-99a-5p and its sensitivity and specificity for stroke diagnosis.

RESULTS: We demonstrated that miR-99a-5p expression was upregulated in neutrophils of both ischemic stroke and hemorrhage patients, while was only increased in the lymphocytes of hemorrhage patients. ROC analysis revealed that the miR-99a-5p level in neutrophils and lymphocytes had a moderate diagnostic value for stroke. Moreover, a positive correlation existed between plasma miR-99a-5p levels and neutrophil numbers or neutrophil/lymphocyte ratio. Meanwhile, miR-99a-5p levels in neutrophils were negatively correlated with thrombin time, while positively correlated with D-dimer and urea levels. Lymphocytic miR-99a-5p levels were positively correlated with platelet mean volume and distribution width.

CONCLUSION: This study demonstrated that miR-99a-5p levels in circulating immune cells might facilitate the diagnosis of ischemic stroke.

Keywords:

Acute stroke, biomarker, lymphocyte, miR-99a-5p, neutrophil

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Introduction

Acute ischemic stroke (AIS) is one leading cause of morbidity and mortality in humans, worldwide. In some cases, early diagnosis warrants the immediate initiation of reperfusion therapy to reduce the risk of mortality. At present, stroke diagnoses depend on clinical examination and various neuroimaging techniques. However, some hospitals are not equipped with magnetic resonance imaging (MRI) and computed tomography (CT) capabilities, and many patients cannot afford to undergo these procedures. Thus, it is critically important to find effective biomarkers for early AIS diagnosis. Circulating biomarkers are considered to be the most valuable adjunct to routine clinical examination and imaging data. The immune system responds rapidly following cerebral ischemia, but new biomarkers in circulating immune cells that are able to give an early prediction with more accuracy and sensitivity are needed.[1,2]

MicroRNAs (miRNAs) are readily detectable in body fluids and are important epigenetic regulators of the immune

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system.[3] Following the identification of miRNA expression profiles in cerebrospinal fluid and blood from AIS patients,[4,5] emerging clinical studies have confirmed the differential expression of specific miRNAs in circulation following stroke.[6-8] It has been reported that elevated hsa-miR-106b-5p and hsa-miR-4306, and decreased hsa-miR-320e and hsa-miR-320d in plasma may be novel biomarkers for the early detection of AIS in humans.[6] It has also been indicated that miR-124, miR-9, and miR-219 in serum are suppressed in AIS, facilitating neuroinflammation and brain injury.[7] Furthermore, it was shown that the atherosclerosis-related circulating miR-17 level was elevated in AIS and associated with future stroke recurrence.[8] The noninvasive nature of circulating miRNA measurement and its sensitivity and specificity in different diseases has encouraged a pursuit of miRNA biomarkers for AIS.[9,10]

Our previous clinical data showed that plasma miR-99a level was significantly changed in ischemic stroke patients within 72 h after stroke onset compared with control subjects, demonstrating that the miR-99a level is clinically significant in the context of stroke. Moreover, our experimental work showed that brain miR-99a levels dramatically changed within hours after ischemia onset and plays an essential role against ischemic brain injury.[11] Therefore, in this study, we investigated the diagnostic potential of miR-99a for ischemic stroke in the hyperacute stage (≤6 h after symptom onset) by further detecting alterations in miR-99a levels in circulating neutrophils, lymphocytes, and plasma, as well as analyzing correlations between miR-99a levels and diagnostic and prognostic biochemical indications for AIS.

Materials and Methods

Patient inclusion and clinical variables

This prospective study analyzed 21 AIS and 8 cerebral hemorrhage patients in the emergency department or neurology ward of Xuanwu Hospital of Capital Medical University (Beijing, China) from March to December 2015. Ischemic stroke was diagnosed by neurologists based on patient medical histories, physical examinations, and radiologic diagnoses on admission, in accordance with guidelines formulated in 2014.[12] The inclusion criteria consisted of: (1) first ischemic stroke and admission within 6 h after symptom onset; (2) National Institute of Health Stroke Scale <25 points; (3) sudden occurrence of a focal neurological deficit in the ischemia lesion that could be observed on CT or MRI scans; and (4) adequate access to patient information. The exclusion criteria were: (1) recurrent stroke; (2) hematological system diseases, malignant tumors, renal or liver failure; (3) history of mental disorders, severe dementia, or coronary artery disease; and (4) other diseases affecting the hemogram. Eight control healthy volunteers who did not have any focal neurological deficit or antecedents of central nervous system disease were recruited from Medical Examination Center of Xuanwu Hospital, Capital Medical University, China. Volunteers in the control group were age- and sex-matched to the patients in the acute stroke groups. The inclusion of human subjects was approved by the Institutional Review Board of Capital Medical University, Beijing, China. All subjects gave their informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of Capital Medical University.

Neurological deficit scores and magnetic resonance imaging

All patients underwent standard neurological and general medical evaluations and assessments using the modified Rankin scale (mRS) and Barthel Index (BI) at admission, and 7 days after stroke onset. MRIs were acquired on a 3.0T Magnetom Verio syngo (Siemens, Germany). Radiologists with over 15 years of experience who were blinded to the clinical histories, physical findings, patient identities, and final diagnostic results used diagnostic workstations with the Unisight system to interpret and measure infarct volumes. The total infarct volume was calculated by the infarct size on the diffusion-weighted imaging sequence and then multiplied by the thickness. All imaging data were standardized by Cross-Sectional Area Intracranial.[13] The formula of the volume standardization for cerebral infarction is as follows: Infarct volume × mean intracranial cross-sectional area/the intracranial cross-sectional area of the patient.

Detection of clinical biochemical indexes

Blood samples from AIS patients within 6 h after stroke symptom onset were collected into an ethylenediaminetetraacetic acid (EDTA) anticoagulant vacuum tube by venipuncture from all stroke patients at time of admission and immediately preserved at 37°C for routine laboratory assays. The routine blood tests and coagulative parameters/platelet (PLT) indices including PLT count, PLT hematocrit, mean platelet volume (MPV), platelet distribution width (PDW), international normalized ratio, activated partial thromboplastin time, thrombin time (TT), D-dimer, and fibrinogen were evaluated using the samples. All blood samples were measured according to the accredited methods at the Department of Clinical Chemistry in Xuanwu Hospital.

Separation of neutrophils, lymphocytes, and plasma

Blood samples (two 4 ml samples per patient) collected into EDTA-anticoagulant tubes were processed according to the following procedures: (1) samples were immediately centrifuged at 200 ×*g* for 10 min at 4°C to obtain plasma; (2) blood cells were diluted with 8 ml normal saline and slowly added to the surface of the lymphocyte separation medium in two 15 ml centrifuge tube; (3) the tubes were centrifuged at 400 ×*g* for 20 min at 20°C, and lymphocytes were separated and saved; and (4) erythrocytes were dissociated with erythrocyte lysing solution 3 times and discarded, and the remaining neutrophils were saved. Neutrophils, lymphocytes, and plasma were stored at − 80°C in RNase/DNase-free tubes for further tests.

MiR extraction and real-time reverse transcription polymerase chain reaction

Total RNAs from neutrophils, lymphocytes, and plasma were extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol, and miRNA was extracted using mirVana miRNA Isolation Kit (Ambion). To validate miRNA expression, the miScript cDNA synthesis kit (Qiagen) was used followed by quantitative real-time polymerase chain reaction (qRT-PCR) using the miScript SYBR Green PCR kit (Qiagen). The Gene Amp PCR System 9700 (Applied Biosystems) was used for TaqMan-based RT-PCR assays. All primers for the miRNAs and U6 endogenous controls for miRNA assays were purchased from Shanghai Bioligo Technology Co. Ltd. The primers for miR-99a-5p were 5′-GCC AAC CCG TAG ATC CGAT-3′ and 5′-GTG CGT GTC GTG GAG TCG-3′. The primers for U6 were 5′-GCT TCG GCA GCA CAT ATA CTA AAAT-3′ and 5′-CGC TTC ACG AAT TTG CGT GTC AT-3′. Relative gene expression was calculated through the $2^{-\Delta\Delta CT}$ method, normalized and expressed as fold change relative to U6.

Statistical analysis

Statistical analysis was performed using SPSS version 17.0 software (SPSS Inc., Chicago, IL, USA). Values in the text and Table 1 are presented as mean ± standard error of the mean. Independent samples *t*-test was used for two-group comparisons. Qualitative data were given as counts and percentages and were compared with Fisher's exact test. One-way analysis of variance was used for comparisons among several quantitative variables. The correlation between two variables was performed using the Pearson's correlation test. Receiver operator characteristic (ROC) curve analysis was performed to calculate the predictive power of the sensitivity and specificity for diagnosis of ischemic stroke. The overall measure of diagnostic accuracy of the models was assessed using the area under the receiver operating characteristic curve (AUC). Then, to further determine the optimal cutoff with optimized sensitivity and specificity for the identification of ischemic stroke, the exact *P* value was calculated and recorded, and $P < 0.05$ was considered statistically significant.

Results

miR-99a-5p levels were increased in circulating immune cells within 6 h following stroke

A total of 8 healthy volunteers and 21 AIS and 8 cerebral hemorrhage patients were enrolled in this study. To avoid possible bias from patient selection, individuals with no statistically significant differences in age, sex, or risk factors including diabetes, hypertension, and hypercholesterolemia histories were recruited in this study. We first detected miR-99a-5p levels in neutrophils, lymphocytes, and plasma of all patients and healthy controls by RT-PCR. In neutrophils, miR-99a-5p expression was significantly upregulated for both AIS and cerebral hemorrhage patients within 6 h of onset [Figure 1a, $P < 0.05$]. In lymphocytes, miR-99a-5p levels were significantly upregulated only in cerebral hemorrhage patients within 6 h of onset [Figure 1b,

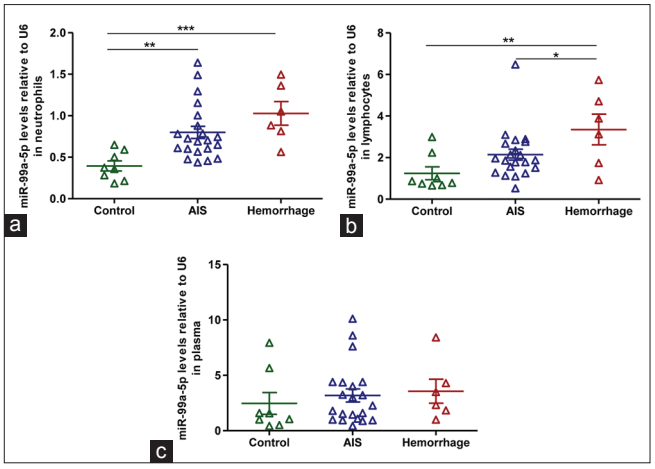

Figure 1: Changes in circulating miR-99a-5p levels from stroke patients. Real-time polymerase chain reaction analysis of miR-99a-5p levels in neutrophils (a), lymphocytes (b), and (c) plasma from AIS patients ($n = 21$), cerebral hemorrhage patients ($n = 8$) and healthy controls ($n = 8$). The data are presented as relative expression following normalization. Data represent mean ± standard error of the mean. *$P < 0.05$ compared to controls. AIS: Acute ischemic stroke

Table 1: Stroke diagnoses on the basis of miR-99a-5p levels within 6 h

Cells	AUC	95%CI	*P*	Cut-off point	Sensitivity	Specificity
Neutrophils	0.896	0.693-1.000	0.001	0.600	0.762	0.875
Lymphocytes	0.768	0.516-1.000	0.028	1.049	0.952	0.750

AUC: Area under the receiver operating characteristic curve, CI: Confidence interval

$P < 0.05$], but not in AIS patients. Plasma miR-99a-5p expression was not significantly changed in either AIS or cerebral hemorrhage patients [Figure 1c].

Correlations between the time of stroke onset and miR-99a-5p levels

To clarify the dynamic variation of miR-99a-5p expression in stroke patients during the hyperacute stage, we analyzed correlations between the two miRs levels and the time of stroke onset, within 6 h. No significant correlation is found between miR-99a-5p levels in neutrophils/lymphocytes and the time of symptom onset [Figure 2a and b]. The opposite trend of gradually increased miR-99a-5p level in plasma and gradually decreased miR-99a-5p level in immune cells indicate that plasma miR-99a-5p might release from neutrophils and lymphocytes.

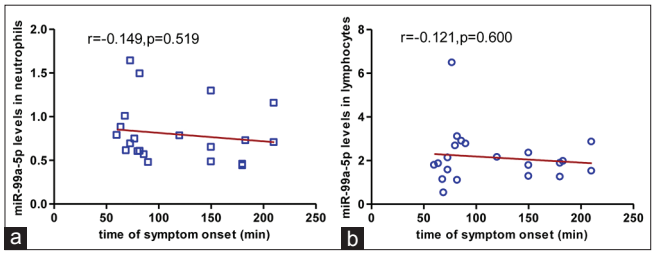

Figure 2: Correlation between miR-99a-5p levels in circulating blood and the time of symptom onset of AIS patients. (a) Correlation between neutrophil miR-99a-5p levels and the time of symptom onset; (b) correlation between lymphocyte miR-99a-5p levels and the time of symptom onset (n = 21). AIS: Acute ischemic stroke

Correlation between miR-99a-5p levels and neurological function score and brain infarct volume

To assess whether miR-99a-5p levels could predict stroke severity and stroke outcome, we analyzed correlations between miR-99a-5p levels and neurological function scores and brain infarct volume at admission and 7 days after stroke onset. No significant correlation existed between miR-99a-5p levels in neutrophils/lymphocytes and mRS or BI score or infarct volume after symptom onset [Figure 3a-f]. No significant correlation existed between miR-99a-5p levels in neutrophils/lymphocytes/plasma and mRS or BI score at 7 days after thrombolytic therapy (data not shown).

Diagnosis of acute ischemic stroke patients within 6 h on the basis of miR-99a-5p levels

To investigate whether miR-99a-5p levels in circulating cells could be AIS biomarkers, ROC curves were constructed to compare the relative concentration of miR-99a-5p in AIS patients and controls, and the AUC for patients were calculated. In general, AUC >0.5 is considered diagnostic, whereas AUC <0.7 indicates a lower diagnostic value; 0.7< AUC <0.9 indicates a moderate diagnostic value; AUC >0.9 indicates a high diagnostic value.[14] The AUC of neutrophil miR-99a-5p was 0.896, indicating it also had moderate diagnostic value for stroke; with a cutoff point of 0.600, we were able to differentiate stroke patients from healthy controls with a sensitivity of 0.762 and a specificity of 0.875 [Figure 4a and Table 1, $P < 0.05$]. The AUC of

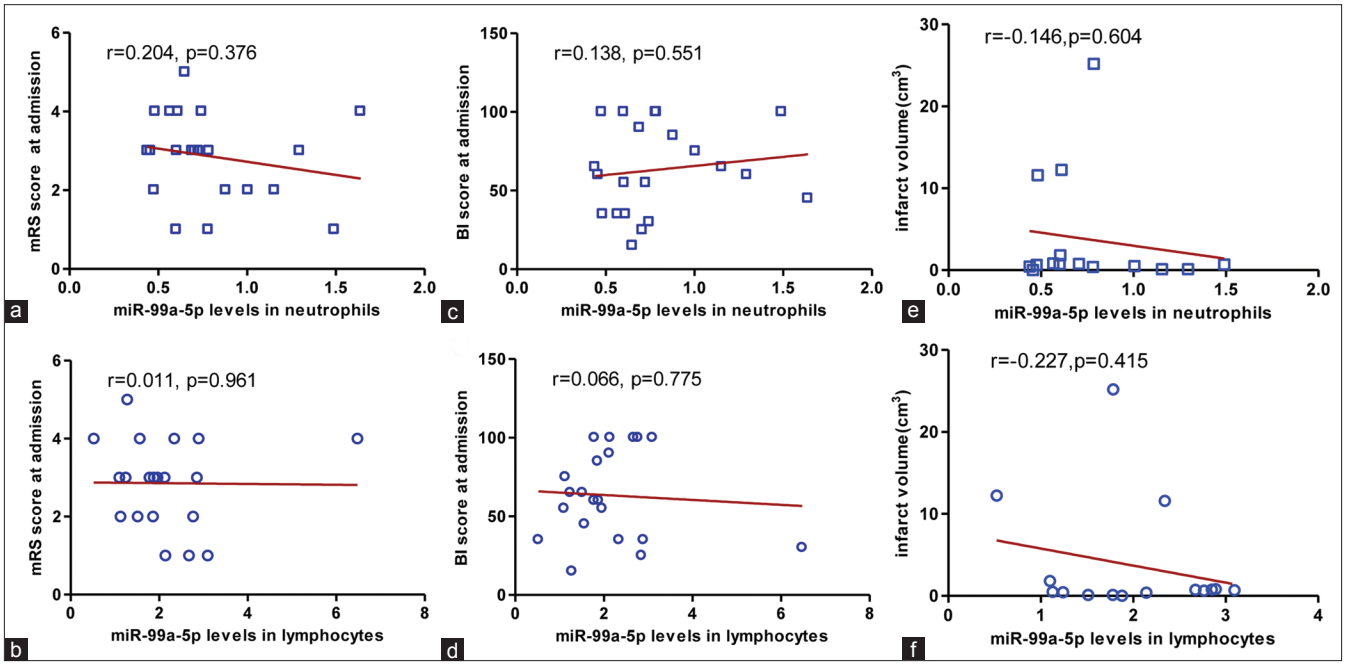

Figure 3: Correlation between miR-99a-5p levels in circulating cells and neurological function score and infarct volume of AIS patients at admission and 7 days after stroke onset. (a-c) Correlation between neutrophil miR-99a-5p levels and mRS, BI and infarct volume in AIS patients at admission. (d-f) Correlation between lymphocytic miR-99a-5p levels and mRS, BI and infarct volume in AIS patients at admission (n = 21). AIS: Acute ischemic stroke, mRS: Modified Rankin scale, BI: Barthel Index

lymphocyte miR-99a-5p was 0.768, indicating it had moderate diagnostic value for stroke; with a cutoff point of 1.049, we were able to differentiate stroke patients from healthy controls with a sensitivity of 0.952 and a specificity of 0.750 [Figure 4b and Table 1, $P < 0.05$].

Correlation between miR-99a-5p levels and number/percentage of circulating immune cells

Our previous data showed that plasma miR-99a levels were associated with some clinical biochemical parameters within 72 h following stroke.[7] Here, we further analyzed correlations between miR-99a-5p levels within 6 h following stroke and number/percentage of circulating immune cells from blood samples of AIS patients. First, the correlation between miR-99a-5p level in neutrophils and the number/percentage of neutrophils in AIS patients was analyzed, and a tendency toward negative correlations is found between miR-99a-5p level in neutrophils and the number/percentage of neutrophils in AIS patients [Figure 5a]. In contrast to miR-99a-5p level in neutrophils, we found a significant positive correlation existed between plasma miR-99a-5p level and the number but not the percentage of neutrophils after symptom onset [Figure 5b, $P < 0.05$]. Tendency toward negative correlations is found between lymphocytic miR-99a-5p level and the number/percentage of lymphocyte in AIS patients [Figure 5c]. Similar to lymphocytic miR-99a-5p level, tendency toward negative correlations is found between plasma miR-99a-5p level and the number/percentage of lymphocyte in AIS patients [Figure 5d]. Correlation between the neutrophil/lymphocyte ratio and miR-99a-5p level in plasma/neutrophil/lymphocyte in AIS patients was also analyzed, and no significant correlation was found [Figure 5e].

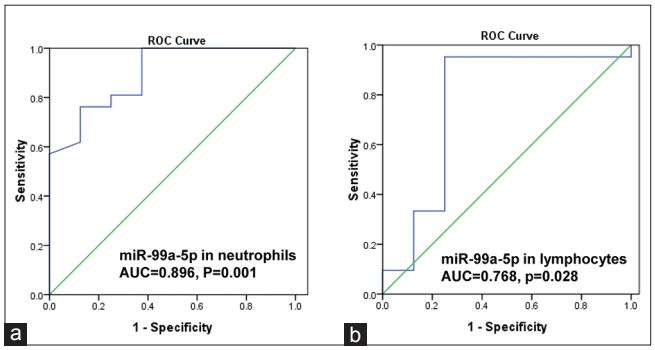

Figure 4: Evaluation of miR-99a-5p levels in circulating blood for the diagnosis of AIS within 6 h after symptom onset. ROC curves were drawn from miR-99a-5p levels in neutrophils (a), and lymphocytes (b) from AIS patients (n = 21). ROC: Receiver operator characteristic, AIS: Acute ischemic stroke

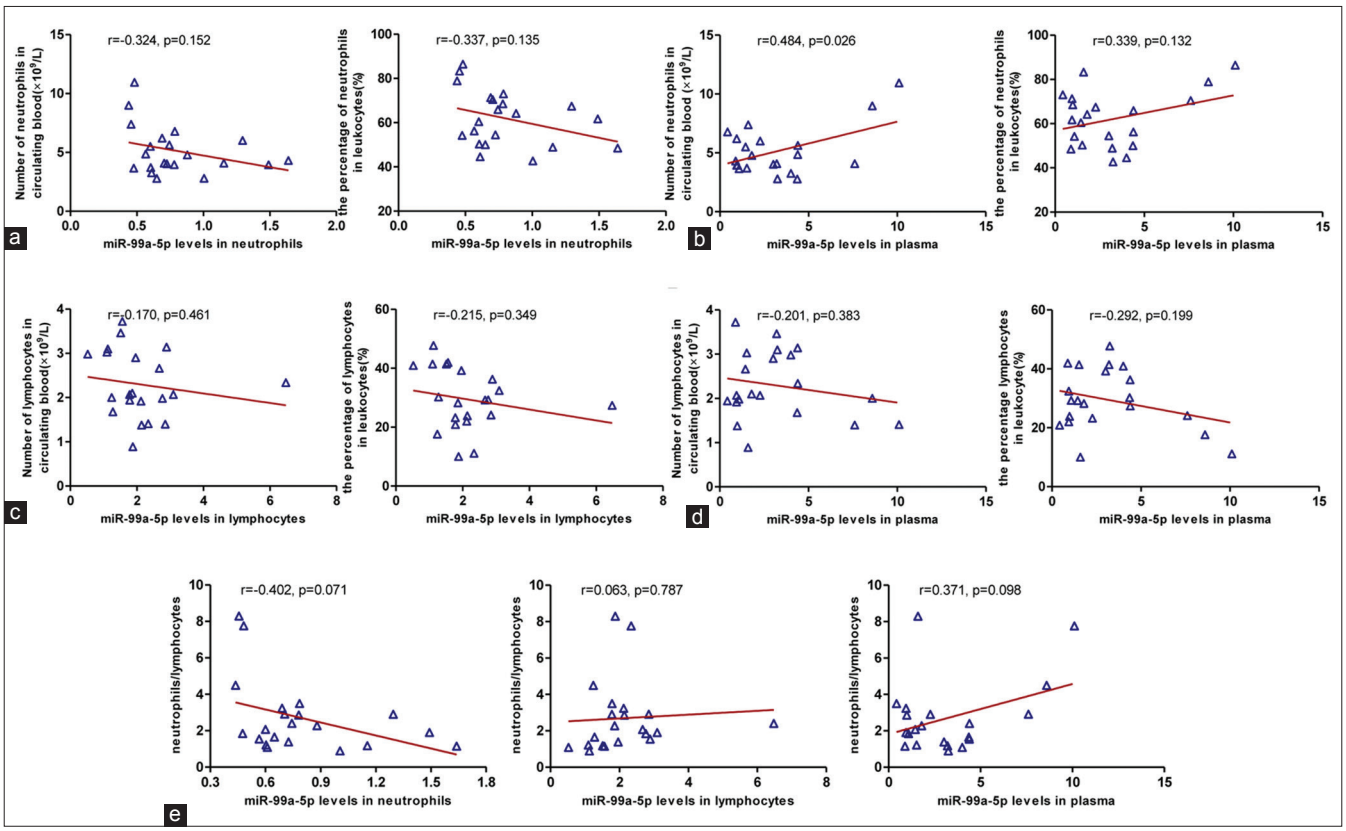

Figure 5: Correlation between circulating miR-99a-5p levels and number/percentage of circulating immune cells of AIS patients. Correlation between (a) miR-99a-5p level in neutrophils and number/percentage of neutrophils; (b) plasma miR-99a-5p level and number/percentage of neutrophils; (c) miR-99a-5p level in lymphocyte and the number/percentage of lymphocyte; (d) plasma miR-99a-5p level and number/percentage of lymphocyte; (e) neutrophil/lymphocyte ratio and miR-99a-5p level in plasma/neutrophil/lymphocyte (n = 21). AIS: Acute ischemic stroke

Correlation between miR-99a-5p levels and coagulative parameters/platelet indices

Correlation between the miR-99a-5p levels in neutrophil/lymphocyte/plasma and nine coagulative parameters/PLT indices in AIS patients was analyzed, respectively. A significant negative correlation existed between miR-99a-5p level in neutrophil and TT-sec, while a significant positive correlation also existed between neutrophil miR-99a-5p levels and D-dimer and urea after symptom onset [Figure 6a, $P < 0.05$]. A significant positive correlation existed between lymphocytic miR-99a-5p level and MPV/or PDW after symptom onset [Figure 6b, $P < 0.05$]. Otherwise, no obvious correlation exist between plasma miR-99a-5p level and nine coagulative parameters/PLT indices in AIS patients (data not shown).

Discussion

In this study, we identified the diagnostic potential of miR-99a-5p in circulating cells from AIS patients. First, miR-99a-5p levels in neutrophils, lymphocytes, and plasma were differently changed in AIS patients and cerebral hemorrhage patients. Second, ROC analysis revealed that miR-99a-5p levels in neutrophil and lymphocyte had moderate diagnostic value for AIS diagnosis. Finally, we found that miR-99a-5p might influence the pathological processes that imbalance immune cell proliferation and coagulation/fibrinolysis during stroke. These data suggest that miR-99a-5p in circulating cells might be a potential biomarker for ischemic stroke diagnosis that regulates the peripheral immune and coagulation/fibrinolysis systems in patients with AIS, and this regulation may relate to the severity of ischemic brain injury.

It is widely believed that miRNAs released from damaged cells or circulating cells lead to the increased expression of serum miRNAs.[15] In this study, plasma miR-99a-5p levels did not change significantly within 6 h after onset, while neutrophil miR-99a-5p levels were significantly upregulated in both AIS and cerebral hemorrhage patients; however, the miR-99a-5p levels in lymphocytes was significantly increased only in cerebral hemorrhage patients but not in AIS patients. The aforementioned data indicated that detection of miR-99a-5p levels in lymphocytes could be used to make a diagnosis of cerebral ischemia or cerebral hemorrhage within 6 h of symptom onset. Although we did not find a significant association between miR-99a-5p levels and brain infarct volume in AIS patients, plasma miR-99a-5p levels showed a time-dependent increase after stroke onset and a linear correlation with neurological functional scores. In addition, ROC analyses revealed that miR-99a-5p in neutrophils and lymphocyte both had a moderate diagnostic role for stroke. Therefore, miR-99a-5p expression in circulating immune cells may be a sensitive biomarker for identifying early stages of ischemic stroke.

In response to acute cerebral ischemia, central and peripheral inflammation is seen in ischemic brain injury between several hours to days after stroke. The neutrophil/lymphocyte ratio has been shown to predict short- and long-term outcomes of ischemic stroke.[16] Recently, a study showed that peripheral neutrophils are potential biomarkers of the outcome when used in conjunction with advanced imaging.[17] In this study, a significant positive correlation existed between plasma miR-99a-5p levels and the number of neutrophils after symptom onset, within 6 h. Our previous study

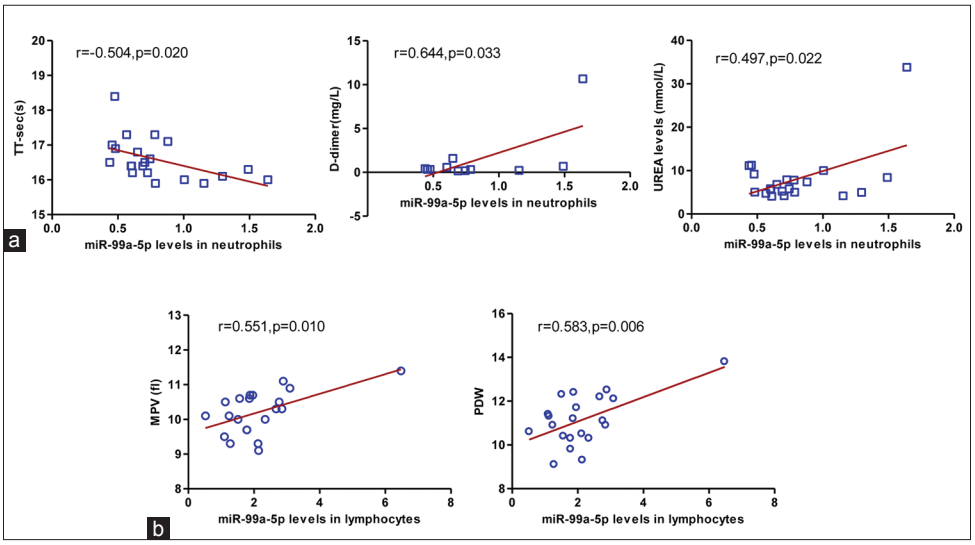

Figure 6: Correlation between circulating miR-99a-5p levels and coagulative parameters/PLT indices of AIS patients. (a) Correlation between miR-99a-5p level in neutrophil and TT-sec, D-dimer and urea levels in AIS patient. (b) Correlation between lymphocytic miR-99a-5p levels and MPV and PDW in AIS patients (n = 21). PDW: Platelet distribution width, MPV: Mean platelet volume, TT: Thrombin time, AIS: Acute ischemic stroke, PLT: Platelet

suggested exogenous miR-99a-5p overexpression in the brain of mice protects against focal cerebral ischemia injury through regulating cell cycle progression of neural cells,[11] which might underlie its involvement in the regulation of neutrophil numbers. Moreover, miR-99a-5p level in plasma and miR-99a-5p level in neutrophils showed contrary correlation with neutrophil numbers as well as neutrophil/lymphocyte ratio, the underlying meaning and mechanisms need further experimental investigation.

The main cause of AIS is the formation of a thrombus, and imbalance in the coagulation/fibrinolysis system plays a crucial role in thrombus formation and progression. In this study, we examined the association between miR-99a-5p levels in circulating blood and nine coagulation/fibrinolysis biomarkers. We found a significant positive correlation between lymphocytic miR-99a-5p levels and MPV/PDW and between neutrophil miR-99a-5p levels and TT-sec/D-dimer within 6 h following stroke. Previous data have demonstrated that circulating MPV and PDW levels are significantly elevated in patients with AIS,[18,19] and that thrombosis biomarkers such as D-dimer are relevant to stroke onset and progression.[20] The data revealed that high MPV and PDW expression are independent predictors of ischemic stroke. Thus, the high miR-99a-5p expression in neutrophil and lymphocyte of AIS patients was always accompanied with a relative high hypercoagulability following a stroke. However, the cause-effect relationship between miR-99a-5p levels and MPV, PDW, TT-sec, and D-dimer still needs further investigations.

Conclusion

Our previous experimental work have indicated that miR-99a reduces neuronal damage following cerebral ischemia/reperfusion through regulating cell cycle progression and preventing apoptosis,[11] and here, we provided the first evidence of the potential value of measuring expression of the neuroprotective miR-99a-5p for AIS diagnosis, by showing changes in miR-99a-5p expression in circulating neutrophils, lymphocytes, and plasma in AIS patients. Because biomarkers for acute stroke are currently lacking, the availability of miRs might facilitate acute stroke diagnosis and evaluating stroke severity. It must be pointed out that this study had a relatively small sample size, and the results should be further validated in larger studies to confirm that miR-99a-5p levels can be used as biomarkers for ischemic stroke in the future. Furthermore, the sensitivity and specificity should be analyzed in larger, longer-term studies. Additional studies are warranted to elucidate the relationship between miR-99a-5p and the numbers of immune cells as well as coagulative parameters/PLT indices and to explore the clinical potential of miRs for stroke treatment.

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Conflicts of interest

There are no conflicts of interest.

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