



Increased Circulating Exosomal miRNA-223 Is Associated with Acute Ischemic Stroke

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Recent studies have demonstrated that exosomal microRNAs (miRNAs) are novel biomarkers and therapeutic targets for various diseases including vascular disease. However, specific exosomal miRNAs expression in stroke patients has not been reported yet. Here, we explored whether circulating exosomal miRNAs can serve as potential biomarkers for the diagnosis of acute ischemic stroke and discussed the potential for clinical application. Blood samples were collected from acute ischemic stroke patients within the first 72 h ($n = 50$). Circulating exosomes were exacted by Exoquick exosome isolation kit and characterized by transmission electron microscopy. Western blot was performed to assess the expression of exosomal protein makers. Exosomal miRNA-223 (miR-223) was detected by RT-PCR assay. The relationship between the expression levels of miR-223 and National Institutes of Health Stroke Scale (NIHSS) scores, brain infarct volume, and neurological outcomes were analyzed. Circulating exosomes were isolated and the size of vesicles ranged between 30 and 100 nm. The identification of exosomes was further confirmed by the detection of specific exosomal protein markers CD9, CD63, and Tsg101. Exosomal miR-223 in acute ischemic stroke patients was significantly upregulated compared to control group ($p < 0.001$). Exosomal miR-223 level was positively correlated with NIHSS scores ($r = 0.31$, $p = 0.03$). Exosomal miR-223 expression in stroke patients with poor outcomes was higher than those with good outcomes ($p < 0.05$). Increased exosomal miR-223 was associated with acute ischemic stroke occurrence, stroke severity, and short-term outcomes. Future studies with large sample are needed to assess the clinical application of exosomal miR-223 as a novel biomarker for ischemic stroke diagnosis.

Keywords: acute ischemic stroke, biomarker, blood, diagnosis, exosomes, humans, microRNA, prognostic

Abbreviations: CE, cardioembolism; DM, diabetes mellitus; LA, large artery atherosclerotic stroke; LACI, lacunar infarct; MCAO, middle cerebral artery occlusion; microRNA, miRNA; miR-223, microRNA-223; mRS, modified Rankin Scale; NIHSS, National Institutes of Health Stroke Scale; OCSF, Oxfordshire Community Stroke Project; PACI, partial anterior circulation infarct; POCI, posterior circulation infarct; ROC, receiver operator characteristic; TACI, total anterior circulation infarct; TEM, transmission electron microscopy; TOAST, Trial of Org 10172 in Acute Stroke Treatment.

INTRODUCTION

Stroke is the second leading cause of death in the worldwide, bringing heavy burden to the society and families (1). Although the morbidity and mortality have been reduced in high-income countries due to the public stroke education and the control of conventional stroke risk factors, the molecular mechanism of the pathogenesis of ischemic stroke still remains unclear.

Generally, the diagnosis of stroke relies on the clinical manifestation of the suspected patients and later confirmation by Computed Tomography (CT) or MR imaging examination. However, it is sometimes difficult for the physicians to make objective judgment when the symptom is slight or hard to differentiate from other neurological and non-neurological diseases. The limitation of brain imaging system in detecting small infarcts should also be noticed. Therefore, abundant studies on biomarkers have been carried out to provide ideal diagnosis for stroke (2–4).

Animal and *in vitro* experiments demonstrated that microRNAs (miRNAs) were involved greatly in stroke pathogenesis by regulating oxidative stress, inflammation, apoptosis, and endothelial function (5–8). Clinical studies also proved that circulating miRNAs were potential biomarkers, which could facilitate the diagnosis of stroke, the identification of subtype, the evaluation of severity, and the prediction of prognosis (9–14).

Exosomes are 30–100 nm vesicles that cells secrete into extracellular space when multivesicular bodies fuse with cell membrane. Initially, exosomes were regarded as cell garbage to remove unwanted proteins. It was not until 2007, when Valadi et al. first identified the existence of RNA and miRNA that important role of exosomes was realized—a novel carrier for intercellular genetic material exchange and communication (15). In recent years, abundant studies provided strong evidence that exosomal miRNAs were novel biomarkers and therapy targets for various diseases such as tumor, neurodegeneration disease, and vascular disease (16–22). However, whether exosomal miRNAs can serve as biomarkers for stroke diagnosis remains largely unknown.

microRNA-223 (miR-223) was shown to be one of the most highly expressed miRNAs in plasma exosomes of healthy human and results about its function in ischemia injury are inconsistent (23). Here, we designed a retrospective case-control study to detect the level of exosomal miR-223 expression in stroke patients and non-stroke subjects. We then analyzed the association between exosomal miR-223 expression and National Institutes of Health Stroke Scale (NIHSS) scores, infarct volume, and modified Rankin Scale (mRS) in stroke patients. We also discussed the potential application of exosomes being novel approach for stroke diagnosis and therapy.

MATERIALS AND METHODS

Subject and Sample Collection

Human project was approved by the committee of Institutional Review Board (IRB) of Shanghai Jiao Tong University, Shanghai, China. Written informed consent was obtained from patients according to the Helsinki Declaration, and the

Helsinki Declaration was followed during the human studies. Peripheral blood was collected from stroke patients who were recruited consecutively from September 2014 to June 2015 in Ruijin Hospital. Control group were enrolled in January 2015 from subjects who came for medical examination and denied previous history of stroke attack. Subjects in each group were matched with age and gender. Demography feature, related previous history including hypertension, diabetes mellitus (DM), hyperlipidemia, cardiopathy, associated laboratory test, and imaging information including blood glucose, blood lipid, electrocardiogram, cardiac ultrasonography, carotid artery ultrasonography, MR imaging, and MR angiography were also collected for analysis. The risk factors were defined as the following: (1) hypertension: blood pressure $\geq 140/90$ mmHg; (2) DM: fasting glucose ≥ 7.0 mmol/L or 2-h postprandial blood glucose ≥ 11.1 mmol/L; (3) hyperlipidemia: triglycerides > 1.7 mmol/L or total cholesterol > 5.7 mmol/L or low-density lipoprotein > 4.3 mmol/L. The exclusive criteria included recurrent stroke or stroke onset longer than 72 h, renal or liver failure, acute infectious disease, tumor, hematologic disease, and patients who are unable to cooperate with physical examination.

The severity of stroke was evaluated with NIHSS scores. The patients were classified into three groups: large artery atherosclerotic stroke (LA), cardioembolism (CE), and small artery stroke (SA) according to the Trial of Org 10172 in Acute Stroke Treatment (TOAST) classification. Patients were also classified into the following four groups: total anterior circulation infarct, partial anterior circulation infarct (PACI), posterior circulation infarct (POCI), and lacunar infarct (LACI) according to Oxfordshire Community Stroke Project (OCSP) definition. The infarct volume was calculated by ABC/2 method (A and B represent the largest diameter of the infarct and its largest perpendicular diameter, while C represents the thickness of the slices where the infarct lesion was visible). 3-month mRS was used to assess the neurologic outcome of stroke patients.

Blood Preparation and Exosomes Isolation

Whole blood was drawn into promoting coagulating tubes and centrifuged at 1,600 g for 10 min. Serum was aspirated and stored at -80°C . ExoQuick exosome precipitation solution (System Biosciences, CA, USA) was used to precipitate serum exosomes. Briefly, centrifuged the serum sample at 3,000 g for 15 min to remove cell debris; Then, added 125 μL ExoQuick exosome precipitation solution to 500 μL serum and mixed well; after refrigerating at 4°C for 30 min, centrifuged the mixture at 1,500 g for 30 min and removed the supernatant; Centrifuged another 5 min to spin down residual fluid and resuspended exosomes pellet by PBS or nuclease-free water. All centrifugations were done at 4°C .

Exosomes Identification

The size and morphology feature of exosomes were examined by transmission electron microscopy (TEM) (TEM, Phillip CM120, 60 kV). Protein were extracted from exosomes resuspension solution with RIPA lysis buffer and separated on 8% polyacrylamide gel before transferring to a nitrocellulose filter

membrane. The blotting membrane was blocked with skim milk and incubated with CD9 antibody (1:1,000 dilution, Abcam, Cambridge, UK)/CD63 antibody (1:200 dilution, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA)/TSG101 (1:500 dilution, Abcam, Cambridge, UK) overnight. After TBST washing, secondary HRP-conjugated antibody was added for 1 h (1:5,000 dilution, HuaAn Biotechnology Inc., Hangzhou, China). The proteins were finally detected using chemiluminescence (Thermo Scientific, Rockford, IL, USA).

RNA Extraction and Reverse Transcription

Total RNA was isolated from exosomes using miRNeasy Mini Kit (Qiagen, Hilden, Germany) mainly according to the manufacturer's instructions. The qiazol volume was 700 μ l, and the final elute volume was 20 μ l. Single-strand cDNA was synthesized using universal cDNA synthesis kit (Exiqon, Vedbaek, Denmark) following the condition: 42°C for 1 h and then 95°C for 5 min.

Real-time PCR

The expression of miRNA was tested by real-time PCR using SYBR Green master mix (Exiqon, Vedbaek, Denmark) on an Applied Biosystems 7900 real-time PCR machine (Life Technologies). The reaction conditions were as follows: 95°C for 10 min followed by 40 cycles of 95°C for 10 s and 60°C for 1 min. The relative expression of miR-223 was normalized to the endogenous control miR-16 expression using the comparative cycle threshold (CT) method. When comparing different groups, the data were logarithm-transformed.

Statistical Analysis

Statistics were calculated using SPSS 19 (IBM SPSS Inc., NY, USA). The Kolmogorov–Smirnov test was used to determine the normality of continuous variables distribution. If normal, Student's *t*-test was used to compare differences between groups, if abnormal, Welch correction was used. Proportions were compared using the chi-square test or Fisher's test.

To test the feasibility of using miR-223 as a biomarker for stroke, we built statistical model by multivariable logistic regression analysis. Forward stepwise selection procedures were adopted. Results were expressed as adjusted odds ratios (OR) with the corresponding 95% confidence intervals (95% CI). Correlations were estimated by Pearson correlation test. Differences were considered significant at $p < 0.05$.

RESULTS

The Identification of Circulating Exosomes

Transmission electron microscopy and western blot were used for identification of circulating exosomes. On the one hand, the yielded vesicles were consistent with exosomes in size and morphology—30–100 nm nano-size and round-shaped (Figure 1A). Some researchers showed TEM picture of cup-shaped exosomes (24). The shape difference is caused by different methods of preparing sample (25). On the other hand, western blot proved the expression of certain exosomal marker proteins, i.e., CD9, CD63, and Tsg101 (Figure 1B). CD9 and CD63 are the most commonly used marker proteins for exosomes identification. Tsg101 involves

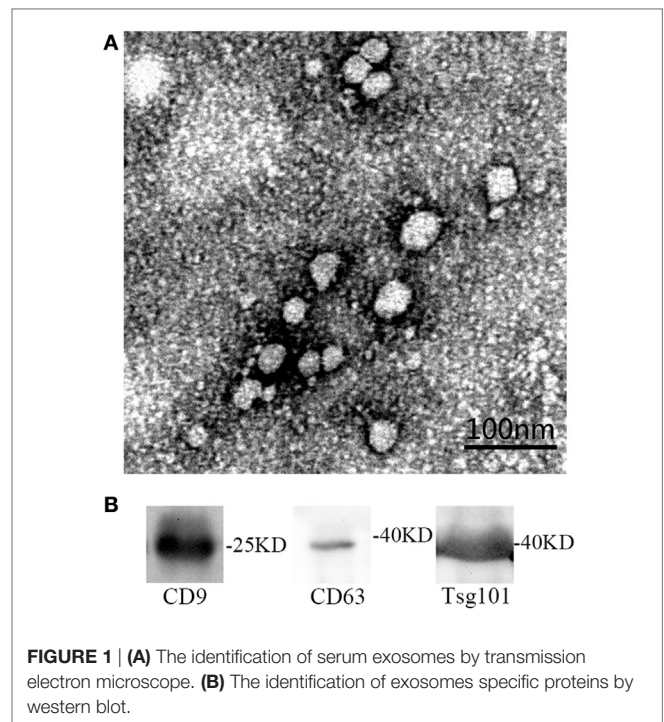


FIGURE 1 | (A) The identification of serum exosomes by transmission electron microscope. **(B)** The identification of exosomes specific proteins by western blot.

in transmembrane cargo inclusion and is important for exosomes biogenesis (26).

Baseline Clinical Characteristics

The clinical characteristics of ischemic stroke patients ($n = 50$) and control subjects ($n = 33$) are shown in Table 1. Mean age and the distribution of race/ethnicity were not different between two groups ($p = 0.67, 1, 1$). The number of male in both groups was about 1.5 times that of female ($p = 0.75$). A greater percentage of risk factors in stroke patients was found including DM 36.0% ($p < 0.05$) and cardiopathy 30.0% ($p < 0.05$). In our study, stroke patients and control group had the similar percentage of hypertension ($p = 0.98$). We considered that this phenomenon may be due to the increased awareness of health care in people with hypertension. They were more willing to participate in our research. The mean NIHSS score of stroke patients was relatively low (3.32). According to the TOAST classification, 50.0% of patients were classified as LA, while cardioembolism 16.0% and SA 34.0%. A smaller percentage of cardioembolism subtype is because that these patients were mostly enrolled to the Department of Cardiology in our hospital. According to the OCSF classification, 22 stroke patients were classified as PACI, while POCI subgroup was 16 cases and LACI subgroup was 11 cases. Patients with good outcome ($mRS \leq 2$) took up 60% and those with poor outcome ($mRS > 2$) accounted for 40%.

Exosomal miR-223 Expression in Acute Ischemic Stroke Patients

In our study, miR-16 was used as internal control. Exosomal miR-16 level was almost the same between the two groups (Table 2, $p = 0.828$). When compared to control group, the expression of

TABLE 1 | Clinical characteristics of ischemic stroke patients and healthy control.

	Stroke	Control	p-Value
Total (N)	50	33	NA
Race (Asian, %)	100%	100%	1
Ethnicity (Han, %)	100%	100%	1
Age (years, mean ± SD)	64 ± 9.4	63 ± 7.9	0.67
Sex (male/female, N)	32/18	20/13	0.75
Hypertension (N, %)	38 (76.0%)	25 (75.8%)	0.98
Hyperlipidemia (N, %)	29 (58.0%)	14 (42.4%)	0.17
Diabetes mellitus (DM) (N, %)	18 (36.0%)	3 (9.1%)	<0.05
Cardiopathy (N, %)	15 (30.0%)	1 (3.0%)	<0.05
NIHSS (mean, min, max)	3.32 (0.14)	NA	NA
TOAST			
LA	25 (50.0%)	NA	NA
CE	8 (16.0%)	NA	NA
SA	17 (34.0%)	NA	NA
OCSF			
TACI	1 (2.0%)	NA	NA
PACI	22 (44.0%)	NA	NA
Posterior circulation infarct	16 (32.0%)	NA	NA
LACI	11 (22.0%)	NA	NA
mRS			
≤2	30 (60.0%)	NA	NA
>2	20 (40.0%)	NA	NA

The difference of age between stroke ($n = 50$) and control group ($n = 33$) were compared with Student's *t*-test. The proportions of race, sex, hypertension, hyperlipidemia, DM, and cardiopathy between two groups were compared with chi-square test.

NIHSS, National Institutes of Health Stroke Scale; TOAST, Trial of Org 10172 in Acute Stroke Treatment; LA, large artery atherosclerotic stroke; CE, cardioembolism; SA, small artery stroke; OCSF, Oxfordshire Community Stroke Project; TACI, total anterior circulation infarct; PACI, partial anterior circulation infarct; LACI, lacunar infarct; mRS, modified Rankin Scale; NA, not available.

TABLE 2 | Exosomal miR-16 and miR-223 levels of ischemic stroke patients and healthy control.

	Stroke	Control	p Value
miR-16 CT value (mean ± SD)	21.88 ± 1.18	21.82 ± 1.51	0.828
miR-223 expression (mean ± SD)	-0.23 ± 0.30	-0.67 ± 0.28	<0.001

Student's *t*-test was used to compare exosomal miR-16/miR-223 levels of stroke patients ($n = 50$) and healthy control ($n = 33$).

CT, cycle threshold.

exosomal miR-223 in the serum of patients with acute ischemic stroke were increased greatly (Table 2; Figure 2A, $p < 0.001$). To explore the changing tendency of miR-223 after stroke attack, we analyzed circulating exosomal miR-223 levels at 24, 48, and 72 h. Exosomal miR-223 expression was not significantly different between subgroups, although we found it had a tendency to increase by time (Figure 2B).

The Relationship between Exosomal miR-223 Expression and Stroke Onset

To eliminate the unmatched risk factors impact (DM and cardiopathy) on exosomal miR-223 expression between two groups, we performed a logistic regression analysis. The result indicated that circulating exosomal miR-223 expression was a possible risk factor of stroke (Table 3, $p < 0.001$). In addition, when the value of exosomal miR-223 increased by 0.1, the risk of stroke occurrence increased by about 0.7-fold. The receiver operating characteristic curve was drawn to evaluate

the diagnostic value of exosomal miR-223 (Figure 3). The area under the curve was 0.859, when circulating exosomal miR-223 = -0.50, had the best sensitivity (84.0%) and specificity (78.8%) for stroke diagnosis.

The Relationship between the Expression of Exosomal miR-223 and Characteristics of TOAST Subtypes, NIHSS Score, Infarct Volume, and mRS

To explore the relationship between circulating exosomal miR-223 and stroke subtypes, we compared exosomal miR-223 expression in stroke patients with different TOAST subtypes (Figure 4A). There was no difference in exosomal miR-223 level among TOAST subtypes, for example, LA (-0.28 ± 0.27 , $n = 26$), cardioembolism (-0.16 ± 0.19 , $n = 7$), and SA (-0.29 ± 0.31 , $n = 17$, $p = 0.53$). Patients who were classified to cardioembolism seemed to have a higher level of exosomal miR-223 but needed further verification with larger sample. To examine the association between circulating exosomal miR-223 and NIHSS score, the Pearson correlation test was performed. Positive correlation was found between the exosomal miR-223 expression and NIHSS score (Figure 4B, $r = 0.31$, $p = 0.03$). As to the relationship between circulating exosomal miR-223 and infarct volume, no significant correlation was found in our study (Figure 4C, $r = 0.20$, $p = 0.19$). To evaluate the relationship between circulating exosomal miR-223 and 3-month prognosis, we compared the exosomal miR-223 level in stroke patients with good outcomes (mRS ≤ 2) and those with poor outcomes (mRS > 2). Stroke patients who had poor outcomes were shown to have a greater expression of exosomal miR-223 than patients who had good outcomes (Figure 4D, -0.1 ± 0.3 vs. -0.3 ± 0.3 , $p = 0.036$).

DISCUSSION

In the present study, we demonstrated that (1) the level of miR-223 in circulating exosomes was elevated after onset of acute ischemic stroke; (2) exosomal miR-223 expression was positively correlated to NIHSS score; (3) stroke patients with poor outcomes inclined to have a greater exosomal miR-223 expression. Therefore, increased exosomal miRNA-223 is associated with acute ischemic stroke.

Abundant studies have demonstrated the extraordinary value of exosomes as strong weapon to diagnose and treat various diseases especially cancer (27–30). As to ischemic disease, previous studies largely focused on exploring the protection role of stem cell-derived exosomes after ischemic injury. Exosomes secreted by stem cells like cardiac progenitor cells, mesenchymal stem cells, and embryonic stem cells alleviated ischemia-reperfusion injury by delivering functional protein and RNAs (31–34). Chopp and colleagues found that systemic administration of mesenchymal stem cell-generated exosomes post stroke onset improved functional recovery with enhanced neurogenesis and angiogenesis (35). This protective role was likely mediated by miR-133 enriched exosomes transferring from mesenchymal stem cells to neurons and astrocytes (36).

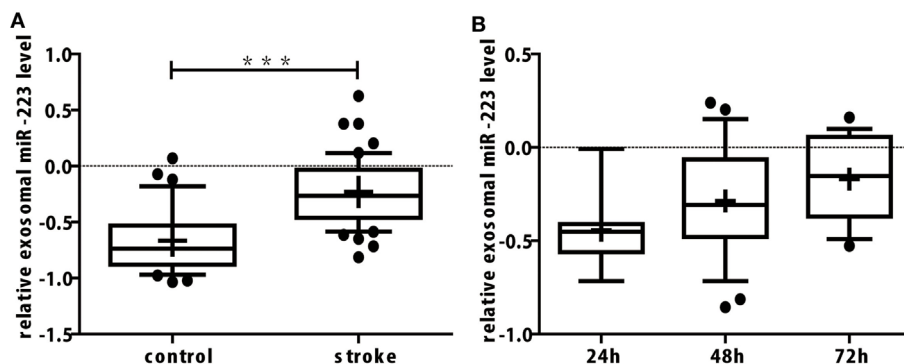


FIGURE 2 | (A) The expression of miR-223 in serum exosomes of acute ischemic stroke patients was upregulated significantly. The difference of exosomal miR-223 level between stroke and control group were compared with Student's *t*-test. Box plots showed the expression of exosomal miR-223 in patients and control. The y-axis indicated miR-223 expression levels by log₁₀ change. ****p* < 0.001, stroke patients vs. control. Data are median (25% percentile, 75% percentile), *n* = 50 in stroke group, *n* = 33 in control group. **(B)** Serum exosomal miR-223 expression of stroke patients between different onset time. Exosomal miR-223 levels of different onset times were compared with one-way ANOVA analysis. Box plots showed the expression of exosomal miR-223 in stroke patients with different onset times. The y-axis indicated miR-223 expression levels by log₁₀ change. Data are median (25% percentile, 75% percentile), *n* = 7 in 24 h subgroup, *n* = 25 in 48 h subgroup, and *n* = 18 in 72 h subgroup.

TABLE 3 | Exosomal miR-223 expression and the risk of stroke.

Factors	Adjusted <i>p</i> -value	OR	95% CI
miR-223	<0.001	1.70	1.31–2.20
Diabetes mellitus (DM)	0.015	7.84	1.50–40.98
Cardiopathy	0.073	7.72	0.83–72.20

Exosomal miR-223 expression together with conventional stroke risks such as hypertension, hyperlipidemia, DM, and cardiopathy were analyzed in a statistical model by multivariable logistic regression.

OR, odds ratios; CI, confidence intervals.

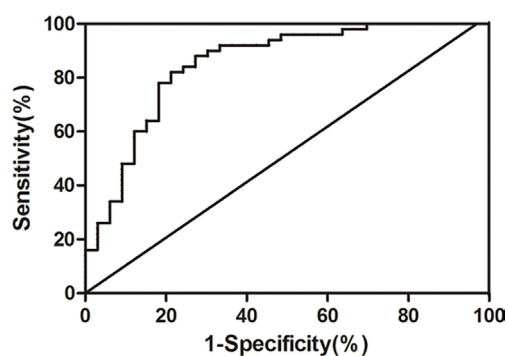
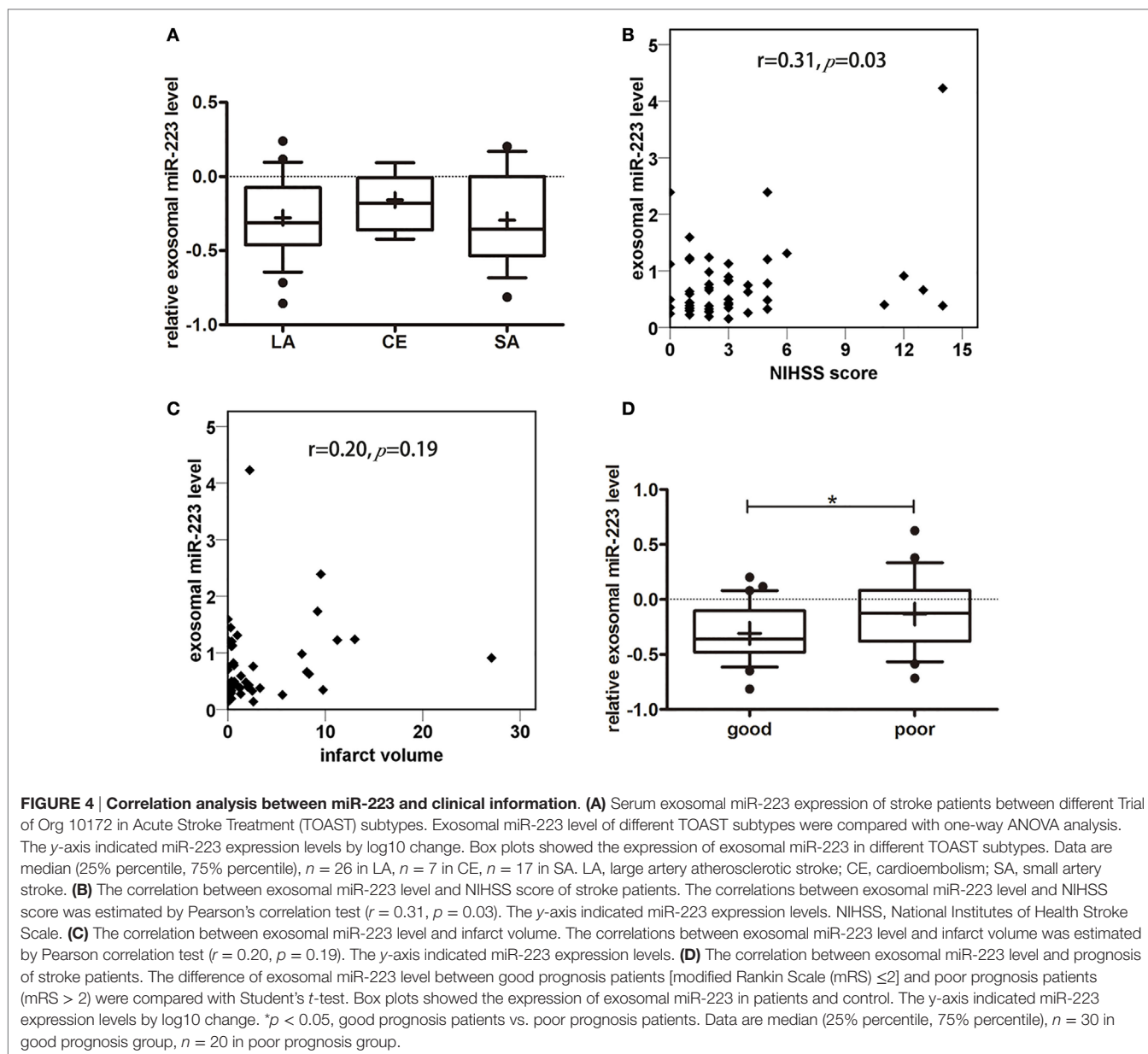


FIGURE 3 | The receiver operating characteristic curve of serum exosomal miR-223 diagnosing ischemic stroke.

So far there are few clinical studies about exosomes with ischemic vascular disease in patients (37, 38). Here, we first reported the existence of exosomes in human circulating blood with acute ischemic stroke. Exosomal miRNAs, compared to total cellular miRNAs or free miRNAs, are considered as more sensitive and specific biomarker since exosomes are secreted into extracellular space in a selective method (39–42). Chen

detected the expression of circulating miR-126 and circulating exosomal miR-126 in transient middle cerebral artery occlusion rats. Exosomal miR-126 decreased significantly 3 h post stroke while circulating miR-126 remained unchanged (40). Our study further explored the possibility of utilizing exosomal miRNAs as novel stroke biomarkers among patients. There was a rise on exosomal miR-223 level post stroke, while no alteration in exosomal miR-21 and exosomal miR-145 level (unpublished data). miR-21, miR-145, and miR-223 are the most well-studied miRNAs that are related to hypoxia or ischemia regulation (13, 43–46).

Previous animal experiments revealed miR-223 as a double-edged sword in functional repair after ischemic injury (47–49). One clinical research also showed the same tendency of miR-223 expression in stroke patients. But the relationship between miR-223 level and NIHSS score was on the contrary to our study (12). The difference might be caused by different sources of RNA sample. The mean NIHSS score of stroke patients in our study was relatively low, indicating that exosomal miR-223 is sensitive for mild stroke. In the prospective population-based Bruneck study, reduced miR-223 and other four miRNAs levels in plasma antedated the manifestation of DM (50). A recent retrospective trail reported that platelet and plasma miR-223 expression were lower in DM patients with ischemic stroke than in DM only patients (51). The study also pointed that platelet miR-223 levels were correlated with plasma miR-223 level, but not with miR-223 level in leukocytes. In our study, we found no difference in serum exosomal miR-223 expression between participants with and without DM, or stroke patients with and without DM. Whether miR-223 expression was a risk factor for stroke in DM patients requires more research. Perhaps different sources of miR-223 play different role in stroke pathogenesis. miR-223 was reported to be elevated in the serum of patients with acute myocardial infarction and angina pectoris (52). But there is no hint whether the alteration is sustained in the long run. Our stroke patients did not contain patients with acute



coronary syndrome since those patients would likely be sent to the Department of Cardiology.

Our study demonstrated that stroke patients with poor outcomes turned to have a greater expression of exosomal miR-223 (53). Future cohort study can be conducted to testify the association between higher miR-223 expression and lower stroke occurrence. Blocking the secretion and uptake of exosomes or regulating exosomal miR-223 expression might be necessary to exam the exact role of exosomal miR-223 in stroke recovery.

Exosomes, representing a novel approach to carry bioactive substances, provides a bright prospect in treating central nervous system diseases including stroke (54). On the one hand, these nano-sized particles can serve as efficient drug delivering system

since they can cross blood brain barrier without much degradation (55). On the other hand, exosomes derived from stem cells may minimize adverse effects of administrating cells such as cell embolism and excessive cell differentiation (56). Endogenous exosomes after hypoxia precondition can also protect the tissue from ischemia-reperfusion injury (57). Hence, stem cell-derived exosomes or genetically modified exosomes are potential stroke treatment.

There are limitations of our study. The sample size was relatively small that we failed to further explore the value of using exosomal miR-223 to differentiate stroke causes. Future study with larger sample is needed. Besides, the blood sample was obtained within 72 h after stroke onset. Perhaps, this is relatively broad time range to detect the alteration of exosomal

miR-223 level. It is more rational to collect the sample from the same patient dynamically at several earlier times such as 1, 3, 6, 12, 24, and 48 h. The correlation between exosomal miR-223 and NIHSS score was relatively weak; thus, it needs further investigation.

CONCLUSION

This study showed that increased circulating exosomal miR-223 is associated with acute ischemic stroke occurrence, stroke severity, and short-term outcome. The clinical value of circulating exosomal miR-223 should be further assessed with large sample research. Functional studies on the role of exosomal miR-223 and its target in ischemic pathogenesis are also essential. We believe exosomal miR-223 has potential as a biomarker and a therapeutic target for ischemic stroke.

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AUTHOR CONTRIBUTIONS

YC and YS designed the work, acquired and interpreted the data, and drafted the manuscript. JH, MQ, YZ, and JG collected and analyzed the clinical data, discussed the results, and participated in the final version. ZZ, JL, and GYY generated the conception of this work, analyzed and interpreted the data, and revised the manuscript. All the authors read and approved the final manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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