Potential Neuroprotective Effect of miR-451 Against Cerebral Ischemia/Reperfusion Injury in Stroke Patients and a Mouse Model

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■ OBJECTIVE: **Recently, microRNAs (miRs) have been reported to be novel regulators in ischemic stroke. In this study, we investigated the pattern of miR-451 expression along with its clinical application in human ischemic stroke and in an in vivo mouse model.**

■ METHODS: **The level of miR-451 was evaluated in patients and mice after ischemic stroke. National Institute of Health Stroke Scale scores and brain infarct volume were analyzed to the correlation of miR-451 expression and clinical information. In addition, blood samples and brain tissues were collected from an established middle cerebral artery occlusion model consisting of 12 adult male mice at 24 hours after the middle cerebral artery occlusion.**

■ RESULTS: **The results showed that miR-451 levels in the circulating blood of ischemic stroke patients were greatly decreased compared with the control. Further correlation analysis revealed a negative association between miR-451 and National Institute of Health Stroke Scale scores ($r = -0.6104$, $P < 0.001$) and infarct volume ($r = -0.5442$, $P < 0.001$). Moreover, miR-451 was downregulated in response to middle cerebral artery occlusion in vivo, along with a negative correlation between miR-451 in brain and blood ($r = 0.9240$, $P < 0.01$). In addition, forced expression of miR-451 weakened ischemic brain infarction and apoptosis levels in focal ischemia-stroked mice, while downregulation of miR-451 significantly augmented ischemic injury.**

■ CONCLUSIONS: **In conclusion, miR-451 displays the neuroprotective effect in ischemic stroke and might serve as a novel therapeutic target of ischemic stroke.**

INTRODUCTION

Stroke is the leading cause of death in the worldwide[1] and accounts for about 10% of all deaths in industrialized counties.[2] Of all strokes, approximately 88% of strokes are ischemic.[3] Ischemic stroke, with high morbidity and mortality, can affect people of all ages.[4] Current therapy in clinic mainly relies on mechanical thrombolysis for ischemic stroke, but unavoidably leads to the ischemia reperfusion injury.[5] Cerebral ischemia/reperfusion injury induces neuronal death and apoptosis and even severely impairs neurologic function.[6] Despite some multiple risk factors for stroke have been proposed, including advanced age, diabetes mellitus, hypercholesterolemia, hypertension, and so forth—the pathophysiologic mechanisms of this deadly disease are not completely clear.[7] Therefore, a better understanding of the molecular mechanism of ischemic injury is necessary to develop effective treatments for ischemic stroke.

MicroRNAs (miRs) are a group of endogenous 18- to 22-nucleotide, short noncoding protein RNA molecules that regulate gene expression by targeting the 3′ untranslated region of the mRNA.[8,9] Previous studies have shown that miRs are correlated with modulation of multiple diseases and pathological processes in the central nervous system, such as stroke occurrence,[10] neuronal cell death,[11] and blood–brain barrier disruption.[12] In addition,

Key words
- Brain ischemia
- miR-451
- MCAO
- Stroke

Abbreviations and Acronyms
CE: Cardioembolism
LA: Large-artery atherosclerosis
MCAO: Middle cerebral artery occlusion
miR: microRNA
NIHSS: National Institutes of Health Stroke Scale
qRT-PCR: Quantitative real-time PCR
SA: Small artery stroke

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in vitro experiments have demonstrated that miRs are involved greatly in stroke pathogenesis and are identified as potential biomarkers to facilitate the diagnosis of stroke.[13-15] Moreover, population-based miRNA profiling analyses show differential expression patterns in young stroke patients and controls.[16] Other microarray analysis by Min et al. found that a total of 15 miRs were upregulated and 44 miRs were downregulated in the ipsilateral ischemic cortex of experimental rats,[17] of which miR-451 was found to be downregulated in the cortex of rats with cerebral ischemia/reperfusion injury compared with rats in the sham group. miR-451 is located on chromosome 17q11.2 and its abnormal expression has been detected in several tumors.[18-20] Additionally, miR-451 exerts its anti-inflammatory effects in chronic inflammatory pain by inhibiting microglia activation-mediated inflammation via targeting TLR4.[21] Moreover, a previous study has reported that miR-451 downregulation impairs ischemic preconditioning mediated cardioprotection.[22] Most importantly, a recent study by Liu et al. demonstrated that miR-451 could protect neurons against oxygen and glucose deprivation/reoxygenation—induced apoptosis and oxidative stress by targeting CUGBP Elav-like family member 2.[23] However, the clinical significance of miR-451 and its functional role in cerebral ischemia/reperfusion injury in vivo still remain unclear.

In this study, we explored the clinical role of miR-451 in ischemic stroke patients by analyzing changes of miR-451 levels in patients and an animal ischemia model. We also evaluated the association between miR-451 expression and National Institutes of Health Stroke Scale (NIHSS) scores and infarct volume in stroke patients, as well as investigated the effect of miR-451 in protecting focal ischemia-stroked mice. Our study suggests an important role for miR-451 in protecting against ischemic stroke.

METHODS

Subject and Sample Collection

A total of 108 clinically and radiologically confirmed ischemia stroke patients were enrolled consecutively from June 2015 to December 2017 at Hainan General Hospital. We also enrolled 97 age- and sex-matched controls who came for medical examination and confirmed no stroke attack. All participants gave informed consent. All ischemic strokes were diagnosed by neurologists according to the medical imaging diagnostic methods (computed tomography and magnetic resonance imaging). The exclusion criteria included receiving thrombolytic therapy, renal or liver failure, tumor, infectious disease, and hematologic disease. Meanwhile, demographic features, associated previous history and imaging information, including hypertension, diabetes mellitus, hyperlipidemia, cardiopathy, blood glucose, cardiac ultrasonography, carotid artery ultrasonography, and magnetic resonance angiography were collected to evaluate the relationship between the expression of miR-451 and these clinical features. All study processes were approved by the institutional review board of Hainan General Hospital.

Finally, the severity of ischemic stroke was evaluated using the NIHSS[24] at 3 months after the stroke onset. The patients were classified into the following groups: large-artery atherosclerosis (LA), cardioembolism (CE), small artery stroke (SA) according to the Trial of Org 10172 in Acute Stroke Treatment (TOAST).[25] Patients were selected with computed tomography or magnetic resonance imaging infarcts present at least 20 hours after ischemic onset and infarct volumes were calculated by the ABC/2 method.[26]

Middle Cerebral Artery Occlusion in Mice

A total of 36 male CD-1 mice (weight range: 25—30 g) were purchased from SLAC Laboratory Animals (Shanghai, China) and housed under a light:dark schedule (12h:12h) with free access to water and food before surgery. Focal cerebral ischemia was produced by establishing an in vivo middle cerebral artery occlusion (MCAO) model, as previously reported.[27] Briefly, mice were anesthetized with an intraperitoneal injection of ketamine/xylazine (100:10 mg/kg) and we gently inserted a silicone-coated 6—0 suture (Doccol Corporation, MA) into the internal cerebral artery through the external cerebral artery, and stopped at the opening of the middle cerebral artery. After 120 minutes of MCAO, the suture was removed to restore blood flow. Then a laser Doppler flow meter (Moor Instruments, UK) was used to verify successful occlusion. Sham-operated mice were manipulated in the same manner except MCAO. Animal care and sacrifice methods were approved by the Institutional Animal Care and Use Committee (IACUC, no. c2301546) of China Medical University.

Preparation of Stroke Patient and Mouse Blood Samples

The peripheral blood sample (5 mL) was collected from stroke patients into tubes containing ethylenediaminetetraacetic acid, and these tubes were immediately centrifuged at 1500 *g* for 10 minutes at 4°C. Then, total RNA was extracted within 12 hours following the manufacturer's instructions (TRIzol, Invitrogen, Carlsbad, CA).

After 24 hours of ischemia, the mice were anesthetized with and intraperitoneal injection of ketamine/xylazine (100:10 mg/kg). When the mouse lost consciousness and was unresponsive to a toe pinch, we decapitated it quickly by cutting the neck with sharp scissors to ensure death. Blood samples were collected from severed necks of decapitated mice into a collection tube.

Intracerebroventricular Injection of miR-451 Mimics and miR-451 Inhibitor

The miR-451 mimics and miR-451 inhibitor were purchased from Ambion (Austin, TX) and administered intracerebroventricular injection, as reported previously.[28] Briefly, mice were deeply anesthetized using pentobarbital sodium and fixed in a stereotaxic apparatus. MiR-451 mimics (GCTGTTTATCTTCCATGCAAACTCGAGTTTG) and miR-451 inhibitor (GCTGTTTATCTTCCATGCAAACTCGAGTTTG) were diluted with Entranster in vivo transfection reagent (Engreen, Beijing, China) and then injected intracerebroventricularly using a microsyringe (Hamilton, Reno, NV) 3 days prior to MCAO.

Measurement of Infarct Volume

Immediately after blood collection, the mouse brains were removed and cut into 1.0-mm—thick coronal sections. The slices were incubated in a solution of 0.5% 2, 3, 5-triphenyltetrazolium chloride (TTC) for 15 minutes at 37°C, and then scanned into a computer. The images of stained slices were analyzed in Image Pro Plus1 6.0 (Media Cybernetics, Silver Spring, MD). The infarct volume was calculated using the formula: 100 × (contralateral hemisphere volume − non-infarct ipsilateral hemisphere volume)/ contralateral hemisphere volume.

Quantitative Real-time PCR

The ischemic brain tissues and blood were collected for total RNA extraction using TRIzol reagent (Invitrogen). The quantitative real-time PCR (qRT-PCR) was used to determine the expression levels of miR-451 between the 2 groups. In brief, 10 ng total RNA was reverse-transcribed to cDNA using the TaqMan miRNA reverse transcription kit (catalogue number 4366596, Applied Biosystems, Foster City, CA). Real-time PCR was performed using a standard SYBR Green PCR kit (QPK-201, Toyobo, Osaka, Japan) and a 7500 Real-Time PCR system (Applied Biosystems) following the manufacturer's instructions. The primer sequences were as follows: miR-451-F 5′-GAACGTCGAAAA-GAAAAGTCTCG- 3′, miR-451-R: 5′-CCTTATCAAGATGCGAACTC-ACA-3'; U6: F 5′-TGTTCGTCATGGGTGTGAAC-3′, U6: R-5′ATG-GCATGGACTGTGGTCAT-3'. The U6 (Applied Biosystems) was used as endogenous control. Relative miRNA expression levels were quantified using the $2^{-\Delta\Delta Ct}$ method. The qRT-PCR protocol consisted of a pre-incubation step at 95°C for 5 minutes; 45 cycles of 60°C for 30 seconds; melting curve of 95°C for 10 seconds, 65°C for 60 seconds, and 65°C for 1 second. PCR was performed in triplicate for each sample.

Western Blotting Analysis

The cerebral cortices of the ischemic brain were collected and lysed with radioimmunoprecipitation assay lysis buffer (Thermo Fisher Scientific, Inc., Waltham, MA) for 20 minutes on ice. The bicinchoninic acid method was used to detect protein concentration. Total protein (30 μg) was separated by 10% SDS-PAGE and transferred to 0.45 μm polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA). After blocking with 5% bovine serum albumin for 2 hours at room temperature, the membranes were incubated with primary antibodies against Bax, Bcl-2, and GAPDH (all from Abcam, Cambridge, UK) at 4°C overnight. After rinsing with Tris-buffered saline-0.01% Tween-20, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody for 1 hour at room temperature. Enhanced chemiluminescence (Pierce; Thermo Fisher Scientific, Inc.) was applied to visualize the bands. Image acquisition was performed using the Tanon-6200 gel imaging system (Tanon Science and Technology Co., Ltd., Shanghai, China). Image processing software (ImageJ; Version 14.8; US National Institutes of Health) was used to analyze the images.

Statistical Analysis

All numerical data are expressed as mean ± standard deviation and performed with triplicate independent test. All statistical calculations were performed using SPSS 13.0 software (SPSS Inc., Chicago, IL). For categorical variables, the χ^2 test was used. Independent samples *t*-test was used for 2-group comparisons. One-way analysis of variance was used to compare groups with more than 3 groups. Correlations were estimated by Pearson correlation test. Differences were considered significant at $P < 0.05$.

Table 1. Clinical Characteristics of the Healthy Control and Ischemic Stroke Patients

Demographic Characteristic	Control	Stroke	*P* Value
Total N	97	108	NA
Race (Han), %	100%	100%	1†
Ethnicity (Yellow), %	100%	100%	1†
Age, years	59 ± 6.4	64 ± 9.2	0.72*
Sex, male/female	45/52	75/33	0.64†
Hypertension	46 (47.4)	58 (53.7)	0.84†
Hyperlipidaemia	57 (58.8)	65 (60.2)	0.57†
Diabetes	4 (4.1)	34 (31.5)	0.002†
Cardiopathy	5 (5.2)	41 (38.0)	0.004†
NIHSS score, mean (range)	NA	3.86 (1, 12)	NA
TOAST LA	NA	56 (51.8)	NA
CE	NA	14 (13.0)	NA
SA	NA	38 (35.2)	NA

Data are presented as n (%) or mean ± standard deviation unless otherwise noted.
NIHSS, National Institute of Health Stroke Scale; TOAST, Trial of Org 10172 in Acute Stroke Treatment; LA, large artery atherosclerotic stroke; CE, cardioembolism; SA, small artery stroke; NA, not available.
*Student's *t* test was used.
†χ^2 test was used.

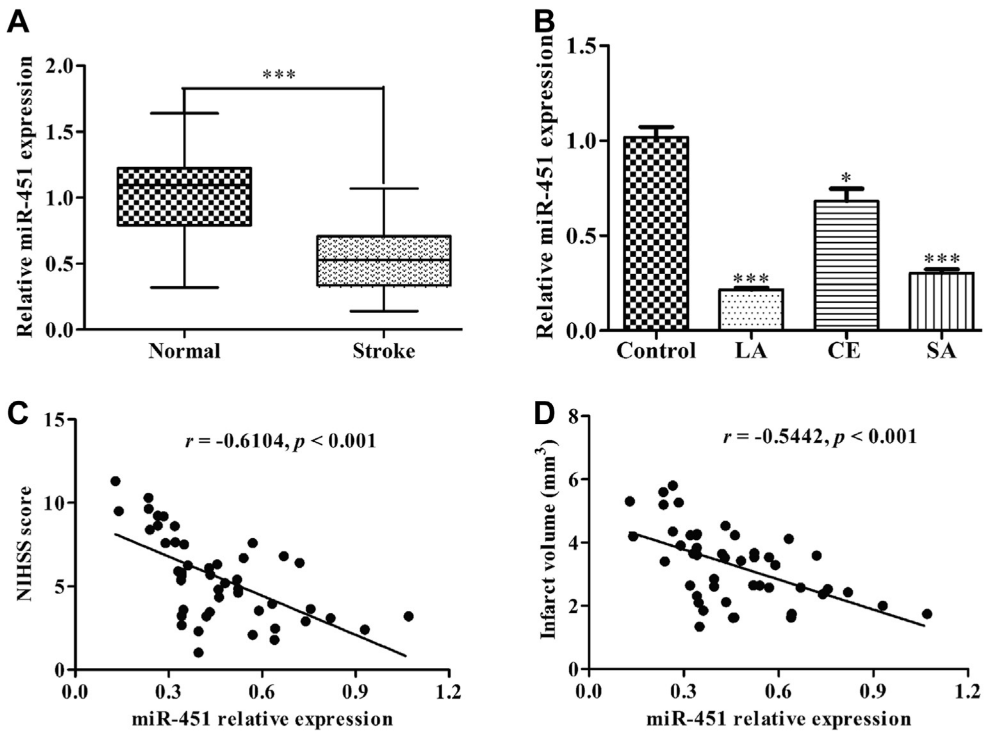

Figure 1. miR-451 expression patterns and the correlation with clinical data. (**A**) miR-451 was significantly decreased in 108 stroke patients compared with 97 controls. (**B**) Subtypes of strokes in our study included 56 large-artery atherosclerosis (LA) patients, 14 cardioembolism (CE) patients, and 38 small artery stroke (SA) patients. miR-451 levels have a negative correlation with (**C**) National Institute of Health Stroke Scale (NIHSS) scores and (**D**) infarct volume of stroke patients. **P* < 0.05; ****P* < 0.001.

RESULTS

Clinical Characteristics of Patients

Ischemic stroke patients were clinically and radiologically diagnosed. The clinical characteristics of patients (n = 108) and control participants (n = 97) are summarized in Table 1. Stroke patients (mean age: 64 years, male/female = 75/33) had no difference to the control group either in age (P = 0.72) or in sex (P = 0.64). However, we found a greater percentage of risk factors in stroke patients including diabetes 31.5% (P < 0.01) and cardiopathy 38.0% (P < 0.01), which might be unmatched risk factors between 2 groups. In addition, a total of 108 stroke patients were classified as LA 51.8%, CE 13.0%, and SA 35.2%. The mean NIHSS score of stroke patients was relatively low (3.86).

Circulating miR-451 Expression Level in Stroke Patients

Using qRT-PCR assay, we determined the expression level of miR-451 in ischemic stroke patients and healthy controls. As shown in Figure 1A, circulating miR-451 was decreased in the stroke group compared with the control group (P < 0.001). We further analyzed the miR-451 expression in the subtypes of ischemic stroke including LA, CE, and SA. Our results demonstrate that miR-451

Table 2. Circulating miR-451 Expression and the Risk of Stroke

Factor	Adjusted *P*-value	Odds Ratio	95% Confidence Interval
Diabetes	0.032	1.94	1.36—2.41
Cardiopathy	0.064	9.12	1.52—26.51
miR-451	0.0009	8.52	1.65—10.62

Statistical model analysis by multivariable logistic regression.

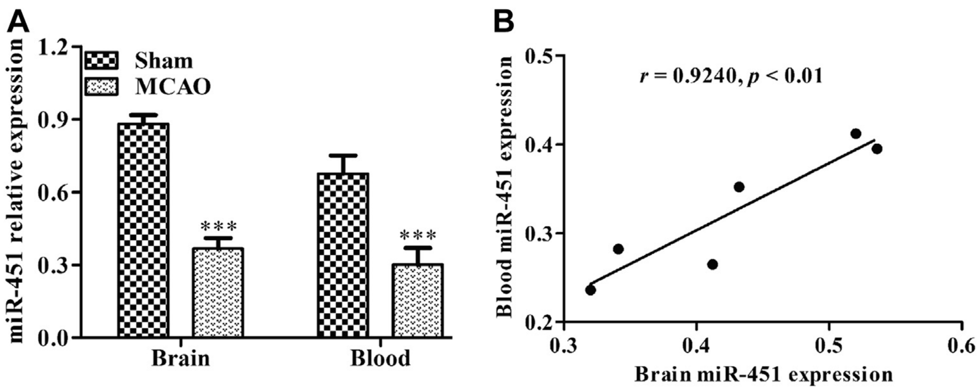

Figure 2. miR-451 levels in brain tissue and blood of ischemic mice. (**A**) The expression of miR-451 in brain tissue and blood of middle cerebral artery occlusion (MCAO) mice at 24 hours after ischemia. $^{***}P < 0.001$, MCAO (n = 6) versus sham (n = 6). (**B**) The positive correlation between brain and blood miR-451 at 24 hours after ischemia. r = 0.9240, $P < 0.01$, n = 6 per group.

expression level was decreased in these 3 subtypes compared with the control group (Figure 1B, $P < 0.05$; $P < 0.001$). Furthermore, the Pearson correlation test was performed to examine the association between miR-451 expression level and NIHSS scores or brain infarct volume. A negative correlation was found between miR-451 expression and NIHSS scores (Figure 1C, $r = -0.6104$, $P < 0.001$) and brain infarct volume (Figure 1D, $r = -0.5442$, $P < 0.001$). In addition, we performed a logistic regression analysis to eliminate the unmatched risk factors (diabetes and cardiopathy) impact on miR-451 expression between the 2 groups. The result indicated that circulating miR-451 expression was a possible risk factor of stroke occurrence (Table 2, $P < 0.001$).

Brain and Blood miR-451 in Ischemic in Vivo Mouse Model

To assess the potential role of miR-451 in stroke pathogenesis, we further established an in vivo model of brain ischemia/reperfusion injury by MCAO and determined the relationship of miR-451 expression between circulating blood and brain tissues. As shown in Figure 2A, miR-451 was significantly downregulated in both brain and blood samples in MCAO mice compared with that of sham mice (brain, MCAO vs. sham, 0.37 ± 0.06 vs. 0.88 ± 0.12, $P < 0.001$; blood, MCAO vs. sham, 0.31 ± 0.14 vs. 0.68 ± 0.06, $P < 0.001$). Further correlation analysis revealed a positive correlation between the circulating blood miR-451 level blood and the brain miR-451 levels using Spearman's correlation analysis (Figure 2B, $r = 0.9240$, $P < 0.01$).

Upregulated miR-451 Attenuated Ischemia-Reperfusion Injury in a Mouse MCAO Model

To investigate the function of miR-451 in ischemic brain injury, we injected miR-451 mimics and miR-451 inhibitor into the cerebral cortex and determined the expression of miR-451 in these 2 treatments by qRT-PCR. As shown in Figure 3A, injection of miR-451 mimics and miR-451 inhibitor could effectively upregulate and downregulate, respectively, miR-451 expression in MCAO mice ($P < 0.001$). Then the infarct volume of ischemic brain treated with miR-451 mimics or miR-451 inhibitor was detected. Obviously, the infarct volume was significantly increased in the MCAO compared with sham group ($P < 0.001$). Of note, miR-451 mimics significantly reduced the infarct volume, whereas this effect was reversed by downregulation of miR-451 (Figure 3B, $P < 0.01$, $P < 0.05$). In addition, we analyzed the effect of miR-451 on apoptosis levels following MCAO in mice. As shown in Figure 4, the upregulation of Bax and downregulation of Bcl-2 levels in MCAO group was reversed by miR-451 mimics treatment, but aggravated by miR-451 inhibitor treatment.

DISCUSSION

Currently, the diagnosis of stroke mainly depends upon clinical grounds and imaging studies. However, these diagnostic methods are usually not reliable for ischemic stroke risk prediction, diagnosis, and outcome prediction. Numerous circulating miRs have been reported to have a potential value in diagnosis of stroke. For instance, miR-99a decreases neuronal damage after focal cerebral ischemia/reperfusion injury in mice.[29] Chi et al. revealed that downregulation of miR-134 induced neuroprotection against ischemic injury in vitro and in vivo.[30] More and more miRs, such as miR-126,[31] miR-30a,[32] miR-124,[33] and miR-138[34] have been reported to be involved in the process of cerebral ischemia/reperfusion injury. These findings suggest that various miRs participate in the ischemic stroke process, which might be reliable and easily detectable circulating biomarkers for ischemic stroke risk and/or outcome prediction.

In the present study, we found circulatory miR-451 expression was decreased in patients compared with controls. The changing expression levels in brain samples were also correlated with blood samples obtained from mice models. Consistently, Min et al. reported that miR-451 was markedly downregulated in the cortex of rats with cerebral ischemia/reperfusion injury compared with rats in the sham group.[17] Additionally, increasing evidence also suggests an important role of miR-451 in the nervous system. MiR-451 is

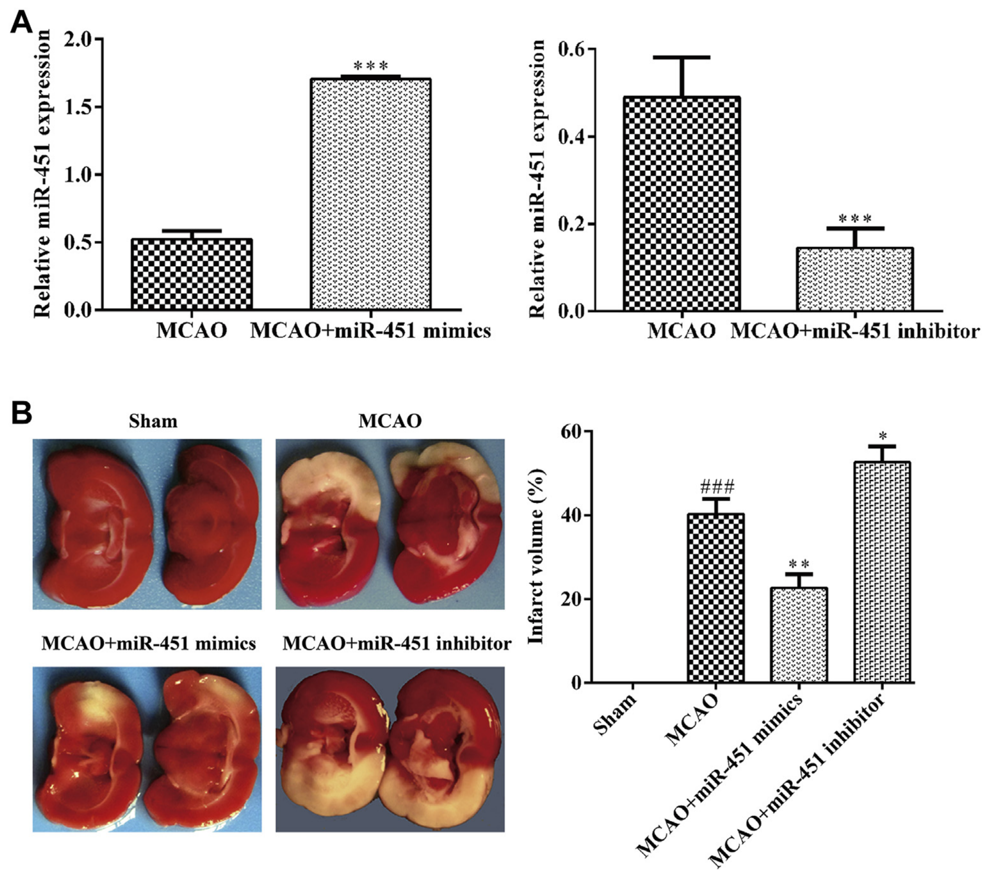

Figure 3. miR-451 upregulation weakened ischemic brain infarction. (**A**) The expression of miR-451 in brain treated with miR-451 mimics or miR-451 inhibitor before middle cerebral artery occlusion (MCAO) for 24 hours, n = 6 per group. (**B**) 2, 3, 5-triphenyltetrazolium chloride staining of representative coronal sections in different groups (**left**) and the relative infarct area percentage was evaluated by observing the unstained infarcted tissue zone (*white*) and the stained normal tissue zone (*red*) (**right**); n = 6 per group. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ versus MCAO; $\#\#\#P < 0.001$ versus sham.

downregulated in Alzheimer disease brain as a contributor the pathogenesis of Alzheimer disease.[35] MiR-451 overexpression leads to increased vulnerability in transfected neurons after experimentally induced traumatic brain injury.[36]

Furthermore, we analyzed miR-451 expression patterns in 3 subtypes of ischemic stroke including LA, CE, and SA. Our results demonstrated that miR-451 expression level was decreased in all of these subtypes compared with controls. Interestingly, we found stroke patients and the control group had a similar percentage of individuals with hypertension ($P = 0.84$), which may be ascribed to the increased awareness of healthcare in people with hypertension. Because the NIHSS score was usually used to assess the severity level of ischemic stroke, we further evaluated the relationship of miR-451 expression level and NIHSS score and found a negative correlation between them.

Whether circulating miR-451 could reflect the response of brain tissue to ischemia was unclear. We thus established an in vivo model of brain ischemia/reperfusion injury by MCAO to explore the role of miR-451 expression in stroke pathogenesis. We first found a positive correlation between the circulating blood miR-451 level and the brain miR-451 levels. Importantly, delivery of miR-451 mimics reduced brain infarct volume, whereas miR-451 downregulation increased brain infarct volume. Small baseline infarct volume was found to be a strong predictor of survival in patients with acute ischemic stroke due to anterior circulation proximal artery occlusion.[37] We speculate that higher miR-451 levels mainly predict a good outcome in stroke patients. Conversely, a low level of miR-451 indicates a prediction of poor outcome.

We found that miR-451 attenuated apoptosis levels after MCAO in mice, as reflected by increased Bcl-2 and decreased Bax expression. In agreement with our data, upregulation of miR-451 protects cells against cardiomyocyte anoxia/reoxygenation and alleviated loss of cardiomyocyte viability.[38,39] MiR-451 overexpression increased oxygen and glucose deprivation/reoxygenation cell viability and

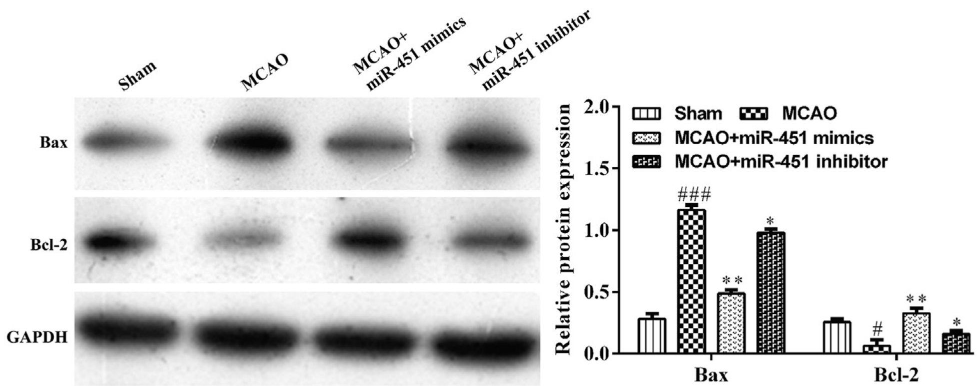

Figure 4. MiR-451 regulated the apoptotic levels in an MCAO mouse model. MiR-451 reduced Bax and increased Bcl-2 expression in middle cerebral artery occlusion (MCAO) mice (n = 6 per group). $*P < 0.05$; $** P < 0.01$ versus MCAO; # $P < 0.05$, ## $P < 0.01$ versus sham.

decreased apoptosis by targeting CUGBP Elav-like family member 2.[23] Our results revealed that insufficiency of miR-451 could exacerbate human ischemia stroke injury, which might be associated with reduced binding activities to the 3′ untranslated region of its target miRNAs.

In summary, the current study demonstrated the significant expressional changes of miR-451 in ischemic stroke occurrence, subtypes, and infarct volume. Our findings reveal miR-451 could induce neuroprotection against ischemic injury and might be a viable molecular predictor in the process of ischemia stroke development. Our study only investigated the role of miR-451 on cerebral ischemia/reperfusion in an in vivo animal model, however. Therefore, further studies on the binding target gene of miR-451 need to be identified by in vitro luciferase process to elucidate the mechanisms of miR involved in the pathogenesis of ischemic stroke.

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