

# Upregulation of MicroRNA-128 in the Peripheral Blood of Acute Ischemic Stroke Patients is Correlated with Stroke Severity Partially through Inhibition of Neuronal Cell Cycle Reentry

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## Abstract

MiR-128, one of the most enriched miRNAs in the human brain, has been reported to protect MCAO mice via inhibiting P38 $\alpha$  MAPK. Whether it is involved in pathogenesis in acute ischemic stroke patients remains to be determined. The present study focused on the clinical importance of miR-128 and its underlying mechanisms. We detected miR-128 levels in the circulating lymphocytes, neutrophils, and plasma of acute ischemic stroke patients by using RT-PCR. miR-128 levels were significantly elevated in circulating lymphocytes, neutrophils, and plasma of patients with acute ischemic stroke. In addition, miR-128 levels in circulating lymphocytes correlated positively with the infarction volume, NIHSS scores at 7 days and mRS at 90 days after ischemic stroke onset. Subsequent KEGG pathway analysis showed that the MAPK signaling pathway and cell cycle are among the pathways targeted by miR-128. Although no correlation was found between miR-128 in plasma and peripheral inflammatory cell numbers, miR-128 decreased in the penumbra and increased in the infarction core of ipsilateral brain tissues in MCAO mice. Moreover, an in vitro study demonstrated that miR-128 antagomir aggravated primary neuronal damage and exacerbated cell cycle reactivation induced by OGD/R stimulation; the underlying mechanism involved increasing cyclin A2, PTEN, and ERK expression and promoting phosphorylation of PTEN and ERK. From the above results, we concluded that the upregulation of miR-128 in circulating lymphocytes of acute ischemic stroke patients was correlated with stroke severity and miR-128 antagomir exacerbated ischemia-reperfusion induced neuronal injury via promoting neuronal cell cycle reentry.

## Keywords

ischemic stroke, microRNA, ischemia-reperfusion, cell cycle

## Introduction

MicroRNAs (miRNAs) are a class of endogenously expressed small noncoding RNAs, which are usually 18–24 nucleotides long and play an important role in various pathophysiological processes via regulating gene expression post-transcriptionally<sup>1</sup>. Because of their master role in gene control and their ease of isolation, dozens of miRNAs isolated from plasma/serum, cerebrospinal fluid, or circulating blood cells of ischemic stroke patients have been identified as potential biomarker candidates<sup>2–7</sup>.

MiR-128 is one of the most enriched miRNAs in the adult mouse and human brain<sup>8,9</sup>. It is encoded by two separate genes, *miR-128-1* and *miR-128-2*, on mouse chromosomes

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1 and 9, or human chromosomes 2 and 3. In mice, germline *miR-128-2* deficiency results in an 80% decrease in miR-128 expression in the forebrain, whereas ablation of the *miR-128-1* gene eliminates only 20% of miR-128 expression<sup>10</sup>. During postnatal development, the expression of miR-128 in the mouse brain increases gradually and peaks in adulthood<sup>11,12</sup>.

The diverse expression of miR-128 throughout regions of the brain suggests its important roles in processes common to various cell-types. Franzoni et al have reported that miR-128 promotes functional neuronal maturation during cortical development via its involvement in neuronal migration, outgrowth, and intrinsic excitability<sup>13</sup>. Chan et al have found that miR-128 governs motor activity by suppressing the expression of various ion channels and signaling components of the extracellular signal-regulated kinase ERK2 network that regulates neuronal excitability<sup>10</sup>. Moreover, miR-128 has also been shown to regulate the proliferation and differentiation of neural progenitor cells, cell-cycle progression in glioblastoma multiforme, and myocardial ischemia/reperfusion injury-induced cardiomyocyte apoptosis through targeting pericentriolar material 1 (PCM1), the transcription factor E2F-3a, or peroxisome proliferator-activated receptor gamma (PPARG)<sup>14-16</sup>. In addition, Mao et al have demonstrated that miR-128 protects against cerebral ischemic injury through decreasing p38 $\alpha$  mitogen activated protein kinase activity in an MCAO mouse model<sup>17</sup>. In the present study, we aimed to further verify the specific effect of miR-128 on acute ischemic stroke patients and to explore its potential regulatory role in neuronal ischemia-reperfusion injury and cell cycle activation following cerebral ischemia.

## Materials and Methods

### Blood Samples of Patients with Acute Ischemic Stroke

The use of the human blood samples for research purposes was approved by the Ethics Committee of Capital Medical University. Written informed consent was obtained from all participants. We enrolled 40 patients with acute ischemic stroke who were treated in the Department of Neurology, Xuanwu Hospital of Capital Medical University. The inclusion criteria were as follows: (1) diagnosis of first ischemic stroke on the basis of clinical information and MRI images; (2) patients aged 55–65 years old; (3) presentation of subjects within 72 h of the event; (4) National Institutes of Health Stroke Scale (NIHSS) score of 4–15; and (5) Trial of ORG 10172 in Acute Stroke Treatment stroke (TOAST) subtype of large-artery atherosclerosis. Equivalent numbers of age-matched patients were enrolled as controls. Blood was collected from each patient/volunteer for analysis.

### Neurological Deficit Scores and Magnetic Resonance Imaging

All patients underwent standard neurological and general medical evaluation and assessment on the basis of the

NIHSS score at 7 days and the modified Rankin scale (mRS) at 90 days after stroke onset.

Magnetic resonance imaging (MRI images were acquired within 72 h of stroke onset on a 3.0 T Magnetom Verio Syngo instrument (Siemens, Munich, Germany). The radiologist used diagnostic workstations with a unisight system for interpretation and measurement of the infarct volume. The analysis was performed blind to the clinical history, physical findings, patient identity, and final diagnostic results. The total infarct volume was calculated on the basis of the infarct size on the diffusion-weighted imaging sequence and then multiplied by the thickness. All image data were standardized according to intracranial cross-sectional area<sup>18</sup>. The formula of the volume standardization of cerebral infarction was as follows: infarct volume  $\times$  mean of intracranial cross-sectional area/intracranial cross-sectional area of patient.

### Separation of Neutrophils, Lymphocytes, and Plasma

Blood samples (2  $\times$  4 ml) were collected into EDTA-anticoagulant tubes and processed according to the following procedures: first, each blood sample was immediately centrifuged at 200 g for 10 min at 4°C, and plasma was obtained. Second, the blood cells were diluted with 8 ml normal saline and slowly added to the surface of the lymphocyte separation medium (Tian Jin Hao Yang Biological Manufacture Co., Beijing, China) in two 15 ml centrifuge tubes. After the tubes were centrifuged at 400 g for 20 min at 20°C, the lymphocytes were separated and saved. Finally, erythrocytes were dissociated with erythrocyte lysis solution three 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 laboratory testing.

### MiR Extraction and Real-Time Reverse Transcription Polymerase Chain Reaction

Total RNA in neutrophils, lymphocytes, and plasma was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and processed as previously described<sup>19</sup>. The PCR primers for miR-128 were 5'-GCGGAGCCGTAGCAC TGT3' and 5'-GTGCGTGTTCG -TGGAGTCG3'. The PCR primers for U6 were 5'-GCTTCGGCAGCACATATAC-TAAAAT-3' and 5'-CGCTTACGAATTTGCGTGTCAT-3'. Relative gene expression was calculated via the  $2^{-\Delta\Delta CT}$  method and expressed as fold change relative to U6 expression.

### Prediction of miR-128 Related Inflammation Pathways and a miRNA-Pathway Network

Targetscan (<http://www.targetscan.org>), miRanda (<http://www.microRNA.org>), and miRBase target prediction database (<http://www.ebi.ac.uk/enrightsrv/microcosm>) were used to determine the predicted target genes of miR-128 as previously described<sup>20</sup>. The overlapping targets predicted by

the above three databases were subjected to Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis; the selection criterion for significant KEGG pathway terms was a  $P$  value  $<0.01$ .

### Mouse Model of Focal Cerebral Ischemia

Male C57Bl/6 mice weighing 20–25 g were purchased from Vital River Laboratory Animal Technology Co. (Beijing, China). All animal experiments in this study were approved by the Institutional Animal Care and Use Committee of Capital Medical University. Mice models of transient focal cerebral ischemia were induced as described previously<sup>21</sup>. To ensure the occurrence of ischemia by MCAO, regional cerebral blood flow was monitored using laser Doppler flowmetry (PeriFlux System 5000, Perimed, Sweden) at a location 0.5 mm anterior and 5.0 mm lateral from the bregma. The rectal temperature was maintained at 37.0°C during and after surgery with a temperature-controlled heating pad (CMA 150; Carnegie Medicin, Sweden).

### Primary Neuronal Culture and Treatment

The primary neurons were dissociated carefully from the cortex of P16–18 C57BL/6 mice. They were seeded at a density of  $5 \times 10^5$  cells/cm<sup>2</sup> and grown in neurobasal medium with 2% B27 supplement (Gibco, Carlsbad, CA, USA), which were replaced half of fresh medium every 3 days. After being cultured for 7 days, the primary neurons were transfected with miR-128 antagomir using MessengerMAX<sup>TM</sup> Reagent (Lipofectamine MessengerMAX<sup>TM</sup>, Invitrogen) for 48 h as pre the manufacturer's protocol. Sense of miR-128 antagomir were synthesized by GenePharma and the sequences were as follows: 5'AAAGAGACCGGUUCACUGUGA3'.

In order to establish the oxygen and glucose deprivation / reoxygenation (OGD/R) cell model, after 48 h transduction, primary neurons were subjected to 2.5 h OGD/24 h R. For OGD treatment, the cells were cultured in glucose-free DMEM and kept in a hypoxic incubator chamber (Billups-Rothenberg, San Diego, CA, USA) filled with 95% N<sub>2</sub>/5% CO<sub>2</sub> at 37°C. After OGD for 2.5 h, the cells were transferred to normal medium under normoxic conditions (5% CO<sub>2</sub>/21% O<sub>2</sub>/74% N<sub>2</sub>) for 24 h.

### Quantification of Lactate Dehydrogenase Activity

Lactate dehydrogenase (LDH) activity was determined as the manufacturer's protocol. Simply speaking, 10  $\mu$ l medium cultured with primary neuron and 190  $\mu$ l working reagent were transferred into 96-well plate and OD<sub>565</sub> were read 0 min (OD<sub>S0</sub>) and 25 min (OD<sub>S25</sub>) after they were mixed briefly; 200  $\mu$ l H<sub>2</sub>O (OD<sub>H2O</sub>) and 200  $\mu$ l Calibrator (OD<sub>CAL</sub>) solution were also transferred into 96-well plate. LDH activity were quantified as the following calculation: LDH activity =  $43.68 \times (OD_{S25} - OD_{S0}) / (OD_{CAL} - OD_{H2O}) \times 10$  (IU/L).

### Cell-Cycle Analysis by Flow Cytometry

At 24 h after reoxygenation, primary neurons were washed with PBS, detached with 0.25% trypsin, and fixed with 70% ethanol overnight. The samples were then resuspended in 0.5 mL PBS, treated with RNase to remove RNA and stained with propidium iodide (Sigma-Aldrich, St. Louis, MO, USA) in the dark for 30 min. The DNA content was measured by fluorescence-activated cell sorting on a FACSCanto flow cytometer with Cellfit software (Becton-Dickinson, San Jose, CA, USA).

### Western Blotting

The ipsilateral penumbra and ischemic core were collected 1 h after ischemia and 1 h, 4 h, and 24 h after reperfusion, then processed for western blotting as previously described<sup>22</sup>. The antibodies (1:1000) specific to cyclin A2, PTEN/P-PTEN, and ERK/P-ERK (Cell Signaling Technology, Boston, MA, USA), and  $\beta$ -actin (Bioworld, Nanjing, China) were used. Blots were detected with horseradish peroxidase-conjugated secondary antibody (1:2000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and an enhanced luminescence kit.

### Statistical Analysis

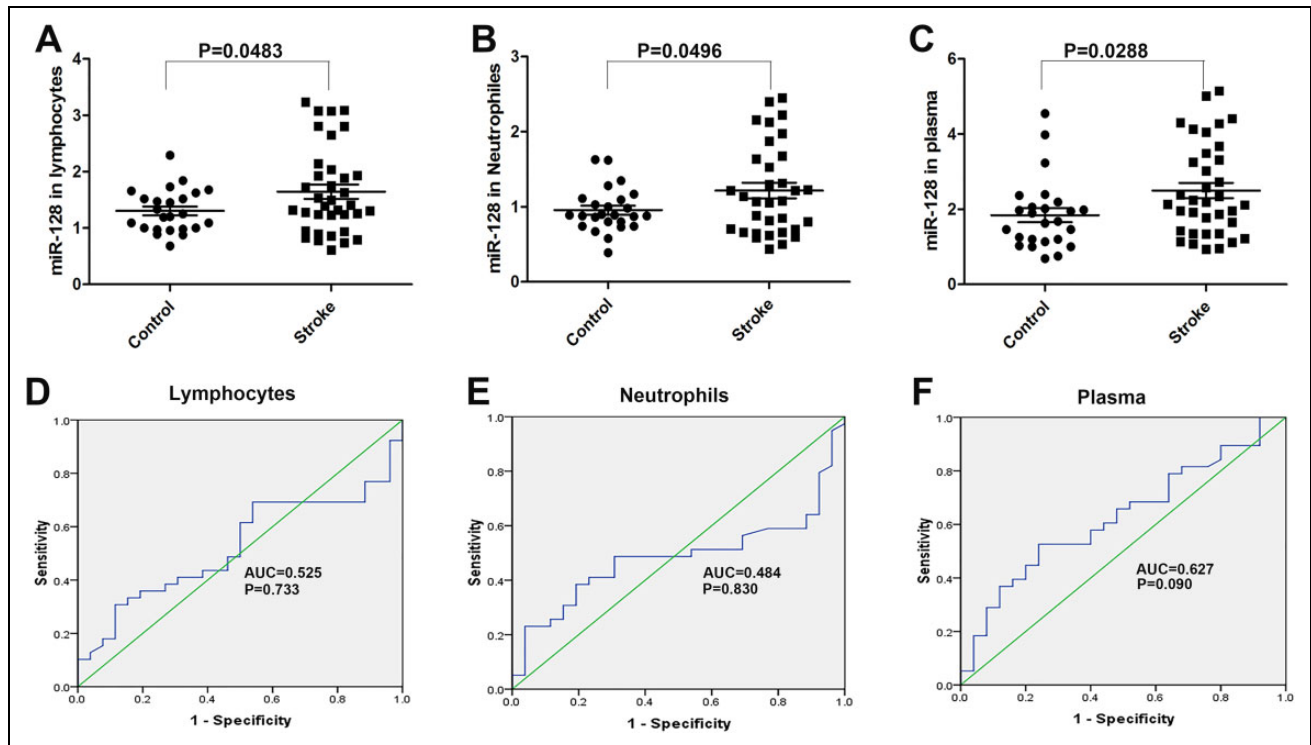
Statistical analyses were carried out using SPSS Version 17.0 software (SPSS Inc., Chicago, IL, USA). Data are expressed as the mean  $\pm$  SEM. Statistical significance between groups was determined with unpaired  $t$ -tests. For correlation analyses, we used the Pearson correlation test.  $P < 0.05$  was considered statistically significant.

## Results

### MiR-128 is Upregulated in the Peripheral Blood (Lymphocytes, Neutrophils, and Plasma) of Acute Ischemic Stroke Patients

A total of 40 acute ischemic stroke patients and 25 healthy controls were enrolled in this study. There were no marked differences between the two groups in age, sex, or risk factors, including histories of diabetes, hypertension, hypercholesterolemia, or smoking. We collected blood samples from all patients and controls, then analyzed miR-128 levels in the circulating lymphocytes, neutrophils, and plasma. RT-PCR analysis revealed that the miR-128 levels in the circulating lymphocytes, neutrophils, and plasma were significantly higher in acute ischemic stroke patients than healthy controls (Fig. 1A–C,  $P < 0.05$ ). These results suggested that miR-128 might be involved in the pathophysiological progression of acute ischemic stroke, although the underlying mechanisms require further study.

To investigate the potential for miR-128 to be used as a diagnostic biomarker of acute ischemic stroke, receiver operating characteristic (ROC) analysis was performed, and the



**Fig 1.** Expression and diagnostic value of miR-128 in the peripheral blood (lymphocytes, neutrophils, and plasma) of acute ischemic stroke patients. (A–C) The miR-128 levels in the circulating lymphocytes ( $n = 24$  in the control group,  $n = 36$  in the stroke group), neutrophils ( $n = 25$  in the control group,  $n = 33$  in the stroke group), and plasma ( $n = 25$  in the control group,  $n = 36$  in the stroke group) in acute ischemic stroke (AIS) patients and healthy controls, as determined by semi-quantitative RT-PCR. U6 was used to normalize the expression levels of target miRNAs in different samples. (D–F) ROC analysis and AUC were used for AIS patients.

area under the curve (AUC) was calculated. Generally,  $AUC > 0.5$  was considered diagnostic,  $AUC < 0.7$  indicated a lower diagnostic value,  $0.7 < AUC < 0.9$  indicated a moderate diagnostic value, and  $AUC > 0.9$  indicated a high diagnostic value. The AUC of miR-128 in lymphocytes was 0.525, and that in plasma was 0.627; both were indicated as weakly diagnostic for acute ischemic stroke (Fig. 1D–F). This result suggested that the upregulation of miR-128 in the peripheral plasma may potentially be used as a biomarker for rapid diagnosis of acute ischemic stroke, but this possibility remains to be further confirmed in more acute ischemic patients.

MiR-128 levels in the circulating lymphocytes were positively correlated with the infarction volume and neurologic scores of acute ischemic stroke patients

To further explore whether miR-128 is involved in acute ischemic stroke progression, we analyzed the correlation of the miR-128 levels in the circulating lymphocytes, neutrophils, and plasma with the infarction volume and neurologic scores of acute ischemic stroke patients. The miR-128 levels in circulating lymphocytes were positively correlated with the infarction volume, NIHSS scores at 7 days, and mRS at 90 days after ischemic stroke onset (Fig. 2A, D and G,  $P < 0.05$ ). These results suggested a bidirectionally regulatory role of miR-128 in acute ischemic stroke. However, the miR-128 levels in the circulating neutrophils and plasma

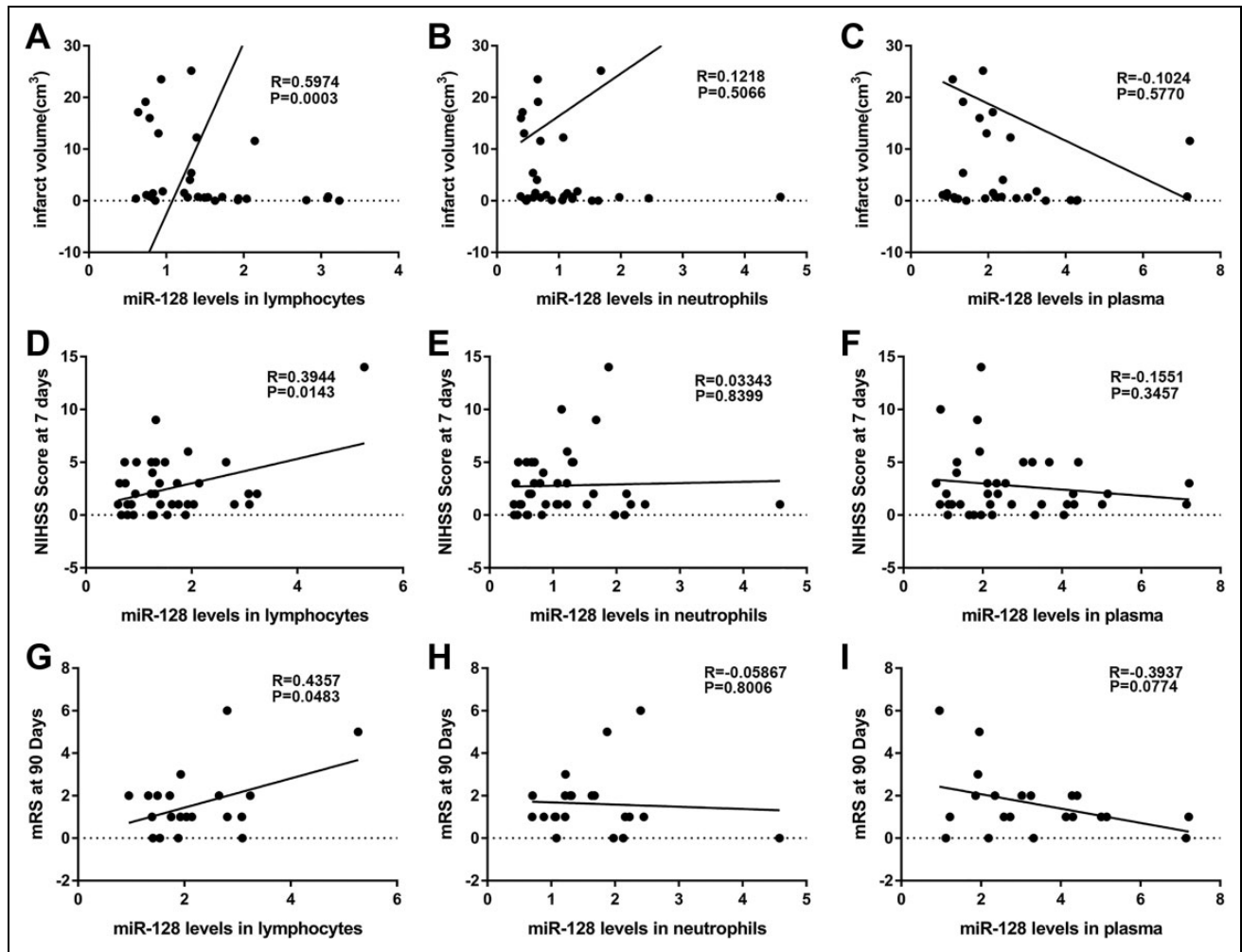
were not correlated with the infarction volume and neurologic score in acute ischemic stroke.

### *Bioinformatics Analysis Showed that miR-128 is Involved in the MAPK Pathway and Cell Cycle Progression*

On the basis of the miRNA microarray analysis (data not shown), we further predicted the inflammation related pathways targeted by deregulated miRNAs in circulating lymphocytes of acute ischemic stroke patients by using KEGG pathway analysis. The results demonstrated that miR-128 played a central role within the miRNA-pathway network (Fig. 3A). Moreover, we determined the miR-128 targeted inflammation related pathways and produced a graphical heat map, which showed that the MAPK signaling pathway and cell cycle related pathways were among those significantly targeted by miR-128 (Fig. 3B).

Plasma miR-128 levels were positively correlated with those in circulating lymphocytes; although they were not correlated with the number of circulating lymphocytes or neutrophils, they were decreased in the ipsilateral penumbra and increased in the ischemic core of MCAO mice.

To determine the origin of the miR-128 plasma expression, we analyzed the correlation between miR-128

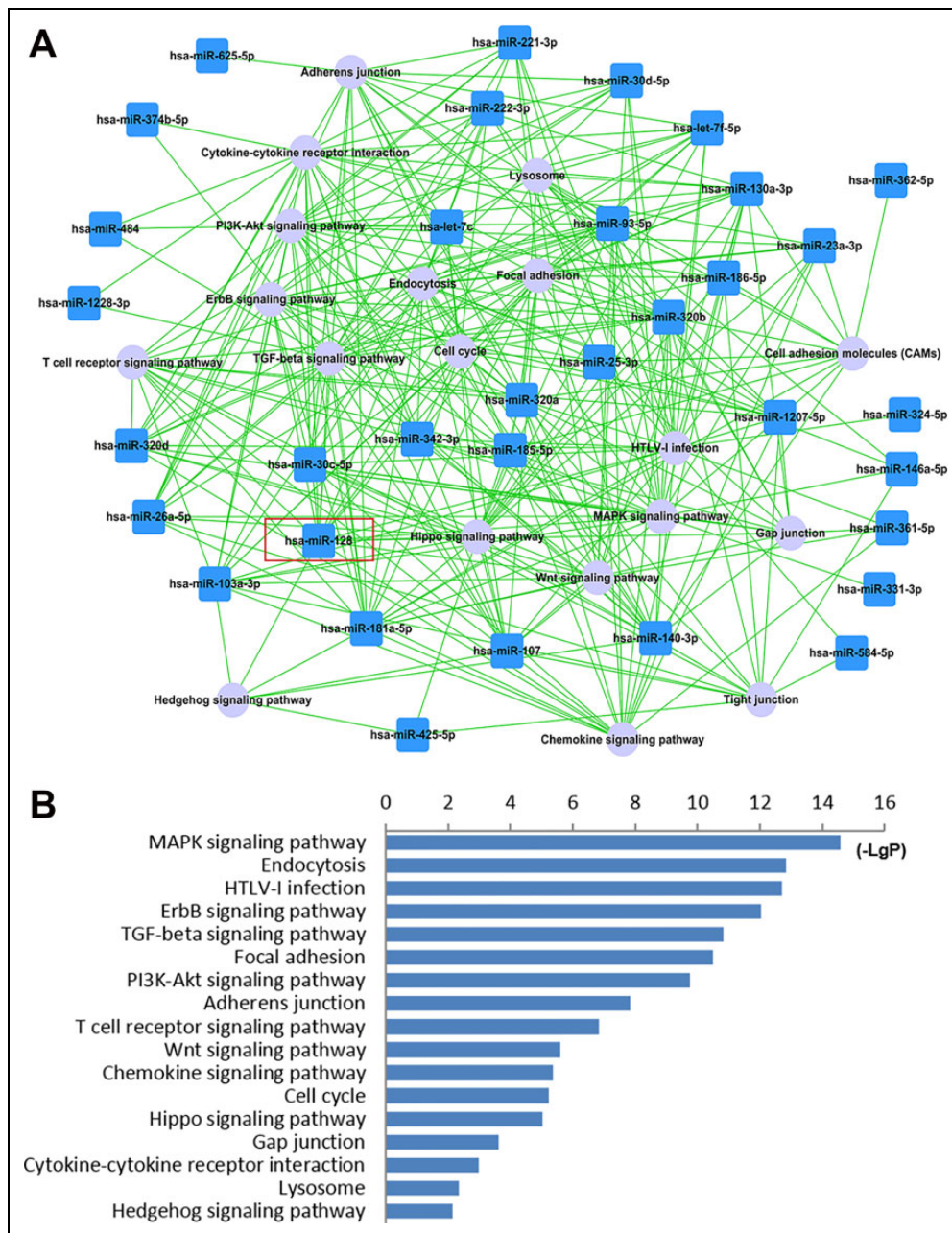


**Fig 2.** Correlations of miR-128 levels in the circulating lymphocytes, neutrophils, and plasma with the infarct volume and neurological scores of acute ischemic stroke. (A–C) Correlations of miR-128 levels in lymphocytes, neutrophils, and plasma with cerebral infarct volume in 32 AIS patients. (D–F) Correlations of miR-128 levels in lymphocytes ( $n = 38$ ), neutrophils ( $n = 39$ ), and plasma ( $n = 39$ ) with NIHSS score at 7 days after AIS onset in AIS patients. (G–I) Correlations of miR-128 levels in lymphocytes, neutrophils, and plasma with mRS scores at 90 days after AIS onset in 21 AIS patients.

expression in plasma and that in circulating lymphocytes and neutrophils. The miR-128 levels in plasma were positively correlated with the miR-128 levels in lymphocytes (Fig. 4A,  $P < 0.05$ ), thus suggesting that miR-128 in plasma partially originated from circulating lymphocytes.

We next sought to determine whether miR-128 upregulation in the peripheral blood influenced the inflammatory cell number through acting on cell cycle progression. To answer this, we analyzed the correlation between miR-128 levels in plasma and the numbers of lymphocytes and neutrophils. Surprisingly, the correlations were not significant (Fig. 4C and D). Given that the miR-128 levels in plasma did not influence the peripheral inflammatory cell numbers, whether miR-128 might enter the brain through a damaged blood brain barrier and might be involved in neuronal injury after cerebral ischemia deserves further study.

To explore the role of miR-128 in neuronal injuries following cerebral ischemia, we first detected its expression in the penumbra and ischemic core in an MCAO mouse model by using real-time reverse transcription PCR (RT-PCR). The miR-128 levels in the penumbra of ipsilateral brain tissues decreased progressively after ischemia for 1 h and reperfusion for 1 h, 4 h, and 24 h, as compared with those observed after sham operation (Fig. 4E,  $P < 0.05$ ). However, after a transient decrease, miR-128 expression in the ischemic core increased progressively and reached 3.5 times that in the sham group after reperfusion for 24 h (Fig. 4F,  $P < 0.05$ ), a result consistent with the upregulation of miR-128 levels in the circulating lymphocytes, neutrophils, and plasma of acute ischemic stroke patients. These results further demonstrate that miR-128 might have bidirectionally regulatory effects on ischemia-reperfusion cerebral injury and thus may be a potential therapeutic target for acute ischemic stroke.

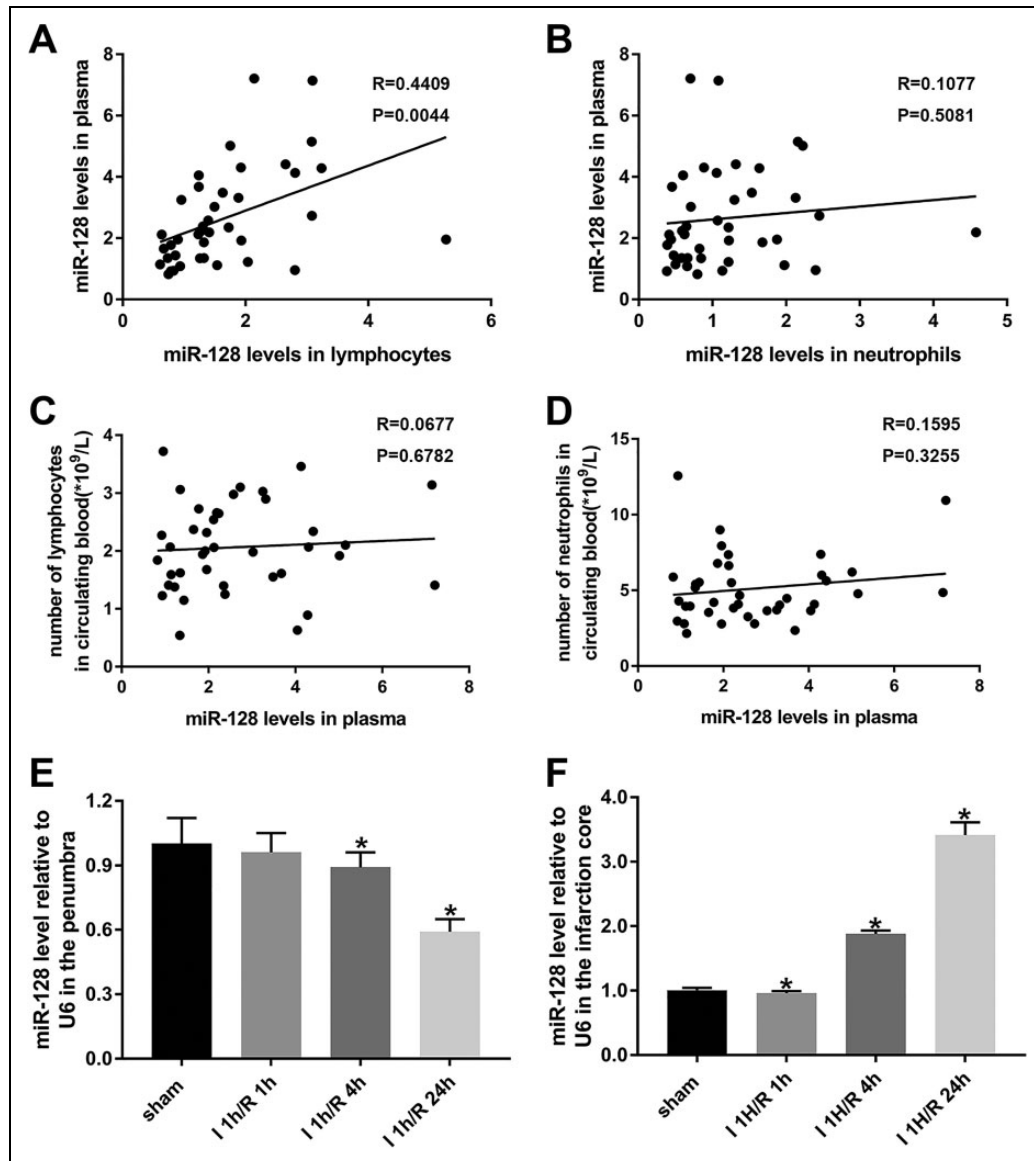


**Fig 3.** Bioinformatic analysis of miR-128 targeted pathways related to the inflammatory response. (A) Dysregulated miRNA-inflammation related pathway network of circulating lymphocytes of AIS patients, based on microarray and KEGG pathway analysis. The blue box nodes represent dysregulated miRNAs, and gray cycle nodes represent KEGG pathways. Edges show the effects of miRNAs on KEGG pathways. The red box marks the inflammation related pathways targeted by miR-128. (B) Inflammation related pathways significantly targeted by miR-128. The vertical axis is the pathway category, the horizontal axis is the significance of pathways represented by  $-\log(P \text{ value})$  (LgP), and  $P < 0.01$  is considered significant.

### *MiR-128 Antagomir Exacerbates OGD/R Induced Primary Neuronal Injury Partially Through Promoting Cell Cycle Reentry*

LDH is an endocellular enzyme and can be released outside the cells upon cell damage, so it is most often measured to evaluate the presence of tissue or cell damage<sup>23</sup>. To

investigate the potential mechanisms of miR-128, we assessed the LDH activity in primary neurons after miR-128 antagomir and OGD/R treatment using an LDH kit. The results demonstrated that LDH activity was significantly upregulated after only OGD/R stimulation, whereas miR-128 antagomir plus OGD/R treatment induced a marked increase in LDH activity (Fig. 5A,  $P < 0.05$ ). These results

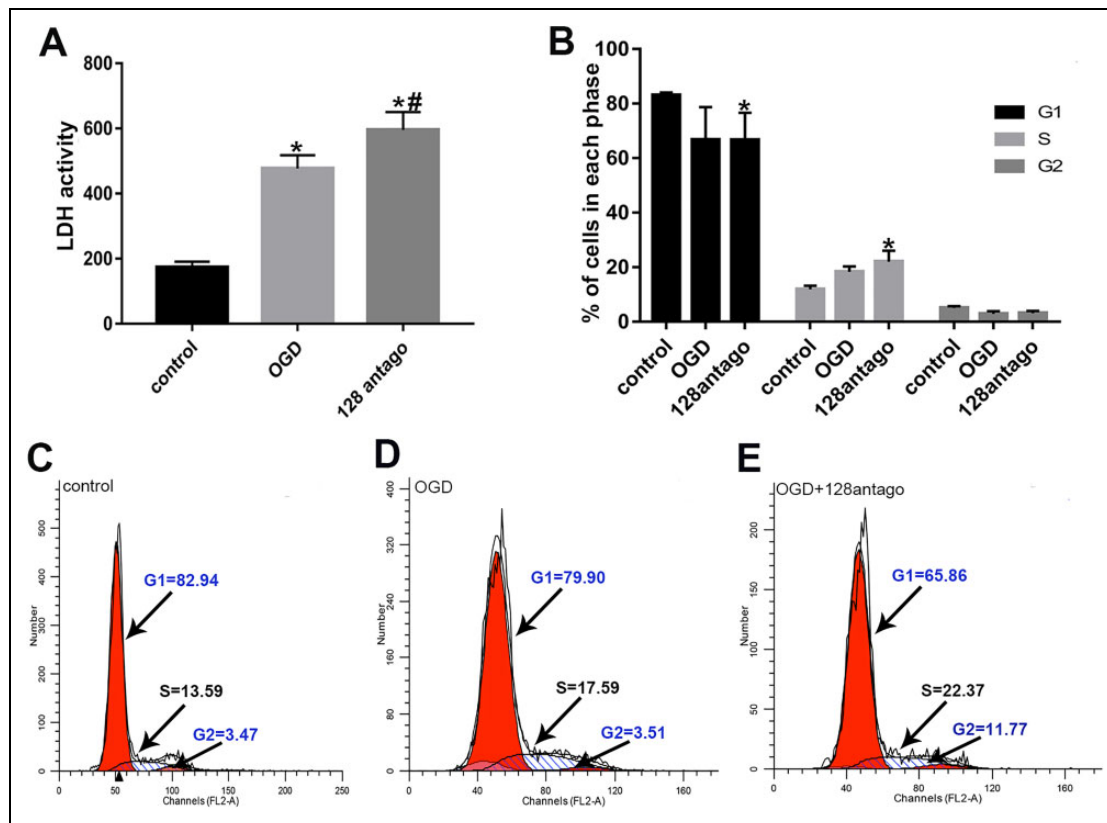


**Fig 4.** Correlations between plasma miR-128 levels and lymphocyte or neutrophil miR-128 levels and the circulating blood number of AIS patients; time-dependent changes in miR-128 expression in the MCAO mouse model. (A and B) Correlations between plasma miR-128 levels and lymphocyte or neutrophil miR-128 levels in AIS patients. (C and D) Correlations between plasma miR-128 levels and the numbers of lymphocytes or neutrophils in the circulating blood of AIS patients.  $N = 40$ . (E and F) Real-time PCR analysis of miR-128 expression changes in the penumbra and ischemic core in an MCAO mouse model after ischemia for 1 h with different reperfusion durations of 1 h, 4 h, and 24 h and in the sham-operated group. U6 was used to normalize expression levels of target miRNAs in different samples. \* $P < 0.05$  versus sham;  $n = 3$  per group.

demonstrated that miR-128 antagomir aggravated primary neuronal injury induced by OGD/R stimulation.

Neuronal cell cycle reentry is required for the neuronal injury evoked by cerebral ischemia<sup>24</sup>. In addition, we demonstrated that cell cycle progression was among the inflammation related pathways targeted by miR-128 using KEGG pathway analysis. To verify whether miR-128 acts on cell cycle progression, we performed flow cytometry assays to explore the cell cycle changes in primary neurons after OGD/R stimulation and miR-128 antagomir

transfection. We found a tendency of proportionately fewer primary neurons in G1 phase but more cells in the S phases after OGD/R stimulation in the treated cells compared with the controls (Fig. 6B). These results confirmed cell cycle reentry induced by OGD/R. However, miR-128 antagomir plus OGD/R treatment exacerbated this cell cycle activation, i.e., markedly fewer cells were found in G1 phase and significantly more primary neurons were in S phase than were observed in control groups (Fig. 6B,  $P < 0.05$ ). These results suggested that miR-128 antagomir may aggravate



**Fig 5.** Effects of miR-128 antagomir on primary neuronal injury and cell cycle after OGD/R treatment. (A) Quantification and analysis of LDH activity in primary neurons under OGD/R and miR-128 antagomir treatment. LDH, lactate dehydrogenase; antago, antagomir; OGD, OGD/R.  $N = 6$ . (B) Flow cytometry analysis and semi-quantification of the effects of miR-128 on the cell cycle of OGD/R induced primary neurons; \* $P < 0.05$  vs. control group;  $n = 4$ . (C–E) Representative flow cytometry of cell cycle in control, OGD/R and OGD/R plus miR-128 antagomir groups.

neuronal injury via promoting ischemia-reperfusion induced cell cycle reentry.

To further explore how miR-128 acts on cell cycle progression, we used western blotting to detect the expression of cell cycle-related signaling molecules including PTEN and ERK and the cell cycle regulatory protein cyclin A2 in primary neurons after miR-128 antagomir and OGD/R treatment. The results demonstrated that compared with control groups, the cyclin A2 levels was upregulated markedly while protein expression and phosphorylation of PTEN decreased significantly under OGD/R stimulation (Fig. 6B, D and F,  $P < 0.05$ ). Moreover, cyclin A2 expression were further increased after miR-128 antagomir plus OGD/R treatment and both the protein expressions and phosphorylation of ERK and PTEN were upregulated as well (Fig. 6B–F,  $P < 0.05$ ).

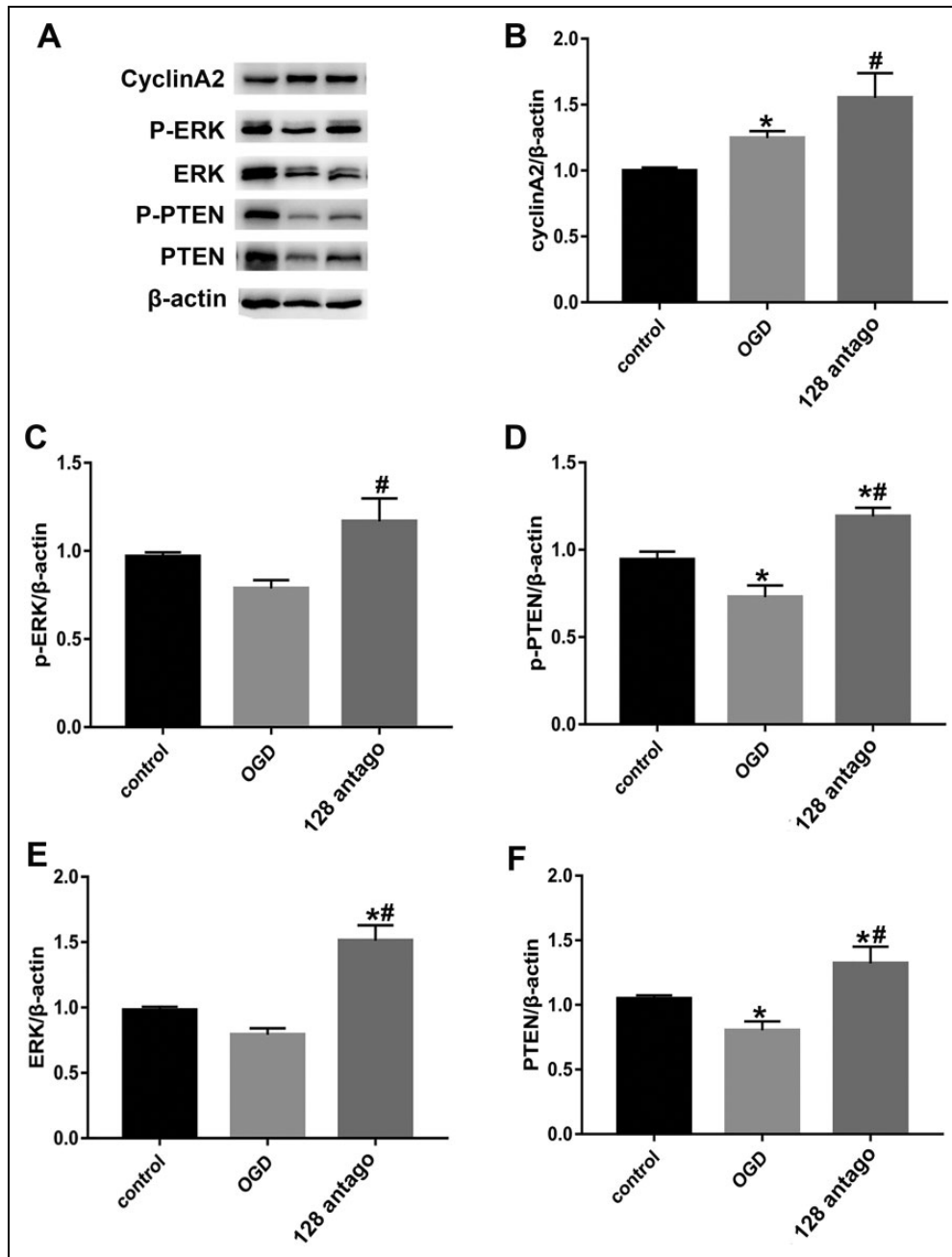
These results suggested that miR-128 antagomir exacerbated ischemia-reperfusion induced cell cycle reentry via upregulating protein expressions of both PTEN and ERK, promoting their phosphorylation and increasing cyclin A2 expression, thereby potentially aggravating neuronal injury after cerebral ischemia.

## Discussion

Although sustained efforts have been made to find the proper target for its prevention and therapy, ischemic stroke remains a major public health problem, owing to its high morbidity and narrow therapeutic window<sup>25,26</sup>. MiRNA-128, a small brain-enriched RNA, has been shown to be involved in neurogenesis, neuronal differentiation, apoptosis, and brain development in humans<sup>27–29</sup>. In addition, miR-128 levels have been reported to be deregulated in the brain in many diseases such as prion-induced neurodegeneration, Huntington's Disease, Parkinson's Disease and Alzheimer's Disease, thus suggesting a possibility of exploiting miR-128 for diagnosis, prognosis, and development of novel therapies<sup>30–32</sup>. Moreover, miRNA-128 has been reported to be increased in the brain tissue in MCAO mice compared with sham-operated mice, and it attenuates ischemic injury through decreasing protein expression of P38 $\alpha$  MAPK<sup>17</sup>. However, its role in ischemic stroke patients remains to be determined.

In the present study, we investigated the expression of miR-128 in acute ischemic stroke patients and explored its potential role in primary neuronal injury induced by OGD/R





**Fig 6.** Effects of miR-128 antagomir on expression and phosphorylation of ERK, PTEN, and cyclinA2 in primary neurons after OGD/R treatment. (A–F) Western blot analysis and semi-quantitation of the effects of miR-128 on the expression of p-PTEN, p-ERK, and cyclin A2, p-ERK, p-PTEN, ERK, and PTEN in OGD/R induced primary neurons. Values represent the mean  $\pm$  SEM. \* $P < 0.05$  vs. control group; # $P < 0.05$  vs. control+OGD/R group,  $n = 5$ .

and cell cycle reentry. The results showed that the miR-128 levels increased in the peripheral blood of acute ischemic stroke patients and were positively correlated with infarction volume, NIHSS score at 7 days, and mRS at 90 days after stroke onset. Together, these results suggested that miR-128 might be involved in the progression of cerebral ischemic injury. Further bioinformatics analysis and *in vitro* studies confirmed that miR-128 antagomir exerted its neurotoxic

effect partially by aggravating OGD/R induced neuronal injury and promoting neuronal cell cycle activation.

Quantitative RT-PCR showed that miR-128 levels increased in the circulating lymphocytes, neutrophils, and plasma of patients with acute ischemic stroke. Because obtaining a blood sample for measurement of miRNA is more convenient and less invasive than other auxiliary examinations such as computed tomography (CT) or MRI,

plasma miR-128 levels may serve as a diagnostic biomarker for acute ischemic stroke. Moreover, we found significantly positive correlations between miR-128 levels in the circulating lymphocytes and the infarction volume, the NIHSS score at 7 days, and the mRS at 90 days after stroke onset. From KEGG pathway analysis, we predicted and found that the MAPK signaling pathway and cell cycle related pathways were among the inflammation related pathways targeted by miR-128. These findings provided clues as to the underlying mechanisms of miR-128 potentially acted following cerebral ischemia. The subsequent correlation analysis suggested that miR-128 in plasma might originate partially from circulating lymphocytes. However, plasma miR-128 levels did not influence the number of peripheral inflammatory cells.

To answer whether miR-128 in the plasma enters the brain and is involved in neuronal injury after cerebral ischemia, we detected its expression in the brain tissues of MCAO mice and explored its underlying mechanisms *in vitro*. In accordance with the clinical data, after a transient decrease, the miR-128 levels were markedly upregulated in the infarction core in MCAO mice, in agreement with the changes in the ipsilateral brain tissues in an MCAO mouse model reported by Mao et al<sup>17</sup>. The results from both the patients and animal models suggested a critical role of miR-128 in the pathogenesis of ischemic stroke.

OGD/R is commonly used to mimic cerebral ischemia and reperfusion *in vitro*. To address the involvement of miR-128 in neuronal injury, miR-128 antagomir was transfected into primary neurons and was found to significantly exacerbate OGD/R induced neuronal injury. In agreement with our results, Mao et al have reported that miR-128 may protect neurons against cell apoptosis by targeting p38 $\alpha$  in an early stage of cerebral ischemia<sup>17</sup>. Yolanda et al also has shown that miRNA-128 overexpression downregulates genes that induce apoptosis and upregulates genes implicated in cell survival<sup>33</sup>. Contrary to our findings, Zeng et al have reported that miR-128 leads to cardiomyocyte apoptosis during myocardial ischemia-reperfusion, through activating PPARG<sup>16</sup>. In addition, several independent studies have illustrated the pro-apoptotic role of miRNA-128 in various cell types<sup>34,35</sup>. From the results of the present study, we concluded that miRNA-128 antagomir has neurotoxic functions upon OGD/R induced primary neuron. However, depending on the cell types on which miR-128 acts, further investigation is warranted to develop it as a therapeutic target for ischemic brain injury.

Inappropriate activation of cell cycle proteins is implicated in neuronal death induced by various pathologic stresses, including ischemia and oxidative stress<sup>36</sup>. Inhibiting the reactivation of cell cycle regulatory proteins such as cyclin D/Cdk4 and transcription factor E2F4 have been identified as a therapeutic target for ischemic injury<sup>24,37,38</sup>. Multiple articles have reported a role of miR-128 in cell cycle progression<sup>39,40</sup>. In the present study, we found that OGD/R stimulation induced a tendency of less neuron in G1 phase and more cells in S phase, a result consistent with cell cycle

reactivation following ischemia. However, miR-128 antagomir aggravated OGD/R induced cell cycle reentry significantly via upregulating protein expression of cyclinA2, ERK and PTEN, as well as increasing phosphorylation of both ERK and PTEN. Thereby this demonstrated that miR-128 antagomir potentially exerted neurotoxic effects after cerebral ischemia through reactivating cell cycle entry and thus exacerbating neuronal injury.

## Conclusions

In summary, the present study demonstrated that miR-128 was involved in the pathogenesis of acute ischemic stroke and miR-128 antagomir exerted neurotoxic effects against ischemia-reperfusion injury through activating cell cycle reentry by promoting ERK and PTEN phosphorylation and increasing ERK, PTEN and cyclinA2 expression. These results suggest that miR-128 holds promise as a miRNA-based therapeutic target that may be exploited for treating ischemic stroke.

## Ethical Approval

The study was approved by the Ethics Committee of Capital Medical University.

## Statement of Human and Animal Rights

All animal care complied with the guide for the Care and Use of Laboratory Animals. Human blood samples were obtained using protocols approved by the Ethics Committee of Xuanwu Hospital, Capital Medical University.

## Statement of Informed Consent

We confirm that guidelines on patients consent have all been met and any details of informed consent obtained are indicated within the text of the submitted manuscript.

## Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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