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MiR-182 promotes apoptosis of neural cells in cerebral infarction rats by PI3K/AKT signaling pathway

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ABSTRACT

To explore the effects of micro ribonucleic acid (miR)-182 on the proliferation and apoptosis of neural cells in cerebral infarction rats and its underlying mechanism. The rat model of cerebral infarction was established, and neural cells were extracted accordingly. The cell proliferation ability was detected *via* cell counting kit-8 (CCK8) assay. In addition, the apoptosis rate was determined through flow cytometry and the activity of active caspase-3. Furthermore, the interaction between miR-182 and PI3K was explored *via* dual luciferase reporter assay, and the protein expression levels were observed *via* Western blotting. The neural cells in mouse brain tissues significantly decreased in the model group compared with that in the control group *via* HE stain. Additionally, the expression level of miR-182 was significantly increased in the model group compared with that in the control group. Furthermore, overexpression of miR-182 could inhibit the proliferation of neural cells through inducing cell apoptosis. Besides, the results of the luciferase reporter assay showed that the relative luciferase activity in neural cells could be inhibited by the transfection with miR-182 ($P<0.05$). Overexpression of miR-182 significantly reduced the protein expression levels of phosphatidylinositol 3-hydroxy kinase (PI3K) and p-AKT. MiR-182 induces apoptosis of neural cells through inhibiting the PI3K/AKT signaling pathway, which plays an important regulatory role in the apoptosis of neural cells in cerebral infarction rats.

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Introduction

Cerebral thrombosis is an ischemic cerebrovascular disease (1), which is an important part of atherosclerosis, accounting for 70-80% of all stroke patients. About 90% of cerebral thrombosis develops from cerebral atherosclerosis (2), so it is often called atherosclerotic cerebral thrombosis. Atherosclerosis, a chronic progressive disease, develops during childhood, but it has clinical symptoms in middle-aged and elderly people.

Pathological changes in atherosclerosis mainly involve the aorta and moderately elastic artery (3), especially the coronary artery and cerebral artery. In addition, multiple organs are involved in the lesion. In the last century, several representative theories have been developed with increasing detailed studies on atherosclerosis, including lipid infiltration, thrombosis, smooth muscle cell cloning, injury response and chronic inflammation (4).

Micro ribonucleic acids (miRNAs) are a class of small endogenous non-coding single-stranded RNAs, as well as one of the most important regulators of gene expression, which interact with specific sequences of the target gene to inhibit the activity or promote the degradation of the target gene at the post-transcriptional level (5). In addition, miRNAs regulate the expression of target genes and participate in biological processes, including cell proliferation, differentiation, apoptosis and metabolism (6). Brain-specific miRNAs are abundantly expressed in the central nervous system but have no or little expression in most other organs. MiRNAs are extremely important in the development and function of the nervous system and it is reported that they can be passively transferred from the damaged cells or infiltrating cells or actively secreted from the damaged cells into the blood circulation (7).

The phosphatidylinositol 3-hydroxy kinase (PI3K)/protein kinase B (AKT) signal transduction pathway is an important pathway for intracellular membrane receptor signal transduction (8), which is the key to maintaining cell survival and inhibiting apoptosis. In addition, it can affect the activation of effector molecules, including downstream apoptosis-related proteins and cell cycle regulatory proteins. Therefore, PI3K/AKT is essential for inhibiting apoptosis and promoting cell proliferation (9). This study aims to explore the regulatory role of plasma miR-182 in neuroinflammation and apoptosis in cerebral infarction and its possible mechanism.

Materials and Methods

Establishment of animal model of middle cerebral artery occlusion (MCAO)

A total of 30 healthy male C57BL/6 rats aged 8-10 weeks old and weighing 20-30 g were purchased from the Beijing Laboratory Animal Center (Beijing, China), and fed in an environmental chamber under a constant temperature of 20°C, 60% humidity and 12/12 h light/dark cycle.

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The animal experiments were approved by the Laboratory Animal Ethics Committee of our hospital. All rats had free access to water and a sterile standard diet.

Cerebral ischemia-reperfusion was induced *via* MCAO. SD rats were anesthetized with 35 mg/kg pentobarbital (ip) and randomly divided into a control group and an MCAO model group. A midline neck incision was made, and the left common carotid artery and the external carotid artery were separated and ligated with the microvascular clamp. 8-0 silicon resin-coated nylon monofilament (180-190 μm) was inserted into the common carotid artery through the small incision and pushed towards the carotid bifurcation for 9 mm, thus inducing MCAO. In the control group, the rats were anesthetized with 35 mg/kg pentobarbital only without MCAO. After 1 h, the monofilament was withdrawn, followed by reperfusion. After 1 d, SD rats were anesthetized with 35 mg/kg pentobarbital and sacrificed for later study.

Cell transfection

The miR-182 mimic/negative control (NC) mimics and the miR-182/NC inhibitor were synthesized by GenePharma (Shanghai China). The cells were transfected using Lipofectamine 3000 (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's program.

Reverse transcription-polymerase chain reaction (RT-PCR)

The brain tissue samples were taken and the hippocampal tissues were isolated, from which the total RNA was isolated using TRIzol reagent (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Then the RNA was reversely transcribed into cDNA using the RT system (Takara Biotechnology, Dalian, China), followed by qPCR using 100 ng of cDNA on the Applied Biosystems 7500 Fast Real-Time PCR System with SYBR Green PCR Kit (TransGen, Beijing, China). The reaction conditions are as follows: denaturation at 95°C for 10 min, 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s for a total of 40 cycles. The primer sequences are shown in Table 1.

Cell proliferation and apoptosis assay

The cells were inoculated into a 96-well plate (1×10^3/well), followed by MTT analysis at 37°C for 4 h. The crystals were dissolved with DMSO at 37°C for 20 min, and then the absorbance was measured at 492 nm using a microplate reader (BioTek Instruments Inc., Vinooski, VT, USA).

After transfection, the cells proliferated in a 6-well plate for 48 h, washed once with PBS and resuspended in binding buffer, followed by staining using 5 μL of fluorescent dye-conjugated Annexin V and 5 μL of PI (BD Biosciences, Franklin Lakes, NJ, USA). Finally, apoptosis was detected using a flow cytometer (FACSCanto, BD Biosciences, Franklin Lakes, NJ, USA).

Gene expression microarray

The RNA (500 ng) in hippocampal tissues was amplified using the fluorescence complementary RNA and RNA labeling kit (Arraystar Inc., Rockville, MD, USA) according to the manufacturer's instructions, followed by array hybridization based on the Agilent monochrome microarray-based gene expression analysis protocol. Then the cDNA samples were labeled with Cy3 using the SureTag DNA Labeling Kit (Agilent Technologies Inc., Rockville, MD, USA). Finally, the microarray was scanned using the feature extraction software (v.10.7.3.1, Agilent Technologies Inc., Rockville, MD, USA).

Western blotting

The total protein was obtained from cultured cells via radioimmunoprecipitation assay (RIPA) lysis (Beyotime, Shanghai, China) and centrifuged at 10,000 g and 4°C for 5 min. The protein concentration was measured using the bicinchoninic acid (BCA) method (Beyotime, Shanghai, China). After separation via 8-12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), 50 μg of protein samples were transferred onto a nitrocellulose membrane, sealed with 5% bovine serum albumin (BSA) and incubated with the following primary antibodies at 4°C overnight: Bcl-2 associated X protein (Bax, Cat. No.: sc-6236, 1:1,000 Santa Cruz Biotechnology, Santa Cruz, CA, USA), PI3K (sc-1331, 1:1,000, Santa Cruz Biotechnology, Santa Cruz, CA, USA), phosphorylated (p)-AKT (sc-7985-R, 1:1,000, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and glyceraldheyde 3-phosphate dehydrogenase (GAPDH) (sc-293335, 1:2,000, Santa Cruz Biotechnology, Santa Cruz, CA, USA). After the membrane was washed with tris buffered saline-tween (TBST), the protein samples were incubated again with the horse radish peroxidase (HRP)-conjugated goat anti-rabbit IgG (sc-2004, 1:5,000, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or HRP-conjugated goat anti-mouse IgG secondary antibody (sc-2004, 1:5,000, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 37°C for 1 h. Finally, the protein bands were observed using electrochemiluminescence (ECL) Western blotting reagent (Thermo Fisher Scientific Inc., Waltham, MA, USA).

Dual-luciferase reporter assay

When the cell density reached 70%, the cells were co-transfected with 0.2 μg of luciferase reporter vector and

Table 1. Primer sequences.

Gene	Primer sequence
miR-183	Forward: 5'-CAGTCTCAAACCAGCACC-3'
	Reverse: 5'-TATGGTTGTTCACGACTCCTTCAC-3'
U6	Forward: 5'-CTCGCTTCGGCAGCACA-3'
	Reverse: 5'-AACGCTTCACGAATTTGCGT-3'
PI3K	Forward: 5'-ATGCCAGAGGAAGGAGGAGC-3'
	Reverse: 5'-GAGCCCACAGTGACAGAATAGG-3'
β-actin	Forward: 5'-AGTGTGACGTGGACATCCGCAAAG-3'
	Reverse: 5'-ATCCACATCTGCTGGAAGGTGGAC-3'

miR-182 mimic or control. The assay was normalized with 0.05 μg of red fluorescent protein expression vector pDsRed2-N1 (Clontech, Mountain View, CA, USA). After 48 h, the cells were lysed with RIPA lysis buffer (0.15 M NaCl, 0.05 M Tris/HCl, pH 7.2, 1% Triton X-100 and 0.1% SDS). The fluorescence intensity of luciferase and red fluorescent protein was measured using the F-4500 fluorescence spectrophotometer (Hitachi, Tokyo, Japan).

Statistical analysis

Statistical Product and Service Solutions (SPSS) 17.0 software (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Differences between the two groups were analyzed by using the Student's t-test. Comparison among multiple groups was done using a One-way ANOVA test followed by a Post Hoc Test (Least Significant Difference). A two-sided 95% confidence interval (CI) was adopted in all tests. P<0.05 suggested the statistically significant difference.

Results

Expression of miR-182 in model group and control group

Firstly, we found that the neural cells of mouse brain tissue significantly decreased in the model group compared with that in the control group via HE staining. To explore the expression level of miR-182 in the model group and control group, RT-qPCR was performed. As shown in Figure 1, the expression level of miR-182 was significantly increased in the model group compared with that in the control group.

Cell proliferation assay

To detect the effect of miR-182 on the proliferation of neural cells in rats with cerebral infarction, neural cells were transfected with miR-182 mimics, miR-182 inhibitor and corresponding NC, respectively, and the cell proliferation ability was detected by CCK-8. The results of CCK-8 showed that the cell proliferation ability was significantly weakened after transfection with miR-182 mimics. All these results suggest that overexpression of miR-182 can inhibit the proliferation of neural cells (P<0.05) (Figure 2).

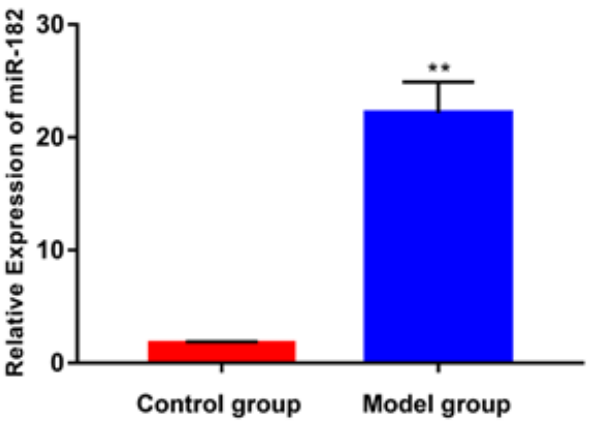

Figure 1. The expression level of miR-182 detected *via* RT-qPCR: The expression level of miR-182 is increased in the model group compared with that in the control group (**P<0.01).

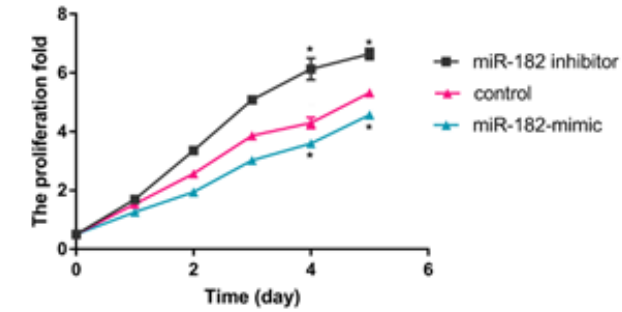

Figure 2. Cell proliferation assay (CCK-8 assay): Overexpression of miR-182 inhibits neural cell proliferation (*P<0.05).

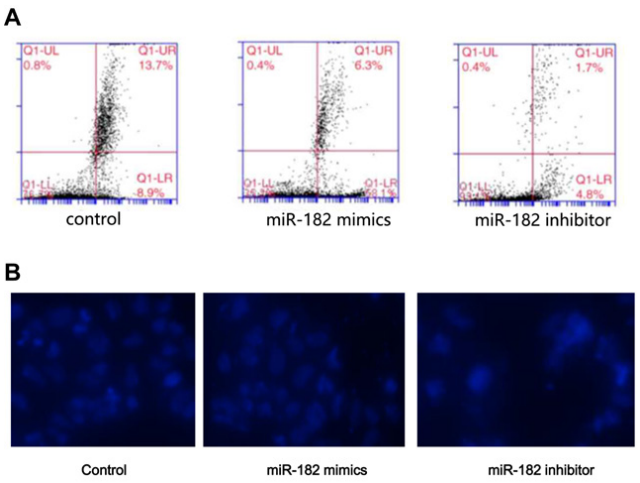

Figure 3. Overexpression of miR-182 inhibits the proliferation of neural cells through inducing cell apoptosis. (A) Apoptosis rate detected via flow cytometry: The apoptosis rate is (21.68±2.62)%, (62.77±1.28)% and (7.42±1.35)% in the control group, miR-182 mimic group and miR-182 inhibitor group, respectively (P<0.05). (B) DAPI staining (100×). The expression level of capsase-3 is significantly increased in neural cells after transfection of miR-182 mimics compared with that in the control group. The expression level of capsase-3 is significantly decreased in the miR-182 inhibitor group, compared with that in the control group.

Apoptosis assay

To further explore the effect of miR-182 on the apoptosis of neural cells, flow cytometry was applied. The results showed that compared with that in the control group, neural cell apoptosis was promoted by up-regulation of miR-182, and the difference was statistically significant (P<0.05). Meanwhile, the down-regulation of miR-182 significantly inhibited neural cell apoptosis (P<0.05) (Figure 3A). In addition, we found that the expression level of capsase-3 was significantly increased in miR-182 mimics group, but significantly decreased in miR-182 inhibitor group compared with that in control group (Figure 3B). The above results suggest that overexpression of miR-182 can inhibit the proliferation of neural cells though inducing cell apoptosis.

Overexpression of miR-182 down-regulating PI3K/AKT signal transduction pathway

The targets for miR-182 were predicted using online tools TargetScan and PicTar, and the conserved binding sites for miR-182 in PI3K 3'-untranslated region (3'UTR) were identified (Figure 4A). The results of the dual lucife-

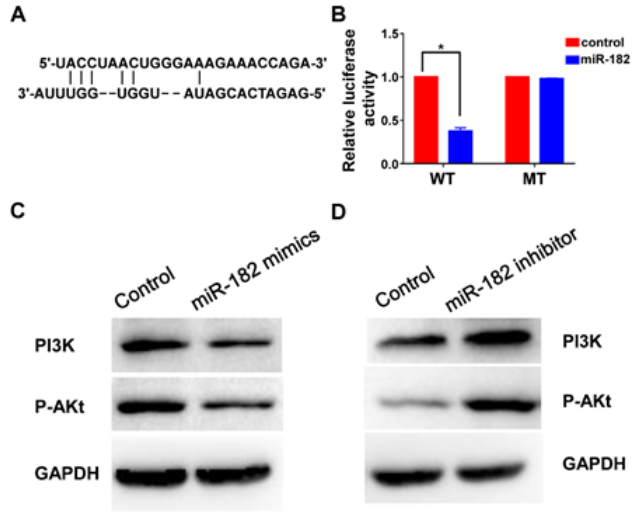

Figure 4. Overexpression of miR-182 inhibits the PI3K/AKT signaling pathway. (A) MiR-182 directly targets PI3K: Binding sites for miR-182 in PI3K 3'UTR; (B) Interaction between miR-182 and PI3K determined using luciferase reporter assay (*P<0.05). (C-D) The protein expression of PI3K and p-AKT are detected by Western blot. The results indicate that miR-128 negatively regulates the PI3K/AKT signaling pathway in neural cells.

rase reporter assay showed that transfection with miR-182 could significantly suppress the relative luciferase activity in neural cells. These results indicate that miR-182 can regulate the expression of PI3K through interacting with 3'UTR of PI3K (Figure 4B). After transfection with miR-182 mimic for 48 h, the expression of PI3K and p-AKT was lower than that in the control group (Figure 4C). Meanwhile, the protein expression of PI3K and p-AKT was significantly up-regulated after down-expression of miR-182 (Figure 4D). All these results suggest that overexpression of miR-182 can promote neural cell apoptosis via down-regulation of the PI3K/AKT signaling pathway.

Discussion

Acute cerebrovascular disease is a common disease in middle-aged and elderly people, and it has high disability and mortality rates. With the aging population and changes in living and social environment, the incidence rate of atherosclerosis has shown an increasing and younger trend year by year (10,11). In the present mouse experiments, we found that the expression level of miR-182 significantly increased in the cerebral infarction model group compared with that in the control group.

It is difficult to detect or repeatedly use miRNAs in human tissues in the clinic. Stable miRNA molecules have been found in peripheral blood in previous studies (12). In addition, different diseases are associated with different miRNA expression profiles, which provide a new idea about the non-invasive diagnosis of disease using peripheral blood miRNAs (13). Currently, peripheral blood miRNAs with specific expression changes can be observed in a variety of diseases, including tumors, diabetes, myocardial infarction, Parkinson's disease and Alzheimer's disease (14). In this study, our findings manifested that overexpression of miR-182 could inhibit the proliferation of neural cells through inducing cell apoptosis. Sun et al. (15) showed that miR-182 can also reduce proliferation and induce apoptosis of cervical cancer cells.

Caspase-3 is the most important apoptosis protease during apoptosis, and its activation depends on the release of cytochrome C. Bcl-2 and Bax genes, two members of the Bcl-2 family, are the most important apoptosis-regulating genes currently known, which can mediate the release of substances through the mitochondrial pathway. Many previous studies have found that Bcl-2 and Bax can act as upstream regulators of caspase-3 and regulate the caspase-3 activity (16,17). In addition, they can be considered as direct substrates to act on downstream caspase-3, which are interrelated and constrained mutually during apoptosis (18,19). In the present study, it was found that overexpression of miR-182 could significantly increase the expression level of capsase-3 in neural cells.

It was previously reported that the PI3K/AKT pathway exerts a neuroprotective effect in ischemic stroke, and it is a central signal transduction pathway regulating cell growth, survival and metabolism. The activation of PI3K/AKT has an important protective effect against ischemic and hypoxic neuron injury, attracting more and more attention (20). The PI3K/AKT signal transduction pathway is an important pathway for intracellular membrane receptor signal transduction, which is the key to maintaining cell survival and inhibiting apoptosis (21-25). It can also affect the activation of effector molecules, including downstream apoptosis-related proteins and cell cycle regulatory proteins. Therefore, PI3K/AKT plays an important role in inhibiting apoptosis and promoting cell proliferation. Besides, PI3K/AKT has an anti-apoptotic effect in cerebral ischemia, and inhibiting this pathway can aggravate neural cell apoptosis induced by cerebral ischemia. In this study, our findings confirm that overexpression of miR-182 can induce neural cell apoptosis *via* down-regulation of PI3K/AKT signaling pathway.

Conclusions

In conclusion, the expression level of miR-182 significantly rises in the cerebral infarction model group compared with that in the control group. In addition, overexpression of miR-182 can inhibit the proliferation of neural cells through inducing cell apoptosis. Furthermore, overexpression of miR-182 can induce neural cell apoptosis *via* down-regulation of the PI3K/AKT signaling pathway. The novelty of this study is that miR-182 may serve as a potential target in the clinical treatment of cerebral infarction in the future.

Conflict of Interest

The authors declared no conflict of interest.

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