

MiR-195-5p Ameliorates Cerebral Ischemia-Reperfusion Injury by Regulating the PTEN-AKT Signaling Pathway

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Background: MiR-195-5p has been shown to play crucial roles in tumor inhibition, but its biological functions in cerebral ischemia-reperfusion (I/R) injury are unclear.

Methods: To mimic cerebral I/R injury, mice were induced by transient middle cerebral artery occlusion (MCAO). Human brain microvascular endothelial cells (HBMVECs) were treated with oxygen-glucose deprivation (OGD) to mimic I/R injury in vitro. The expression of miR-195-5p and PTEN was detected by qRT-PCR or Western blot. Cell viability was evaluated by CCK-8 assay. Cell apoptosis was detected by flow cytometer. Cell death was detected using specific lactate dehydrogenase (LDH) cytotoxicity kit. Infarct volume in mice brains was evaluated by TTC staining. Histopathological analysis was performed by HE staining and TUNEL staining. The interaction between miR-195-5p and PTEN was determined by TargetScan and luciferase reporter assay.

Results: MiR-195-5p was significantly downregulated and PTEN was upregulated during cerebral I/R injury both in vitro and in vivo. Overexpression of miR-195-5p efficiently enhanced cell viability, while reduced LDH release and apoptotic rate of OGD-treated HBMVECs in vitro. MiR-195-5p could negatively regulate the expression of PTEN by directly binding to its 3'-UTR. Overexpression of PTEN obviously attenuated the protective effect of miR-195-5p mimics on cell viability, LDH release and apoptosis in OGD-treated HBMVECs. Meanwhile, overexpression of miR-195-5p increased the expression levels of p-AKT in OGD-treated HBMVECs, while this effect was reversed by overexpression of PTEN. Moreover, overexpression of miR-195-5p efficiently ameliorated brain injury of mice after MCAO treatment in vivo.

Conclusion: Overexpression of miR-195-5p ameliorated cerebral I/R injury by regulating the PTEN-AKT signaling pathway, providing a potential therapeutic target for cerebral I/R injury.

Keywords: cerebral I/R injury, miR-195-5p, PTEN, AKT signaling pathway

Introduction

Stroke, a severe and acute disease in central nervous system of body, is mainly caused by cerebral ischemia.¹ It has been reported that there are approximately 13.5 million new cases of stroke, and 5 million deaths every year worldwide.² Currently, the efficient treatment for cerebral ischemia-caused injury is the restoration of blood flow (reperfusion) in the ischemic region of the brain as quickly as possible.³ However, further brain damage, called ischemia/reperfusion (I/R) injury, always occurs after artery recanalization.⁴ At present, specific drugs to protect I/R injury are not available in clinical practice due to the lack of efficient molecular

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targets.⁵ Therefore, it is more urgent to understand the complex pathogenic mechanism during cerebral I/R injury.

With the development of sequencing technologies, an increasing number of microRNAs (miRNAs), which are a class of non-coding RNAs with approximately 22 nucleotides in length, are identified.⁶ It has been reported that miRNAs repress gene expression via degrading target mRNAs or inhibiting translation.⁷ Recently, a series of miRNAs involved in cerebral I/R injury have been well studied. For example, miR-124 regulates cell pyroptosis during cerebral I/R injury by targeting STAT3.⁸ Cerebral ischemic injury and apoptosis levels are reduced in I/R stroke mouse model and OGD-induced N2A cell model with the overexpression of miR-183-5p.⁹ MiR-187-3p inhibitor attenuates cerebral I/R injury through modulating Seipin-mediated autophagic flux.¹⁰ MiR-211 protects brain against I/R injury by suppressing cell apoptosis.¹¹ MiR-124-5p reduces ROS production and improves the inflammatory microenvironment to protect against cerebral I/R injury by targeting NOX2.¹² All these findings indicate that miRNAs play important roles in cerebral I/R injury and may be considered as potential therapeutic targets for ischemic stroke.

MiR-195-5p is a member of the miR-15/107 family, which has been widely investigated in different types of cancer and identified as a tumor suppressor.¹³ For example, miR-195-5p suppresses the cell migration and invasion of cervical carcinoma by directly suppressing ARL2.¹⁴ MiR-195-5p inhibits proliferation and induces apoptosis of non-small cell lung cancer cells by targeting CEP55.¹⁵ In addition, miR-195-5p was also found to alleviate acute kidney injury by inhibiting inflammation and oxidative stress by targeting vascular endothelial growth factor A.¹⁶ However, the role of miR-195-5p in cerebral I/R injury remains unclear.

Phosphatase and tensin homolog (PTEN) is located on human chromosome 19 and encodes a dual protein phosphatase enzyme.¹⁷ Increased expression levels of PTEN have been observed in MCAO mouse model or OGD-induced cell model in ischemic stroke,¹⁸ confirming that PTEN closely participates in the ischemic stroke progression. Recently, PTEN is found to function as the target of miRNAs in various human diseases including cerebral I/R injury. For example, knockdown of miR-216 suppresses the progression of osteosarcoma by targeting PTEN.¹⁹ MiR-183-5p attenuates cerebral ischemia injury by negatively regulating PTEN.⁹

In this study, we found that miR-195-5p was significantly downregulated during cerebral I/R injury. Moreover, overexpression of miR-195-5p efficiently inhibited the proliferation and induced apoptosis of human

brain microvascular endothelial cells (HBMVECs) *in vitro*, and also protected brain against I/R injury in mouse model *in vivo*. Therefore, our study demonstrated that miR-195-5p functioned in cerebral I/R injury through regulating the AKT signaling by directly targeting PTEN.

Materials and Methods

Mouse Model of Cerebral Ischemia

Male C57BL/6J mice (approximately 8–10 weeks) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. Mice were kept in the standard environment with a humidity of 45–55% and 21–23°C under a 12h/12h light/dark cycle. Cerebral ischemia model was established using transient middle cerebral artery occlusion (MCAO) as previously described.²⁰ For detection of the expression levels of miR-195-5p and PTEN in ischemic brain tissues, after anesthesia with pentobarbital sodium (30 mg/kg) by intraperitoneal injection, mice underwent 2 h of MCAO followed by 6, 12, 24 and 48 h reperfusion. To explore the role of miR-195-5p, miR-195-5p agomir and agomir NC were obtained from Genepharma (Shanghai, China), and then 100 µM of miR-195-5p agomir or agomir NC was injected into mice by intra-cerebroventricular injection via a stereotaxic apparatus on day 1 before MCAO. Stereotaxic coordinates: anteroposterior, 0.8 mm; mediolateral, 1.5 mm; depth, 3.5 mm. Then, mice were submitted to 2 h of MCAO followed by 24 h reperfusion. Sham-operated mice underwent the same procedure except MCAO. Mice in the negative control (NC) group were injected with the same concentrations of normal saline. There were five mice in each group. Subsequently, neurological deficit of mice was evaluated. Finally, mice were sacrificed by decapitation and brain tissues were collected for subsequent experiments.

TTC Staining

Infarct volume of brain in mice after 24 h of reperfusion was evaluated using TTC staining as previously described.²¹ In brief, the brain tissues were cut into 2-mm-thick sections and then incubated with 2% TTC solution (Sigma-Aldrich) at 37°C for 20 min. The sections were fixed with 4% paraformaldehyde for 2 h and photographed using a digital camera. The infarct volume was analyzed using Image-Pro Plus 6.0: total infarct volume/total brain volume × 100%.

Neurological Deficit Evaluation

Neurological deficit was evaluated as previously described using modified Longa score.²² 0 indicates no observable deficit; 1 indicates failure to fully extend the left forepaw; 2 indicates difficulty in circling to the left; 3 indicates failing to the left side; 4 indicates no spontaneous walking with decreased level of consciousness.

Histopathological Analysis

The pathological histological damage of the whole brain in mice was evaluated by using Hematoxylin and eosin (HE) staining as previously described.²³ Briefly, after 24 h of reperfusion, the brain tissues were fixed with 4% paraformaldehyde, dehydrated by alcohol and embedded in paraffin. The brains were cut into 5- μ m-thick sections and incubated with HE reagents, followed by photographed under a light microscopy in five randomly selected fields.

TUNEL Staining

The apoptosis of cortical neurons was evaluated using terminal deoxynucleotidyl-transferase-mediated 2'-deoxyuridine 5'-triphosphate nick-end labeling (TUNEL) assay as previously described.²⁴ In brief, the brain tissues were fixed in 4% paraformaldehyde, cut into 10 μ m sections and stained with 50 μ M TUNEL reagent (Roche Applied Science, Penzberg, Germany) at 37°C for 60 min in the dark. Then the sections were incubated with DAPI solution for 20 min to stain the nucleus. The positive cells were observed and photographed using a fluorescence microscope (Olympus, Tokyo, Japan). The apoptosis of cortical neurons was calculated based on the following formula: the TUNEL-positive cells/the total cells \times 100%.

Cell Culture and OGD Model

Human brain microvascular endothelial cells (HBMVECs) were obtained from the ATCC and cultured in DMEM supplemented with 10% FBS at 37°C with 5% CO₂. To mimic the cerebral ischemia condition in vivo, HBMVECs were induced by oxygen-glucose deprivation (OGD) as previously described.²⁵ Briefly, HBMVECs cells were exposed to glucose-free DMEM and cultured at 37°C with 95% N₂/5% CO₂ for 2 h. Then the glucose-free DMEM was replaced with glucose-containing DMEM and HBMVECs were transferred to normal condition for 24 h to receive re-oxygenation. Cells in the Sham group were still cultured under normal conditions. For detection of the expression levels of miR-195-5p and PTEN, cells

received 2 h of OGD followed by 6, 12, 24 and 48 h re-oxygenation. To explore the role of miR-195-5p, cells achieved 2 h of OGD followed by 24 h re-oxygenation.

Cell Transfection

The miR-195-5p mimics, miR-195-5p inhibitor and corresponding negative controls (miR-NC and inhibitor NC) (GeneChem Corp., Shanghai, China) were transfected into HBMVECs using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific). To overexpress PTEN, the full length of PTEN was cloned into the backbone vector pcDNA3.1 (GeneChem Corp., Shanghai, China) to generate the overexpression plasmid pc-PTEN, and the empty vector was used as the negative control (pcDNA3.1). Then, 10 nM of pc-PTEN and pcDNA3.1 were transfected into HBMVECs using Lipofectamine 2000. After transfection for 48 h, the transfection efficiency was confirmed by qRT-PCR.

qRT-PCR

Total RNAs were extracted from brain tissues and cultured HBMVECs using Trizol reagent (Invitrogen). Then, quantitative real-time RT-PCR was performed on an ABI 7500 Fast Real-time PCR system (Applied Biosystems) using SYBR Green mixture (Invitrogen). The relative expression changes of targets were calculated using the $2^{-\Delta\Delta Ct}$ method. GAPDH was used as an internal reference for expression levels of mRNA, and U6 for miRNA. The primers used for qRT-PCR were as follows: miR-195-5p (human) forward: 5'-GCAGCACAGAAATATTGG-3', reverse: 5'-GAACATGTCTGCGTATCTC-3'; miR-195-5p (mouse) forward: 5'-AGCAGCACAGAAATATTGGC-3', reverse: 5'-GAACATGTCTGCGTATCTC-3'; U6 forward: 5'-ACAGATCTGTCGGTGTGGCAC-3', reverse: 5'-GGCCCCGGATTATCCGACATC-3'; PTEN (human) forward: 5'-TGAGTTCCTCAGCCGTACCT-3', reverse: 5'-GAGGTTTCCTCTGTCCTGGTA-3'; PTEN (mouse) forward: 5'-TGAGTTCCTCAGCCATTGCCT-3', reverse: 5'-GAGGTTTCCTCTGGTCCTGGTA-3'; GAPDH forward: 5'-ATCCACGGGAGAGCGACAT-3', reverse: 5'-CAGCTGCTTGTAAGTGGAC-3'.

Western Blot

Total proteins were extracted from brain tissues and cultured HBMVECs using RIPA lysis buffer (Beyotime Biotechnology). Approximately equal amounts of protein samples were separated by 12% SDS-PAGE and then transferred onto PVDF membranes (Millipore). After blocking with 5% skim milk, the membranes were

incubated with specific primary antibodies including anti-PTEN (1:500, Abcam, Cambridge, MA, USA), anti-p-AKT (1:1000, Abcam), anti-AKT (1:1000, Abcam), anti-cleaved caspase-3 (c-Cas-3) (1:500, Abcam), anti-Bcl-2 (1:500, Abcam), anti-Bax (1:500, Abcam) and anti-GAPDH (1:1000, Abcam) at 4°C overnight. On the following day, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:10,000, Abcam) at room temperature for 2 h. Finally, the protein signals were observed using ECL reagents and the gray values were analyzed by Image-Pro Plus 6.0 software.

Cell Counting Kit-8 (CCK-8) Assay

Approximately 1×10^4 OGD transfected or un-transfected HBMVECs were treated with or without OGD incubation, and then seeded into 96-well plates. Cells were cultured for 24 h, and 10 μ L of CCK-8 solution (Sigma-Aldrich) was added and incubated for another 2 h. The optical density (OD) was detected by a microplate reader (Bio-Tek, Winooski, USA) at an absorbance of 450 nm.

Cell Apoptosis

Cell apoptosis of HBMVECs was evaluated by an Annexin-V/propidium iodide (PI) apoptosis detection kit (Beyotime Institute of Biotechnology) as previously described.⁹ In brief, HBMVECs were harvested and stained with Annexin V-FITC and PI for 20 min in the dark following the manufacturer's instructions. Subsequently, cell apoptosis was detected by flow cytometer (BD FACSCalibur; BD Biosciences). The rate of apoptosis was defined as the total percentage of early and late apoptotic cells.

Luciferase Reporter Assay

The putative binding site between miR-195-5p and the 3'-UTR of PTEN was predicted by TargetScan v.7.2. The wild type (WT) and mutant (MUT) of PTEN containing the putative binding site with miR-195-5p were synthesized by Genechem (Shanghai, China) and cloned into pmirGLO dual luciferase reporter vector (Promega) to generate the recombinant luciferase reporter vectors of PTEN WT and PTEN MUT. These luciferase reporter vectors were co-transfected with miR-195-5p mimics or miR-NC into HBMVECs. The relative luciferase activity was detected by the Dual-luciferase reporter assay system (Promega) at 48 h after transfection.

Detection of LDH Release

Cell death of HBMVECs was detected using the lactate dehydrogenase (LDH) Cytotoxicity Assay Kit (Jiancheng, Nanjing, China). In brief, after transfection or OGD treatment, the LDH release in the cultural supernatant of HBMVECs was detected following the manufacturer's instructions.

Statistical Analysis

Data were presented by means \pm standard deviation (SD), and each experiment was repeated for 3 times. Statistical analyses were performed by GraphPad Prism 8.0 (GraphPad Software, Inc.). Difference between two groups was determined by Student's t-tests, and difference between multiple groups was tested by one-way ANOVA followed by Tukey's test. $P < 0.05$ was considered as the significant threshold.

Results

MiR-195-5p Was Downregulated and PTEN Was Upregulated Following Cerebral I/R Injury Both in vivo and in vitro

To explore the role of miR-195-5p in cerebral I/R injury, mice were induced by 2 h of MCAO, followed by 6, 12, 24 and 48 h reperfusion. The results of qRT-PCR showed that the expression levels of miR-195-5p were obviously reduced in brain tissues of mice after reperfusion for 12 h ($p < 0.01$), 24 h ($p < 0.01$) and 48 h ($p < 0.01$) (Figure 1A). Meanwhile, the expression of PTEN was significantly upregulated in brain tissues of mice after reperfusion for 12 h ($p < 0.01$), 24 h ($p < 0.01$) and 48 h ($p < 0.01$) (Figure 1B). Similarly, the protein expression levels of PTEN in brain tissues were confirmed by Western blot (Figure 1C). To determine their expression, HBMVECs were exposed to 2 h of OGD followed by 6, 12, 24 and 48 h of re-oxygenation. Similarly, miR-195-5p was downregulated (all $p < 0.05$) and PTEN was upregulated (all $p < 0.05$ except for 6 h of re-oxygenation) in OGD-treated HBMVECs (Figure 1D and E). The protein expression levels of PTEN in HBMVECs were also confirmed by Western blot (Figure 1F). In addition, TTC staining results showed that MCAO/R treatment obviously increased the infarct volume of brain in mice (Figure 1G). These results suggested that miR-195-5p and PTEN may play important roles in cerebral I/R injury.

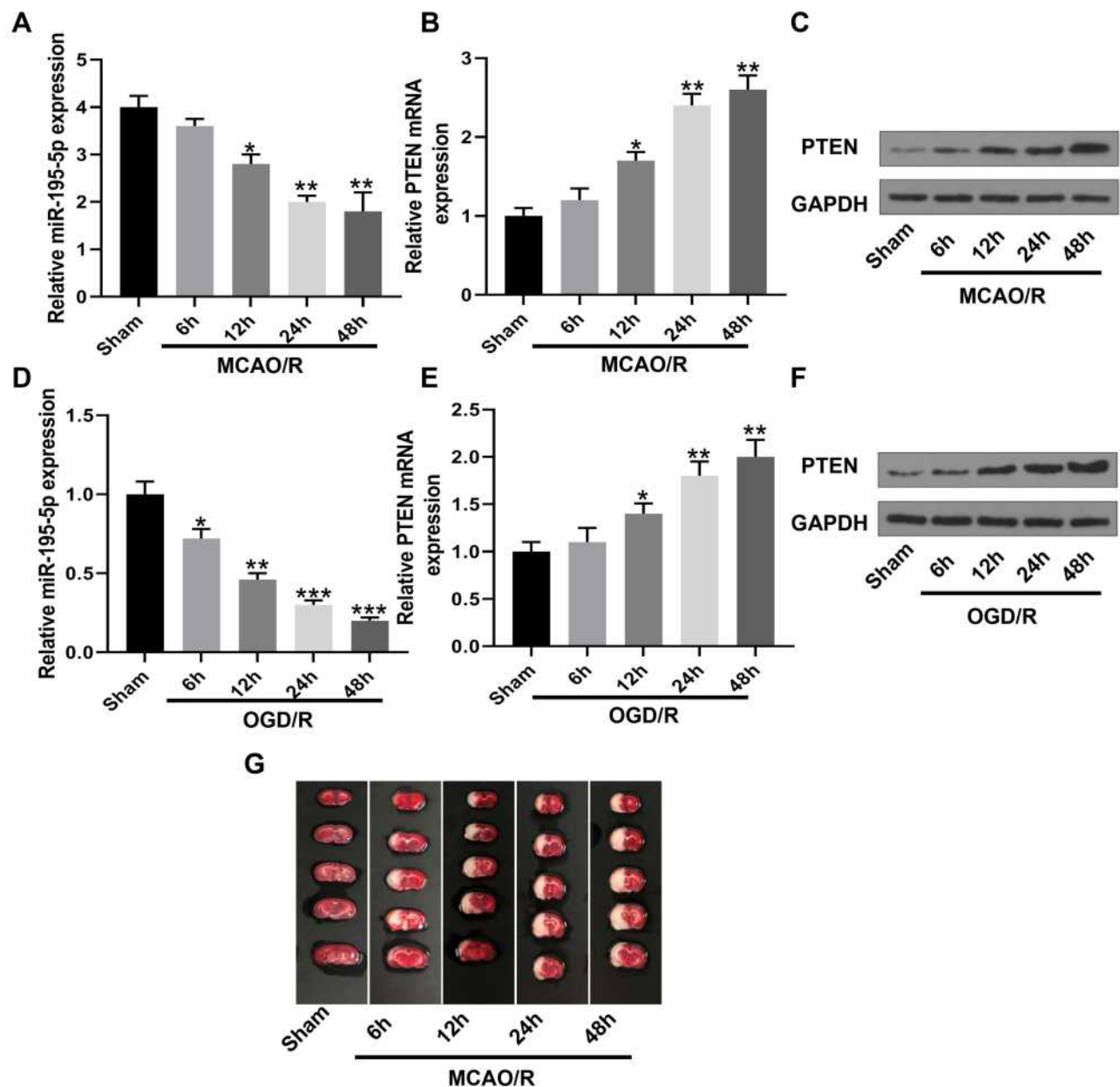


Figure 1 MiR-195-5p was downregulated and PTEN was upregulated following cerebral I/R injury. (A–C) Mice were treated by 2 h of MCAO and reperfusion for 6, 12, 24, and 48 h. The expression of miR-195-5p (A) and PTEN (B) in brain tissues was detected by qRT-PCR. (C) The protein expression levels of PTEN in brain tissues were detected by Western blot. (D–F) HBMVECs were treated by 2 h of OGD and re-oxygenation for 6, 12, 24, and 48 h. The expression of miR-195-5p (D) and PTEN (E) was detected by qRT-PCR. (F) The protein expression levels of PTEN were detected by Western blot. (G) The infarct volume in brains was assessed by TCC staining. Data were presented as means \pm SD. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Overexpression of miR-195-5p Improved Cell Viability and Attenuated OGD/R-Induced Apoptosis in HBMVECs

To further determine the role of miR-195-5p, miR-195-5p mimics was transfected into HBMVECs to overexpress miR-195-5p, and qRT-PCR results showed that OGD/R treatment significantly reduced the expression levels of miR-195-5p, and miR-195-5p mimics significantly increased the expression

levels of miR-195-5p in OGD/R-treated HBMVECs compared with that of miR-NC ($p < 0.01$, Figure 2A). CCK-8 assay revealed that OGD/R treatment significantly reduced HBMVECs viability compared with that in the control group, and overexpression of miR-195-5p obviously enhanced cell viability of OGD/R-treated HBMVECs compared with miR-NC ($p < 0.01$, Figure 2B). Meanwhile, OGD/R treatment significantly increased the release of LDH in cultural

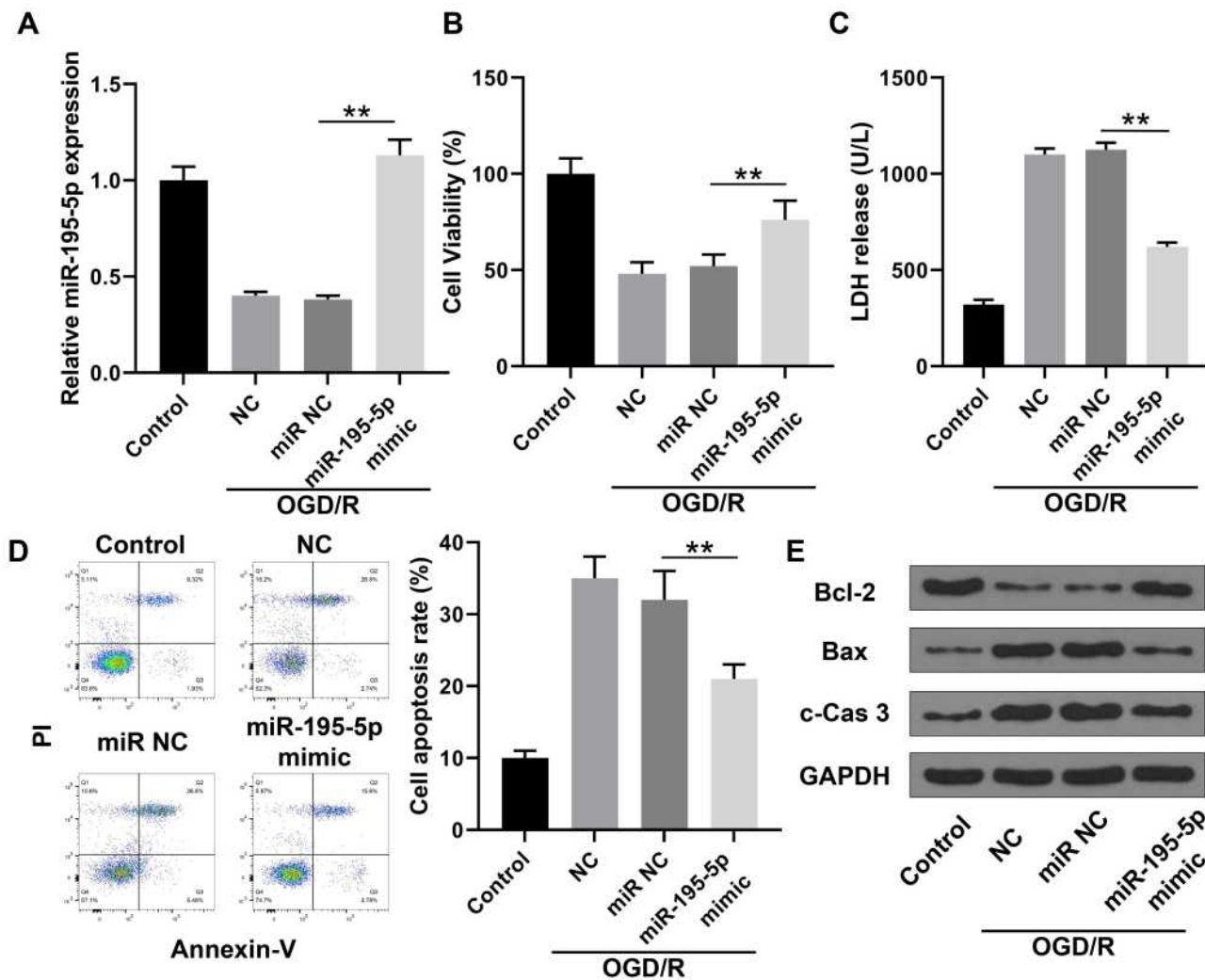


Figure 2 Overexpression of miR-195-5p improved cell viability and attenuated OGD/R-induced apoptosis in HBMVECs. HBMVECs were transfected with miR-195-5p mimics and miR-NC, and then received 2 h of OGD followed by 24 h of re-oxygenation. (A) The transfection efficiency was determined by qRT-PCR. (B) Cell viability was evaluated by CCK-8 assay. (C) The release of LDH was detected by specific cytotoxicity assay kit. (D) Cell apoptosis was evaluated by flow cytometer. (E) The protein expression of apoptosis-related makers was detected by Western blot. Data were presented as means \pm SD. $**p < 0.01$.

supernatant and induced apoptosis of HBMVECs compared with that in the control group, while overexpression of miR-195-5p obviously attenuated these OGD/R-treatment caused effects compared with miR-NC ($p < 0.01$, Figure 2C and D). In addition, the expression levels of apoptosis-related makers were detected by Western blot and the results indicated that OGD/R treatment obviously increased the expression levels of Bax and c-Cas 3, while reduced the expression levels of Bcl-2 in HBMVECs compared with that in the control group, and overexpression of miR-195-5p obviously reversed the effects of OGD/R treatment in HBMVECs (Figure 2E). These results suggested that overexpression of miR-195-5p could efficiently improve cell viability and attenuate OGD/R-induced apoptosis in HBMVECs.

PTEN Was a Target of miR-195-5p

It has been reported that PTEN may be a potential target of miR-195-5p.²⁶ To determine the relationship between miR-195-5p and PTEN, Targscan was applied and the prediction showed that there was a putative binding site between miR-195-5p and the 3'-UTR of PTEN (Figure 3A). Then, miR-195-5p mimics and miR-195-5p inhibitor were transfected into HBMVECs and the transfection efficiency was confirmed by qRT-PCR (Figure 3B). Luciferase reporter assay was performed to determine their relationship and the results revealed that overexpression of miR-195-5p significantly reduced the relative luciferase activity of PTEN WT compared with miR-NC in HBMVECs ($p < 0.05$), but no change was observed on the relative luciferase activity of PTEN MUT (Figure 3C). Meanwhile, overexpression of

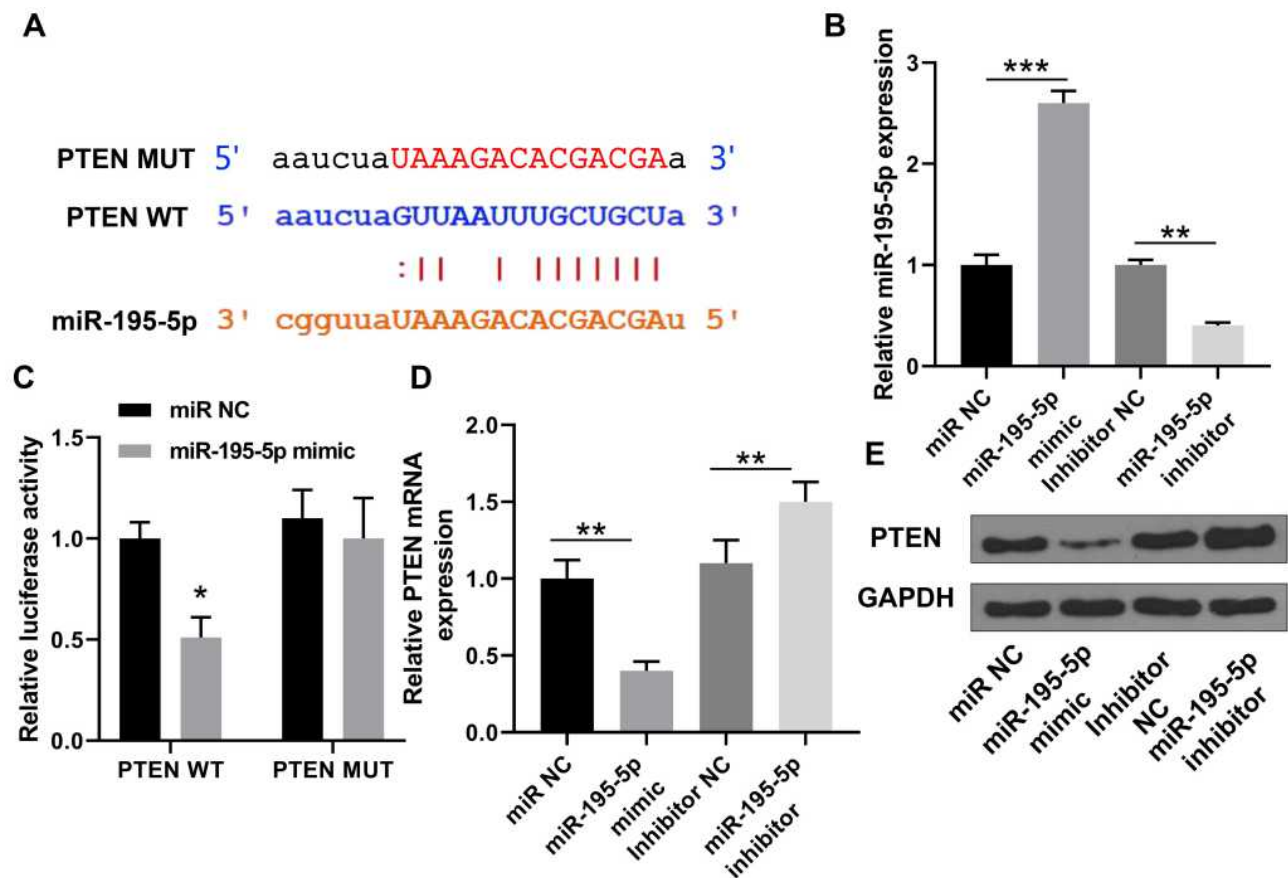


Figure 3 PTEN was a target of miR-195-5p. (A) The potential interaction between miR-195-5p and PTEN was predicted by TargetScan. (B) HBMVECs were transfected with miR-195-5p mimics/inhibitor and corresponding negative controls. The transfection efficiency was confirmed by qRT-PCR. (C) The relative luciferase activity of PTEN WT or MUT was detected by dual luciferase reporter system. (D and E) HBMVECs were transfected with miR-195-5p mimics/inhibitor and corresponding negative controls. The expression of PTEN was detected by qRT-PCR (D) and Western blot (E). Data were presented as means \pm SD. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

miR-195-5p significantly decreased the expression levels of PTEN ($p < 0.01$), and downregulation of miR-195-5p increased the expression levels of PTEN ($p < 0.01$) (Figure 3D and E). These results demonstrated that PTEN is a direct target of miR-195-5p.

Overexpression of miR-195-5p Improved I/R Injury in vitro by Regulating the PTEN-AKT Signaling Pathway

To determine whether the function of miR-195-5p was mediated by PTEN, HBMVECs were transfected with miR-195-5p mimics, or co-transfected with miR-195-5p mimics and pc-PTEN, and then induced by 2 h of OGD followed by 24 h of re-oxygenation. Overexpression of miR-195-5p significantly enhanced cell viability ($p < 0.01$) and reduced the release of LDH in the supernatant ($p < 0.05$) of OGD/R-treated HBMVECs compared with miR-NC, and these effects were obviously reversed by overexpression of PTEN ($p < 0.05$, Figure 4A and B). Overexpression of miR-

195-5p obviously increased the protein expression levels of p-AKT and Bcl-2, while decreased the expression levels of Bax and c-Cas 3 in OGD/R-treated HBMVECs compared with miR-NC, which was also reversed by overexpression of PTEN (Figure 4C and E). In addition, miR-195-5p mimics significantly reduced apoptotic rate of OGD/R-treated HBMVECs compared with miR-NC ($p < 0.01$), and this inhibitory effect was attenuated by overexpression of PTEN ($p < 0.01$, Figure 4D). These results suggested that miR-195-5p/PTEN affected the development of I/R injury by regulating the AKT signaling pathway.

Overexpression of miR-195-5p Ameliorated Cerebral I/R Injury in vivo

To further confirm the protective effect of miR-195-5p in cerebral I/R injury in vivo, mice were injected with miR-195-5p agomir and NC agomir, and then received 2 h of MCAO followed by 24 h reperfusion. The expression levels of miR-195-5p in brain tissues of NC group were significantly

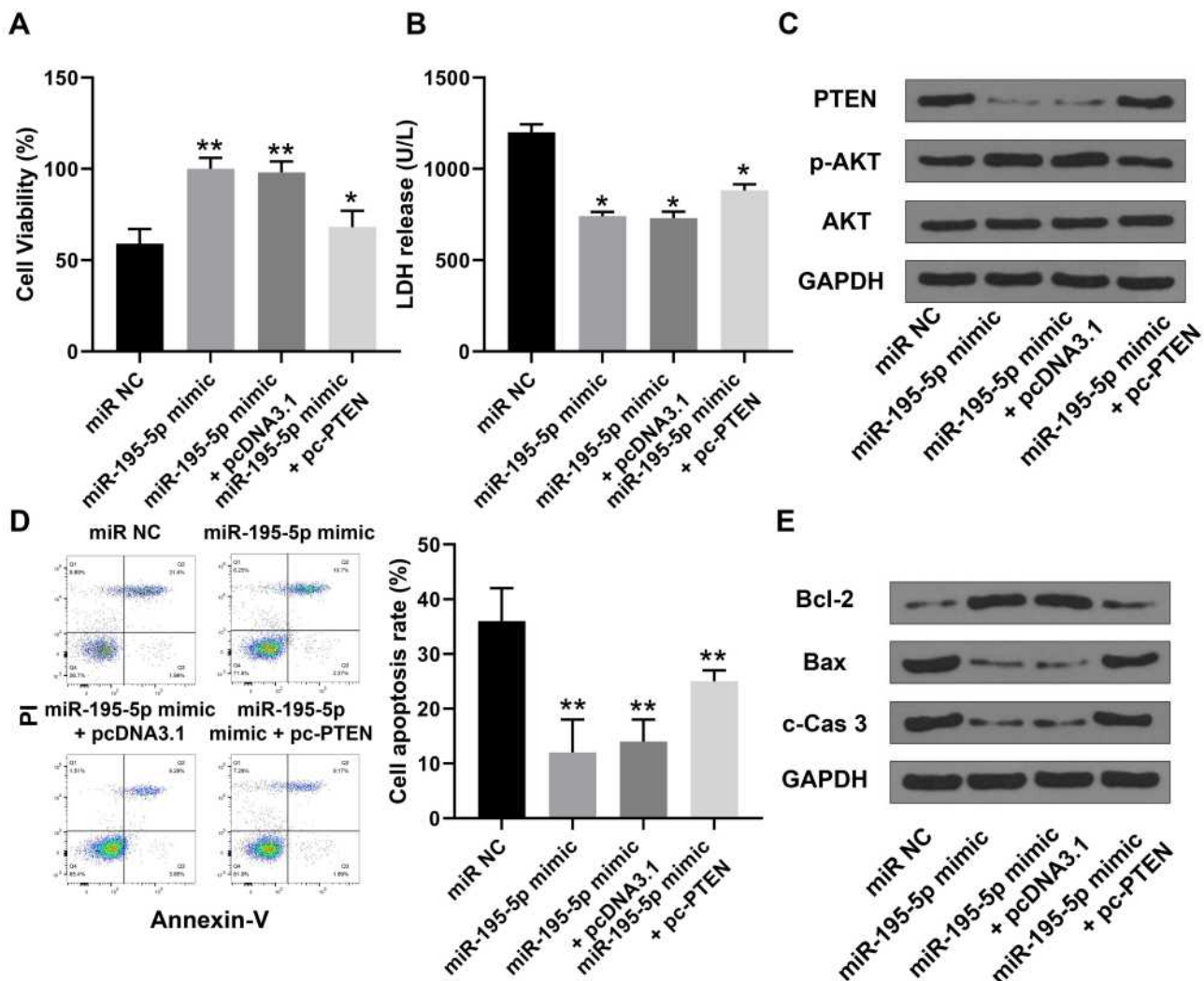


Figure 4 Overexpression of miR-195-5p improved I/R injury in vitro by regulating the PTEN-AKT signaling pathway. HBMVECs were transfected with miR-195-5p mimics, or co-transfected with miR-195-5p mimics and pc-PTEN, and then induced by 2 h of OGD followed by 24 h of re-oxygenation. (A) Cell viability was evaluated by CCK-8 assay. (B) The release of LDH was detected by specific cytotoxicity assay kit. (C) The expression of PTEN, AKT and p-AKT was detected by Western blot. (D) Cell apoptosis was evaluated by flow cytometer. (E) The protein expression of apoptosis-related makers was detected by Western blot. Data were presented as means \pm SD. * $p < 0.05$, ** $p < 0.01$ vs miR-NC.

reduced compared with that in the Sham group ($p < 0.001$), and the expression levels of miR-195-5p in miR-195-5p agomir group were obviously increased compared with that in NC agomir group ($p < 0.01$, Figure 5A). The infarct volume of brain in NC group was significantly increased compared with that in the Sham group ($p < 0.001$), whereas infarct volume in miR-195-5p agomir group was obviously reduced compared with that in NC agomir group ($p < 0.01$, Figure 5B). HE staining also confirmed the protective role of overexpression of miR-195-5p in cerebral I/R injury (Figure 5C). The apoptosis of cortical neurons was evaluated by TUNEL staining and the results indicated that MCAO/R treatment significantly induced cortical neurons apoptosis compared with Sham operation ($p < 0.001$), and the number of TUNEL positive

cells in miR-195-5p agomir group was significantly reduced compared with that in NC agomir group ($p < 0.01$, Figure 5D). In addition, the neurological deficit score was evaluated and it showed that MCAO/R treatment significantly increased the neurological deficit score of mice compared with that in the Sham group ($p < 0.001$), and miR-195-5p agomir obviously reduced the neurological deficit score of MCAO/R treated mice compared with agomir NC ($p < 0.001$, Figure 5E). Western blot results in brain tissues showed that MCAO/R treatment significantly increased the expression levels of PTEN ($p < 0.001$) and decreased the expression levels of p-AKT ($p < 0.001$) compared with that in the Sham group, while miR-195-5p agomir reduced the expression levels of PTEN ($p < 0.01$) and increased the expression levels of p-AKT ($p < 0.01$) in

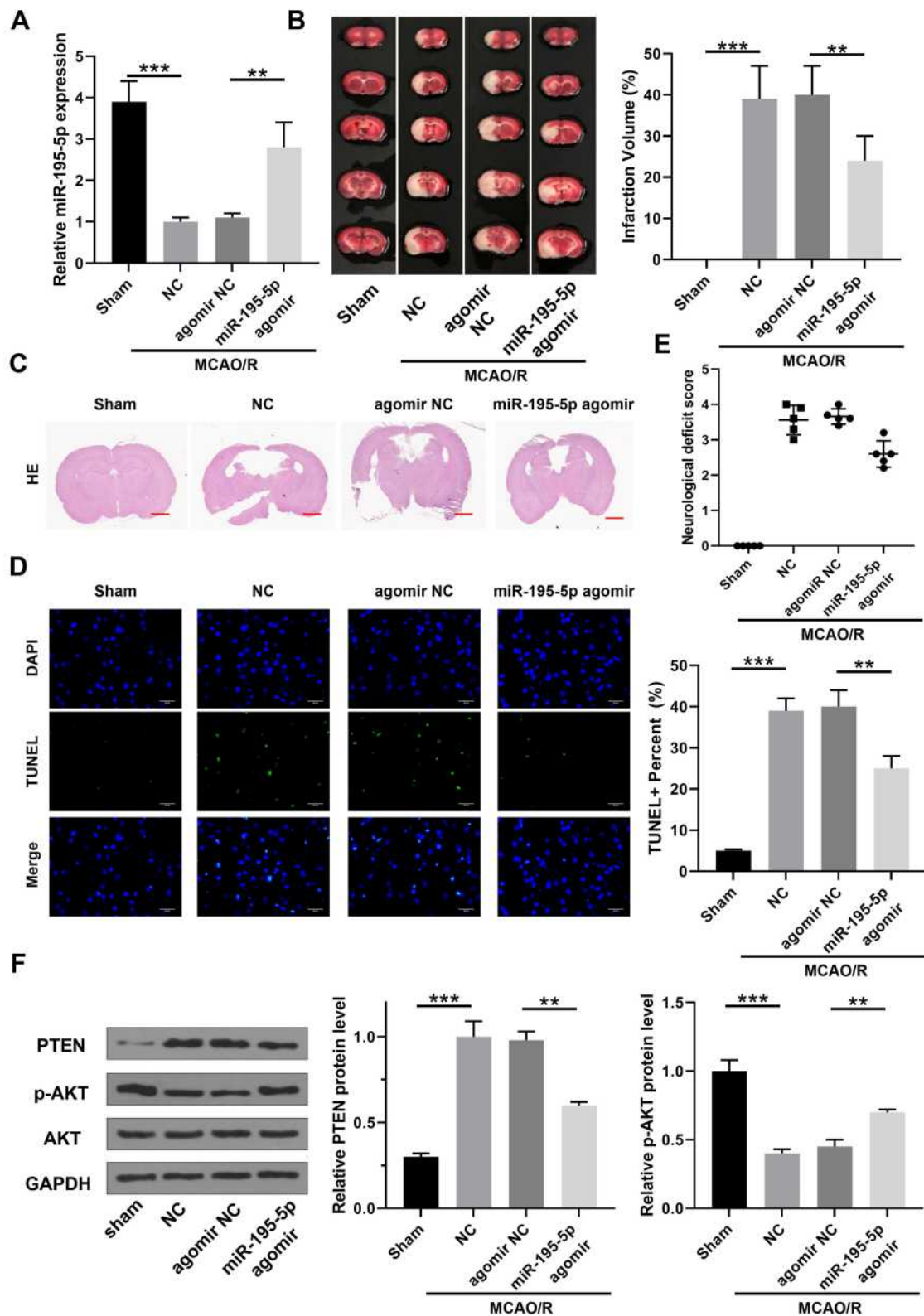


Figure 5 Overexpression of miR-195-5p ameliorated cerebral I/R injury in vivo. MiR-195-5p agomir or agomir NC (100 μ M) was injected into mice, and mice then received 2 h of MCAO followed by 24 h of reperfusion. (A) The expression of miR-195-5p in brain tissues was evaluated by qRT-PCR. (B) The infarct volume in brains was assessed by TTC staining. (C) H&E staining of whole brain in mice. Scale bar = 400 μ m. (D) The apoptosis of cortical neurons was evaluated by TUNEL staining. TUNEL positive cells (green) and DAPI positive cells (blue), and images were merged by using fluorescence microscope. Scale bar = 50 μ m. (E) Neurological deficit score. (F) The expression of PTEN, p-AKT and AKT in brain tissues was evaluated by Western blot. Data were presented as means \pm SD. ** p < 0.01 and *** p < 0.001.

MCAO/R treated mice compared with agomir NC group (Figure 5F). These results suggested that miR-195-5p ameliorates cerebral I/R injury by regulating the PTEN-AKT signaling pathway *in vivo*.

Discussion

Due to the unsatisfied therapeutic effects for ischemic stroke,²⁷ the identification of efficient targets in cerebral I/R injury becomes more necessary. In the last decades, non-coding RNAs including long non-coding RNAs (lncRNAs) and miRNAs have become promising therapeutic targets in ischemic stroke.^{28,29} In the present study, we explored the role and underlying molecular mechanism of miR-195-5p in cerebral I/R injury, expecting to provide novel insights into stroke treatment. We found that low expression levels of miR-195-5p were closely associated with cerebral I/R injury both *in vivo* and *in vitro*. Specifically, miR-195-5p affected the development of cerebral I/R injury by regulating the PTEN-mediated AKT signaling pathway. As expected, our study demonstrated that miR-195-5p might be considered as potential diagnostic and therapeutic targets for I/R injury in brain.

Apoptosis has been demonstrated to play a crucial role in the complicated pathologies leading to ischemic brain injury and also the subsequent reperfusion damage.³⁰ Extensive studies have reported various miRNAs that are affecting apoptosis signaling in cerebral I/R injury and may serve as potential targets. For example, overexpression of miR-224-3p alleviates apoptosis and then protects against cerebral ischemia reperfusion injury.³¹ MiR-1247-3p protects brain stroke through inhibiting apoptosis.³² MiR-25 negatively regulates cerebral ischemia/reperfusion injury-induced cell apoptosis.³³ Here, our study demonstrated that overexpression of miR-195-5p not only inhibited the apoptosis in OGD/R-treated HBMVECs *in vitro*, but also reduced the apoptosis of cortical neurons in MCAO/R-treated mice *in vivo*. Our study identified a new miRNA that is involved in cerebral I/R injury.

Previous studies have revealed that miRNAs always exert regulatory functions by directly binding to the 3'-UTR of target mRNAs in cerebral I/R injury.⁶ Upregulation of miR-219a-5p attenuates cerebral ischemia/reperfusion injury *in vitro* by targeting Pde4d.³⁴ In this study, we identified that PTEN was a potential target of miR-195-5p, and luciferase reporter assay determined that miR-195-5p could regulate PTEN by directly binding to its 3'-UTR. Previous studies have reported that PTEN

was abnormally expressed in various human cancers including prostate cancer,³⁵ endometrioid endometrial cancer,³⁶ and breast cancer.³⁷ Moreover, a recent study reported that PTEN was significantly upregulated in ischemic stroke.¹⁸ Here, we confirmed that PTEN was significantly upregulated during cerebral I/R injury. Overexpression of PTEN obviously attenuated the protective effects of overexpression of miR-195-5p in cerebral I/R injury *in vitro*. Our study also confirmed a previous prediction that PTEN might be a potential target of miR-195-5p. In addition, previous studies have identified that miR-195-5p has some additional targets involved in various biological processes. For example, miR-195-5p alleviates acute kidney injury through repressing inflammation by targeting VEGFA.³⁸ MiR-195-5p modulates the sensitivity of paclitaxel-resistant prostate cancer cells by targeting FKBP1A.³⁹ MiR-195-5p regulates the stemness of colon cancer cells by targeting MCM2.⁴⁰ Whether these genes mediated the role of miR-195-5p in cerebral I/R injury needs to be investigated in future studies.

The AKT signaling is a critical element that involves in apoptosis regulating the repair and survival of ischemic nerve cells in cerebral ischemia.⁴¹ It has been reported that PTEN can modulate the AKT signaling pathway to affect the development of cerebral I/R injury.⁴² Here, we detected the expression of AKT signaling members p-AKT and AKT, and found that overexpression of miR-195-5p increased the expression levels of p-AKT and had no obvious effect on the expression of AKT. Moreover, overexpression of PTEN significantly attenuated the inhibitory effect of miR-195-5p mimics on HBMVECs apoptosis. However, the effects of miR-195-5p on AKT signaling downstream genes should be investigated in the future.

Conclusion

In conclusion, our study demonstrated that miR-195-5p played a potential protective role in cerebral I/R injury by regulating the PTEN/AKT signaling pathway, suggesting that miR-195-5p might be a novel therapeutic target for cerebral I/R injury.

Availability of Supporting Data

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to their containing information that could compromise the privacy of research participants.

Ethical Approval and Consent to Participate

All animals were maintained according to the American Animal Protection Legislation. This study was approved by the animal Ethics Committee of Tianjin First Central Hospital. Procedures operated in this research were completed in keeping with the standards set out in the principles on ethical animal research outlined in the Basel Declaration.

Consent to Publish

Not applicable.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Funding

There is no funding to report.

Disclosure

All other authors have no conflicts of interest. We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

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