

MicroRNA-19a mediates neuroprotection through the PTEN/AKT pathway in SK-N-SH cells after oxygen-glucose deprivation/reoxygenation injury

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Abstract. Ischemic stroke is one of the most common public health problems worldwide. The aim of the present study was to investigate the role of microRNA-19a (miR-19a) and its possible target genes in SK-N-SH cells subjected to oxygen-glucose deprivation/re-oxygenation (OGD/R) injury. SK-N-SH cells are a suitable model for host transfection. SK-N-SH cells were transfected with miR-19a mimic or inhibitor and PTEN-small interfering (si) RNA in order to alter the expression of miR-19a, PTEN and AKT. The expression changes in acute cerebral ischemic injury (ACII) were verified using RT-qPCR and Western blotting. Expression changes and the association between miR-19a and PTEN following OGD/R were also assessed using a double luciferase analysis. In addition, cell viability and apoptosis were measured using an MTT and flow cytometry. miR-19a was downregulated; however, PTEN was markedly increased following OGD/R injury. miR-19a mimics increased cell viability, decreased cell apoptosis of SK-N-SH cells following OGD/R, which effects was similar to PTEN siRNA; however, miR-19a inhibitor had the opposite roles with miR-19a mimics. The present study provides novel information about the cell apoptosis and invasion mechanisms associated with the miR-19a/PTEN/AKT pathway and may present a potential therapeutic approach for OGD/R injury.

Key words: Oxygen-glucose deprivation/reoxygenation — microRNA-19a — PTEN — Neuroprotection

Abbreviations: ACII, acute cerebral ischemic injury; miR-19a, microRNA-19a; OGD/R, oxygen-glucose deprivation/reoxygenation; PTEN, phosphatase and tensin homolog deleted on chromosome 10.

Introduction

Stroke is a leading cause of mortality in a number of countries and the second leading cause of mortality in China (Hankey et al. 2010; Mortality and Causes of Death 2016).

Disability and consciousness disorders following stroke are a major burden for patients and their families. The majority of incidences of stroke are ischemic stroke (Tian et al. 2018). Ischemic injury to brain tissue following the inflammatory cascade response provoked by an oxygen-glucose deprivation/re-oxygenation typically causes ischemic brain damage and secondary brain injury (Wang et al. 2016). Despite developments in medical science, there is currently no effective therapeutic strategy for acute cerebral ischemic injury (ACII). It is therefore of great interest to develop novel treatments for ACII in order to reduce the morbidity and mortality.

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MicroRNAs (miRNAs/miRs) are small noncoding RNAs with length of 17–22 nucleotides. miRNAs are produced by a two-step pathway mediated by the RNaseIII enzymes Droscha and Dicer, which yield the mature miRNA molecules incorporating into a polyprotein complex called RNA-induced silencing complex and subsequent complete or incomplete binding to the 3'-untranslated region (UTR) of their respective target mRNAs that negatively regulate target gene expression at the post-transcriptional level by inhibiting translation and degrading target mRNAs (Bartel 2004; Bentwich et al. 2005). miRNAs have been suggested to play a key role in cerebral ischemic injury (Khoshnam et al. 2017b). miRNAs have been identified as important regulators of several biological processes, including cell differentiation, growth, proliferation, migration and apoptosis (Cho 2010; Aigner 2011; Rottiers et al. 2011). miR-19a is encoded by the oncogenic miR-17-92 cluster, which is considered to serve a role in a number of different cancers (Zhang et al. 2017). The oncogenic effect of the miR-17-92 cluster in B cell lymphoma has been demonstrated (Ventura et al. 2008; Mu et al. 2009) and several pro-apoptotic genes, including Phosphatase and tensin homolog deleted on chromosome 10 (*PTEN*), have been confirmed as target genes of miR-17-92 (O'Donnell et al. 2005; Novotny et al. 2007). However, the function and role of miR-19a in acute cerebral ischemic injury remain unclear. *PTEN* is a novel tumor suppressor gene whose double phosphatase activity has been identified in recent years (Stumpf and den Hertog 2016). It is considered to be one of the most important tumor suppressor genes along with p53, and is located on autosomes 10q23.3 (200 kb) (Fulci et al. 2000; Tate et al. 2009).

Dephosphorylating of protein kinase B (AKT) at Ser473 but not at Thr308 is mediated by one or both of the pleckstrin homology domain leucine-rich repeat protein phosphatase family of phosphatases (Crotty et al. 2013). Kroner et al. (2000) reported that the phosphorylation of Thr308 and Ser473 may be performed in either order independently of each other, although phosphorylation of Ser473 is required to fully activate AKT. As such, the level of phosphorylated (p)-AKT (Ser473) is the primary marker of AKT activation and reflects the overall amount of p-AKT (Chua et al. 2009; Riaz et al. 2012). AKT, the serine-threonine kinase, is a key molecule that serves a role in the phosphoinositide (PI3K)/AKT pathway and is able to inhibit apoptosis to promote cell survival (Ge et al. 2019). Previous studies have reported that apoptosis occurs in the peripheral penumbra of ischemia (Hou and MacManus 2002; Broughton et al. 2009) and p-AKT may inhibit apoptosis. It has also been reported that the expression of p-AKT is temporarily upregulated at the onset of focal cerebral ischemia and downregulated at 24 h following reperfusion; however, no significant changes in AKT expression were observed (Zhao et al. 2006). AKT phosphorylation promotes cell survival following cerebral ischemic

insult *via* inducing the phosphorylation and subsequent inactivation of several pro-apoptotic proteins, including glycogen synthase kinase (GSK) 3 β (Endo et al. 2006). The PI3K/AKT pathway mediates neuronal survival following cerebral ischemia and reperfusion (Endo et al. 2006; Zhu et al. 2013), which suggests that p-AKT308 and p-AKT473 may serve a role in cerebral ischemia-reperfusion injury.

Human SK-N-SH, a neuroblastoma cell line, is considered to be a core cell model of cerebrovascular tissues for *in vitro* assessments (Maggi et al. 1998). In order to assess the overall prognosis for brain tissue, these human neuroblastoma cells were selected for *in vitro* experiments in the present study. Cerebral infarction is caused by interrupted blood flow, leading to oxygen and glucose deprivation in the brain, which in turn results in infarction due to cell dysfunction and death (Verklan 2009; Pearson-Smith and Patel 2017). The oxygen-glucose deprivation/re-oxygenation (OGD/R) cell model is a classical *in vitro* cell model of stroke (Irmady et al. 2014). The ultimate consequence of neuronal glucose deprivation is neuronal death by apoptosis or necrosis. Necrosis is a passive death process that occurs in cells that lack specificity, whereas apoptosis is a form of programmed cell death and is an active, regulated process (Gong et al. 2014). Apoptosis is associated with the aging process throughout life, making it a focus of experimental research. In the present study, an OGD/R model was constructed based on SK-N-SH cells to simulate cerebral ischemia-reperfusion injury (Li et al. 2017). The cell model was transfected with a miR-19a mimic or inhibitor and *PTEN* small interfering (si) RNA to investigate the potential role of miR-19a in OGD/R injury *in vitro* and to identify potential target genes of miR-19a, which may be associated with mechanisms of OGD/R injury and be potential targets for therapy.

Materials and Methods

Ethics approval and consent to participate

The present study protocol was approved by the Ethics Committee of the First Affiliated Hospital of Soochow University and carried out following the NIH guidelines. All patients provided written informed consent.

Clinical experiments and grouping

A total of 30 paired peripheral blood samples were collected from 20 patients with acute cerebral infarction (18 male, 12 female; mean age, 54 years; age range, 36–72 years) (cerebral infarction group) and 20 normal healthy volunteers (17 male, 13 female; mean age, 55 years; age range, 35–75 years) (normal group) at the First Affiliated Hospital of Soochow University (Suzhou, China) between June 2015 and

June 2016. The National Institutes of Health (NIH) Stroke Scale system was used to evaluate the severity of ischemic stroke. Patients were excluded if any other central nervous system disease was identified. Venous blood samples (5 ml) were drawn from all participants. The serum was isolated by centrifuging at $1,000 \times g$ at 4°C for 5 min and then stored in liquid nitrogen. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was used to detect the expression of miR-19a in the samples.

Cell lines and culture

Human SK-N-SH cells lines were purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured as a monolayer in 25-cm^2 flasks containing Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin-streptomycin. All cells were maintained in an incubator containing 5% CO_2 at 37°C and underwent passage twice weekly.

OGD/R model

The normal cell culture medium was removed and SK-N-SH cells were washed three times with PBS. The culture medium was replaced with serum/glucose-free DMEM and cells were placed into a hypoxic chamber at 37°C (95% N_2 and 5% CO_2) for 16 h. Cells in the Control group were cultured under normoxic conditions for the same time. All cells were subsequently re-perfused for 12 h under normal culture conditions at 37°C (95% O_2 and 5% CO_2).

RNA isolation

Total RNA was isolated from the cultured cells using TRIzol according to the manufacturer's protocol (Invitrogen; Thermo Fisher Scientific, Inc.). The concentration and purity of the RNA was detected using a NanoDrop™ Spectrophotometer 2000 (Thermo Fisher Scientific, Inc., Pittsburgh, PA, USA) at 260 nm.

RT-qPCR

The expression of miR-19a in the Control and OGD/R groups was determined using RT-qPCR, performed using TaqMan miRNA assays (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Total RNA was extracted using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.) and cDNA synthesis was performed with a reverse transcription kit (Promega Corporation, Madison, WI, USA). The PCR primers used were as follows: PTEN, forward 5'-TTTGAAGACCATAACCCACCAC-3' and reverse

5'-ATTACACCAGTTCGTCCCTTTC-3'; and GAPDH, forward 5'-CTGGGCTACACTGAGCACC-3' and reverse 5'-AAGTGGTTCGTTGAGGGCAATG-3'. The thermocycling conditions were as follows: Initial denaturation for 10 min at 95°C followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. All RT-PCR reactions were performed 3 times. qPCR was performed with SYBR Premix Ex Taq II (Takara Bio, Inc., Otsu, Japan) using a Light Cycler 480 system (Roche Diagnostics, Basel, Switzerland). The $2^{-\Delta\Delta\text{C}_q}$ method was used to quantify the relative RNA expression (Livak and Schmittgen 2001). U6 was used as a reference gene for miRNA quantification, and GAPDH was used as a reference gene for proteins quantification.

Transfection and grouping

Following OGD/R, SK-N-SH cells were transfected with miR-19a mimic, miR-19a inhibitor, PTEN siRNA or the corresponding negative control (NC) using DharmaFect 2000 Transfection Reagent (Thermo Fisher Scientific, Inc.) as previously described (Bentwich et al. 2005). The SK-N-SH cells were collected 24 h following transfection and groups were designed as follows: OGD/R+NC group, OGD/R+miR-19a mimic group, OGD/R+miR-19a inhibitor group and OGD/R+PTEN siRNA group.

Measurement of cell viability

The viability of SK-N-SH cells was measured with an MTT assay. The MTT working solution (1:10 dilution of the 5 mg/ml stock; Promega Corporation, Madison, WI, USA) was filtered through a $0.22\text{-}\mu\text{m}$ filter and stored at 4°C . SK-N-SH cells were washed with warm RPMI-1640 medium (Thermo Fisher Scientific, Inc.) without phenol red, following which the cells were seeded at a density of 8×10^3 cells/well in 96-well plates containing DMEM with 10% fetal bovine serum, and cultured at 37°C for 12 h in a 5% CO_2 incubator. A total of 20 μl MTT working solution was added into each experimental well, following which plates were incubated at 37°C for a further 2 h. Then MTT solution was removed, and dimethyl sulfoxide was added to each well to dissolve the purple formazan. The absorbance of each well was read at a wavelength of 570 nm using a microplate reader. Absorbance was assumed to be directly proportional to the number of surviving cells and the viability of the control group was assumed to be 100%. A total of 4 independent experiments were performed, in quadruplicate.

Evaluation of miR-19a and PTEN using a double-luciferase targeting assay

miR-19a has previously been demonstrated to target PTEN. For the luciferase reporter assays, a pGL3 Firefly

Luciferase reporter vector (Promega Corp., Madison, WI, USA), with the 3'UTR fragment of human PTEN cDNA containing the putative target site for miR-19a and serving as an endogenous transfection control. A QuickChange Multi Site-Directed Mutagenesis kit (Stratagene; Agilent Technologies, Inc., Santa Clara, CA, USA) was used to mutate the human-miR-19a binding site in the PTEN 3'-UTR. The mutated (MUT) 3'-UTR sequence was used to create a luciferase plasmid, luc-PTEN-MUT, without a complementary miR-19a binding sequence. The SK-N-SH cells were transfected with the PTEN 3'UTR reporter or the MUT 3'UTR (30 ng/ml) using Lipofectamine[®] 2000[®] reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Furthermore, a Renilla luciferase construct was also co-transfected to normalize transfection efficiency. At 24 h post transfection, a dual-luciferase assay (Promega Corp.) was performed to assess the relative luciferase activities under a FL500 microplate fluorescence reader (BioTek Instruments, Inc., Winooski, VT, USA), according to the manufacturer's protocol. A minimum of three independent experiments was performed as previously described (Bartel 2004).

Measurement of SK-N-SH cell apoptosis using flow cytometry

The SK-N-SH cells were digested with Trypsin-EDTA (0.25%), phenol red (Thermo Fisher Scientific, Inc.) and 1×10^5 cells/ml were seeded in 6-well plates. Apoptotic cells were detected using an Annexin V-fluorescein isothiocyanate (FITC)/PI flow cytometry assay (BD Biosciences) and Cell Quest Software (version 3.1) according to the manufacturer's protocol. In brief, cells transfected with PTEN siRNA and miR-19a mimic for 48 h were fixed in pre-cooled 70% ethanol at 4°C overnight. The cells were harvested, washed twice with cold PBS and suspended in cold Annexin V-FITC binding buffer. Following the addition of 5 μ l Annexin V-FITC and 5 μ l PI, the cells were incubated in the dark at room temperature for 30 min. Each sample was subsequently incubated with 400 μ l binding buffer. The samples were analyzed using the FACSCalibur flow cytometer (BD Biosciences). A total of three independent experiments were performed.

Western blotting

SK-N-SH cells were transfected with an NC, miR-19a mimic, miR-19a inhibitor or PTEN siRNA. At 48 h after miRNA transfection, the SK-N-SH cells were homogenized in a lysis buffer (50 mM Tris-HCl, pH 8.0; 10 mM NaCl; 1 mM DTT; 2mM EDTA; 1% NP-40; 0.1% SDS and protease and phosphatase inhibitor cocktails) and total protein was extracted using radio immunoprecipitation

assay buffer (Thermo Fisher Scientific, Inc.). The protein concentration was measured using a BCA kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). For Western blot analysis the protein lysates (20 μ g/lane) were separated on 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes. Then 10% skimmed milk was used to block the membranes for 1 h at room temperature. The membrane was probed with primary antibodies against PTEN (ab32199; 1:10,000), AKT (ab8805; 1:500), p-AKT308 (ab38449; 1:500) or p-AKT473 (ab8933; 1:500; all Abcam, Cambridge, UK). GAPDH (ab8245; 1:1,000, Abcam) overnight at 4°C. GAPDH served as the loading control. The membranes were subsequently incubated with horseradish peroxidase-conjugated secondary antibodies (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at room temperature for 2 h. The protein bands were scanned using a Chemi Doc XRS+ Imaging System and quantified using Quantity One v4.6.2 software (both Bio-Rad Laboratories, Inc.).

Statistical analysis

Data are expressed as the mean \pm standard error of the mean. Differences between the means were analyzed using a Student's *t*-test. Differences between multiple groups were assessed by one-way analysis of variance followed by the Dunnett's *post-hoc* test. Each experiment was repeated ≥ 3 times. Calculations were performed using Excel software (Microsoft Corporation, Redmond, VA, USA) and the data were plotted using GraphPad Prism 6.0 software (GraphPad Software, Inc., La Jolla, CA, USA). A value $p < 0.05$ was considered to indicate a statistically significant difference.

Results

miRNA-19a expression in peripheral blood samples from patients with acute cerebral infarction and normal healthy volunteers

RT-qPCR results indicated that the expression of miR-19a was significantly downregulated in the patients with acute cerebral ischemic injury when compared with that in normal healthy volunteers (Fig. 1A; $p < 0.01$).

Expression of miRNA-19a and PTEN in SK-N-SH cells following OGD/R

To confirm the differential expression of miRNA-19a following OGD/R, the expression of miRNA-19a and PTEN was examined using RT-qPCR and Western blotting. The results revealed that miRNA-19a mRNA was significantly

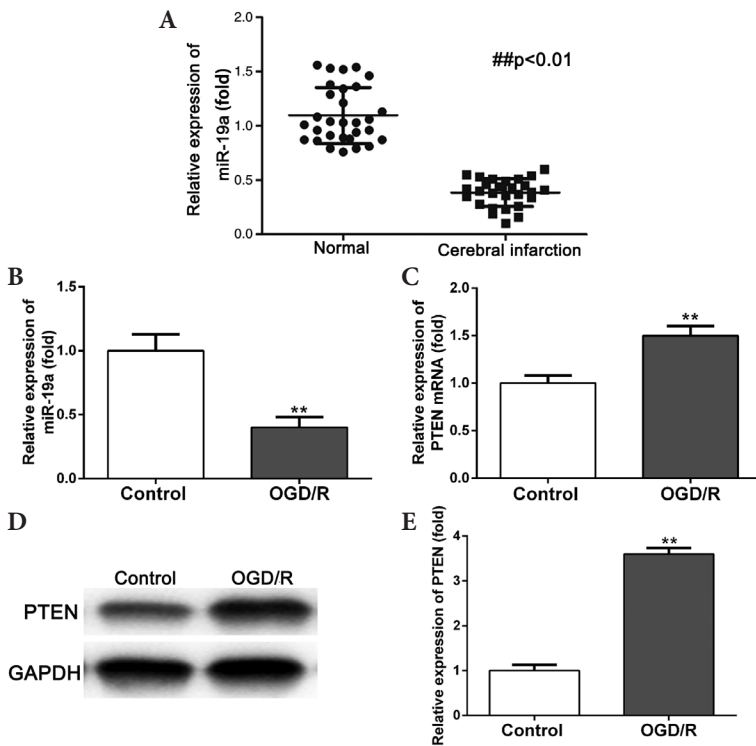


Figure 1. Comparison of miR-19a expression in peripheral blood samples from the normal healthy volunteers and the patients with acute cerebral ischemic injury (A), and in the SK-N-SH cells of Control and OGD/R treatment groups (B). The level of PTEN mRNA (C) and the protein expression of PTEN (D) in the Control and OGD/R groups. E. The relative protein levels of PTEN in the Control and OGD/R groups. $## p < 0.01$ vs. Normal healthy volunteers. $** p < 0.01$ vs. Control group. miR, microRNA; OGD/R, oxygen-glucose deprivation/reperfusion.

downregulated in the OGD/R group compared with the Control group (Fig. 1B; $p < 0.01$). However, the expression of PTEN mRNA was significantly elevated in the OGD/R group compared with the Control group (Fig. 1C; $p < 0.01$). Similarly, Western blotting revealed that the expression of PTEN protein was significantly upregulated in the OGD/R group compared with the Control (Fig. 1D and E; $p < 0.01$), which is consistent with the results of RT-qPCR.

Expression of miRNA-19a and PTEN in transfected SK-N-SH cells following OGD/R

Following OGD/R and transfection with miR-19a mimic/inhibitor, PTEN siRNA or NC, the expression of miRNA-19a and PTEN in SK-N-SH cells was assessed using RT-qPCR and Western blotting. The results of RT-qPCR revealed that miRNA-19a was upregulated in the miR-19a mimic and PTEN-siRNA groups compared with the NC group (Fig. 2A; $p < 0.01$), while miRNA-19a expression was downregulated in the miR-19a inhibitor group (Fig. 2A; $p < 0.05$). The expression of PTEN mRNA was increased in the miR-19a inhibitor group compared with the NC group (Fig. 2B; $p < 0.05$); however, PTEN mRNA expression was reduced in the miR-19a mimic and PTEN-siRNA groups compared with the NC group (Fig. 2B; $p < 0.05$). The expression of PTEN at the protein level was detected using Western blotting (Fig. 2C) and the results demonstrated

that PTEN protein expression was significantly upregulated in the miR-19a inhibitor group compared with the NC group (Fig. 2D; $p < 0.01$).

PTEN is a direct target of miR-19a

A schematic illustration of miR-19a binding to the wild-type (WT) PTEN 3'UTR is presented in Fig. 3A. Cells co-transfected with miR-19a mimic and PTEN 3'UTR-WT exhibited less cell luciferase activity compared with those co-transfected with NC+PTEN 3'UTR-WT or Control+PTEN 3'UTR-WT (Fig. 3A; $p < 0.05$). No significant differences in luciferase activity were observed in cells co-transfected with miR-19a+PTEN 3'UTR-MUT, NC+PTEN 3'UTR-MUT or Control+PTEN 3'UTR-MUT (Fig. 3A). These results suggest that PTEN is negatively regulated by miR-19a.

miRNA-19a affects cell proliferation of SK-N-SH cells following OGD/R

MTT results demonstrated that optical absorption in the miR-19a inhibitor group was significantly decreased compared with the NC group (Fig. 3B; $p < 0.05$ or $p < 0.01$). By contrast, transfection with miR-19a mimic or PTEN-siRNA significantly increased cell viability compared with NC (Fig. 3B; $p < 0.05$ or $p < 0.01$).

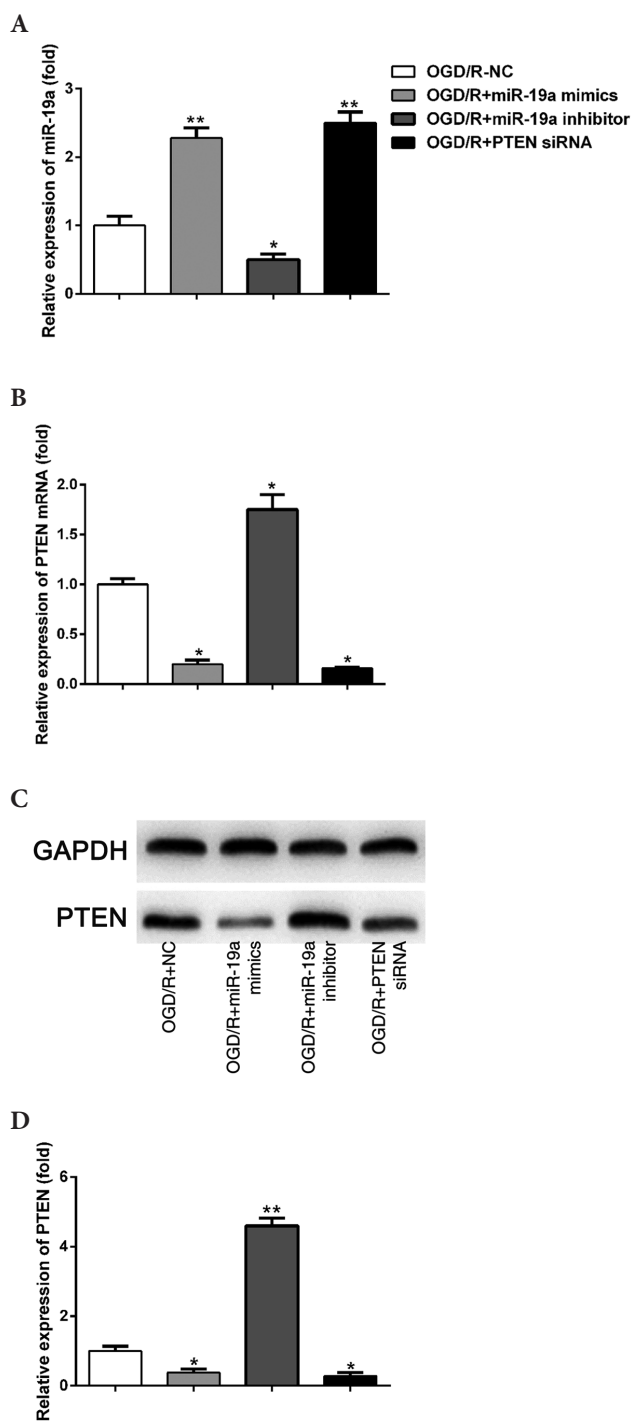


Figure 2. Expression of miR-19a (A) and PTEN (B) in SK-H-NH cells subjected to OGD/R treatment and transfected with miR-19a mimic, miR-19a inhibitor or PTEN siRNA. Protein expression (C) and relative protein levels (D) of PTEN in SK-H-NH cells transfected with miR-19a mimic was detected using Western blotting miR-19a inhibitor or PTEN siRNA after OGD/R. * $p < 0.05$ and ** $p < 0.01$ vs. the OGD/R+NC group. miR, microRNA; OGD/R, oxygen-glucose deprivation/reperfusion; NC, negative control.

miRNA-19a overexpression affects the cell apoptosis of SK-N-SH cells following OGD/R

Flow cytometry was performed to investigate the effects of exogenous miR-19a on cell apoptosis. The number of apoptotic cells was significantly higher in the miR-19a inhibitor group compared with the NC group (Fig. 4; $p < 0.05$). Conversely, the number of apoptotic cells was significantly reduced in the miR-19a mimic and PTEN-siRNA groups (Fig. 4; $p < 0.01$). This suggests that miR-19a upregulation or PTEN knockdown is able to promote cell cycle progression and suppress apoptosis in SK-N-SH cells.

Expression of AKT, p-AKT308 and p-AKT473 in SK-N-SH cells following OGD/R

The expression of p-AKT308 and p-AKT473 in the miR-19a mimic and PTEN-siRNA groups was significantly

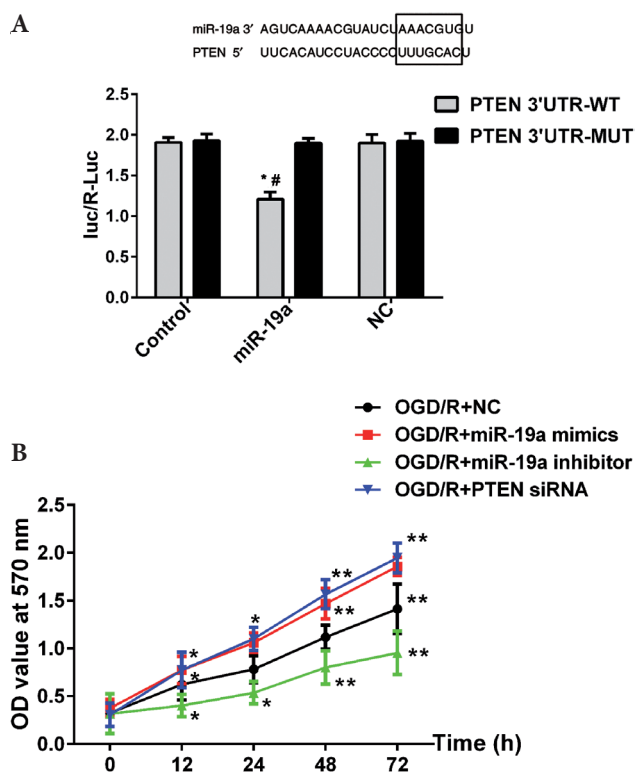


Figure 3. miR-19a targets PTEN. A. Putative binding of miR-19a to the PTEN gene 3'-UTR. SK-H-NH cells were co-transfected with luciferase plasmids containing the luc-PTEN-WT or luc-PTEN-MUT and the relative luciferase activities in different groups were determined. * $p < 0.05$ vs. NC+PTEN 3'UTR-WT and # $p < 0.05$ vs. Control+PTEN 3'UTR-WT group. B. MTT growth assays. * $p < 0.05$, ** $p < 0.01$ vs. OGD/R+NC group. UTR, untranslated region; WT, wild type; MUT, mutant; siRNA, small interfering RNA; OD, optical density. For more abbreviations, see Fig. 2.

increased compared with the NC group (Fig. 5A and B; $p < 0.01$). Similarly, the expression of p-AKT308 and p-AKT473 in the miR-19a inhibitor group was higher compared with the NC group (Fig. 5A and B; $p < 0.05$). No significant differences were observed in AKT between groups (Fig. 5C).

Discussion

Ischemic stroke is the third leading cause of death in developing countries and is a leading cause of disability in developing countries and China (Norrvig and Kissela 2013). The morbidity of stroke has increased with increased life expectancy. There are multiple risk factors for stroke, including genetics and the environment (Vinters et al. 2018). However, the pathogenesis of stroke remains poorly understood. It is therefore important to explore the pathogenic and protective mechanisms associated with stroke.

In recent years, it has been reported that cerebral ischemic injury may alter the expression of several miRNAs in ischemic brain tissue (Khoshnam et al. 2017a). miRNAs serve roles in development, differentiation and apoptosis in the nervous system (Nowak and Michlewski 2013; Tielking et al. 2019), as well as a number of nervous system diseases, including cerebral cancer, Alzheimer's disease, Parkinson's disease and ischemic stroke (Kalani et al. 2013; Roth et al. 2016; Quinlan et al. 2017). The level of miRNAs in cerebral ischemic tissue and the peripheral blood may be associated with the conditions of ischemic stroke (Khoshnam et al. 2017a). In addition, cerebral ischemia is known to alter

the miRNA profile in a spatial-temporal manner (Zou et al. 2019). The present study demonstrated that there was a marked reduction in miR-19a expression in the blood of patients who had experienced cerebral infarction. The results of the present study demonstrated that miR-19a was decreased in SK-H-NH cells subjected to OGD/R compared with normal control cells. Following OGD/R, miR-19a levels were decreased in SK-H-NH cells with a corresponding increase in PTEN expression. This suggests that miR-19a may act as a significant regulator of PTEN expression and so may have potential as a therapeutic agent for reducing neuronal apoptosis following ischemic injury. Furthermore, PTEN protein expression was significantly decreased in SK-N-SH cells transfected with miRNA-19a mimic or PTEN siRNA, whereas transfection with miRNA-19a inhibitor resulted in increased PTEN protein expression. These results suggest that miRNA-19a may serve a crucial role in the response to OGD/R and the regulation of PTEN.

In the present study, cell apoptosis was assessed in SK-H-NK cells following OGD/R treatment using flow cytometry. The results demonstrated that miR-19a knockdown accelerates cell apoptosis and enhances OGD/R injury, whereas miR-19a overexpression reduces cell apoptosis and has a protective effect. Collectively, these findings indicate that miR-19a upregulation may serve an important neuroprotective role in OGD/R injury. Furthermore, transfection with miR-19a mimic reduced luciferase activity in cells expressing WT but not MUT vectors, while overexpression or suppression of miR-19a resulted in a decrease or increase in PTEN expression during OGD/R injury, respectively. These data suggest that PTEN is a direct target gene of miR-19a. Ap-

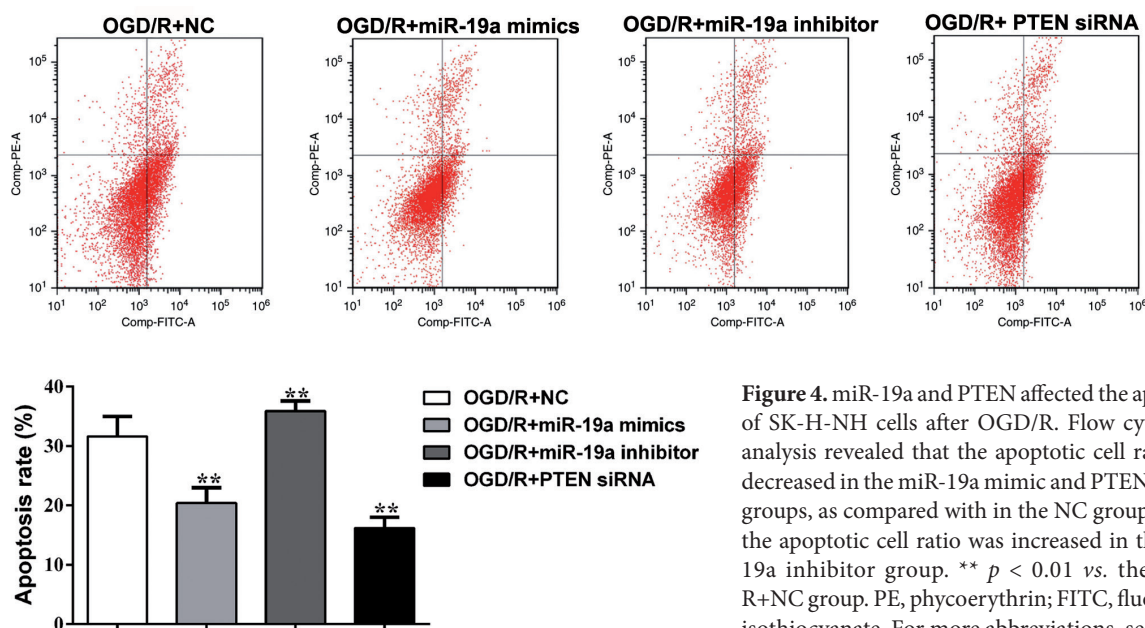


Figure 4. miR-19a and PTEN affected the apoptosis of SK-H-NH cells after OGD/R. Flow cytometry analysis revealed that the apoptotic cell ratio was decreased in the miR-19a mimic and PTEN-siRNA groups, as compared with in the NC groups, while the apoptotic cell ratio was increased in the miR-19a inhibitor group. ** $p < 0.01$ vs. the OGD/R+NC group. PE, phycoerythrin; FITC, fluorescein isothiocyanate. For more abbreviations, see Fig. 3.

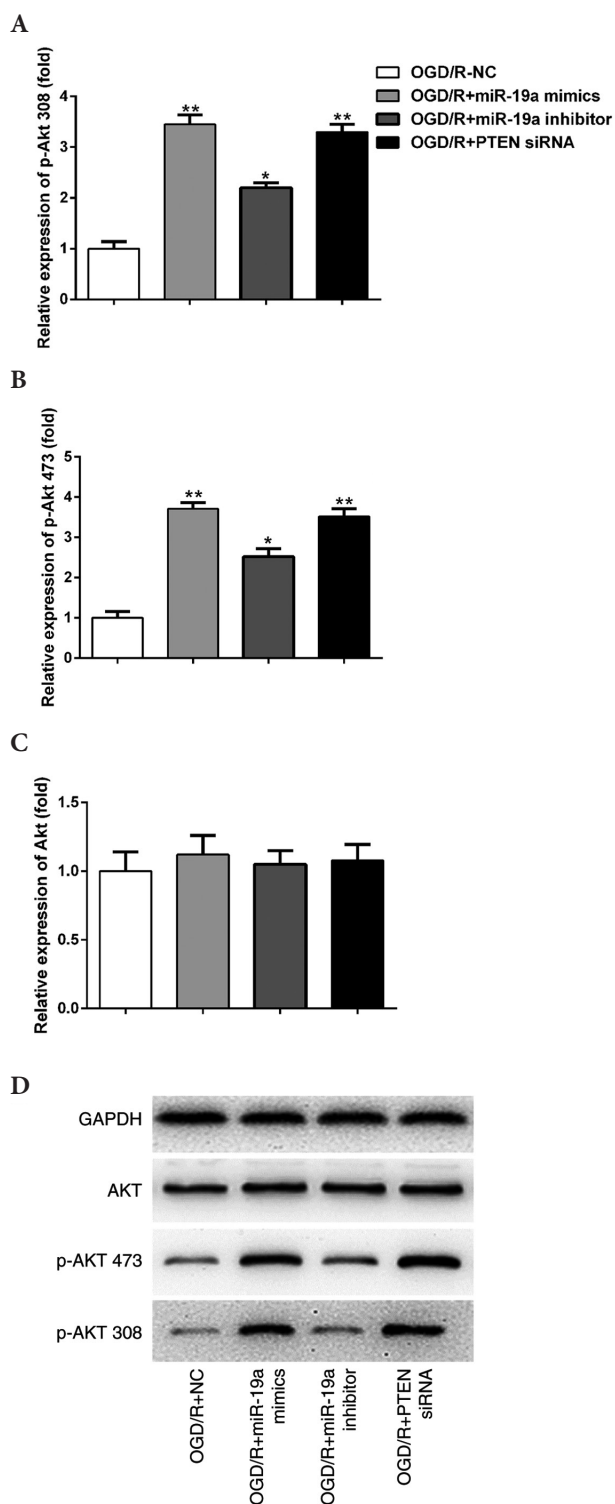


Figure 5. Relative levels of p-AKT 308 (A), p-AKT 473 (B) and AKT (C) in the OGD/R+NC, OGD/R+miR-19a inhibitor, OGD/R+miR-19a mimic and OGD/R+PTEN siRNA groups were determined using Western blotting (D). * $p < 0.05$ and ** $p < 0.01$ vs. the OGD/R +NC group. p, phosphorylated; AKT, protein kinase B. For more abbreviations, see Fig. 3.

apoptosis and autophagy programmed cell death are the main routes by which nerve cell death occurs in cerebral ischemia-reperfusion injury (Wei et al. 2016) and are regulated by apoptosis-related genes and their products.

The results of the present study suggest that transfection with miR-19a inhibitor results in decreased viability of SK-N-SH cells and induces cell death. As a further experiment, we focused on the targets of miR-19a, since the translation of the mRNA targets can be inhibited or the mRNAs degraded by miRNA to prevent their functions. In order to accurately predict the target genes of miR-19a, we evaluated miRecords, and identified that PTEN could be used as a target gene for the subsequent experiments. The results of Western blotting indicate that miR-19a overexpression decreased the expression of PTEN protein, whereas miR-19a knockdown had the opposite effect. In addition, the phosphorylation of AKT is a primary target of PTEN. The results of the present study indicate that the expression levels of p-AKT308 and p-AKT473 are increased by miR-19a overexpression and decreased by the miR-19a inhibition. Luciferase reporter assays also confirmed that PTEN is a direct target of miR-19a in SK-N-SH cells. SK-N-SH cells transfected with miR-19a inhibitor underwent OGD/R, which decreased the expression of miR-19a and upregulated the expression of its target gene PTEN, as well as increasing the expression of p-AKT308 and p-AKT473 protein. However, transfection with miR-19a mimic or PTEN siRNA suppressed the expression of PTEN while upregulating p-AKT308 and p-AKT473. These results suggest that miR-19a-PTEN-p-AKT is an important pathway in OGD/R injury. These findings suggest that miR-19a overexpression protects SK-N-SH cells from OGD/R injury by downregulating PTEN expression as a neuroprotective strategy. MiR-19a mimics may have potential as neuroprotective pharmacological agents for the treatment of cerebral ischemic injury. Further investigation into the opposing functions of miR-19a and PTEN may contribute to our understanding of the complex regulatory mechanisms of miRNAs in OGD/R injury.

In conclusion, the present study revealed that miR-19a targets PTEN mRNA directly and functions as an oncogene *via* the PTEN/AKT signaling pathway in OGD/R injury. However, it has certain limitations. The molecular mechanism of miR-19a in regulating OGD/R injury was studied *in vitro* only; further *in vivo* studies are required to verify these results. In addition, miRNAs have a complex mechanism of action in the regulation of human diseases, and miR-19a has a large number of target genes. The present study only investigated the role of miR-19a in targeting PTEN in OGD/R injury; whether other pathways or mechanisms are associated with OGD/R injury requires further study.

Conflict of interests. The authors declare that they have no competing interests.

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