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ORIGINAL ARTICLE

Expression and regulation of miR-449a and AREG in cerebral ischemic injury

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Abstract

Rodent focal ischemia models are widely used to mimic and examine human strokes. To the best of our knowledge, no investigation has systematically examined the expression changes of microRNA (miR)-449a and Amphiregulin (AREG) as well as their biological relationship during middle cerebral artery occlusion (MCAO) and oxygen and glucose deprivation/reperfusion (OGD/R). The present study examined the histological and behavioral outcomes of MCAO and the function of miR-449a and AREG in cerebral ischemic injury. Rats were subjected to 2 h MCAO, which was followed by reperfusion. miR-449a and AREG were examined in the injury tissues of MCAO rats and the OGD/R cell line by reverse transcription-quantitative polymerase chain reaction. Protein expressions of AREG in the injury tissues of MCAO rats was measured using an immunohistochemistry and the protein expression levels of AREG, epidermal growth factor receptor (EGFR), phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) and the phosphorylation level of Akt (p-Akt) were analyzed by western blotting. Cell apoptosis was examined following the knock down and subsequent overexpression of AREG in a human OGD/R neuronal cell line by small interfering RNAs (siRNAs) and plasmid transfection. Luciferase reporter assays were used to validate the target of miR-449a. The expression changes and regulatory mechanisms of miR-449a and AREG in an ischemia/reperfusion (I/R) injury model were examined in vivo and in vitro. The neurological deficit score, brain edema volume, cerebral infarct area, and the number of apoptosis cells in ischemic rats were all markedly elevated, than that in the control rats. The expression of miR-449a was decreased and AREG was increased in the MCAO rats and human OGD/R neuronal cell line. miR-449a inhibition or AREG overexpression in OGD/R cells resulted in a significant decrease in apoptotic cells, and AREG was revealed to be one of the direct targets of miR-449a. Molecular recovery was observed following transfection with miR-449a mimics and AREG knockdown in an OGD/R model in vitro. The present study demonstrated that miR-449a was downregulated while AREG was upregulated in cerebral ischemic injury, and the recovery of neurological function can be obtained following the overexpression of miR-449a and the knockdown of AREG in an I/R injury model. miR-449a functions in ischemic stroke via directly targeting AREG. These findings suggest a novel mechanism involving in cerebral I/R injury model and may aid investigators in gaining a deeper understanding of strokes in a clinical setting.

Keywords MCAO · miR-449a · AREG · Ischemia · Stroke · OGD/R

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Introduction

Ischemic stroke is a leading cause of mortality in developed countries and brings not only economic pressure but also the burden of care (Dirnagl et al. 1999). However, the underlying mechanisms of the disease remain largely unknown and the treatment options for acute stroke remain limited (Group* TNIoNDaSr-PSS 1995; Green and Shuaib 2006). Although neuroprotective agents such as AMPA and NMDA antagonists have been developed to cure ischemic stroke, the results in a clinical setting were not satisfactory (Besancon et al.

2008). Therefore, it is urgent to explore the underlying mechanisms of the disease in order to identify novel strategies.

The MCAO model is a widely used focal ischemia models (Zhang et al. 1997). In addition, neurological deficits such as infarct volume and the number of apoptosis cells is often used for the evaluation of ischemic stroke in the MCAO model in rodents (Zhang et al. 1997; Overgaard et al. 1992). Following I/R injury in vitro, the majority of cells undergo apoptosis (Broughton et al. 2009). Hundreds of microRNAs exist in the mammalian genome and regulate approximately 30% of human genes (Ambros 2004; Lewis et al. 2005). MicroRNAs are non-coding RNAs, 18–25 nucleotides (nt) in length (Yu et al. 2014). miR-449a, a member of miR-449 family, is expressed highest during the proliferative phase of embryonic neurogenesis (Barca-Mayo and De Pietri Tonelli 2014). The expression of mir-449a can be detected in the brain tissue of mice and it is essential for normal brain development, and the inactivation of miR-449a caused underdeveloped basal forebrain structures (Wu et al. 2014). miR-449a is a well-known tumor suppressor and previous studies (Yao et al. 2015) have reported that miR-449 functions as a tumor suppressor by inducing senescence and apoptosis. Recently, Yang et al. (2018) reported an inhibitory effect of miR-449a on neuronal differentiation, which indicated that miR-449a might be related to the regulation of nerve. However, to the best of our knowledge, few studies have investigated its function in ischemic stroke and bioinformatics software (TargetScan Human 7.1) predicts that miR-449a has binding sites with AREG 3′UTR. Thus, we speculated that the role of AREG in the injury following ischemic stroke might be related to miR-499a. AREG is a member of the epidermal growth factor family (Berasain and Avila 2014). It is an autocrine growth factor as well as a mitogen for astrocytes, Schwann cells and fibroblasts. AREG is associated with epidermal growth factor (EGF) and transforming growth factor alpha (TGF-alpha) (Eckstein et al. 2008). Moreover, AREG acts as an autocrine survival factor for adult sensory neurons and is a mitogen for adult neural stem cells (Falk and Frisen 2002). It is presently unclear how the AREG are regulated and expressed in an ischemic stroke, and these processes therefore require further clarification. However, it has previously been established that the AREG functions as the ligand of EGF receptor (EGFR), which is linked to the PI3K/AKT and Raf/mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK1/2) signaling cascades that either protect cells or potentiate cell injury (Zhou et al. 2015).

Recent studies (Chen et al. 2018) have demonstrated that following the activation of EGFR/MAPK signaling cascades, cell proliferation and neurogenesis can be promoted in transient cerebral ischemic brains. This process could further improve the repair of neurological functions in post-ischemic stroke rats (Chen et al. 2018). The activation of the PI3K/Akt/NF-κB pathway can increase the expression of tight junction proteins to stabilize blood-brain barrier (BBB) integrity and increase the alleviation of inflammation via decreasing inflammatory mediators in stroke and neurodegenerative diseases (Zhu et al. 2018). Autophagy was also generated in this pathway in order to exert a protection function (Yan et al. 2014). The present study aimed to investigate whether the protective role of miR-449a was mediated through EGFR/MAPK signaling or by activating the PI3K/Akt pathway in cerebral ischemia-reperfusion injury and in cultured cells.

As few studies have investigated whether miR-449a participates in the pathological process of ischemia and the underlying mechanisms, in the present study, a rat MCAO model and cell OGD/R model were established to detect the expression level of miR-449a in vivo and in vitro. The results provided evidences that miR-449a regulates cell cycle and reduces cell death in ischemia. In addition, the present study demonstrated that AREG is a target of miR-449a in ischemia. The data also revealed that miR-449a regulates EGFR and the phosphorylation level of PI3K/Akt. The findings of the present study provide the possibility that miR-449a may serve as molecular target for therapy of cerebral ischemic injury.

Materials and methods

Surgical procedures

The present study was approved by the SPF Animal Welfare Ethics Committee (Beijng, China). Harlan Sprague Dawley rats (SD rats, male, purchased from SPF (Beijing) Biotechnology Co. Ltd., weighing 260-280 g, were used for the preparation of MCAO model. Because higher estrogen levels of female rats, while estrogen has the nerve protective effect, and the secretion of estrogen is cyclical. During the experiment, the experimental personnel couldn't make accurate calculations of the rat estrogen level, which caused some difficulties for the apply to cerebrovascular disease animal model of female animals. Female animals is not conducive to the stability of the model, so it is more suitable for the preparation of MCAO model in the male rat.

Rats were prepared for MCAO according to the protocol outlined in a previous study (Longa et al. 1989) with some modifications. Briefly, the rats were fasted overnight prior ischemic injury; however, had free access to water. The rats were then anesthetized with an intraperitoneal injection of 4% chloral hydrate (0.3 mg/100 g), a midline

ventral neck incision was performed, and the right common carotid artery was identified. In recent years, there are indeed many controversies about the application of chloral hydrate, but we think it is still applicable in animal experiments. We also refer to many literatures and find that most researchers still use chloral hydrate in the application of MCAO rat model (Zhang et al. 2011; Fan et al. 2018; Qian et al. 2019). The right common carotid artery (CCA), external carotid artery (ECA) and internal carotid artery (ICA) were exposed. Occlusion of the common carotid artery and external carotid artery were ligated through a ventral midline neck incision. Ischemia was induced with a 3–0 nylon monofilament suture interleaved via the ECA into ICA in order to block MCA in rats. The filament was maintained in place for 2 h, and the monofilament was removed on order to restore the ICA-MCA blood perfusion. Reperfusion was permitted for approximately 24 h. The body temperature of the animals was kept constant at 37 °C using a thermostatically controlled heating pad until the animals recovered from surgery. Rats with neurological function score ≥ 2 can be regarded as the success of MCAO model. Rats with no acute neurological deficit or hemorrhage were excluded and new rats were ready for the MCAO model. Rats were assigned into a MCAO model group ($n = 32$) and a normal group (n = 32), in which rats underwent sham surgery without line embolism and the remainder of the procedure was the same as that of the MCAO model group.

Neurologic assessment

A screening neurologic examination was performed at 24 h after reperfusion following use of a 5-point scoring system according to ZeaLonga (Longa et al. 1989). The score correlated was with neurobehavioral defects and a higher score represents more severe defects in neural function ($n = 32$). The assessment was based on criterion including, spontaneous activity, balance, symmetry of movements, symmetry of forelimbs, climbing ability, muscular co-ordination, reaction to touch, and response to vibrissae touch. The animals were scored on a five point scale as follows: 0 (no deficit) - normal; 1-mild deficit; 2-moderate deficit; 3-severe deficit; and 4-serious deficit. Observers were blinded to group assignment and were experienced in performing this scoring system and a single observer scored all animals in order to minimize variability in application of the scoring system between groups.

Assessment of cerebral edema

The rats were anesthetized and decapitated, and the brain was then isolated rapidly following neurologic assessment. The whole brain was rinsed with clean water and dried with filter paper. The wet weight of the brain was measured by an electronic balance. The brain was then dried for 48 h in an oven and weighted, and the dry weight was recorded. The cerebral edema degree was assessed by the ratio: (wet weight - dry weight) / wet weight * 100%. Eight rats were randomly selected from each group ($n = 8$).

Cerebral infarct volume

Following the neurological function score of assessment, the rats were anesthetized quickly and the brain was isolated and stored at −20 °C for 10 min. The olfactory bulb and lower brain stem were removed. The rest of the brain was cut into continuous backward slices and evenly with a coronal 2 mm thick section. The slices were placed into 2% 2, 3, 5 - triphenyltetrazoliumchloride (TTC) and incubated at 37 °C for 30 min without light to induce a chromogenic reaction. Images were taken after slices were stored for 24 h in 4% formaldehyde at 4 °C and the cerebral infarction area was calculated by Image J software (National Institutes of Health, Bethesda, MS, USA). The corrected cerebral infarction volume was calculated by the ratio, [total infarction volume - (ischemic side brain tissue volume - contralateral brain tissue volume)] / contralateral brain tissue *100% ($n = 8$).

TUNEL analysis for tissue apoptosis

The rats were perfused with 4% formaldehyde for 24 h following blood perfusion. Then the brain were isolated and fixed with 4% formaldehyde. Following gradient ethanol dehydration, the brain was made transparent with xylene. The tissue was embedded with paraffin and the brain was cut into continuous slices with a 4 μm coronal section. The nucleus of apoptotic cells was staining using DeadEnd TM fluorometric TUNEL cell apoptosis detection kit (Promega Corporation, Madision, WI, USA) according to the manufacturer's protocol. The apoptotic neurons in the cerebral cortex on the ischemic side were observed and counted with a ×400 optical microscope. A total of 6 fields of view were randomly selected to calculate the number of apoptotic cells. Eight rats for analysis in normal and MCAO groups were randomly selected ($n = 8$).

Immunohistochemistry (IHC) staining

The IHC staining was carried out on immunostaining instruments (Roche Ventana Discovery or LEICA BondMax) following manufacturer's guidelines. The following antibodies were used in this study: anti-AREG (ab33558, Abcam).

Human neuronal cell line culture

A human neuron cell line (Neur) was purchased from ScienCell Research Laboratories, Inc. Cells were cultured in

a basic medium consisting of DMEM (GIBCO; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 10% FBS and penicillin-streptomycin (both GIBCO; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Cells were subcultured when they were 80–90% confluent. 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 1, 2, 4 and 6 h. Cells in the control group were cultured under normoxic conditions for the same amount of time. All cells subsequently underwent reperfusion for 16 h under normal culture conditions at 37 °C (95% O_2 and 5% CO_2). Then, CCK-8 kit (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) was performed to detect the cell viability according to the manufacturer's protocol.

siRNA, miR-499a mimics and transfection

For subculture and the transfection experiments, cells were trypsinized by TrypLE Select (Invitrogen; Thermo Fisher Scientific, Inc.), centrifuged for 5 min at 500 rpm, resuspended, and plated in 24-well plates. Neur cells were transfected with AREG siRNA (5'-GGAUUUGAGGUUACCUCAATT-3′), miR-449a mimics and a negative control (NC) vector using Lipofectamine 2000 following OGD/R. Additionally, the sequences of miR-449a mimics were 5′-UGGCAGUGUAUUGUUAGCUGGU-3′ (sense) and 5′-CAGCUAACAAUACACUGCAAUU-3′ (antisense) and the sequence of NC was 5′-ACGUGACA CGUUCGGAGAAUU-3′ (sense) and 5′-AAUUCUCCGAACGUGUCACGU-3′ (antisense), respectively. Following a 48 h incubation, the cells were used for subsequent experiments. The groups comprised of a Control group (Neur cells without treatment), OGD/R group, NC group, miR-449a mimics group and AREG siRNA group.

MTT cell proliferation assay

After the transfection was stabilized, Neur cells were plated in 96-well plates at a density of 1×10^5 cells/well containing DMEM supplemented 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. The MTT solution was then removed, and dimethyl sulfoxide was added to each well to dissolve the purple formazan. Cell viability was subsequently analyzed at a wavelength of 570 nm using a micro plate reader.

Flow cytometry

Neur cells were collected and centrifuged for 10 min at 3000 xg. Ethanol was added to mix the cell pellets and RNA was removed following treatment with an RNase. The stained cells were brown and they were analyzed on FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

The total RNA from brain tissue of the cerebral infarct area and Neur cell, which was extracted by TRIZOL regent (Life Technologies; Thermo Fisher Scientific, Inc.). In the MCAO model group, three rats were selected randomly for RNA extraction. The concentration and purity of total RNA were detected by ultraviolet absorption detector and the formaldehyde degeneration electrophoresis. The transcription expression level of miR-449a and AREG were detected by RT-q PCR. cDNA was synthesized using PrimeScript RT reagent Kit (TaKaRa Biotechnology Co., Ltd., Dalian, China). Reverse transcription-quantitative PCR analyses for mRNA expression by using SYBR Premix Ex Taq (TaKaRa Biotechnology Co., Ltd., Dalian, China), U6 and GAPDH were used as an internal control for miR-449a and the proteins. The expression levels of miR-449a and other genes mRNA levels were normalized using the threshold cycle (Cq) of U6 and GAPDH, respectively.

Luciferase assay

The mutant AREG was used to compare the fluorescence intensity of wild-type AREG combined with mir-449a in the detection of dual luciferase reporter gene, which proved AREG is a direct target of miR-449a in Neur cells. For 3'-UTR assays, Neur cells, stably expressing miR-449a, were cultured in 96-well plates and co-transfected with 50 ng wild-type (AREG-3'-UTR-WT) or mutant (AREG-3'-UTR-MT) and control luciferase reporter, 10 ng pRL-CMV Renilla luciferase reporter by using Lipofectamine 2000. The cell lysates were collected at 48 h, and the firefly and Renilla luciferase activities were measured by using a dual-luciferase reporter assay system (Promega Corporation). Transfection efficiency was normalized to the control luciferase.

Western blot

The proteins were extracted from brain tissue of the cerebral infarct area and Neur cell. The level of AREG,

EGFR, PI3K/Akt and the phosphorylation level of PI3K/Akt were detected by western blot analysis. The tissues or cells were lysed in RIPA buffer plus protease inhibitors (Roche Diagnosis, Basel, Switzerland) and lysates were electrophoresed on 10% SDS-PAGE gels and blotted onto nitrocellulose membrane (Qi et al. 2014). Following blocking with 5% nonfat milk in Tris-buffered saline with Tween (TBST; Invitrogen; Thermo Fisher Scientific, Inc.) for 2 h at room temperature, and the membranes were then incubated overnight with primary antibody anti-AREG (ab180722, Abcam, Cambridge, UK), anti-EGFR (ab40815, Abcam), anti-PI3K (#4249), anti-p-PI3K(#4228), anti-Akt (#4691) and anti-p-Akt (#4060) and visualized with the ECL Detection Reagents (Pierce; Thermo Fisher Scientific, Inc.).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA). The results were presented as mean ± SD. A Student's t test was used to evaluate the statistical significance of difference between two groups, while differences between multiple groups were assessed by one-way analysis of variance (ANOVA) followed by the Dunnett's post hoc test. The correlation between the levels of miR-449a and AREG was measured using Pearson's correlation analysis. All experiments were performed at least three times with triplicate samples. $P < 0.05$ was considered to indicate a statistically significant.

Results

Establishment and evaluation of the MCAO model

A MCAO model is a widely used strategy to examine ischemic stroke in rats (Zhang et al. 1997). The present study initially established the transient MCAO model in rats and evaluated the situation at different levels were used. The neurological score of MCAO rats was calculated according to the 5-point scoring system and revealed that the scores of the MCAO rats were significantly higher than that in normal group (**$p < 0.01$, Fig. 1a), indicating the neurological deficits were present in MCAO rats. The present study further detected the cerebral edema and the infarct volume of MCAO rats, and the results demonstrated that the extent of cerebral edema and infarct volume were also higher than that in normal group (Fig. 1b and c). The number of cells number undergoing apoptosis in the stroke area was then calculated. Apoptosis was determined by terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL) staining, and it was observed that the MCAO rats had a markedly elevated number of apoptotic cells (Fig. 1d). Therefore, the results demonstrated that the MCAO rats were successfully established and exhibited the appropriate symptom of ischemic injury.

miR-449a is downregulated in cerebral ischemic injury model

In order to investigate whether miR-449a participate in the biological process of MCAO rats, the present study first examined the expression of miR-449a by RT-qPCR with three rats in the MCAO model compared with normal tissue. The results revealed that miR-449a was evidently downregulated in MCAO rats compared with rats in the normal control group (Fig. 2a). To further confirm the downregulation of miR-449a in OGD/R cells, a human neuron cell line (Neur) was purchased to establish the model of oxygen and glucose deprivation/reoxygenation (OGD/R). A CCK-8 viability assay kit was then used to examine cell viability in the OGD/R model for an optimum time. In Neur cells, OGD/R for 1 h (1 h OGD followed by 12 h reperfusion) increased cell death by 20% vs. the control group (normal Neur cell line without OGD/R), and cells underwent OGD/R for 2 h (2 h OGD followed by 12 h reperfusion) with and cell viability decreased to more than 50% of control (Fig. 2c). Therefore, the present study established a successful OGD/R for 2 h model with a moderate cell death number in order to conduct the following experiments. The expression of miR-449a in OGD/R (2 h OGD followed by 12 h reperfusion) cells was detected by RT-PCR, and the results demonstrated that the fold change of miR-449a in OGD/R group was significantly decreased than compared with that in the control group (Fig. 2b). These results indicated that miR-449a is downregulated in ischemic stroke in vivo and in vitro.

AREG is upregulated in cerebral ischemic injury model

To confirm the relationship between miR-449a and its target AREG, the present study detected the expressions of AREG in a rat MCAO model. The RT-qPCR results revealed that the fold change of AREG was significantly higher than that of the control, indicating the upregulation of AREG in the MCAO model (Fig. 3a), and this trend was also observed in the OGD/R group, when compared

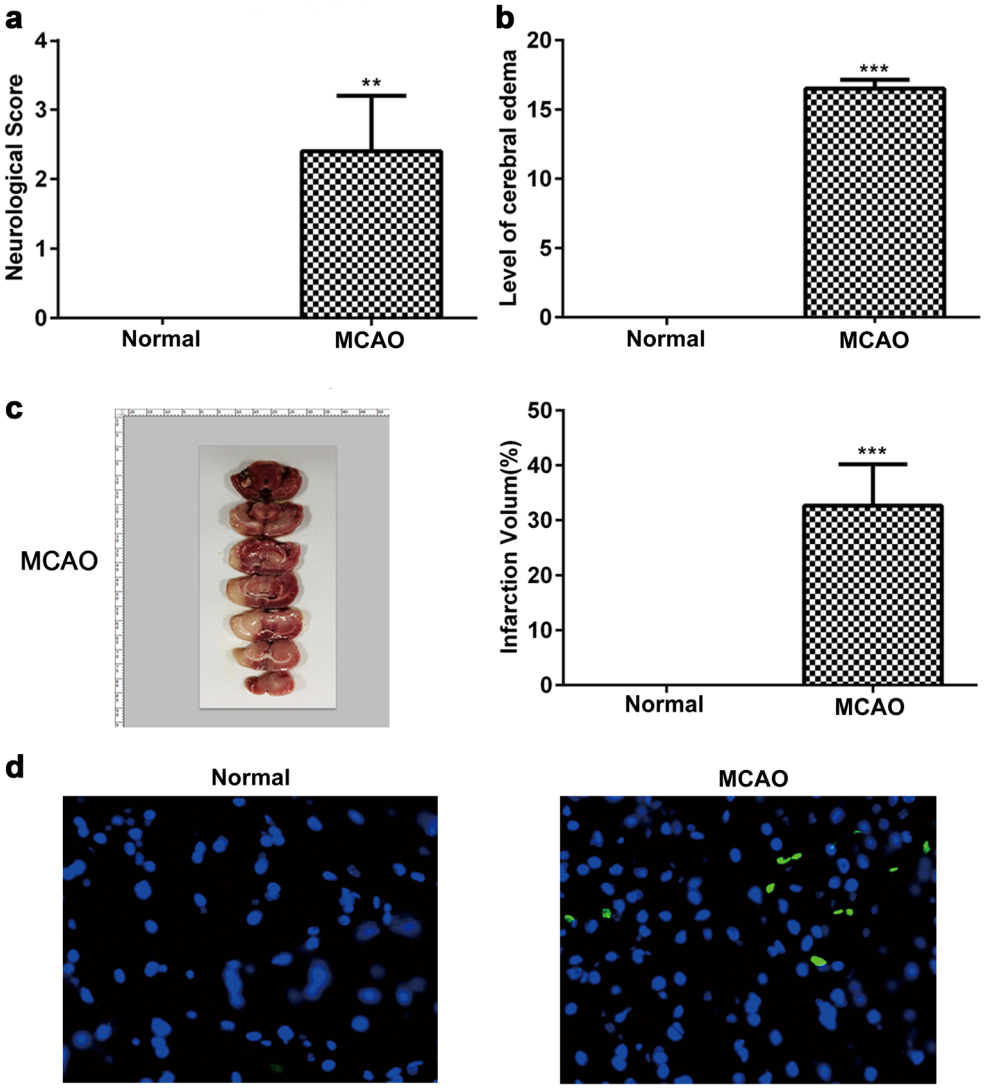

Fig. 1 The change of indexes was measured following ischemia-reperfusion injury. a The neurological score is elevated significantly of rats in MCAO group comparing to the rats in normal group. **b** The cerebral edema is increased significantly of rats in MCAO group when compared with that in normal group. **c** Rats that were exhibited considerably more infarct volume as compared to normal rats. **d** The number of neuronal cell death in MCAO was elevated remarkably when compared with normal group. Values are represented as mean ± SD, $n = 8$. $p < 0.05$ as determined by Student's t test.$^{**}p < 0.01$,$^{***}p < 0.001$ vs normal

with the control group (Fig. 3b). In order to examine the protein level of AREG, western blotting was conducted in ischemic cerebral cortex tissue and it was observed that AREG expression was markedly upregulated in the MCAO group. Immunohistochemical staining was performed in order to detect the positive protein expression rate of AREG. The positive expression of AREG protein was indicated by the appearance of brown particles (Fig. 3c) and there were more cells with positive AREG in the brain tissue of MCAO rats than those found in the normal rats. The correlation between miR-449a and AREG expression was assessed using Pearson's correlation analysis and the results revealed that miR-449a was negatively correlated with AREG (Fig. 3d). In addition, the expression levels of EGFR and the phosphorylated Akt and PI3K were up-regulated in MCAO rats compared with the control group (Fig. 3e). The results demonstrated that miR-449a is negatively correlated with the expression of AREG in MCAO rats.

AREG is a direct target of miR-449a

MicroRNAs exert their function by downregulating their downstream target gene expression. In the present study, miR-449a was downregulated while AREG upregulated in cerebral ischemic injury. To investigate whether AREG serves as a target of miR-449a, the dual-luciferase reporter system was performed, and revealed that AREG is a direct

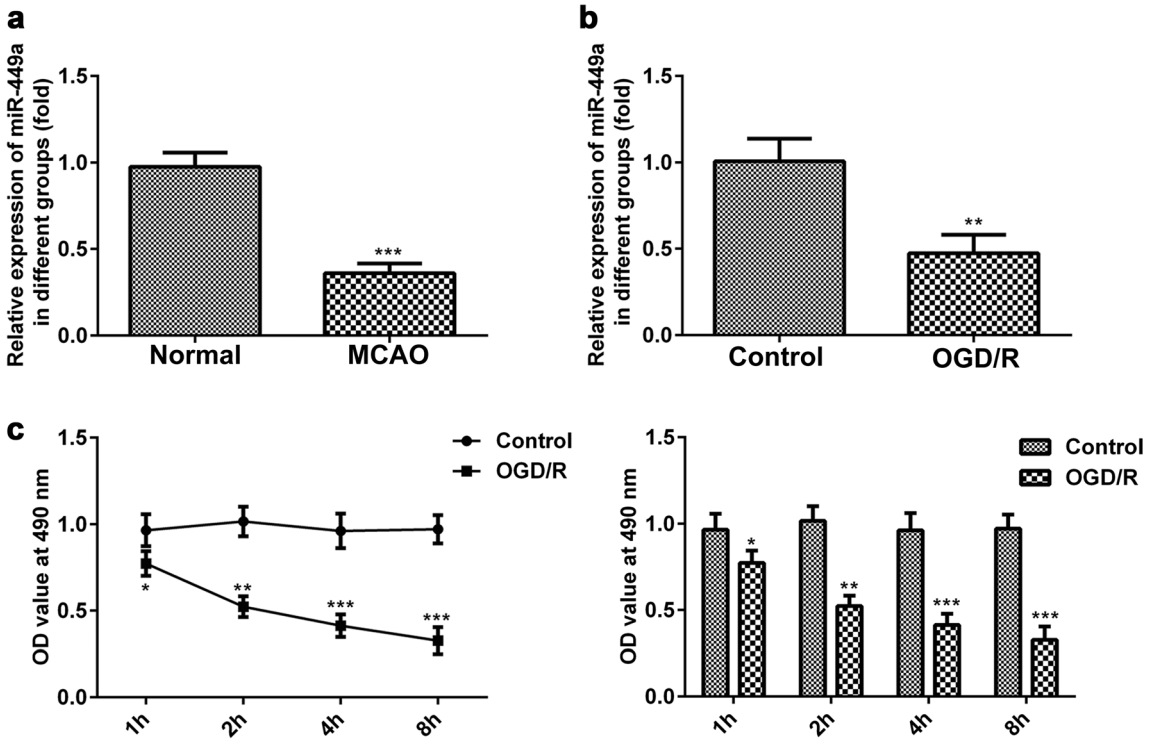

Fig. 2 miR-449a expression was downregulated following (**a**) MCAO of rats and (**b**) OGD/R of Neur cells. **c** Results from CCK-8 viability assay indicated that the viability of cell under OGD for 1 h, 2 h, 4 h and 8 h, followed by 12 h reperfusion. Neur cells were underwent 2 h OGD followed by 12 h reperfusion with the viability declined to more than 50% of control. Values are represented as mean ± SD, $n = 8$. $p < 0.05$ as determined by Student's t test.*$p < 0.05$, **$p < 0.01$,***$p < 0.001$ vs normal or control

target of miR-449a (Fig. 4a). Therefore, the results indicated the regulatory function of miR-449a on the expression level of AREG that serves as one of the miR-449a direct targets. However, further analysis with western blotting revealed that not only AREG was upregulated, but also the protein levels of EGFR, the phosphorylated Akt and PI3K were higher in the OGD/R group than that of control group; however, the total Akt and PI3K level were not significantly altered (Fig. 4b). This indicated that the PI3K/Akt pathway was activated. Transfection of miR-449a mimics vector and AREG siRNA resulted in a decrease in the expression level of EGFR, the phosphorylated Akt and PI3K when compared with the OGD/R and NC groups.

miR-449a overexpression and AREG interferon reduces injury in OGD/R model

The previous results demonstrated that miR-449a is downregulated while AREG is upregulated in OGD/R neuronal cells, indicating their negative correlation in vitro. The transfection resulted in an increase of miR-449a and a decrease of AREG among the miR-449a mimics group and AREG siRNA group when compared with the OGD/R and NC groups according to the RT-qPCR results (Fig. 4d). The cell viability was markedly elevated in the OGD/R cells transfected with the miR-449a mimics and AREG siRNA groups according to the MTT assay (Fig. 4c). Then, the number of apoptotic cells in Neur cells were investigated using a flow cytometer at 24 h following OGD/R. The percentage of apoptotic cells were increased in the OGD/R group compared with the control group, yet the miR-449a mimics and siRNA AREG in OGD/R cells resulted in a significant decrease in apoptotic cells when compared with the OGD/R and NC groups (Fig. 5). Furthermore, the percentage of cells in the G0/G1 phase in OGD/R cells was significantly higher than that in control group, while the percentage of cells in S phase was much lower than that in the control group (Fig. 6). In the group transfected with miR-449a mimics and AREG siRNA, the percentage of cells in the G0/G1 phase was decreased while the percentage of cells in S1 stage was markedly increased (Fig. 6). Collectively, these results indicate that the overexpression of miR-449a and AREG reduces cell injury.

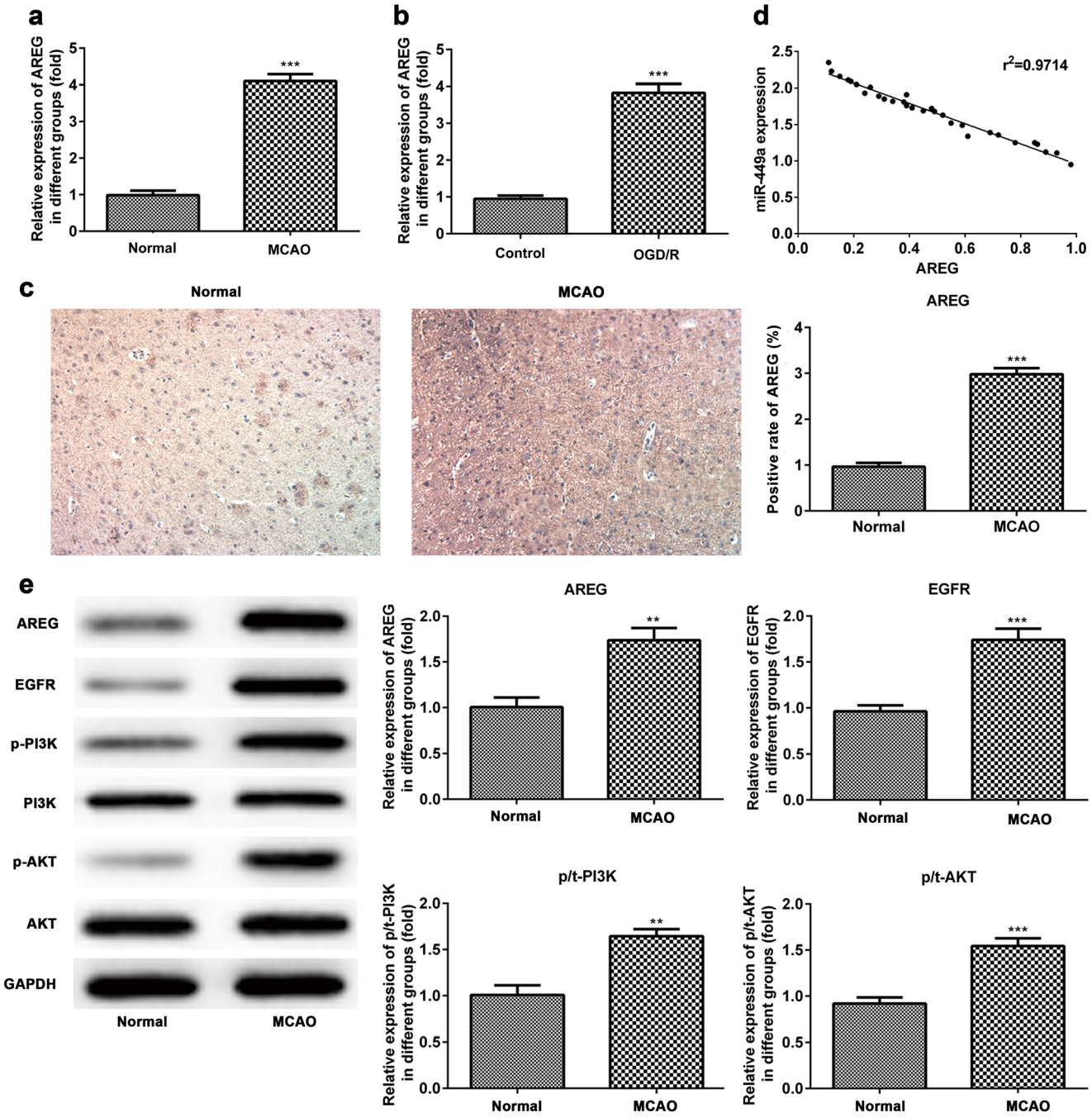

Fig. 3 AREG expression was upregulated following (**a**) MCAO of rats and (**b**) OGD/R of Neur cells. **c** The representative images of immunohistochemistry (IHC) staining for AREG in brain tissues (×200). Analysis of IHC staining for AREG in normal and MCAO tissues (left). In comparison with rats in the normal group, the brain tissue of rats in the MCAO group presented with an increased number of cells that had a positively-expressed AREG (right). **d** Pearson's correlation analysis of miR-449a expression with AREG mRNA in brain tissues of MCAO rats. $r^2 = 0.9714$. (E) The protein levels of EGFR and PI3K/Akt signaling following MCAO increased significantly comparing to normal rats. Values are represented as mean ± SD, $n = 8$. $P < 0.05$ as determined by Student's t test.*$P < 0.05$, **$P < 0.01$,***$P < 0.001$ vs normal or control

Discussion

In the present study, a MCAO stroke model was constructed in rats and the expression levels and regulatory functions of miR-449a and AREG were detected. The MCAO model was first evaluated using different criterion, including the deficient neurological behavior score, the level of cerebral edema, the cerebral infarct volume,

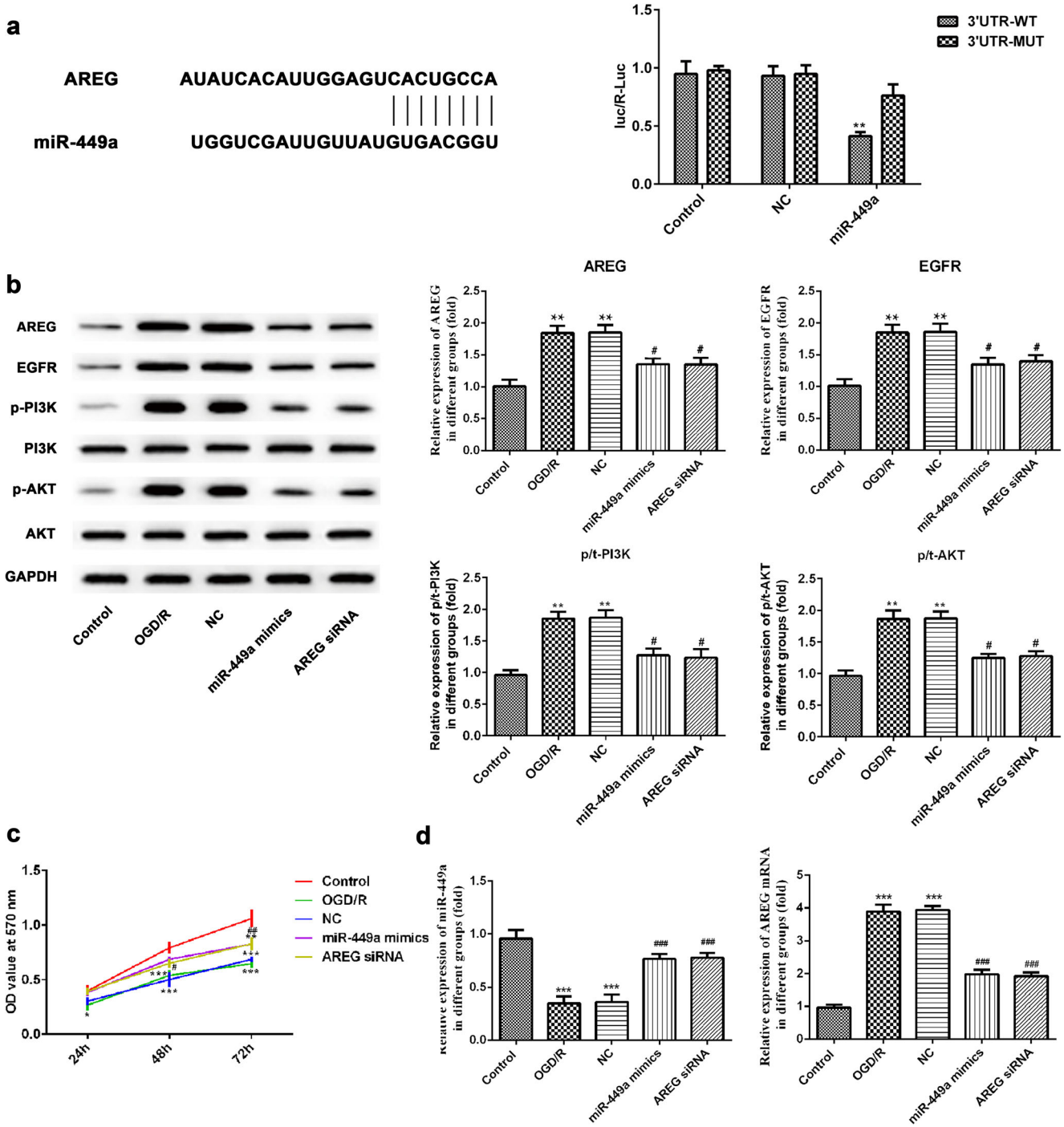

Fig. 4 Putative binding of miR-449a to the AREG gene 3′-UTR (**a**). Neur cells were co-transfected with luciferase plasmids containing the luc-AREG-WT or luc-AREG-MUT and the relative luciferase activities in different groups were determined. **P < 0.01 vs NC and control 3'UTR-WT. **b** Relative levels of AREG, EGFR, p/t-AI3K and p/t-Akt in different groups were determined using western blotting. **c** MTT growth assays. **d** The mRNA levels of miR-449a and AREG in different groups. Values are represented as mean ± SD, $n = 8$. $p < 0.05$ as determined by one-way ANOVA analysis. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs control; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs OGD/R and NC

the number of apoptotic cells, the transcription level of miR-449a and the expression level of AREG and the associated signaling pathway. To further demonstrate the function of miR-449a and AREG in cultured cells, the present study used a OGD/R model in human neuronal cell lines for the transfection experiments. The results demonstrated that miR-449a was downregulated while AREG was upregulated in the OGD/R cell line model,

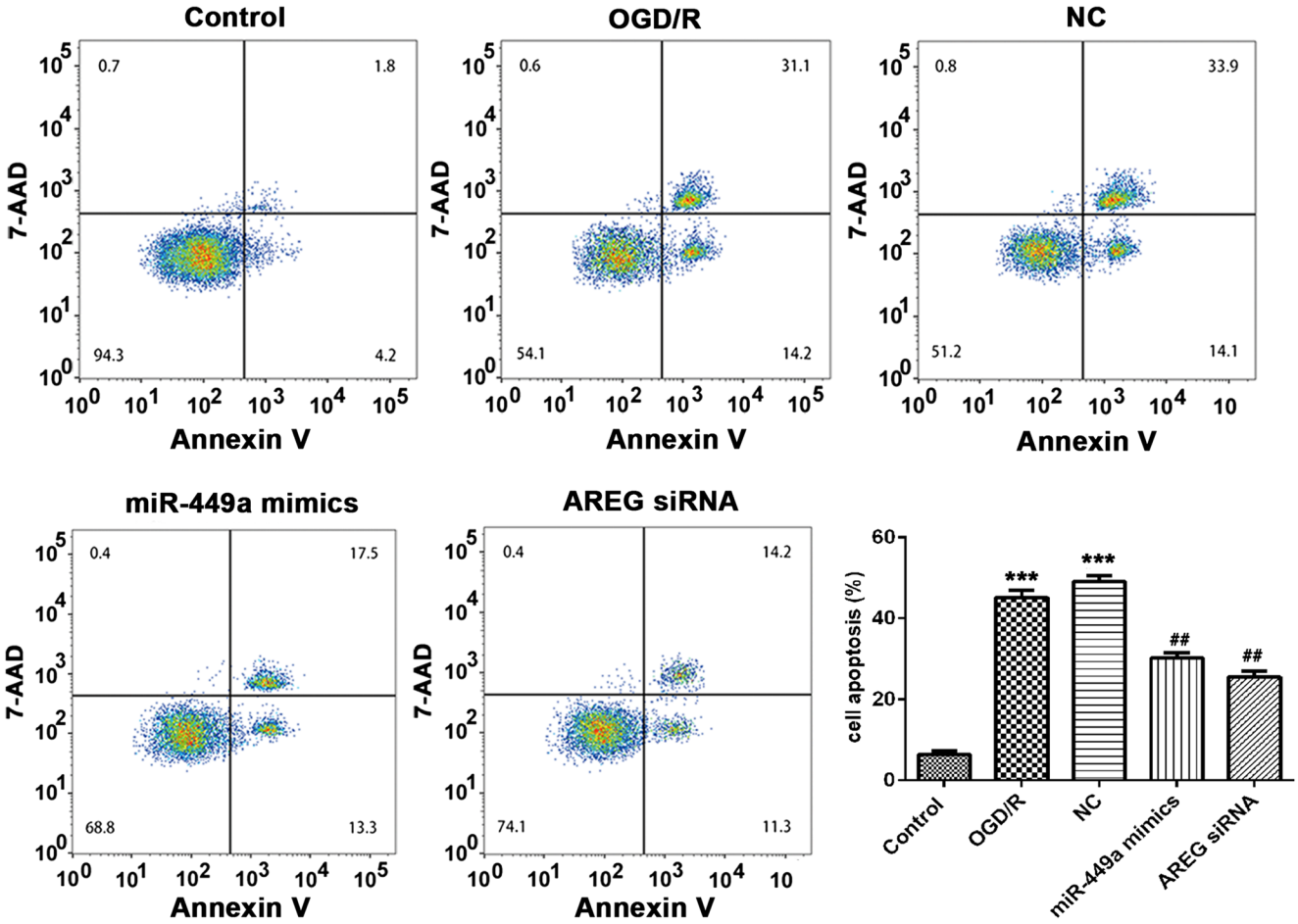

Fig. 5 Flow cytometry analysis revealed that the apoptotic cell ratio was increased significantly following OGD/R, but miR-449a mimic and AREG siRNA could significantly inhibit cell apoptosis. Values are represented as mean ± SD, $n = 8$. $p < 0.05$ as determined by one-way ANOVA analysis. ***$p < 0.001$ vs control; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs OGD/R and NC

and transfection with the miR-449a mimic vector or AREG siRNA rescued the symptoms of ischemic cells. These findings provided a novel therapeutic method for the treatment of acute stroke and laid the foundation to examine the function of miR-449a in ischemic stroke.

At present, the role of miR-449a in cerebral ischemia has not been fully elucidated. The present study demonstrated showed that OGD/R significantly decreased the expression of miR-449a, and increased the expression of AREG, and the protective role of miR-449a was examined in an overexpression experiment. A previous study reported that EGFR is associated with the PI3K/Akt and Raf/MAPK/ERK1/2 signaling pathways (Zhou et al. 2015) and a crosstalk was evident between the two protective pathways during brain ischemia/reperfusion. In the present study, the protein expression level of p-PI3K and p-Akt were also increased, indicating the activation of PI3K/Akt pathway.

Numerous studies have reported that drugs such as peptidase inhibitors, peptides and enzymes have been used to attenuate neuronal injury via the activation of PI3K/Akt cascade or MAPK/ERK1/2 pathways via BDNF or EGFR mediated signaling (Qi et al. 2014; Zhang et al. 2014; El-Marasy et al. 2018; Huang and Hu 2018), while the present study demonstrated that miR-449a primarily targeted AREG. These indicated the protective function of miR-449a was most likely through the EGFR-mediated PI3K/Akt pathway. It is important to explore the mechanisms underlying the PI3K/Akt pathway (Lopez-Morales et al. 2018), which may protect the blood-brain barrier and decrease the inflammatory mediators in order to successfully reduce inflammation (Zhu et al. 2018).

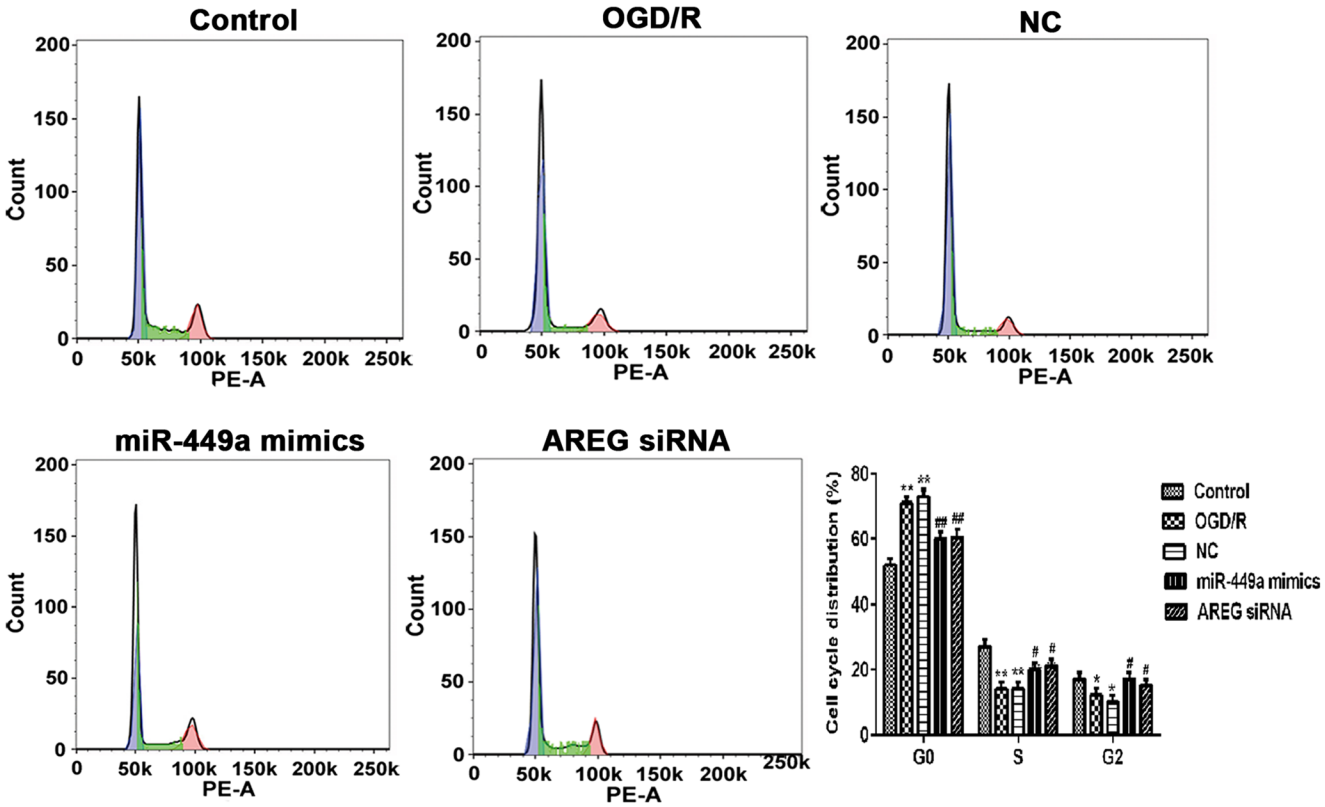

Fig. 6 Flow cytometry analysis. miR-449a mimic and AREG siRNA groups had a greater number of G1-phase cells and fewer S-phase cells. Values are represented as mean ± SD, $n = 8$. $p < 0.05$ as determined by one-way ANOVA analysis. $^{*}p < 0.05$, $^{**}p < 0.01$ vs control; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs OGD/R and NC

In addition, the results of flow cytometry assay revealed that miR-449a could decrease cell death in OGD/R. The expression changes of miR-449a and AREG were involved in ischemia period or reperfusion period needs further conformed, however, they did influence cell apoptosis and cycle in Neur OGD/R model. Autophage also participages in the process, which was closely related to apoptosis. Whether autophage also decreased needs to be further detected. There are studies elucidated the relationship between the PI3K/Akt pathway and the proliferation of neural progenitor cells (Kisoh et al. 2018) or the involvement of other cell types. Our results were consistent with previous research about the cell proliferation.

Conclusion

In conclusion, we have identified an important role of miR-449a, which was frequently downregulated in rat ischemia brain tissues of MCAO model and human neuron cell lines of OGD/R. Overexpression of miR-449a inhibited the injury of ischemic in vitro and in vivo through directly regulation of AREG expression. miR-449a may be as a novel target for treatment for cerebral ischemic injury.

Compliance with ethical standards

Conflict of interest No conflict of interest to declare.

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