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Original Article

MiR-199a-5p inhibition protects cognitive function of ischemic stroke rats by AKT signaling pathway

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Abstract: To explore the effect of miR-199a-5p and AKT signal pathway on cognitive function and neuronal cells in rats with ischemic stroke. Sprague-Dawley rats were divided into 6 groups: Normal group (normal rats), Sham group (rats received sham operation), Model group (MCAO rats), miR-199a-5p inhibitor group (model rats treated with miR-199a-5p inhibitor), IGF-1 group (model rats treated with AKT signaling pathway activator), miR-199a-5p inhibitor + IGF-1 group (model rats treated by miR-199a-5p inhibitor and AKT signaling pathway activator). Rat behavior and cerebral infarction area were observed. TUNEL fluorescence staining was used to detect neuronal apoptosis in hippocampal CA1 region of rats. The dual luciferase reporter assay validated the targeting relationship between miR-199a-5p and AKT. qRT-PCR and WB were used to detect the expression level of miR-199a-5p, (p)-AKT and (p)-mTOR, apoptosis-related proteins Bax and Bcl-2. Compared with the normal group, the expression of miR-199a-5p was increased in the Model group, and the expression levels of AKT, mTOR, p-AKT, and p-mTOR were decreased (all $P < 0.05$); the cognitive function of the rats in the Model group was thereby significantly lower ($P < 0.05$). miR-199a-5p was targeted to inhibit AKT. Compared with the Model group, miR-199a-5p inhibition combined with IGF-1 showed more significant effects on improving cognitive function and protecting neuronal cells of rats. In conclusion, silencing miR-199a-5p can effectively improve cognitive function in ischemic stroke rats and decrease neuronal apoptosis in hippocampus by activating the AKT signaling pathway.

Keywords: AKT signaling pathway, cognitive function, ischemic stroke, miR-199a-5p, neurons

Introduction

Ischemic stroke has high disability rate and high mortality rate [1]. Neuronal damage usually accompanies with patients with ischemic stroke, and impairs cognitive function in them [2]. As Chinese aging society is coming, the incidence of ischemic stroke is increasing annually. Meanwhile, ischemic stroke adds a serious burden to the patients and their families. However, the pathogenesis of ischemic stroke remains unclear [3, 4]. Therefore, exploration of its pathogenesis can provide new insights for ischemic stroke treatment.

Currently, among the possible pathological mechanisms related to ischemic stroke, AKT signaling pathway has been reported to be involved in the protective mechanism of ischemic injury [5, 6]. Upon activation, AKT can not only inhibit the expression of pro-apoptotic factors to further inhibit the apoptosis of neurons, but also inhibit the expression of downstream complexes to up-regulate the expression of mammalian target of rapamycin (mTOR) [7-9]. However, the mechanism for the upstream regulatory of the AKT signaling pathway needs to be further explored.

microRNA can regulate downstream target genes and we found that there is a binding site between miR-199a-5p and AKT1 by bioinformatics prediction. At present, the researches on miR-199a-5p are mainly in the field of oncology, including cervical cancer, papillary thyroid carcinoma and non-small cell lung cancer [10-15]. There are few studies aiming to explore the regulation mechanism of miR-199a-5p in ischemic stroke Furthermore, only an article reported that the α-tocopherol had a protective effect on neurons in the ischemic stroke mice and the expression of miR-199a-5p was also significantly reduced after treatment [16]. Therefore, we hypothesized that miR-

199a-5p may affect cognitive dysfunction and nerve damage after ischemic stroke by regulating AKT signaling pathway.

In our study, we constructed a rat model with ischemic stroke and provide different treatments to explore the effects of miR-199a-5p on cognitive function and neuronal cells in rats with ischemic stroke.

Methods

Animals

One hundred and twenty healthy SPF SD male rats with a weight of 254-287 g were purchased from Animal Experimental Research Center of Zhejiang Chinese Medicine University. Breeding conditions: in SPF-class rat house, 22±1°C, 40%-60% relative humidity, 12 h light and dark lighting cycle. This study was approved by the animal ethic committee of Tianjin Huanhu Hospital.

Preparation of rat model with middle cerebral artery occlusion (MCAO)

Rats were anesthetized by intraperitoneal injection of 1% sodium pentobarbital (Shanghai Rongchuang Biotechnology Co., Ltd., China) at a dose of 0.06 g/kg. The scalp of the rat was cut open to expose the skull over the ear for the convenience of cerebral blood flow detector. With the help of a 1 cm long longitudinal incision from the mandible to the manubrium, the left carotid sheath, the common carotid artery, the external carotid artery, and the internal carotid artery were exposed. The common carotid artery and external carotid artery were double-ligated and the internal carotid artery was clamped. After that, the cerebral blood flow detector was reattached to the external carotid artery, and the arterial clip was removed to unclasp the internal carotid artery. The signal change of the cerebral blood flow was detected. And 1 h after 20% drop, the common carotid artery and the external carotid artery were double-ligated again, and the common carotid artery was disconnected to restore part of the blood flow until the cerebral blood flow detector signal returned to 100%. Finally, the wound was sutured [26]. In the sham-operated (Sham) group, the middle artery was not ligated. Body temperature maintainance during operation and free access to drinking and eating after operation should be both carefully provided.

Grouping

Rats were grouped into Normal group (normal rats), Sham group (rats received sham operation), Model group (MCAO model rats), miR-199a-5p inhibitor group (MCAO model rats transfected with miR-199a-5p inhibitor), IGF-1 group (MCAO model rats treated with AKT signaling pathway activator), miR-199a-5p inhibitor + IGF-1 group (MCAO model rats transfected with miR-199a-5p inhibitor and treated with AKT signaling pathway activator). miR-199a-5p inhibitor and IGF-1 were purchased from MedChemExpress LLC. In miR-199a-5p inhibitor group, IGF-1 group and miR-199a-5p inhibitor + IGF-1 group, rats were injected with drugs accordingly into the lateral ventricle (which was located in 1 mm posterior to the ventricle, 2.5 mm outside the midline, 3 mm at the ventral side of the skull) 30 min after MCAO modeling, once every 3 days. The subsequent experiments were performed 7 days after MCAO modeling.

Dual luciferase reporter assay

AKT1 was predicted as the target gene of miR-199a-5p in biological prediction website (http://www.targetscan.org/vert_72/), which was then verified by dual luciferase reporter assay. The synthetic AKT1 3'UTR gene fragment was inserted into pMIR-reporter (WT) via endonuclease sites SpeI and Hind III (Beijing Huayueyang Biotechnology Co., Ltd.). A mutation site in the complementary sequence of the target sequence (MUT) was designed basing on AKT1 wild type, and the target fragment was inserted into the pMIR-reporter plasmid by T4 DNA ligase. The correctly sequenced luciferase reporter plasmids WT and MUT were co-transfected with the miR-199a-5p into the cell HEK-293T (Shanghai Beinuo Biotechnology Co., Ltd.). After 48 h of transfection, the cells were collected and lysed, and the luciferase activity was measured using a luciferase assay kit (K801-200, Biovision) and Glomax 20/20 luminometer fluorescence detector (Promega). All the steps were in accordance with the kit operating instruction, and each experiment was repeated for three times.

Table 1. Primer sequences

Gene	Forward (5' to 3')	Reverse (5' to 3')
miR-199a-5p	GGGCCCAGTGTTCAGACTAC	GTGCAGGGTCCGAGGT
U6	GCTTCGGCAGCACATATACTAAAAT	CGCTTCACGAATTTGCGTGTCAT
AKT	GAAGCTGAGCCCACCTTTCA	CATCTTGATCAGGCGGTGTG
mTOR	TCTTCTTCCAGCAAGTTCAGC	GAATCAGACAGGCACGAAGG
GAPDH	GGATGCAGGGATGATGTTCT	AAGGGCTCATGACCACAGTC

Animal behavior analysis

On the 3rd day post-operation, rat behaviors were tested using the Pole Test and the Foot Fault Test [27, 28]. Before test, all the rats were trained 3 times a day, for 3 days, including climbing up, turning and climbing down in the Pole Test as well as climbing over the overhead net from one side to another in The Foot Fault Test. Three days after the training, in the Pole Test, the time for turning 180° to head down was recorded as T_{turn}, and the time when all the rats reached the ground was recorded as T_{total}; in the Foot Fault Test, the number of missed steps on the overhead net of both the injured and uninjured sides were recorded to calculate the percentage the injured side took in total step numbers.

Triphenyltetrazolium chloride (TTC) staining to calculate cerebral infarct size

Brain tissues were taken from 6 rats in each group to detect the infarct size of each group by TTC staining. The rats' brain tissues were obtained after neurobehavioral test and frozen at -20°C for 20 min. The brain tissues were then made to coronal sections, which were stained by PBS solution containing 0.5% TTC (Beijing Solabao Technology Co., Ltd., China) at 37°C in a dark surrounding for 20 min. The scanning of those brain tissue sections was archived. The images were analyzed by Image-pro plus software (Media Cybernetics, USA). The proportion of cerebral infarct area = the area of white unstained part/total area * 100% [29].

TUNEL fluorescence staining

Brain tissues were taken from 6 rats in each group to detect apoptosis rates in hippocampus by TUNEL fluorescence detection kit (11684817910, Roche, Switzerland). The paraffin section of hippocampus tissues was placed on the poly-lysine coated slides (Shanghai Duke Biotechnology Co., Ltd., Shanghai, China). The experiment was operated according to kit instruction, in which TUNEL positive cells were stained in green and the nuclei were stained with DAPI. Five slices were prepared for each sample, and 5 fields were randomly selected from each slide. The TUNEL-positive cells and total cells in the sections were counted and analyzed by Image-Pro Plus 6.0 software. The ratio of TUNEL-positive cells = TUNEL positive cell number/total cell number * 100%.

qRT-PCR

Hippocampus tissues from three rats in each group were taken to exact total RNA by Trizol (Cat. No. 16096020, Thermo Fisher Scientific, USA) and 5 μg of total RNA from each sample was taken for the synthesis of cDNA according to qRT-PCR kit (ABI, USA). PCR reaction system (25 μL) including 300 ng cDNA, 1× PCR buffer, 200 μmol/L dNTPs, forward and reverse primers 80 pmol/L respectively, 0.5 U Taq enzyme (S10118, Shanghai Yuanye Biotechnology Co., Ltd., China). The reaction conditions were pre-denaturation at 94°C for 5 min, denaturation at 94°C for 30 s, annealing at 54.5°C for 30 s, extension at 72°C for 30 s, for a total of 30 cycles, and finally 72°C for 10 min. The product was preserved at 4°C. The primer sequences of miR-199a-5p, U6, AKT and GAPDH were showed in **Table 1**. The primers were purchased from Wuhan Shenggong Bioengineering Co., Ltd., U6 was the internal reference of miR-199a-5p and GAPDH was the internal reference of other genes. $2^{-\Delta\Delta Ct}$ was used to calculate the relative expression level of the target gene. $\Delta\Delta CT = \Delta Ct_{study\ group} - \Delta Ct_{control\ group}$ ($\Delta Ct = Ct_{target\ gene} - Ct_{reference\ genes}$.

Western blot

Hippocampus tissues from three rats in each group were taken and washed twice with PBS. Then they were lysed by lysis buffer (R10010, Beijing Solarbio Technology Co., Ltd., China), mixed on vortex (Hefei Abson Scientific Instrument Co., Ltd., China), and centrifuged at

12,000 xg for 30 min at 4°C. The supernatant was taken and the total protein concentration was measured by BCA kit. Then, 50 μg of protein was dissolved in 2× SDS loading buffer, and boiled at 100°C for 5 min. The protein sample was separated by 10% SDS-PAGE gel electrophoresis, transferred to PVDF membrane, blocked by 5% skim milk at room temperature for 1 h, and incubated with primary antibodies including rabbit anti-GAPDH (ab-181602, 1:3,000, abcam, China), AKT (ab-179463, 1:3,000, abcam, China), p-AKT (ab8933, 1:500, abcam, China), mTOR (ab-2732, 1:2,000, abcam, China), p-mTOR (ab-137133, 1:3,000, abcam, China), Bcl-2 (ab196495, 1:2,000, abcam, China), Bax (ab32503, 1:3,000, abcam, China). After washing with TBST for 3 times, the membrane was then incubated with HRP-labeled secondary antibody for 1 h. After rinsing with TBST, the membrane was developed by ECL Fluorescence Detection Kit (Cat. No. BB-3501, Ameshame, UK), photographed by Bio-Rad Image Analysis System (BIO-RAD, USA), and analyzed by Quantity One v4.6.2 software. The relative protein content = the gray value of the corresponding protein band/the gray value of the GAPDH protein band.

Statistical analysis

All data were processed using SPSS 21.0 statistical software. The measurement data were expressed as mean ± standard deviation. The comparison between two groups adopted t test. The comparison between multiple groups used one-way ANOVA combined with Bonferroni post hoc test. $P < 0.05$ indicated that the difference was statistically significant.

Results

Expression of mir-199a-5p and AKT signaling pathway in ischemic stroke rats

The biological prediction website (http://www.targetscan.org/vert_72/) showed that miR-199a-5p had a specific binding site to AKT (**Figure 1A**), which was verified by the dual luciferase assay (**Figure 1B**). Compared with the NC mimic group, miR-199a-5p mimic can decrease the luciferase activity in the AKT wild type 3'-UTR group, but had no effect on luciferase activity in AKT mutant 3'-UTR group, indicating that miR-199a-5p specifically can bind to AKT-3'-UTR and down-regulated AKT gene expression.

The mRNA and protein expression of miR-199a-5p and factors in AKT signaling pathway in each group was detected by qRT-PCR and Western blot (**Figure 1C-E**). The results showed that compared with the Normal group, the Sham group showed no significant difference in the expression of miR-199a-5p and factors in AKT signaling pathway ($P > 0.05$). Compared with the Normal group or the Sham group, the model group had increased expression of miR-199a-5p, but decreased expression of AKT and mTOR mRNA, p-AKT/AKT and p-mTOR/mTOR ($P < 0.05$). Compared with the Model group, miR-199a-5p inhibitor group showed decreased miR-199a-5p expression, while miR-199a-5p inhibitor group and IGF-1 group showed increased AKT and mTOR mRNA, p-AKT/AKT and p-mTOR/mTOR ($P < 0.05$). miR-199a-5p inhibitor + IGF-1 group had higher expression of AKT and mTOR mRNA, p-AKT/AKT and p-mTOR/mTOR than either miR-199a-5p inhibitor group or IGF-1 group ($P < 0.05$).

Down-regulation of miR-199a-5p or activation of AKT signaling pathway contributed to improved cognitive function in ischemic stroke rats

To investigate the mechanism of miR-199a-5p and AKT signaling pathways working on cognitive function in ischemic stroke rats, rats received miR-199a-5p silencing or AKT activator after which their behaviors were analyzed (**Figure 2**). Compared with the Normal group or the Sham group, Model group had prolonged T_{turn} and T_{total} as well as the number of missed steps of injured limbs ($P < 0.05$). Opposite results occurred in the miR-199a-5p inhibitor group and the IGF-1 group when compared to the Model group. The shorted T_{turn} and T_{total} along with the decreased number of missed steps of injured limbs were observed in miR-199a-5p inhibitor + IGF-1 group when compared to the miR-199a-5p inhibitor group or the IGF-1 group ($P < 0.05$). These results suggested that silencing miR-199a-5p or activating AKT can alleviate cognitive dysfunction in ischemic stroke rats. The combination of miR-199a-5p inhibitor and IGF-1 much more improved the cognitive function in ischemic stroke rats.

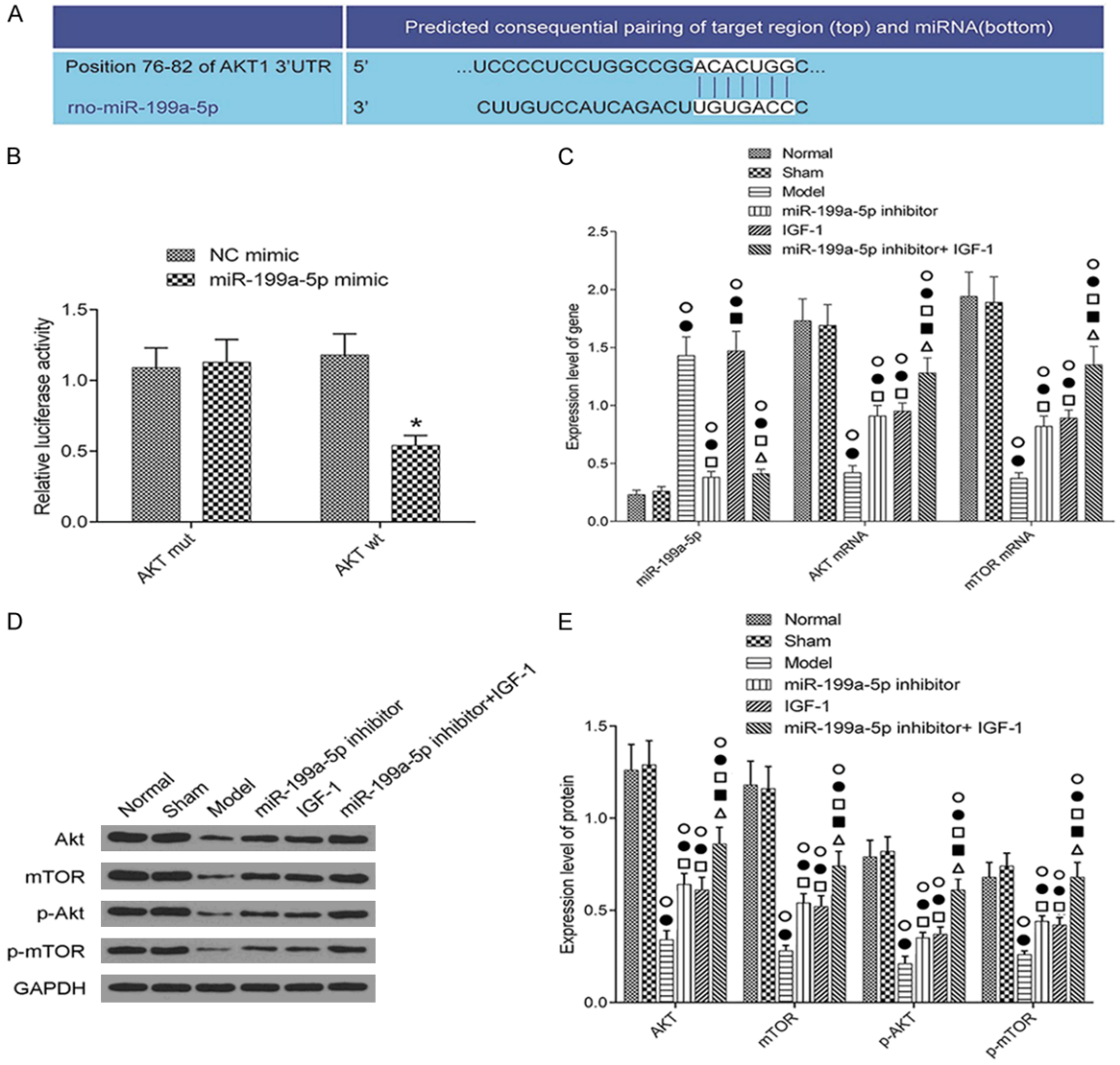

Figure 1. Expression of miR-199a-5p and AKT signaling pathway in ischemic stroke rats. (A) Bioinformatics prediction of binding sites on the 3'UTR between miR-199a-5p and AKT. (B) Dual luciferase reporter assay; *P < 0.05, compared with the NC mimic group. The mRNA (C) and protein (D, E) expression level of miR-199a-5p, AKT and mTOR mRNA in each group of rats were detected. ○P < 0.05, compared to the Normal group; •P < 0.05, compared with the Sham group; □P < 0.05, compared with the Model group; ▪P < 0.05, compared with the miR-199a-5p inhibitor group; ΔP < 0.05, compared with the IGF-1 group.

Down-regulation of miR-199a-5p or activation of AKT signaling pathway reduced cerebral infarction area in ischemic stroke rats

Cerebral infarction occurred in rats 1 h after MCAO treatment. Compared with Model group, the infarct volume was significantly decreased in miR-199a-5p inhibitor group or IGF-1 group ($P < 0.05$). Compared with the miR-99a-5p inhibitor group or IGF-1 group, the cerebral infarction volume was significantly decreased in miR-199a-5p inhibitor + IGF-1 group ($P < 0.05$; **Figure 3**). These results indicated that inhibition of miR-199a-5p or activation of AKT signaling pathway reduced cerebral infarction volume in ischemic stroke rats.

Down-regulation of mir-199a-5p or activation of AKT signaling pathway can inhibit neuronal apoptosis in ischemic stroke rats

The apoptosis rate of neuronal cells in hippocampal CA1 region was detected by TUNEL fluorescence staining (**Figure 4A**, **4B**). There was

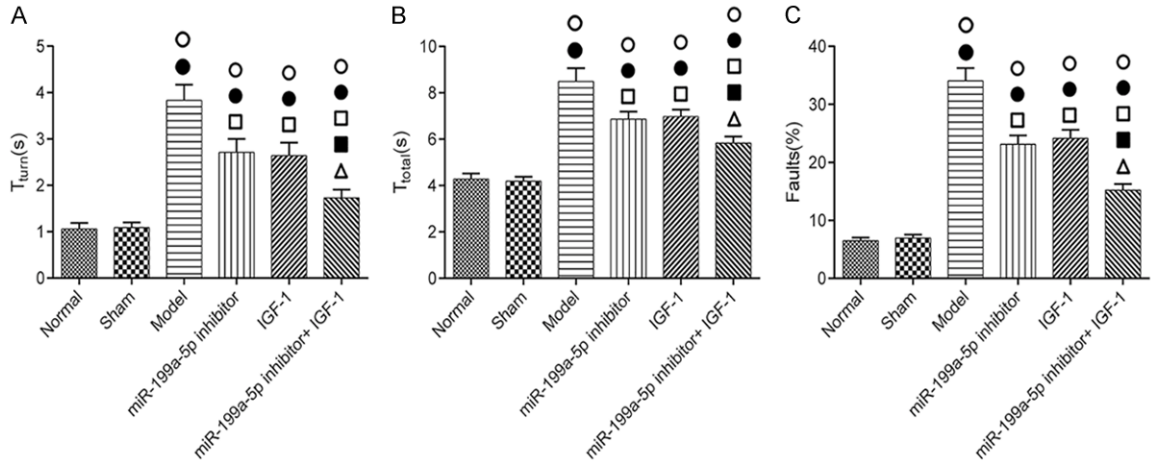

Figure 2. Down-regulation of miR-199a-5p or activation of AKT signaling pathway improves the behaviors of ischemic stroke rats. A. Statistical analysis of the time used by the rats in each group at 180° head down. B. Statistical analysis of the time taken by each group of rats to reach the ground. C. Results of the trial of the rats in each group. °P < 0.05, compared to the Normal group; •P < 0.05, compared with the Sham group; □P < 0.05, compared with the Model group; ▪P < 0.05, compared with the miR-199a-5p inhibitor group; ΔP < 0.05, compared with the IGF-1 group.

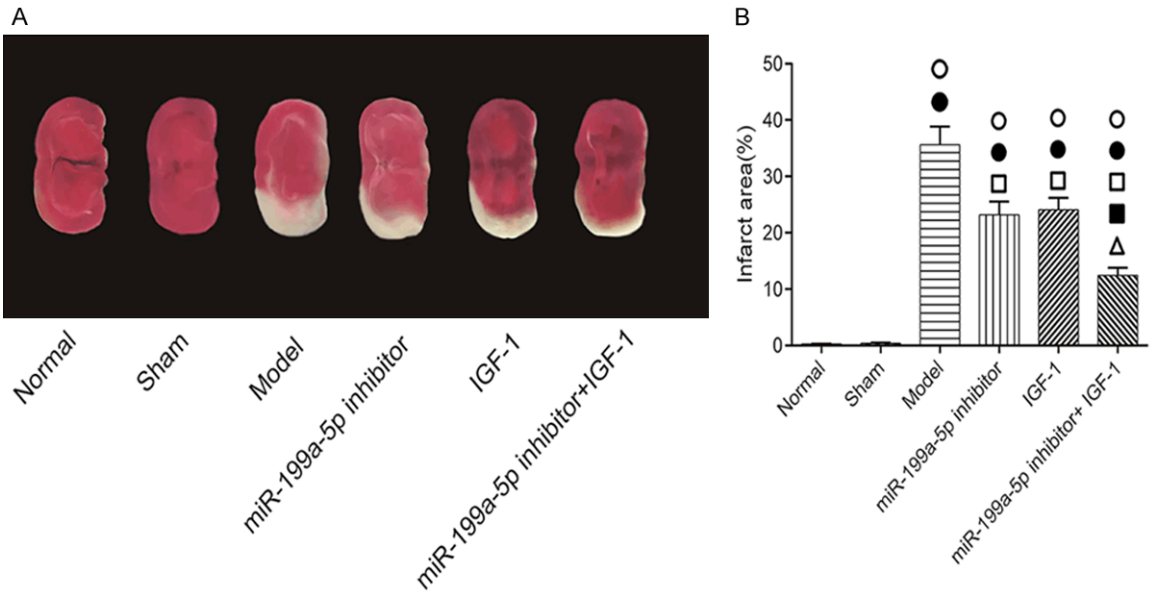

Figure 3. Down-regulation of miR-199a-5p or activation of AKT signaling pathway reduce cerebral infarction area in ischemic stroke rats. A. Brain tissues stained by TTC in each group; B. Cerebral infarct volume of each group. °P < 0.05, compared to the Normal group; •P < 0.05, compared with the Sham group; □P < 0.05, compared with the Model group; ▪P < 0.05, compared with the miR-199a-5p inhibitor group; ΔP < 0.05, compared with the IGF-1 group.

no significant difference in neuronal apoptosis rate of the hippocampal CA1 region between the Normal group and the Sham group (P > 0.05). The apoptotic rate of neuronal cells in hippocampal CA1 region was significantly increased in the Model group when compared to the Normal group or the Sham group, but significantly decreased in the miR-199a-5p inhibitor group or the IGF-1 group when compared to the Model group (P < 0.05). No matter the miR-199a-5p inhibitor group or the IGF-1 group had the significantly higher neuronal apoptosis rate than the miR-199a-5p inhibitor + IGF-1 group (P < 0.05).

Western blot was used to detect the expression of Bax and Bcl-2 proteins in each group (**Figure 4C**, **4D**). Compared with the Normal

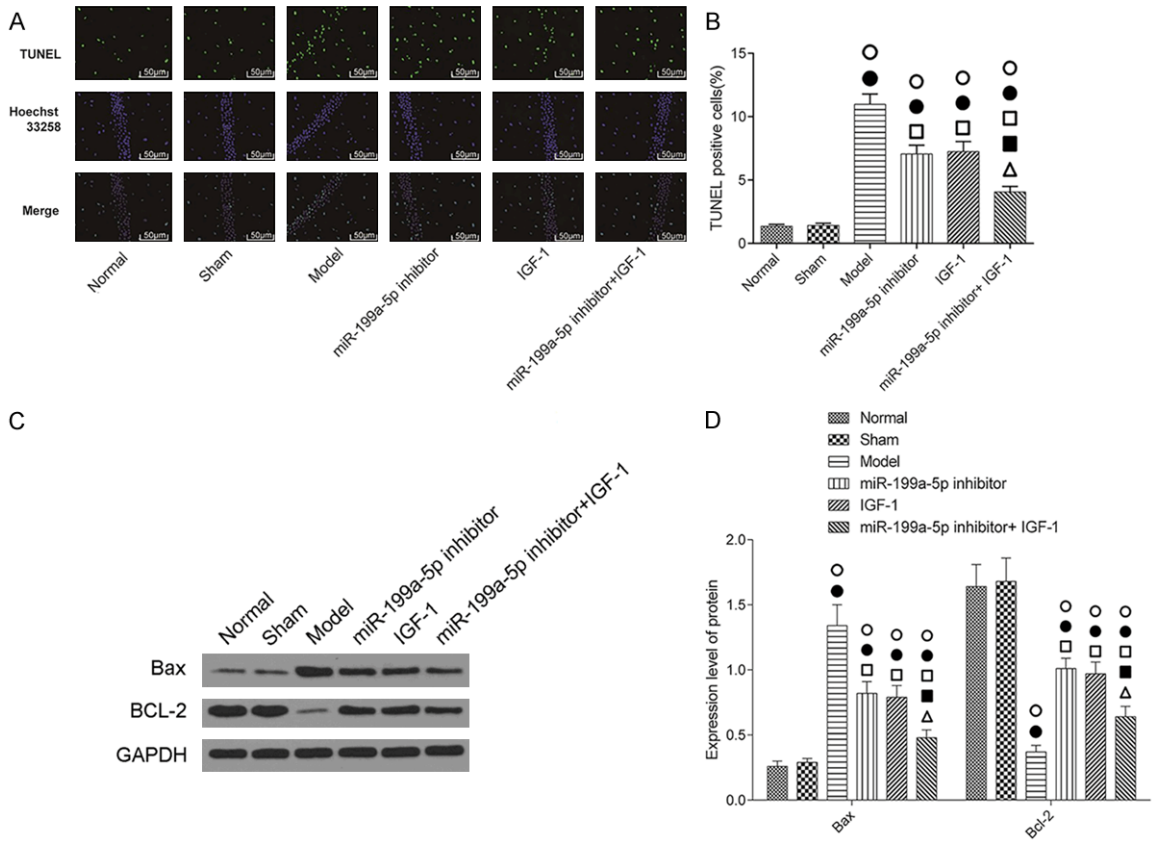

Figure 4. Down-regulation of miR-199a-5p or activation of AKT signaling pathway can inhibit neuronal apoptosis in ischemic stroke rats. A. TUNEL fluorescence staining results of hippocampal CA1 area in each group (200×). B. Apoptosis rate of hippocampal CA1 region in each group of rats. C. Expression of apoptosis related proteins. D. Quantification results of protein expression. °P < 0.05, compared to the Normal group; •P < 0.05, compared with the Sham group; □P < 0.05, compared with the Model group; ▪P < 0.05, compared with the miR-199a-5p inhibitor group; ΔP < 0.05, compared with the IGF-1 group.

group or the Sham group, the Model group showed the significantly increased expression of Bax protein but the significantly decreased expression of Bcl-2 protein (P < 0.05). Opposite results occurred in the miR-199a-5p inhibitor group or IGF-1 group when compared to the Model group. In the miR-199a-5p inhibitor + IGF-1 group, the expressions of Bax protein and Bcl-2 protein were decreased and increased, respectively, in comparison to the miR-199a-5p inhibitor group or the IGF-1 group (P < 0.05). These results indicated that inhibition of miR-199a-5p or activation of AKT signaling pathway inhibited neuronal apoptosis in hippocampal CA1 neurons of rats with ischemic stroke.

Discussion

Ischemic stroke is a common disease in the elderly with an increasing incidence, which can lead to physical dysfunction and cognitive dysfunction in patients, bringing great difficulties in clinical treatment [17-19]. Currently, there is no effective strategy for the prevention and treatment of ischemic stroke patients. Therefore, exploring the pathogenesis of ischemic stroke can lay a theoretical foundation for clinical treatment.

Previous studies have reported that AKT signaling pathway activation can improve ischemic stroke [20-22]. In our study, we also found that the expression levels of AKT and mTOR mRNA, p-AKT and p-mTOR were significantly decreased in ischemic stroke rats, indicating that the AKT signaling pathway was inhibited in ischemic stroke rats. In order to determine whether AKT activation improves cognitive function and neuronal damage in ischemic stroke rats, we administered AKT sig-

naling pathway activator IGF-1 in model rats. The results indicated that the cognitive function of IGF-1 treated rats was significantly improved, and the area of cerebral infarction was significantly reduced. AKT activation promotes the expression of downstream mTOR and regulates the expression of apoptosis-related factors, thereby improving cognitive dysfunction in ischemic stroke rats [23-25]. We have also confirmed that after treatment, the expression of mTOR was increased, the expression of pro-apoptotic factor Bax protein was significantly decreased, and the expression of anti-apoptotic factor Bcl-2 protein was significantly increased.

Articles from Li et al. and Yu et al. reported that high level of miR-199a-5p had beneficial effects on cerebral ischemic injury or apoptosis and inflammation in neural cells [1, 2]. However, Zhong et al. found that overexpression of ANRIL or inhibition of miR-199a-5p could protect cells against ischemia induced injury by elevating cell viability through CAV-1 mediated MEK/ERK pathway [3]. In this study, to investigate the regulatory mechanism of miR-199a-5p in ischemic stroke rats, we first examined the expression of miR-199a-5p in normal and ischemic stroke rats. It's showed that the expression of miR-199a-5p was significantly up-regulated in model rats. After silencing miR-199a-5p, the cognitive function of the rats was significantly improved, the area of cerebral infarction was significantly decreased, and the neuronal cell apoptosis was inhibited. All these results proved the protective effect of miR-199a-5p on cognitive function and neuronal cells in ischemic stroke rats. However, the expression and function of miR-199a-5p may be different in different sites and cells and different disease models.

In addition, the bioinformatics analysis predicted the target relationship between miR-199a-5p and AKT1, which was confirmed by dual luciferase reporter assay. The combined treatment of miR-199a-5p silencing and AKT signaling activation in ischemic stroke rats showed better treatment effects than single drug treatment.

After a series of experiments, we identified the role of miR-199a-5p in cognitive function and neuronal cell protection in rats with ischemic stroke, and the downstream regulatory mechanism of miR-199a-5p, which could target and silence the AKT signaling pathway. MicroRNA can regulate variety of life activities, mainly through post-transcriptional regulation of downstream target genes. At present, we only determine the regulatory relationship between miR-199a-5p and AKT signaling pathway in ischemic stroke rats; however, the regulatory network of miR-199a-5p in ischemic stroke rats is unclear. What's more, the specific molecular mechanism of post-transcriptional regulation of AKT by miR-199a-5p has not been fully elucidated.

miR-199a-5p expression is up-regulated in ischemic stroke rats, and silencing of miR-199a-5p activates AKT signaling pathway, thereby improving cognitive function and neuronal cell protection in rats.

Disclosure of conflict of interest

None.

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