MiR155 Affect the Cerebral Ischemic Injury by Rheb/mTOR Signaling Pathway

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Abstract

Ischemic stroke remains to be a leading cause of death and disability worldwide at present. Cerebral ischemia/reperfusion injury (CIRI) is a critical pathogenesis leading to a poor prognosis for ischemic stroke patients. Recent studies have found that miR155 may be involved in the occurrence of CIRI after stroke, but its role and specific mechanism are not completely clear. Therefore, this study investigated the effects of miR155 expression levels on mortality, cerebral infarction volume and neuronal apoptosis in MCAO/R rats by changing the expression level of miR155 in the ischemic penumbra (IP) region of rats. QRT-PCR and Western Blotting were used to detect the expression changes of Rheb, mTOR, 4EBP1 and S6K1 under different miR155 expression levels. The results showed that inhibition of miR155 could reduce the mortality, the volume of cerebral infarction and the number of neuronal apoptosis in MCAO/R rats. The mRNA and protein expressions of Rheb, mTOR, 4EBP1 and S6K1 were increased after inhibition of miR155, and vice versa. It suggested that inhibition the expression of miR155 could alleviate CIRI in the IP by activating Rheb/mTOR signaling at the transcriptional level. At the same time, we found that the phosphorylation level of mTOR signaling pathway were also increased after inhibition of miR155, indicating that autophagy level regulated by mTOR signaling pathway may be involved in the CIRI.

Introduction

Stroke is the second leading cause of death and the first cause for acquired long-term disability worldwide, including China[1]. Statistics demonstrated that stroke attacks approximately 800,000 people worldwide annually, and the incidence shows a gradual increase trend following the accelerated population aging[2, 3], leading to a global annual economic burden. There are two types of stroke: ischemic stroke(IS) and hemorrhagic stroke(HS). In pathogenesis, IS is the general call of the cerebral tissue necrosis and neurocyte apoptosis caused by insufficient blood supply due to stenosis or occlusion of the responsible blood-supply artery, which constitutes almost 60-70% of all strokes[4]. At present, all clinical therapies for IS can be summarized as two types: re-building of blood flow and neuroprotection. Systemic thrombolysis using recombinant tissue type plasminogen activator (rt-PA) is the only therapy that believed to be effective and approved by FDA of USA for treating IS patient since 1995[5] until 2015 when more sophisticated clinical trials showed robust outcomes for endovascular therapy (EVT)[6]. However, both thrombolysis therapy and EVT shows limitations in clinical application as a result of the certain time window, relative high prices, increased risk of hemorrhage complications and the following ischemic re-perfusion damage[7]. While neuroprotection therapy can be used alone or together with thrombolysis therapy and EVT, which is primarily aimed to rescue the brain tissue of the boundary zone of the ischemic core, also called ischemic penumbra (IP)[8]. Given the very limited treatment approaches for IS currently, it is urgently needed to explore new therapeutic target for this devastating disease. Many molecular mechanisms were involved in cerebral ischemia/reperfusion injury (CIRI) of IS, one of which was the mammalian target of rapamycin (mTOR) signaling pathway, having the ability to prevent apoptosis of neuronal cells, to inhibit cell death due to autophagy, to promote neurogenesis, and to accelerate angiogenesis. Thus it was believed may have the potential of preventing the ischemic neuronal

death and promoting the neurological recovery and was thought as a promising intervention target of IS[9].

Mammalian target of rapamycin (mTOR) was first proved and cloned in 1933 by Brown and his colleagues[10]. MTOR is an atypical serine/threonine protein kinase belonging to phosphatidylinositol kinase-related kinase(PIKK) protein family whose molecular weight is 289kDa and highly conserved among species and plays extremely widely roles in regulating cell growth, proliferation, apoptosis, autophagy and cell cycle. The activity of mTOR was maintained at the degree of its normal function of equilibrium by combining several proteins to form two complexes, named as mTORC1 and mTORC2[11], to realize the biological function of mTOR. Compared with mTORC2, mTORC1 has been found to be more sensitive to rapamycin [12]. Ras-homolog enriched in brain (Rheb) is a necessary protein in phosphorylating of mTOR for activation[13]. It regulated the survival, growth, and differentiation of cells by enhancing the signaling of the mTORC1 pathway[14]. As far as our knowledge at present, the downstream target proteins of mTOR primarily include ribosomal protein S6 kinase1 (S6K1) and eukaryotic initiation factor 4E (eIF4E)-binding protein 1(4EBP1). Activated mTOR play a role in regulating translation initiation and cell growth by phosphorylating to activate these downstream target proteins[15]. Activated S6K1 promotes mRNA production, ribosomal protein translation and cell growth. Activated Rheb-GTP can directly combine with Raptor and further activate mTORC1 and regulate 4EBP1 to combine with mTORC1[16]. When mTORC1 is inhibited, nonphosphorylated 4EBP1 competitively binds with eukaryotic translation initiation factor 4G (eIF4G) to eIF4E. The combination of eIF4G with eIF4E is essential for initiating translation by interacting with the 5'-mRNA cap structure. The mTORC1 phosphorylates 4EBP1 and further causes the latter to dissociate from eIF4E, thus allowing eIF4G to combine with eIF4E and accelerate the initiation of translation[17].

MicroRNA(miRNA) is a series of long-chain non-coding RNA that has a length of about 20-22bp of nucleotides, characterized by the wide acting range and high regulating efficacy, attracting more and more interest in various diseases. They are highly conserved among various eukaryotes. MiRNA and their corresponding target mRNAs have complementary pairing ending non-coding regions, the target mRNA molecules are degraded or translation inhibited after pairing with the miRNAs, mainly degraded, thus further participate in the regulation of cell growth, differentiation, energy metabolism, apoptosis, and other important and basic cellular physiological processes. One miRNA may have multiple target mRNAs and a mRNA can be regulated by several miRNAs. MicroRNA155 (miR155) is a member of the miRNA family, having all characteristics of miRNA, is proved to be involved in many diseases such as cancer, inflammation and myocardial infarction and development. A recent study demonstrated that under hypoxia condition, miR155 inhibited the activity of mTOR signaling pathway by targeting the mRNA of multiple molecules (including Rheb, RPS6KB2, RICTOR, etc.) in mTOR signaling pathway, leading to their degradation or translation inhibition, and further induced the autophagy process and promote apoptosis of cells, which indicated that miR155 may play a role in the pathology of cerebral ischemic damage. Similarly, Guo Ping Xing etc. reported that inhibition of miR-155 may play a protective role in ischemic stroke by acting on Rheb mRNA and further phosphorylating S6K. However, they didn't explore whether there were the same changes in mTOR and 4EBP1, which is necessary to clarify the mechanism of

miR155 and mTOR signaling pathway in ischemic stroke. Thus, we will explore this problem in MCAO/R rats, and further verify the effect of miR155 on Rheb/mTOR signaling pathway in vivo.

Materials And Methods

Animals

Adult male Sprague–Dawley (SD) rats (Laboratory Animal Center of Xi'an Jiao Tong University) with an average age of 8–12 weeks and average weight of 220–280 g were used in this study. They were randomly divided into five groups: sham group (only artery was dissociated), blank group (MCAO/R group), miR155 overexpressed group (LV-miR155+ MCAO/R group, stereotactically intracranial injection LV-miR155 a week before MCAO/R conducted), miR155 inhibited group (LV-miR155 sponge group + MCAO/R group, stereotactically intracranial injection LV-miR155 sponge a week before MCAO/R conducted), negative control group (LV-scrambled-miR155 + MCAO/R group, stereotactically intracranial injection LV-scrambled-miR155 a week before MCAO/R conducted). All rats were raised in a special pathogen-free animal house under standard conditions with a 12-h light–dark cycle, $60\% \pm 5\%$ humidity, and $22^{\circ}\text{C} \pm 3^{\circ}\text{C}$ ambient temperature for 3 days, during which enough water and pelleted food were supplied and all harmful stimulations were avoided.

Stereotactically Intracranial Injection

Carrier of lentivirus (LV) can integrate purpose gene to the genes of cells of host and express continuously and efficiently, moreover, host has a good tolerance for LV. So we chose LV as the carrier to packing miR-155 (over expression), miR-155 Sponge (inhibited expression) and scrambled miR-155 (negative control), conducted by Shanghai Genechem. To build the above model of regulated miR-155 expression, Stereotactically Intracranial Injection were conducted, the specific operation procedures are as follows:

1. Weighting and anaesthetizing: after forbidden of food or drink for 12 h and weighted, the male SD rats were anesthetized by an intraperitoneal injection of 10% chloral hydrate with a dose of 400 mg/kg.
2. Fixing and disinfecting: brain stereotaxic apparatus was located on the horizontal desk to horizontally aligned. The SD rats were fixed on the stereotaxic frame at prone position, clamp the teeth, slowly and gently insert the ear stick in the external auditory canal, adjusting the vernier caliper of the ear stick and the incisor to assure that the rat brain is on horizon. Using the shaving knife eliminated the hairs on the parietal cranium and using the iodophor disinfected skin.
3. Perforate on the skull: using the scalpel to make an one centimeter incision on the median sagittal line, blunt dissected layer by layer till the parietal bone was fully exposed and cleaned the view with disinfect swabs. Choose the frontal suture as the zero point, adjust the rulers to aim at the drilled hole. In the present experiment, three drilled holes was made, the specific coordinate was: +1.0 mm before frontal suture, +3mm on the right side of the median line, -1.3mm in deep; -6.0 mm after frontal suture, +3mm on the right side of the median line, -1.3mm in deep; -2.5mm after frontal suture, +3mm

on the right side of the median line, -1.3mm in deep. Perforate the skull slowly by electric drill till reach the Dura matter.

4. Injection—install the microinjector containing the lentivirus on the stereotaxic apparatus, aimed at the performed hole, let the needle tip of the microinjector down to the surface of the dura mater, then slowly insert the needle along Z axis with the speed of 0.1 μ l/min, stand for 10 minutes after finishing injection and then slowly withdrawn the needle.
5. Postoperative nursing—when the stereotaxic intracranial injection finished—cleaned the oozing blood on the wood with the cotton swabs, sutured the skin and disinfected with iodophor. Slowly loosen the bilateral ear stick and the teeth fixator—put the rats back into the rat cage, warm the rat with electric blanket till it wake.

Middle cerebral artery occlusion/reperfusion (MCAO/R)

The MCAO/R model of SD rats was constructed according to the instructions of previous reports of Zea-Longa (E Z Longa et al, 1989). After adaptation for 3 days in the animal house, the male SD rats were fasted and drink freely for 12 h before surgery. They were anesthetized by an intraperitoneal injection of 10% chloral hydrate with a dose of 350 mg/kg and then fixed on the laboratory table. A surgical midline incision was performed on the abdominal side of the neck, and then the right common carotid artery (CCA), the internal carotid artery (ICA), and the external carotid artery (ECA) were exposed and isolated. ECA was ligated at the bifurcation of ICA and ECA, CCA was ligated at the near-heart end, a knot was made at the beginning of ICA, and a “v”-shape mouth was cut below the bifurcation of ICA and ECA. Afterward, a nylon suture (poly-L-lysine-coated monofilament nylon suture, 0.36 ± 0.02 mm diameter) was gently inserted into the right CCA lumen and then gently injected into the ICA till the beginning of the middle cerebral artery for approximately 1.8–2.0 cm when feeling a sense of resistance, implying that the blood flow of the middle cerebral artery was blocked. The previous knot at the beginning of ICA was turned into a firm knot to fix the suture, and then the skin of the neck incision was sewn up. After 90 min of blocking, the nylon sutures were gently removed from the ICA and reperfusion for 24h was performed. Once the aforementioned steps were successfully implemented, the neck incision was closed. An automatic homeothermic blanket control unit was used throughout the surgical procedure and post-surgery recovery to constantly monitor and maintain the body temperature of rats at 37°C. The rats in the sham group endured the same surgical operations except inserting the monofilament nylon suture.

3, 7-Triphenyltetrazolium chloride staining for evaluating cerebral infarction in animal models

After ischemia for 90 min, the rats were anesthetized by an intraperitoneal injection of 10% chloral hydrate (350 mg/kg) at 24 h of the reperfusion time. The rat brains were rapidly and gently removed and frozen at -20°C for 20 min. Frozen brain tissues were dissected and sliced into 2-mm coronal sections. The slices were stained with 2% 2, 3, 7-triphenyltetrazolium chloride (TTC, Sigma, USA) at 37°C for 15 min in the dark; then, they were fixed in 10% formaldehyde buffer for 30 min. Next, they were placed on a medical blue sheet and photographed using a digital camera. The ImageJ 1.46R software (NIH, USA) was used to determine the cerebral infarction area, model hemisphere volume, and contralateral hemisphere

volume so as to reduce the influence of brain edema on the infarction volume. The adjusted formula was used to calculate the infarction volume, which was expressed as a ratio: percentage of corrected infarct volume = {[infarct volume - (ipsilateral hemisphere volume – contralateral hemisphere volume)]/contralateral hemisphere volume} × 100%.

TUNEL Staining

After ischemia for 90 minutes and reperfusion for 24 hours, the rats were anesthetized by an intraperitoneal injection of 10% chloral hydrate (400 mg/kg). The SD rats were fixed on the operating table in supine position and make the heart fully exposed. Perfusion the heart with 200ml physiological saline and 4% Paraformaldehyde(PFA) in sequence. Then the rats were executed by pulling the neck, carefully prize up the skull using curved forces and gently take out the brain entirely and putting into 4% PFA to fix for 24h. Furthermore, dehydrate gradiently under 4 °C with 10% saccharose for 2 days, 20% saccharose for 2 days, 30% saccharose 7 days till the brain tissue settlement to the bottom. Embed the brain tissue using OCT solution after precooling for 1 hour and avoid to produce bubble. Then using a frozen slicer to slice in coronal section of 10um in thickness and fix the slices on the glass slide embedded with poly-L-lysine and stored in -20°C refrigerator. TUNEL staining was conducted strictly according to the instructions of the TUNEL kit box. Observe the slices under the fluorescence microscope. TUNEL-positive cells exhibit green fluorescence under blue ray and the nucleus exhibit blue under purple ray. Randomly choose view and take photo under 10x40 microscope, analysis the photos by image J, counting the cells and calculate the rate of TUNEL-positive cells (cell apoptosis rate = TUNEL-positive cells/DAPI-positive cells × 100%).

Western Blot

Aliquots (15 µl) of 10 % brain homogenates were separated on SDS-PAGE and electronically transferred onto a PVDF membrane with a semidry blotting system. Membranes were blocked with 5 % (w/v) nonfat milk in 1× Tris-buffered saline containing 0.2 % Tween 20 (TBST) at room temperature (RT) for 2 h and probed with various primary antibodies at 4 °C overnight, including 1:1,000-diluted anti-Rheb mAb (Cell Signaling Technology, 13879S), 1:5000-diluted anti-mTOR mAb (Proteintech, 66888-1-1g), 1:1,000-diluted anti-S6K1 mAb (Cell Signaling Technology, 2708S), 1:1,000-diluted anti-4EBP1 mAb (Cell Signaling Technology, 9644S), 1:100,000-diluted β-actin mAb (Abclonal, AC026), 1:1000-diluted anti-pmTOR mAb (Invitrogen, 44-1125G), 1:1,000-diluted anti-pS6K1 mAb (Cell Signaling Technology, 9205S) and 1:1,000-diluted anti-p4EBP1 mAb (Cell Signaling Technology, 2855S), respectively. After washing with TBST, blots were incubated with 1:25,000-diluted horseradish peroxidase (HRP)-conjugated goat anti-mouse or rabbit IgG (Thermo Fisher Scientific, USA), at RT for 2 h. Blots were developed using the Enhanced ChemoLuminescence system and visualized on autoradiography films. Images were captured by ChemiDocTMXRS+ Imager.

RNA isolation

After reperfusion for 24, 48, and 72 h, the rats were anesthetized by an intraperitoneal injection of 10% chloral hydrate (350 mg/kg), and the brain tissues were rapidly taken out on the ice. Afterward, the ischemic core, boundary zone, and relative contralateral brain tissues were divided and weighed separately. The corresponding matched cerebral tissues were taken out in the sham groups. As suggested, 1 mL of TRIzol reagent (Invitrogen, USA) was added to every 100 mg cerebral tissue and homogenized rapidly. Total RNA was isolated from tissues following the manufacturer's protocol of TRIzol reagent. The NanoDrop ND-1000 (Thermo Fisher Scientific, USA) ultraviolet spectrophotometer was used to determine the concentration and the purity of the RNA, and the agarose gel electrophoresis method was used to test the integrity of the RNA.

Reverse transcriptase–polymerase chain reaction

Quantitative reverse transcriptase–polymerase chain reaction (qRT-PCR) was used to detect the miR-155 level in different brain tissues. Total RNA that proved to be well qualified was reverse transcribed into cDNA of miR-155 and U6 snRNA following the manufacturer's protocol of TaqMan MicroRNA Reverse Transcription Kit, and the cDNA was stored at -20°C for further use. Then, the qRT-PCR was performed to detect the miR-155 and U6 levels following the manufacturer's protocols of TaqMan MicroRNA assays and TaqMan® Universal PCR Master Mix II, without UNG (Applied Biosystems, USA). Three identical panels were designed for each reaction. Also, U6 was chosen as the internal reference of miR-155 expression. QRT-PCR of the SYBR green method was used to detect the mRNA levels of Rheb, mTOR, S6kb1, and 4Ebp1 in different brain tissues. Briefly, RT-PCR was carried out using the PrimeScript™ RT reagent Kit with gDNA Eraser and SYBR® Premix Ex Taq™ II (Tli RNaseH Plus) (TaKaRa, China) with Bio-Rad iQ5 Real-Time PCR System (Bio-Rad, USA). The target gene expression was normalized to the expression of β -actin, and the $2^{-\Delta\Delta CT}$ method was used to quantify the target gene expression.

Statistical analysis

Statistical analyses were conducted using the SPSS 21.0 software (IL, USA), and the data were expressed as mean $\pm$ standard deviation. Differences in continuous variables between two groups were investigated using the two-tailed Student *t* test in which statistical significance was determined when the *P* value of the corresponding statistical tests was less than 0.05.

Results

1. Overexpression and inhibition of miR155 in the cerebral of model rats

To test the efficiency of the re-combined LV- miR155 and LV- miR155 sponge, which intending to make the miR155 over-expressed or under-expressed after stereotaxic intracranial injection distinguishly, we tested the miR155 levels by qRT-PCR in different groups, including sham group, blank control group (MCAO/R group), over-expression group (MCAO/R+LV-miR155 group), down-regulated group (MCAO/R+ LV- miR155 sponge) and negative control group (MCAO/R+scrambled LV- miR155 group) (Figure 1). As we

expected, compared to the sham group, the miR155 levels increased (&, $p < 0.05$), meanwhile, compared to the MCAO/R group, miR155 level in the MCAO/R+LV- miR155 group was up-regulated (*, $p < 0.05$) and in the MCAO/R+ LV- miR155 sponge was down-regulated (*, $p < 0.05$). Moreover, miR155 level had no change in the MCAO/R+scrambled LV- miR155 group ($p > 0.05$).

2. MiR155 negatively influence the survival rate of the model rats

In order to estimate the influence of miR155 on the survival rate of model rats, the survival rates of different groups were calculated (Table 1). All 20 rats in the sham group were survived, that is, the survival rate in the sham group is 100%. While in the MCAO/R group, only 26 out of 53 rats survived, thus, the survival rate was 49%. Interestingly, only 14 out of 42 rats survived in the miR155 over-expressed group, nevertheless, 12 out of 20 rats survived in the miR155 inhibited group, with the survival rate was 33% and 60% respectively. In the negative control group, 11 out of 21 rats were survived, meaning the survival rate was 52%. In summary, the data above showed that knocking down of miR155 increased the survival rate of model rats, while over-expressed miR155 leading to the survival rate declined. In one word, miR155 negatively influences the survival rate of the model rats.

3. MiR155 positively influence the infarct volume of the cerebra of the rats enduring MCAO/R.

To estimate the influence of miR155 on infarct volume of the cerebra of the rats enduring MCAO/R, the infarct volume and its percentage of different groups were calculated. The infarct volume of the cerebra was estimated by TTC staining of the brain slices of rats in different groups (Figure 2). The pictures in panel A were the results of the TTC staining, the red part of every slice represents normal brain tissue while the white part represents the infarcted brain tissue. The panel B showed the infarct percentages of different groups. Compared with the sham group, the infarct percentages of other groups increased (*, $p < 0.05$). Compared with the blank control group (MCAO/R group), the infarct percentage increased in the miR155 over-expressed group (#, $p < 0.05$), while the infarct percentage declined in the miR155 inhibited group (#, $p < 0.05$). In conclusion, the results described above showed that knocking down of miR155 decreased the infarct volume of model rats, while over-expressed miR155 leading to the survival rates increased, that is, miR155 positively influences the infarct percentage of the model rats, the differences has statistical significance.

4. MiR155 positively influence the TUNNEL-positive cells of the cerebra of the rats enduring MCAO/R.

To estimate the influence of miR155 on cell apoptosis of the cerebra of the rats enduring MCAO/R, the TUNNEL staining of the brain slices of different groups were conducted and the percentage of TUNNEL-positive cells in each group were calculated (Figure 3). Panel A showed the results of TUNNEL staining of different groups, in which the blue fluorescence signal represent the cell nucleus and the green fluorescence signal represent the cells undergoing apoptosis. Panel B is the analysis of the percentage of TUNNEL-positive cells in different groups. It revealed that compared to the sham group, all the other groups had more TUNNEL-positive cells, and compared to the blank control group (MCAO/R group), the percentage of TUNNEL-positive cells increased in the miR155 over-expressed group (#, $p < 0.05$) but

decreased in the miR155 inhibited group(#, $p < 0.05$). Moreover, there was no difference between the blank control group and the negative control group($p > 0.05$). In summary, the results described above showed that knocking down of miR155 decreased the percentage of TUNNEL-positive cells of model rats, while over-expressed miR155 led to the percentage of TUNNEL-positive cells increased, that is, miR155 positively influences the TUNNEL-positive cells of the cerebra of the rats enduring MCAO/R.

5. Expression of miR155 increased but the mRNA levels of Rheb, mTOR, 4EBP1 and S6K1 decreased after cerebral ischemia.

In order to observe the change of the expression of Rheb/mTOR and miR155 after cerebral ischemia, qRT-PCR was conducted (Figure 4). The results showed that miR155 levels increased in the MCAO/R group compared to the sham group(&, $p < 0.05$). In contrast, the mRNA levels of Rheb, mTOR, 4EBP1 and S6K1 decreased in the MCAO/R group compared to the sham group(&, $p < 0.05$).

6. MiR155 negatively regulated the mRNA levels of Rheb, mTOR, S6K1 and 4EBP1 in the cerebra of the rats enduring MCAO/R.

To estimate the influence of miR155 on the mRNA levels of Rheb, mTOR, S6K1 and 4EBP1 in the cerebra of the rats enduring MCAO/R, the qRT-PCR of the brain tissues of different groups were conducted (Figure 5). Panel A showed the qRT-PCR results of Rheb mRNA of different groups, in which reveals that compared to the sham group, Rheb mRNA level decreased(&, $p < 0.05$), however, compared to the MCAO/R group, Rheb mRNA level decreased in the miR155 over-expression group(*, $p < 0.05$) while increased in the hypo-expression group(*, $p < 0.05$), besides, there was no difference in the negative control group($p > 0.05$). Panel B showed the qRT-PCR results of mTOR mRNA of different groups, in which reveals that compared to the sham group, mTOR mRNA level decreased(&, $p < 0.05$), however, compared to the MCAO/R group, mTOR mRNA level decreased in the miR155 over-expression group(*, $p < 0.05$) while increased in the down-regulated group(*, $p < 0.05$), besides, there was no difference in the negative control group($p > 0.05$). Panel C showed the qRT-PCR results of S6K1 mRNA of different groups, in which reveals that compared to the sham group, S6K1 mRNA level decreased(&, $p < 0.05$), however, compared to the MCAO/R group, S6K1 mRNA level decreased in the miR155 over-expression group(*, $p < 0.05$) while increased in the down-regulated group(*, $p < 0.05$), besides, there was no difference in the negative control group($p > 0.05$). Panel D showed the qRT-PCR results of 4EBP1 mRNA of different groups, in which reveals that compared to the sham group, 4EBP1 mRNA level decreased(&, $p < 0.05$), however, compared to the MCAO/R group, 4EBP1 mRNA level decreased in the miR155 over-expression group(*, $p < 0.05$) while increased in the down-regulated group(*, $p < 0.05$), besides, there was no difference in the negative control group($p > 0.05$). In summary, the results described above showed that knocking down of miR155 increased the mRNA expression of Rheb, mTOR, S6K1 and 4EBP1 in the brain tissue of model rat, while over-expressed miR155 decreased the mRNA expression of Rheb, mTOR, S6K1 and 4EBP1 in the brain tissue of model rat, that is, miR155 negatively influences the mRNA expression of Rheb, mTOR, S6K1 and 4EBP1 in the cerebra of the rats enduring MCAO/R.

7. MiR155 negatively regulated the protein levels of Rheb,mTOR, S6K1 and 4EBP1 in the cerebra of the rats enduring MCAO/R.

To estimate the influence of miR155 on the protein levels of Rheb,mTOR,S6K1 and 4EBP1 in the cerebra of the rats enduring MCAO/R, the western blot of the brain tissues of different groups were conducted (Figure 6). Panel A showed the western blot results of Rheb,mTOR,S6K1 and 4EBP1 of different groups,in which β -actin was used as inner reference. Panel B was the analysis of the western blot signal of the aforementioned molecules in different groups, reveals that Panel B(a) shows the analysis of Rheb protein in different groups,which reveals that compared to the sham group, Rheb protein level decreased(&, $p<0.05$),however,compared to the MCAO/R group, Rheb protein level decreased in the miR155 over-expression group(*, $p<0.05$) while increased in the down-regulated group(*, $p<0.05$),besides,there was no difference in the negative control group($p>0.05$).Panel B(b) showed the analysis of mTOR in different groups,which reveals that compared to the sham group, mTOR protein level decreased(&, $p<0.05$),however,compared to the MCAO/R group, mTOR protein level decreased in the miR155 over-expression group(*, $p<0.05$) while increased in the down-regulated group(*, $p<0.05$),besides,there was no difference in the negative control group($p>0.05$). Panel B(c) showed the analysis of S6K1 protein in different groups, which reveals that compared to the sham group, S6K1 protein level decreased(&, $p<0.05$),however,compared to the MCAO/R group, S6K1 protein level decreased in the miR155 over-expression group(*, $p<0.05$) while increased in the down-regulated group(*, $p<0.05$),besides,there was no difference in the negative control group($p>0.05$). Panel B(d) showed the analysis of 4EBP1 protein in different groups,which reveals that compared to the sham group, 4EBP1 protein level decreased(&, $p<0.05$),however,compared to the MCAO/R group, 4EBP1 protein level decreased in the miR155 over-expression group(*, $p<0.05$) while increased in the down-regulated group(*, $p<0.05$),besides,there was no difference in the negative control group($p>0.05$). In summary,the results described above showed that knocking down of miR155 increased the protein expression of Rheb,mTOR, S6K1 and 4EBP1 in the brain tissue of model rat,while over-expressed miR155 decreased the protein expression of Rheb,mTOR, S6K1 and 4EBP1 in the brain tissue of model rat,that is, miR155 negatively influences the protein expression of Rheb,mTOR, S6K1 and 4EBP1 in the cerebra of the rats enduring MCAO/R.

8. MicroRNA155 negatively regulated the protein levels of p-mTOR, p-S6K1 and p-4EBP1 in the cerebra of the rats enduring MCAO/R.

To estimate the influence of miR155 on the protein levels of p-mTOR, p-S6K1 and p-4EBP1 in the cerebra of the rats enduring MCAO/R, the western blot of the brain tissues of different groups were conducted (Figure 7). Panel A showed the western blot results of p-mTOR, p-S6K1 and p-4EBP1 of different groups,in which β -actin was used as inner reference. Panel B was the analysis of the western blot signal of the aforementioned molecules in different groups, reveals that compared to the sham group, the protein level of p-mTOR, p-S6K1 and p-4EBP decreased(&, $p<0.05$),however,compared to the MCAO/R group, the protein level of p-mTOR, p-S6K1 and p-4EBP decreased in the miR155 over-expression group(*, $p<0.05$) while increased in the down-regulated group(*, $p<0.05$),besides,there was no difference in the negative control group($p>0.05$). In summary,the results described above showed that knocking down of miRNA155

increased the protein expression of p-mTOR, p-S6K1 and p-4EBP in the brain tissue of model rat, while over-expressed miRNA155 decreased the protein expression of p-mTOR, p-S6K1 and p-4EBP in the brain tissue of model rat, that is, miRNA155 negatively influences the protein expression of p-mTOR, p-S6K1 and p-4EBP in the cerebra of the rats enduring MCAO/R.

Discussion

This study investigated the effect and mechanism of miRNA155 on the CIRI of the MCAO/R model rat. First, by regulating the miRNA155 expression level in the brain of rats through Stereotactic intracranial injection, we found that over-expression of miRNA155 enhanced the CIRI, exhibiting relatively higher death rate, larger infarction volume and more cells enduring apoptosis. On the contrary, downregulated miRNA155 in the brain of model rats led to alleviate CIRI, exhibiting relatively lower death rate, smaller infarction volume and less cells enduring apoptosis. Then we studied the expression change of the primary molecules of mTOR signaling pathway, including Rheb, mTOR, S6K1 and 4Ebp1. The results demonstrated that inhibition of miR155 increased the expression of the above molecules in CIRI, and vice versa, which exhibited not only on the transcriptional levels but also the translational levels as well as the phosphorylation levels.

Ischemic cerebral stroke is an acute disease threatening human health, which is caused by the local brain blood supply obstacles and is influenced by multiple pathophysiological process factors, with the characteristics of high morbidity and high fatality. miRNAs are endogenous small noncoding regulatory RNA that modulate protein expression by interacting with target mRNAs and participate in a variety of pathological and physiological processes. Specific stroke-induced miRNA expression profiles have been reported in both the blood and the brain, and in experimental models and patients at different reperfusion times [18]. The pattern of circulating miRNA expression suggests an early influence of age in stroke pathology, with a later emergence of sex as a factor for stroke severity [19]. Changed inflammation-related miRNA profiles in plasma following IS have been reported [20]. Previous study have found that the polymorphisms of miR-155 (miR-155 rs767649T>A) was related to the risk of IS in a Korean population [21]. Recent study have shown that association of miR-155 with the Risk of IS in a Chinese population [22]. In the plasma of stroke patients, endothelial microvesicles and their carrying miRNA-155 may serve as biomarkers for IS [23]. In addition, the patterns of miRNA expression were used to predict stroke subtypes [18]. MiR155 has been proved to up-regulated in the pathogenesis of IS and related to enhancing the ischemic injury. MiR-155 knockdown improved the neurological function and alleviated ischemia/reperfusion injury [24]. The expression of miR-155 increased in the cerebral tissues of MCAO rats with ischemia. Exogenous miR-155 inhibitors down regulated miR-155 expression but up regulated Rheb and mTOR expression, showing a protective effect in the injury process of IS. This protective role was characterized by a decrease in the infarct size and decreased apoptosis rate [25]. lncRNA Oprm1 decreased the Infarct size and improved neurological score in MCAO induced injury by regulating the MiR155/GATA3 axis [26]. Consistent with previous studies, our study found that the expression of miR155 in IP brain tissue was significantly increased at 24 hours after stroke, which suggested that miR155 is likely involved in the ischemic cerebral injury. In addition, this study demonstrated that inhibition the

expression of miR155 resulted in a decrease in the number of apoptotic cells, the size of cerebral infarction and mortality rate, which indicated that inhibition of miR155 has a protective effect on CIRI. Therefore, therapeutic approaches that targeted miR-155 probably worked in IS[27]. However, the specific mechanism of miR155 in CIRI has not yet been conclusion.

In this study, it was found that not only the expression of miR155 was increased, but also the expression of Rheb, mTOR, S6K1 and 4Ebp1 were decreased in the brain of MCAO rats 24 days after reperfusion. Several studies demonstrated that regulating mTOR activity had the potential of neuroprotection during IS. The application of estradiol to adult female ovariectomized rats before focal cerebral ischemia obviously reduced infarct volumes and cell apoptosis in the cerebral cortex, and prevented the decline in the expression of phosphorylated mTOR and p70S6K induced by ischemia[28]. The down-regulation of S6K1 accelerated injury in astrocytes induced by oxygen–glucose deprivation (OGD), an in vitro model of ischemia; on the contrary, the up regulation of S6K1 through adenoviral infection relieved cell injury. Furthermore, the knockout of S6K increased the infarct volume and the mortality of mice with focal cerebral ischemia[29]. Moreover, the down-regulated mTOR activity induced by rapamycin made cell survival tough and facilitated apoptotic injury in neural cells enduring OGD[30, 31]. Erythropoietin has been demonstrated to defend microglia from OGD by promoting the mTOR activity and inhibiting the release of mitochondrial cytochrome C because mTOR inhibition through rapamycin silences the cell-protective function of erythropoietin[31]. Rapamycin can also enlarge the brain infarct size and increase the neurological deficit score in rats with focal cerebral ischemia, indicating that promoting mTOR activation may lead to lighter ischemic brain injury and better behavior recovery. Rheb is a necessary protein in phosphorylating of mTOR for activation. Previous study found that Rheb mRNA levels were down-regulated in parietal cortex and Lateral striatum in MCAO rats after 24h, which was consistent with the results of the present study[32]. Our study found that Rheb expression was decreased and miR155 expression was increased on 24 h after reperfusion in MCAO/R rats. Recent study has demonstrated that miR-155 might regulate the expression of Rheb at the post-transcriptional levels by directly act on Rheb mRNA in vitro[25]. These results suggest that miR155 may inhibit mTOR signaling pathway by targeting Rheb in IS. The protective effect of inhibiting miR155 on ischemic stroke may be achieved by removing the inhibition of Rheb and mTOR signaling pathways.

In order to confirm the mechanism of miR155 and Rheb/mTOR signaling pathways in CIRI, we regulated the expression level of miR155 in rat of MCAO/R. The results showed that the mRNA and protein levels of Rheb, mTOR, 4Ebp1 and S6k1 were increased after inhibition the expression of miR155 in the ischemic brain tissues, and vice versa. It indicated that miR155 participate in CIRI by inhibiting the Rheb/mTOR signaling pathway. In fact, previous studies have found that miR155 targets Rheb to inhibit mTOR signaling in vitro. In human nasopharyngeal cancer and cervical cancer cells, miR155 contribute to hypoxia-induced autophagy by directly interacting with the 3' UTRs of Rheb to inhibit mTOR signaling[33]. Guoping Xing et al found that miR155 was involved in hypoxic-induced apoptosis by inhibiting the expression of Rheb and mTOR in BV2 cells[25]. Our study further verified the above results in vivo, and found that miR155 inhibited the Rheb/mTOR signaling pathway at the transcriptional level. In addition, We demonstrated that phosphorylation levels of substrate proteins (S6k1 and 4EBP1) of mTOR also were

increased after inhibition the expression of miR155 in the brain tissues. In mammals, mTORC1 inhibits autophagy initiation through Phosphorylation[34]. In human umbilical vein endothelial cells, miR155 promotes ox-LDL-induced autophagy by targeting the PI3K/Akt/mTOR pathway[35]. Mrigya Babuta et al found the mTOR pathway is inhibited in mice and in Alcoholic liver disease patients after chronic alcohol consumption. Then, they demonstrated miR155 induces autophagy in macrophages and hepatocytes by inhibiting the Rheb/mTOR pathway[36]. MiR155 may regulated cell autophagy to participate in IS by inhibiting the phosphorylation levels of mTOR and it's substrate proteins in our study.

IS constitutes almost 80% of the stroke cases, while the remaining 20% are hemorrhage strokes. Once the blood flow to the brain is insufficient, the cells experience a series of molecular programs, including the toxicity of excitatory neurotransmitters, dysfunction of mitochondria, acidosis, imbalance of ions, oxidative stress, and inflammation. Oxidative stress in the brain results in ischemic injury can eventually initiating programmed cell death pathways, including apoptosis, autophagy, and necroptosis. Previous studies have demonstrated that the miRNA155 mainly participate in neuroinflammation[37]. MiR155 shows high expression in brain injury diseases such as cerebral ischemia and glioma, and has been found to regulate the activity of the inflammatory processes. In the serum of patients with ischemic stroke, the expression of miR-155 were positively correlated with TNF- α and could be used as potential inflammatory biomarker for acute IS[38]. In the MACO mouse brain tissues, miR155 knockdown inhibited the expressions of IL-1 β , IL-6, TNF- α , iNOS, and COX-2 and alleviated inflammation response[24]. Wei Chen et al. found that miR155 induced cell apoptosis and damaged neural function by activating inflammatory cytokines TLR4/MyD88[39]. Inhibition of miR-155 promoted recovery after Experimental Mouse Stroke in vivo by suppression the expression of inflammatory process[40]. In addition to the inflammation, miR155 has also been found to be involved in the regulation of autophagy. MiR155 activates autophagy to modify inflammatory responses through regulating TLR4/NF-B pathway in ischemic cerebral tissues[41]. In fact, It has been found that miR155 is involved in some diseases by regulating autophagy and inflammation through the mTOR signaling pathway. It's reported that the miR155 expression was decreased in dendritic cells (DCs) from Behcet's disease (BD) patients and increased miR155 decreased the production of TNF- α , IL-6, and IL-1 β by DCs through inhibition of the Akt/mTOR signaling pathway and by inducing the process of autophagy[42]. In this study, we think miR155 may induce autophagy and promoted inflammation responses by inhibiting the Rheb/mTOR signaling pathway in cerebral ischemia/reperfusion injury.

In conclusion, the significance of this study is that we found that inhibition of miR155 alleviates the CIRI, the mechanism is to enhanced the expression and phosphorylation level of the Rheb/mTOR signaling pathway and leading to inhibition of the autophagy and further alleviated the inflammatory reaction due to ischemic stroke. MiR155 regulates Rheb and mTOR signaling pathways at the transcriptional level in vivo. Some limitations of the current study are the lack of exploration of the specific target point deserve further study, as there were different voices on this problem in the past researches. What needs to be further investigated is whether or not there are changes in the biomarkers of autophagy and inflammation in the brain of model rats.

Declarations

Authors' contributions Conceptualization:Y-E Y and Z-L D; Methodology: L C and Y-Y X; writing - original draft preparation: Y-E Y and L C; writing review and editing: X-R Z,Y J, Y M, F Y and Y-Y J; Funding acquisition:Y-E Y, Z-L D,N-B G and J X; resources:Y-E Y, Z-L D; supervision:ZL D,Y-E, XR Z,ZQ L,Y T and H G.All authors have read and agreed to the published version of the manuscript.

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Availability of data and material All data supporting the conclusions of this manuscript are provided in the text, fgures and tables.

Ethics approval The questionnaire and methodology for this study was approved by the Ethical Committee of the Xi'an Central Hospital, Medical College of Xi'an Jiao Tong University, Xi'an Shaanxi, China, under protocol LW-2021-002, for the use of animal specimens. Animal raising and experimental protocols followed the Chinese Regulations for the Administration of Affairs Concerning Experimental Animals.

Consent to participate Not applicable

Consent for publication Not applicable

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Tables

Tab.1 Knocking down of microRNA155 increased the survival rate of model rat.

After ischemia for 90min and re-perfusion for 24h ,the survival rate of different groups we analyzed. It demonstrated that compared to the sham group, the survival rate of both the MCAO/R group and the MCAO/R+LV miR-155 Scrambled group declined. But compared to the MCAO/R group, the survival rate of MCAO/R+LV miR-155 group was declined, the survival rate of MCAO/R+LV miR-155 Sponge group was increased. compared to the sham

Groups	amount	survival	livability	
Sham	20	20	100%	
MCAO/R	53	26	49%	*
MCAO/R+LV miR-155	42	14	33%	* #
MCAO/R+LV miR-155 Sponge	20	12	60%	* #
MCAO/R+LV miR-155 Scrambled	21	11	52%	*

group,**P*<0.05; compared to the MCAO/R group,#*p*<0.05.

Figures

Figure 1

Regulation of miR155 by stereotactic intracranial injection of recombinant lentivirus. Panel A: The TTC staining of the brain slices of rats enduring MCAO/R with the injection mark shown. Panel B: The relative expression levels of the MirRNA155 in different groups. The statistical significance compared with the sham group was marked as &. The statistical significance compared with the MCAO/R group was marked as *.The data are presented as the mean $\pm$ SD;p<0.05.

Figure 2

The infarction volume of the MCAO/R rats shrinks after knocking down the microRNA155 expression. Panel A: Result of the TTC staining of different groups(three rats in each group),the red parts of the brain slices was the normal cerebral tissue while the white part was the infarcted cerebral tissue. Panel B: the percentage of the infarction volume of different groups.The statistical significance compared with the sham group was marked as *. The statistical significance compared with the MCAO/R group was marked as #. The data are presented as the mean $\pm$ SD; $p < 0.05$.

Figure 3

The apoptosis rate of the MCAO/R rats declines after knocking down the microRNA155 expression. Panel A: Results of the TUNEL staining of IP area of rat brain slices of different groups. DAPI stains the nuclei of cells, TUNEL stains the plasma of cells enduring apoptosis. Panel B: Analysis of the percentage of TUNEL-positive cells in different groups. The statistical significance compared with the sham group was

marked as *. The statistical significance compared with the MCAO/R group was marked as #. The data are presented as the mean±SD;p<0.05.

Figure 4

After ischemia for 90min and re-perfusion for 24h, the level of miR155 and mRNA levels of Rheb,mTOR,4EBP1 and S6K1 were detected. compared with the sham group, the microRNA155 expression increases while the Rheb,mTOR,4EBP1 and S6K1 were declined.The statistical significance compared with the sham group was marked as &.The data are presented as the mean±SD;p<0.05.

Figure 5

The alteration of mRNA levels of Rheb,mTOR,4EBP1 and S6K1 under different levels of microRNA155. Panel A: The alteration of mRNA levels of Rheb under different levels of microRNA155. Panel B: The alteration of mRNA levels of mTOR under different levels of microRNA155. Panel C:The alteration of mRNA levels of S6K1 under different levels of microRNA155. Panel D:The alteration of mRNA levels of 4EBP1 under different levels of microRNA155. The data are presented as the mean $\pm$ SD;p<0.05.

Figure 6

The alteration of protein levels of Rheb,mTOR,4EBP1 and S6K1 under different levels of microRNA155. Panel A: Western blot results of β-actin ,Rheb,mTOR,S6K1 and 4EBP1 using the brain tissues of rats of different groups. Panel B: The relative protein expression levels of Rheb,mTOR,S6K1 and 4EBP1 in different groups with β-actin as the inner reference. The statistical significance compared with the sham group was marked as &. The statistical significance compared with the MCAO/R group was marked as *. The data are presented as the mean±SD;p<0.05.

Figure 7

The alteration of protein levels of phosphorylated mTOR, 4EBP1 and S6K1 under different levels of microRNA155. Panel A: Western blot results of β -actin, p-mTOR, p-S6K1 and p-4EBP1 using the brain tissues of rats of different groups. Panel B: The relative protein expression levels of p-mTOR, p-S6K1 and p-4EBP1 in different groups with β -actin as the inner reference. The statistical significance compared with the sham group was marked as &. The statistical significance compared with the MCAO/R group was marked as *. The data are presented as the mean $\pm$ SD; $p < 0.05$.