STUDY ON THE MECHANISM OF MIR-140-5P ON ALLEVIATING CEREBRAL ISCHEMIA-REPERFUSION INJURY IN RATS BY TARGETING TLR4

Li Jian[1], Yu Tianlian[2], Zhou Changrui[2], Li Lijuan[2,*]
[1]Department of Neurology, Dongying Traditional Chinese Medicine Hospital (Dongying Shengli Hospital), Dongying, 257055, China - [2]Department of Laboratory Medicine, Dongying Traditional Chinese Medicine Hospital (Dongying Shengli Hospital), Dongying, 257055, China

ABSTRACT

To investigate the effect and mechanism of miR-140-5p on alleviating cerebral ischemia-reperfusion injury in rats by targeting TLR4.Rat models of cerebral ischemia-reperfusion injury (IRI) and neuronal cell models of oxygen-glucose deprivation and reoxygenation (OGD/R) were established. miR-140-5p expression in brain tissues of cerebral IRI rats was interfered by lentivirus vectors. Transient transfection technique was used to interfere with the miR-140-5p and TLR4 expressions in neuron OGD/R cell model. RT-PCR was used to detect the miR-140-5p and TLR4 mRNA expression. Tetrazole chloride (TTC) staining was used to detect the cerebral infarction area of cerebral IRI rats. Western Blot was used to detect the TLR4 and apoptosis-related proteins expressions in cells. The expression of interleukin -6(IL-6), IL-10 and TNF-α in brain tissue was detected by ELISA. The superoxide dismutase (SOD) and malondialdehyde (MDA) expressions in tissues was detected by electrochemiluminescence immunoassay. Apoptosis was detected by flow cytometry. The relationship between miR-140-5p and TLR4 was verified by double fluorescein reporter enzyme.miR-140-5p was low expressed and TLR4 was high expressed in the cerebral IRI ratsbrain tissues and neurons OGD/R cell model. The development of cerebral IRI will lead to the aggravation of inflammatory reaction and oxidative stress reaction in brain tissue and increase the cerebral infarction area. Over-expression of miR-140-5p can effectively relieve inflammatory reaction and oxidative stress reaction in brain tissue of cerebral IRI rats and reduce cerebral infarction area. Over-expression of miR-140-5p in neuron OGD/R cells or inhibition of TLR4 expression can effectively inhibit the apoptosis of Ht22 cells, up-regulate the anti-apoptotic protein Bcl-2expression, and down-regulate the pro-apoptotic proteins Bax and Caspase-3expressions. The double fluorescein reporter enzyme verified the targeted relationship between miR-140-5p and TLR4.miR-140-5p was low expressed in brain tissues of cerebral IRI rats. It can reduce cerebral IRI of rats by targeting TLR4, improve neurological function of rats, and inhibit apoptosis of neuronal cells. It may be a potential therapeutic target for cerebral IRI.

Keywords: *MiR-140-5p, TLR4, cerebral ischemia-reperfusion injury of rats, mechanism, apoptosis.*

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Introduction

Stroke, as the major disease that leads to death in cerebrovascular disease, mainly affects the elderly[(1)]. In recent years, with the emergence of aging population, the incidence, mortality and disability rate of stroke are getting higher and higher[(2)]. The main cause of stroke is brain tissue damage caused by brain tissue ischemia. At present, the main treatment for stroke is ultra earlier thrombolysis. However, recanalization after brain tissue ischemia will further cause tissue damage and dysfunction. This is the cause of cerebral ischemia-reperfusion injury (IRI)[(3,4)]. The development of cerebral IRI can lead to microvascular dysfunction and tissue barrier dysfunction. Currently, there is no particularly effective treatment method for the development of cerebral IRI[(5)]. Therefore, it is of great significance to find a treatment method for brain IRI by exploring the mechanism of IRI.

miRNA, as a non-coding microRNA, mainly realizes its regulatory function on cells by combining

with target mRNA[6]. In recent years, more and more studies[7, 8] have shown that miRNA is bound up with the occurrence of ischemia. For example, researches[9] concluded that the expression of many miRNA in blood and cells of stroke mammals has changed. In addition, research[10] found that miRNA can play a protective role on cerebral ischemia by regulating the signal transmission of inflammatory factors. The miR-140-5p is a miRNA[11] that has been proved to play an vital role in the development and progression of tumors, but in recent years there have been more and more reports on the connection between miR-140-5p and cerebrovascular diseases. For example, earlier studies[12] reported that miR-140-5p can affect cerebrovascular generation after ischemic stroke by targeting VEGFA. Studies[13] clearly pointed out that it has a regulatory effect on the expression of allergic C-reactive protein (Hs-CPR) in acute cerebral infarction, but the regulation and mechanism of inflammatory response in the process of cerebral IRI are not elaborated in detail. TLR4 is a member of the Toll-like receptor family, which is bound up with the development of inflammatory reaction. Previous studies have clearly pointed out that TLR4 plays an vital role in the development of inflammatory reaction in cerebral IRI[14].

We predicted that there was a targeted relationship between the development of miR-140-3p and TLR4 through bioinformatics, so the main purpose of our research is to explore whether miR-140-3p can regulate inflammatory response of cerebral IRI rats by regulating TLR4, as follows.

Materials and methods

Experimental materials

60 SD rats of clean grade (obtained from the Experimental Animal Center of Sun Yat-sen University) with body mass of 260~300 g were selected and fed in an environment with temperature of 20~25°C and relative humidity of 40%~60%, with normal diurnal rhythm alternating and free to eat and drink. There was HT22 cell line of mouse hippocampal neuron (ATCC), rabbit anti-human TLR4, Bax, Bcl - 2 and Caspase-3 monoclonal antibody (Cell Signaling Company, USA), rabbit anti-human GAPDH monoclonal antibody (Pulitzer gene technology co., LTD., Beijing), concentration of BCA protein assay kits (Shanghai weiao biotechnology co., LTD), in situ cell death test kit (Roche Diagnostics, Basel, Switzerland), qPCR kit and reverse transcription kit (TransGen Biotech, Beijing, China) and dual-luciferase reporter assay kit (Solarbio, Beijing, China). miR-140-5p, miRNA NC, internal reference U6 and GAPDH primers were synthesized and planned by Shanghai GenePharma Co.,Ltd. There was real-time quantitative PCR (BioRad, Berkeley, California, USA), interleukin 6 (IL - 6), IL - 10 and TNF-α ELISA kit (Moshake biological technology co., LTD.), CAnnexin V-FITC/PI apoptosis kit (Shanghai beibo biotechnology Co. LTD.), flow cytometry instrument CytoFLEX LX (Beckman, Brea, California, USA), DMEM medium (Gibco, Rockville, MD, USA), fetal bovine serum (FBS), trypsin (Hyclone,Logan,Utah, USA) and Trizol reagent (Applied Invitrogen,Carlsbad, California, USA).

Establishment of rat cerebral IRI model

Rats were indiscriminately sorted into blank control group (BCG), sham operation group (SOG), IRI group (IG), IRI+miR-140-5p-agomir group (IMAG) with 15 rats each. A rat model of middle cerebral artery occlusion (MCAO) was established by reformative Zea-Longa[15].

First, 3% sodium pentobarbital was injected intraperitoneally at a dose of 50mg/kg. After observing that the respiration of the rat smoothly entered the deep anesthesia, the rate was fixed on the operation table in the supine position. The neck skin was subjected to conventional disinfection.

Then, an incision was made in the middle of the neck. The right common carotid artery, external carotid artery and internal carotid artery were separated and fully exposed. Then, the common carotid artery and external carotid artery were ligated, and an incision was made under the bifurcation of the common carotid artery. The nylon line-lock coated with polylysine was inserted into the common carotid artery and extended into the internal carotid artery. When there was a slight resistance, the extension was stopped. After ischemia for 2 hours, the nylon thread was gently pulled out to set up an IRI rat model.

Rats in the SOG were not subjected to artery ligation and nylon thread insertion, and the other operations were the same as before. After ischemia for 2 hours, rats in the IMAG were intraventricular injected with miR-140-5p-agomir with a lentiviral vector concentration of 20umol/L for a total of 10ul, while rats in the other group were injected with the same dose of normal saline. When all rats regained consciousness for 24 hours, the neurological deficits of the rats were evaluated[16].

TTC dyeing method

The rats were narcotized again by intraperitoneal injection of 3% pentobarbital sodium and slain for cervical dislocation after neurological impairment assessment. The brain tissues of the rats were treated with paraffin embedding and sectioning, and incubated in 2% TCC solution for 15min.

Then it was fixed in 4% paraformaldehyde for 24 hours, and then washed 3 times with PBS. The cerebral infarction area was observed under the microscope.

Establishment of cell model and transfection

Ht22 cells were placed in DMEM medium (incorporating 10%FBS and 1% penicillin/ streptomycin mixture) and hatched at 37°C and 5%CO_2. When the cells adhered to the wall and grew to 80%, cells were collected and divided into BCG, oxygen-glucose deprivation and reoxygenation (OGD/R) group, OGD/R+miR-140-5p-agomir group (OMG), miR-NC group (MG), Si-TLR4 group (STG) and Si-NC group (SNG). Cells in BCG were cultured normally. Cells in other groups were cultured in Hank's balanced salt solution of glucose.

The cells were cultured in the hypoxia incubator at 37°C, 94% N2 and 5%CO_2. After 24 hours, cells were transferred to a culture medium containing 4.5g/L glucose and incubated for 3 hours at 37°C, 5% CO_2 to establish an OGD /R model. After the establishment of model, miR-140-5p-agomir, miR-NC, Si-TLR4, and Si-NC were transfected into cells in the OGD/R+miR-140-5p-agomir group and MG for 24h, respectively, while cells in the OGD/R group were not transfected.

RT-PCR detection

Total of RNA was extracted by using Trizol reagent. The purity and concentration of RNA were determined by ultraviolet spectrophotometer.

Then total of RNA of 5μg was taken to reverse transcription cDNA according to the kit instructions. Then, the 1μLsynthesizedcDNA was taken and amplified. Amplification conditions were as follows: PCR reaction conditions: pre-degeneration at 94°C for 45s, degeneration at 94°C for 10s, anneal and extentionat at 60°C for 45s, then followed a total of 40 cycles. 3 repeated wells were established in each sample. The research was carried out for three times. miR-140-5p used U6 as an internal parameter, TLR4 used GAPDH as internal parameter and $2^{-\triangle\triangle ct}$ was used to analyze the data. More details are shown in Table 1 for primer sequence.

Factors	Upstream primers	Downstream primers
miR-140-5p	5'-CAGTGGTTTTACCCTAT-GGTAG-3'	5'-ACCATAGGGTAAAAC-CACTGTT-3'
U6	5'-GCTTCGGCAGCA-CATATACTAAAAT-3'	5'-CGCTTCACGAATTTGC-GTGTCAT-3'
TLR4	5'-AGTCAGAATGAGGACT-GGGTGAG-3'	5'-GTAGTGAAGGCAGAG-GTGAAAGC-3'
GAPDH	5'-CATGTTCGTCATGGGT-GTGAAC-3'	5'-CAGTCTTCTGGGTGG-CAGTGAT-3'

Table 1: Primers sequence table.

Detection of western blot

Cerebral ischemia tissue suspension and cultured Ht22 cells were collected. The total protein was extracted by RIPA lysis. Protein concentration was detected by BCA. The protein concentration was adjusted to 4μg/μL, isolated by 12% SDS-PAGE electrophoresis, transferred membrane to PVDF membrane after ionization, dyed in ponceau working solution, soaked with PBST for 5min, washed, and sealed with 5% skim milk powder for 2h. Then, Bax (1:500), Caspase-3 (1: 500), Bcl-2 (1: 500), TLR4 (1:500) and GAPDH primary antibody (1: 500) were added and blocked overnight at 4°C.

The first antibody was extracted by washing the film, and HRP-conjugated goat anti-rabbit second antibody (1:1000) was added, incubated at 37°C for 1h, and cleansed three times with PBS for 5 min each time. The excess liquid on the film was dried with a filter paper, and the ECL was illuminated and developed.

Determination of inflammatory-related factors and oxidative stress-related factors

Brain tissue suspensions were collected, and the expressions of IL-6, IL-10 and TNF-α were detected according to the ELISA kit instructions. The expression of superoxide dismutase (SOD) and malondialdehyde (MDA) in tissues was detected by electrochemiluminescence immunoassay.

Detection of apoptosis by flow cytometry

The transfected cells were digested with 0.25% trypsin and washed twice with PBS after digestion. 100μL of binding buffer was added to prepare into 1*106 /mL suspension.

AnnexinV-FITC and PI were successively added and incubated at room temperature and in the dark for 5min. FC500MCL flow cytometer system was used for determination. The experiment was repeated for three times to take the average.

Statistical methods

In this study, SPSS19.0 was used for statistical analysis of the collected data. The desired images was drew by GraphPad 6.

Independent t-test was applied for comparison between groups. Single-factor analysis of variance was applied to compare among multiple groups. LSD-t-test was applied for pairwise comparison after the event. When P<0.05, there was a statistical difference.

Results

miR-140-5p and TLR4 expressions in various rat brain tissues and cells

Compared with rats in the BCG and SOG, miR-140-5p was markedly down-regulated in rats in IG and MG, and TLR4 mRNA expression was markedly up-regulated, while miR-140-5p expression was markedly up-regulated and TLR4 mRNA expression was markedly down-regulated in rats brain tissue in IMAG (p<0.05).

Compared with ht22 cells in BCG, miR-140-5p expression in OGD/R group and miR-NC cells was markedly down-regulated, and TLR4 mRNA expression was markedly up-regulated. Meanwhile, miR-140-5p expression in OMG cells was markedly up-regulated, and TLR4 mRNA expression was markedly down-regulated (P<0.05). miR-140-5p and TLR4 mRNA expression were negatively correlated in myocardial tissue (r=-0.689, P=0.004). Figure 1.

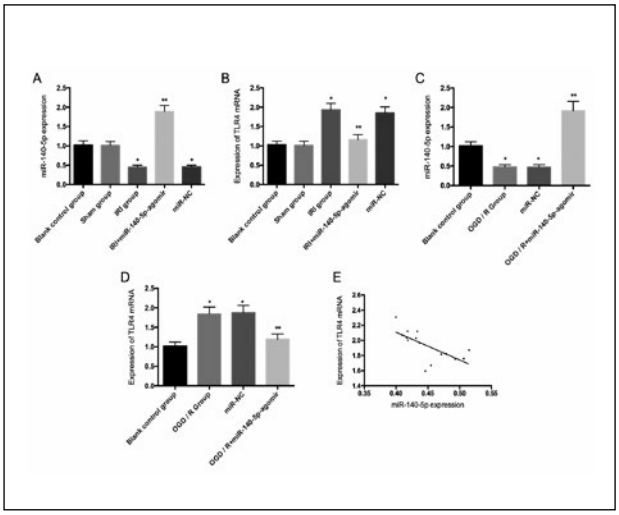

Figure 1: miR-140-5p and TLR4 expressions.
*Figure A: Expression of miR-140-5p in rat brain tissue; Figure B: Expression of TLR4 mRNA in rat brain tissue; Figure C: Expression of miR-140-5p in Ht22 cells; Figure D: Expression of TLR4 mRNA in Ht22 cells; Figure E: Correlation analysis of miR-140-5p and TLR4 expression. *, **compared with Control Group and Sham Group, P<0.05; *compared with **, P<0.05.*

Over-expression of miR-140-5p reduces cerebral infarction area and neurological defect in IRI rats

Compared with BCG and SOG, rats in IG and MG have markedly increased cerebral infarction area (P<0.05) and neurological deficit score (P<0.05), but compared with rats in IG, rats in IMAG have markedly decreased cerebral infarction area and neurological deficit score (P<0.05). Figure 2.

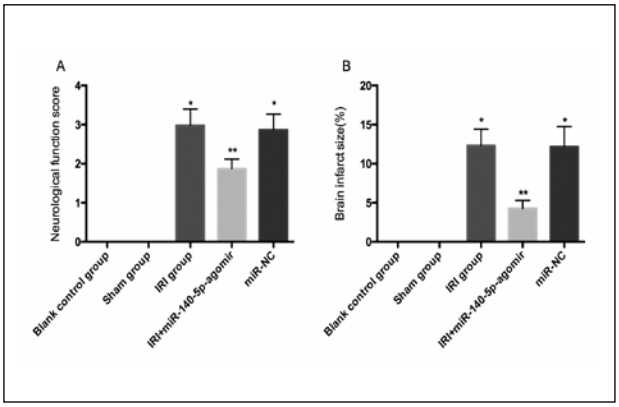

Figure 2: Effect of miR-140-5p on cerebral infarction and neurological function in IRI rats.
*Figure A: Effect of miR-140-5p on neurological deficits in IRI rats; Figure B: Effect of miR-140-5p on cerebral infarction area in IRI rats. *, **compared with Control Group and Sham Group, P<0.05; *compared with **, P<0.05.*

Effects of miR-140-5p over-expression on inflammation and oxidative stress reaction in brain tissue of IRI rats

Compared with BCG and SOG, the expressions of IL-6, TNF-α and MDA activity in brain tissues of rats in IG and MG were markedly increased, and the expressions of IL-10 and SOD activity were markedly decreased (P<0.05). However, compared with rats in IG, the expressions of IL-6, TNF-α and MDA activity of rats in IMAG were markedly reduced, and IL-10 expression and SOD activity were markedly increased (P<0.05). Figure 3.

Effect of miR-140-5p on OGD /R nerve cells

For purpose of further evaluating the protective effect of miR-140-5p on cerebral I/R, we established an OGD /R model in Ht22 cells to simulate cerebral ischemia-reperfusion.

The results demonstrated that compared with the BCG, the apoptosis rate of OGD /R group and MG increased markedly, the pro-apoptotic proteins Bax and Caspase-3 increased markedly, and the anti-apoptotic protein Bcl-2 decreased markedly (P<0.05). Compared with the cells of OGD /R group and MG, the apoptosis rate of miR-140-5p-agomir

group decreased markedly. The pro-apoptotic protein Bax was markedly down-regulated and the anti-apoptotic protein Bcl-2 was markedly up-regulated (P<0.05). Figure 4.

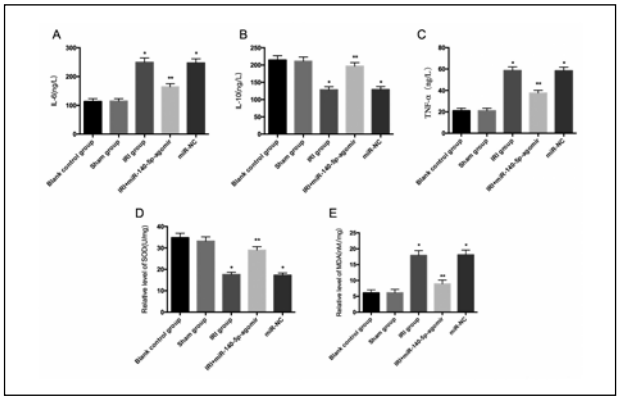

Figure 3: Effect of miR-140-5p on brain inflammatory response and oxidative stress response in IRI rats.
*Figure A: Effect of miR-140-5p on IL-6 expression in brain tissue of IRI rats; Figure B: Effect of miR-140-5p on IL-10 expression in brain tissue of IRI rats; Figure C: Effect of miR-140-5p on TNF-α expression in brain tissue of IRI rats; Figure D: Effect of miR-140-5p on SOD activity in brain tissue of IRI rats; Figure E: Effect of miR-140-5p on MDA activity in brain tissue of IRI rats. *, **compared with Control Group and Sham Group, P<0.05; *compared with **, P<0.05.*

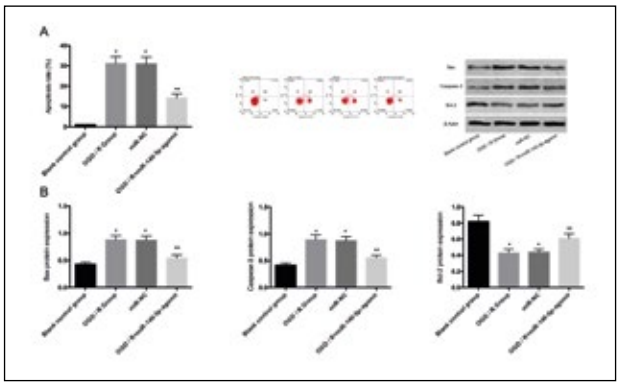

Figure 4: Effect of miR-140-5p on OGD /R nerve cells.
*Figure A: Effect of miR-140-5p on neuronal apoptosis rate; Figure B: Effect of miR-140-5p on apoptosis-related proteins in neuronal cells. *, ** compared with Control Group, P<0.05; *compared with **, P<0.05.*

Influence of inhibition of TLR4 expression on OGD/R nerve cells

We concluded that TLR4 and miR-140-5p expressions was negatively correlated in cerebral ischemia tissues and OGD /R nerve cells, so Si-TLR4 was transfected into OGD /R nerve cells.

The results demonstrated that compared with the BCG, the apoptosis rate of OGD /R group and SNG was markedly increased, the pro-apoptotic proteins Bax and Caspase-3 were markedly up-regulated, and the anti-apoptotic protein Bcl-2 was markedly down-regulated (P<0.05). Compared with OGD /R group and SNG, the apoptosis rate of STG was markedly reduced, the pro-apoptotic protein Bax was markedly down-regulated and the anti-apoptotic protein Bcl-2 was markedly up-regulated (P<0.05). Figure 5.

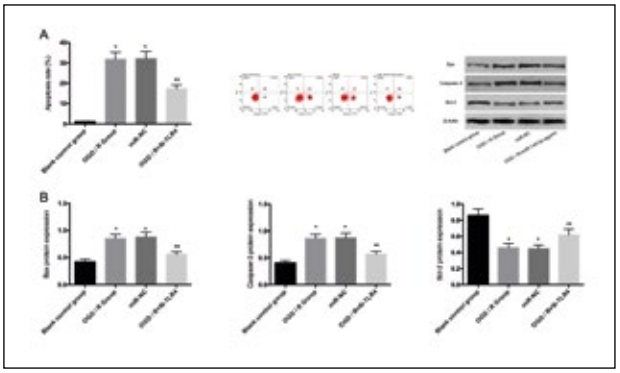

Figure 5: Effect of TLR4 expression on OGD /R nerve cells.
*Figure A: Effect of TLR4 on neuronal apoptosis rate; Figure B: Effect of TLR4 on apoptosis-related proteins in neuronal cells. *, **compared with Control Group, P<0.05; *compared with **, P<0.05.*

Double fluorescein reporter enzyme

In order to confirm the targeting connection between miR-140-5p and TLR4, firstly, the downstream target gene of miR-140-5p was foreseen by Targetscan7.2, and the targeted binding site between TLR4 and miR-140-5p was found. Therefore, we conducted double luciferase activity detection and the consequences indicated that the activity of TLR4-3'UT Wt luciferase was markedly reduced after miR-140-5pover-expression (p<0.05), but it had no effect on the activity of TLR4-3'UTR Mut luciferase (p>0.05). WB detection found that the expression of TLR4 protein in ht22 cells was markedly reduced after transfection of miR-140-5p-agomir (P<0.05). More details are shown in Figure 6.

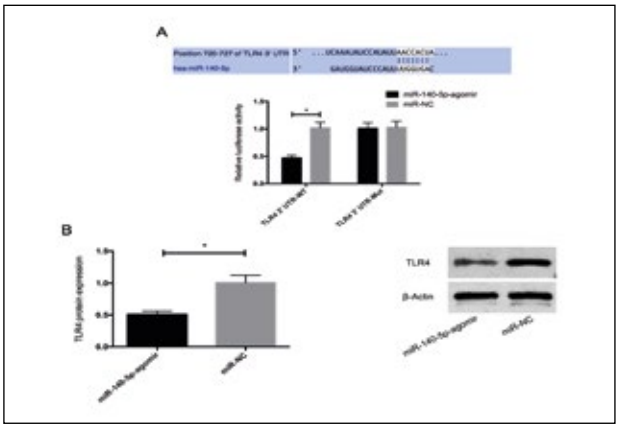

Figure 6: Double fluorescein reporter enzyme.
*Figure A: Effect of miR-140-5p on TLR4 bifluorescin activity; Figure B: Effect of miR-140-5p on TLR4 protein. *indicates P<0.05.*

Discussion

Cerebral IRI is a complicated and serious complication of cerebrovascular diseases. It not only involves the development of inflammation, but also involves a series of pathological processes such as mitochondrial function and impaired metabolism ability, which can lead to the loss of neurons and the formation of brain injury[17]. However, there is currently no effective treatment method for the development of cerebral IRI clinically[18], so it is of great clinical significance to find possible effective treatment methods by exploring the mechanism of cerebral IRI development. In recent years, research[19] demonstrated that miRNA plays an vital role in regulating neuronal apoptosis, and miRNA also plays an important role in the development of cerebral IRI.

miR-140-5p has been reported to play the role of tumor suppressor gene in the past[20], but in recent years there have also been studies[21] pointing out that miR-140-5p has a protective action on hypoxic-ischemic brain damage, which makes us found the potential role of miR-140-5p in cerebrovascular diseases. In our study, we concluded that miR-140-5p was in a low expression state in brain tissues of cerebral IRI rats and OGD /R cell models. When we treated miR-140-5p over-expression of cerebral IRI rats, we found that the cerebral infarction area of cerebral IRI rats was markedly reduced, and inflammatory reaction and oxidative stress reaction were markedly relieved. In vitro experiment has also proved that miR-140-5p can markedly inhibit the apoptosis of ht22 cells after OGD /R. The detection of apoptosis-related proteins showed that the expression of pro-apoptotic proteins Bax and Caspase-3 protein was markedly decreased, and the expression of anti-apoptotic protein Bcl-2 was markedly increased. This suggested that miR-140-5p can alleviate brain injury and neuronal apoptosis caused by IRI. Previous studies have proved that miR-140-5p has a inhibitory effect on the development of inflammatory reactions. For example, it is reported[22] that miR-140-5p can play a protective role in synovial injury rat gonarthritis by inactivating TLR4/Myd88 pathway.

Moreover, other research[23] directly pointed out that miR-140-5p can regulate inflammation through Smad/TGF-β pathway. All these demonstrated that miR-140-5p plays an vital role in regulating inflammation, which is consistent with our results. However, the specific mechanism of miR-140-5p to alleviate cerebral IRI injury is still unclear. TLR4, as a member of TRL family, is mainly expressed by microglia in brain[24]. Previous studies[25] have found that inhibition of TLR4 can improve hippocampus damage in hypoxic-ischemic brain damage rat models. This makes us realize the important role of TLR4 in cerebrovascular diseases, and we have found a targeted relation between miR-140-5p and TLR4 through bioinformatics prediction, which makes us guess whether miR-140-5p has a protective effect on cerebral IRI by regulating TLR4. First of all, we found that the TLR4 expression in brain tissue and OGD /R cells of cerebral IRI rat model was markedly up-regulated, and we found that the cell apoptosis rate, the pro-apoptotic proteins Bax and Caspase-3expressions, and the anti-apoptotic protein Bcl-2 expression were markedly up-regulated after inhibiting the TLR4 expressionin ht22 cells after OGD/R. In the past, many studies have proved that TLR4 plays an vital role in inflammatory response and apoptosis of neuronal cells in rats with cerebral IR injury. For example, studies[26] reported that PRDX1 can enhance inflammatory response and apoptosis of cerebral IR by activating TLR4.

Some research[27] clearly pointed out that inhibition of TLR4/NF-kB signal can improve cerebral IR of rats. This is consistent with our conclusion. Finally, in order to demonstrate the targeting relationship between miR-140-5p and TLR4, we carried out double fluorescein reporter enzyme detection. The results also proved the targeting relation between miR-140-5p and TLR4. This is also the first time that we have proved that miR-140-5p can lessen inflammatory response and brain injury in cerebral IRI rats and inhibit neuronal apoptosis by targeting TLR4.

To sum up, miR-140-5p was low expressed in cerebral IRI rats'brain tissues. It can reduce cerebral IRI of rats by targeting TLR4, improve neurological function of rats, and inhibit apoptosis of neuronal cells. It may be a possible therapy target for cerebral IRI. However, this research also has certain deficiencies. For example, whether there are other targets of miR-140-5p in improving cerebral IRI is still unknown, but we will further start basic experiments in the future to provide more data support for the mechanism of miR-140-5p in cerebral IRI.

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Corresponding Author:
Li Lijuan
Email: tianxing2002@163.com
(China)