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The decreased circular RNA hsa_circ_0072309 promotes cell apoptosis of ischemic stroke by sponging miR-100

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Abstract. – OBJECTIVE: To investigate the expression of circ_0072309 in patients with ischemic stroke (IS) and in *LIFR* humanized mice with middle cerebral artery occlusion (MCAO). Further, we explored the underlying mechanism of circ-0072309 in IS.

MATERIALS AND METHODS: The content of circ-0072309 in serum of patients with IS (n = 70) was measured by qRT-PCR, and the ROC curve was analyzed. *LIFR* humanized mice were used to measure the content of circ-0072309 in ischemic hemisphere by qRT-PCR and the protein expression of cleaved-caspase-3, cleaved-caspase-8 were detected by Western blot. After that, the expression of miR-100 in serum of patients with IS and in ischemic hemisphere of MCAO mice were detected, and then, we analyzed the correlation between the expression of circ-0072309 and miR-100. The binding sites between circ-0072309 and miR-100 were predicted by online database. We detected whether cric-0072309 bind to miR-100 by Dual-Luciferase report in bEnd2. In addition, bEnd2 was treated with oxygen-glucose deprivation (OGD) to simulate injury of cerebral vascular after cerebral ischemia. After treated with miR-100 mimic or miR-100-inhibitor, we detected the cell survival and rate of cell apoptosis, and the content of cleaved-caspase-3 and caspase-8 protein. The target mRNA of miR-100 was predicted by bioinformatics analysis and analyzed by Dual-Luciferase. After treating bEnd2 with circ-0072309 and miR-100 mimic, we analyzed the cell survival and apoptosis to identify the potential regulatory mechanism.

RESULTS: The results of qRT-PCR showed that the expression of circ-0072309 was significantly decreased while the content of miR-100 was significantly increased in the serum of IS patients and in the ischemic hemisphere of MCAO mice. There was a negative correlation between the expression of circ-0072309 and miR-100. The results of Dual-Luciferase showed that circ-0072309 could directly bind to miR-100. After treating bEnd2 with OGD, miR-100-mimic caused a decrease rate of cell survival and an increased rate of apoptosis. Dual-Luciferase showed that miR-100 regulated cell survival and apoptosis by directly binding to mTOR. By comparing treated bEnd2 with circ-0072309, co-transfected bEnd2 with circ-0072309 and miR-100 reduced cell survival and increased apoptosis.

CONCLUSIONS: According to these results, this study revealed that the circ_0072309-miR-100-mTOR regulatory axis could alleviate IS, and it may be a potential target for the treatment of IS.

Key Words:
Circ_0072309, MiR-100, mTOR, Apoptosis, IS.

Abbreviations

IS = ischemic stroke, MCAO = middle cerebral artery occlusion, OGD = oxygen-glucose deprivation, WB = Western blot, BSA = bovine serum albumin, MEM = Modified Eagle's Medium, FBS = fetal bovine serum, qRT-PCR = quantitative Reverse Transcription-Polymerase Chain Reaction, PBS = phosphate-buffered saline, miRNA = MicroRNA, ROC = receiver operating characteristic curve.

Introduction

Stroke is a common cardiovascular and cerebrovascular disease, which has the characteristics of high incidence, high mortality, and high disability rate. It is currently the third leading cause

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of death in the world, ranking second among all cardiovascular and cerebrovascular diseases[1-3].

CircRNA is an exon of endogenous non-coding RNA, mainly from protein-coding genes, which exists widely in animals, plants, and other organisms[4]. Differently from linear RNA structure, it connects 3' and 5' ends to form a complete ring structure through exon cyclization or intron cyclization, so it is more stable and conservative. CircRNA can regulate the occurrence and development of tumors[5-8], cardiovascular, cerebrovascular diseases[9-11], nervous system disease[12], etc.

Hsa_circ_0072309 is the splicing sequence of the exon of *LIFR* (chr5: 38523520-38530768). It has been found that the decreased expression of hsa-circ-0072309 in breast cancer tissue can regulate the progression of cancer[13], and Chen et al[14] have reported that hsa-circ-0072309 affects the development of renal cell carcinoma. However, the role of hsa-circ-0072309 in ischemic stroke has not been reported. MicroRNAs (miRNAs) are a series of translated single-stranded RNA molecules that negatively control target genes *via* translational inhibition or degradation of complementary mRNAs. Abnormal expression of miRNA in exact tissues may be involved in the process of some disease[15]. The classical mechanism of circRNA is that sponge miRNA, prevents miRNA from binding to target genes, thus regulating the expression of target genes.

In this study, we aimed to examine the expression and functional of circ_0072309 in the pathogenesis of IS, as well as to disclose molecular mechanisms. First, we measured the expression of circ-0072309 in the peripheral blood of patients with IS. Considering the species differences in the sequence and structure of circ-0072309, we constructed humanized *LIFR* mice. The MCAO mouse model was used to study the changed expression of circ-0072309 in the ischemic hemisphere during stroke. bEnd2 cells were treated with OGD to simulate the effect of ischemia on microvascular cells[16]. We analyzed the potential target miRNA by online circRNA database, such as circBase, circinteractome, and circRNADb, which may affect the expression of circ-0072309, and verified by Dual-Luciferase reporter genes. After the bEnd2 was treated with miR-100-mimic or miR-100 inhibitor, we observed the cell survival and apoptosis, and detected the expression of apoptosis-related proteins. Then, we verified that miR-100 regulates apoptosis by regulating the expression of p-mTOR.

Materials and Methods

Patients

From February 2013 to February 2016, 75 male patients with AIS were treated in our hospital and 90 in the control group. The inclusion criteria for AIS patients were as follows[17]: (a) male over 18 years old, (b) according to the patient's history, laboratory and neurological examination, computed tomography (CT), magnetic resonance imaging (MRI) and/or diffusion-weighted imaging (DWI) diagnosed as IS; (c) symptoms within 24 hours after admission. The criteria for excluding AIS patients were as follows: (a) cerebral hemorrhage; (b) severe infection, inflammation or autoimmune diseases; (c) hematological malignant tumor or solid tumor history; (d) died within 24 hours after admission; (e) received immunosuppressive therapy within 6 months. The control group was screened from the high-risk group of strokes and defined as at least three risk factors of IS, such as, hypertension, atrial fibrillation or valvular disease, smoking, hyperlipidemia, diabetes, lack of physical exercise, overweight or obesity, and family history of stroke.

This study was approved by the Ethics Committee of our hospital and carried out in accordance with the Declaration of Helsinki. All participants or their guardians signed an informed consent form before registration.

Animal Model

The *LIFR* humanized mice were obtained from the Shanghai Model Organisms Center, Inc. (Shanghai, China). The humanized mice male mice (weight about 25 g, 8 weeks) were randomly divided into sham group or MCAO groups. Then, permanent focal cerebral ischemia was produced by endovascular occlusion of the left middle cerebral artery occlusion (MCAO) as previously described. Sham-operated mice were subjected to the same surgery, but not occlude MCAO. Mice were raised with controlled temperature (22 ± 3°C) and humidity (60% ± 5%) under a 12 h light/dark cycle and were allowed water and food *ad libitum*[18]. This study was approved by the Local Ethics Committee of our hospital.

Cell Culture

The mouse brain microvascular endothelial cell line bEnd.3 was purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). Oxygen-glucose deprivation (OGD) injuries were used to mimic post-stroke ischemic

damage as previously described. In brief, bEnd.3 cells were cultured in glucose-free Dulbecco's Modified Eagle's Medium (DMEM) and incubated in a 37°C humidification chamber containing 5% CO_2 and 95% N_2 for 6 h.

CCK-8 Assay

Cell Counting Kit-8 (CCK-8, Dojindo Molecular Technologies, Kumamoto, Japan) was used to evaluate cytotoxicity as previously described. In brief, cell suspensions (1×10^4 cells, 100 µl/well) were seeded in a 96-well plate. The determination of CCK-8 was as follows: the 10 µl reagent was added to 100 µl of the DMEM medium in each well and co-cultured in darkness at 37°C for 2 hours. The 96-well plate was placed at an absorbance of 450 nm. The whole experiment was repeated three times.

Apoptosis Assay

The cells apoptotic rates were measured by Annexin V-FITC/PI apoptosis detection kit (Beijing Biosea Biotechnology, China) combined with flow cytometer (Beckman Coulter, Brea, CA, USA). bEnd2 cells inserted into the 6-well plate with 10^5 cells/well density and cultured at 37°C for 24 h, were washed and resuspended with PBS. 5 µl Annexin V-FITC were added to the cell suspension, which was gently mixed at 2-8°C and then incubated for 15 minutes in the dark. After adding 10 µl Pl, it was gently mix well and incubated for 5 minutes under the condition of 2-8°C in the dark. The data were analyzed depending on FlowJo software (Stanford University, Palo Alto, CA, USA)[19].

RNA Extraction and Real-time Quantitative PCR Assays

Total RNAs from serum of male patients with IS and ischemic hemisphere of mice were obtained by using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) and quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR) was performed using PrimeScript™ RT reagent Kit (TaKaRa, Dalian, China), according to the manufacturer's protocol. The levels of mRNA expression were quantified by standard Real Tme-PCR protocol with SYBR Premix Ex Taq (TaKaRa, Dalian, China). Reactions and signal detection were measured using a Real-time PCR system (Bio-Rad. Hercules, CA, USA). β-actin/U6 was used as a reference gene. The gene specific primers were as Table I.

Luciferase Activity

We inserted the full-length of human circ-0072309 into phBAD (HanBio, Shanghai, China) vector. bEnd2 were seeded in a 24-well plate. After 24 hours, miR-100 mimics or miR-NC were co-transfected with circ-0072309-wt (mTOR-wt) or circ-0072309-mut (mTOR-mut) into bEnd2 cells using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). After transfecting 48 h, Dual-Luciferase reporter assay system (Promega, Madison, WI, USA) was applied according to the manufacturer's instructions.

Western Blot

The total cell lysate was prepared by destroying cells with RIPA buffer containing protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific, Waltham, MA, USA) and centrifuged with 12000 rpm at 4°C for 10 mins. BCA protein analysis reagent (Thermo Fisher Scientific, Waltham, MA, USA) was used to detect the concentration of protein. The same amount of different protein samples was separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The polyethylene difluoride (PVDF) film was sealed with 0.1% Tris-Buffered Saline and Tween-20 (TBST) containing 5% skim milk powder for 1 h. Next, the different protein blots were incubated with the primary antibodies overnight at 4°C and

Table I. Primers for selected genes.

Gene name	Primers	
	Sense	Antisense
circ_0072309	TCCACACCGCTCAAATGTTA	ATCCAGGATGGTCGTTTCAA
miRNA-100	GAGCCAACCCGTAGATCCGA	GTGCAGGGTCCGAGGT
β-actin	CTCCATCCTGGCCTCGCTGT	CTCCATCCTGGCCTCGCTGT
U6	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT

subsequently with secondary antibodies at room temperature over 2 h. The primary antibodies were as follows: Anti-p-m-TOR (Abcam, Cambridge, MA, USA) diluted at 1:1000, anti-cleaved-caspase 3 (Abcam, Cambridge, MA, USA) and anti-cleaved-caspase 8 (Abcam, Cambridge, MA, USA) diluted at 1:800, anti-β-actin (Abcam, Cambridge, MA, USA) diluted at 1:2000. Horseradish peroxidase (HRP) coupled with secondary antibody (Abcam, Cambridge, MA, USA; dilution at 1:10000) was used for the detection of primary antibody. ECL chemiluminescence system (Thermo Fisher Scientific, Waltham MA, USA) was used to visualize the binding antibodies[20].

Statistical Analysis

The values were expressed as the mean ± standard deviation (SD). SPSS 17.0 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 5.0 (San Diego, CA, USA) were used for statistical assay. The significance between groups was analyzed by Student's *t*-test. p-value<0.05 was considered statistically significant.

Results

Comparison of Circ_0072309 Expression Between Patients with AIS and Controls

Firstly, we detected the expression of circ-0072309 in peripheral blood of male IS group (n = 90) and in control group (n = 50) by qRT-PCR. The expression of circ_0072309 in IS patients was significantly lower than that in the control group (Figure 1). ROC curve displayed that circ-0072309 had a nice predictive value for IS risk [AUC = 0.9505 (95% CI: 0.9187-0.9823)] (Figure 2B), with the best cutoff point value of 0.329, where the AUC reached the maximum value, and the sensitivity as well as specificity at best cutoff point was 98.23% and 91.87%, respectively.

Circ_0072309 Can Regulate Cell Apoptosis In Vivo of MCAO Mice

In order to further study the effect of circ-0072309, we constructed humanized *LIFR* mice to product hsa-circ-0072309 and employed MCAO model to mimic IS. Firstly, we detected the expression of circ-0072309 in the Sham group and MCAO by qRT-PCR. The expression of circ-0072309 in Sham group was significantly higher than IS group (Figure 2A). The total proteins were extracted from the ischemic hemisphere from MCAO mice and sham mice using the TRIzol reagent. The relative protein expression levels cleaved caspase-3, and cleaved caspase-8 in MCAO group was lower than that in Sham (Figure 2B, 2C). These data implied that circ-0072309 might alleviate the risk of apoptosis in IS.

The Expression of MiR-100 in Serum of Patients and Mice

A novel mechanism has been found in which cross-connection between circRNAs and mRNAs exist by competing for shared microRNAs (MiRNAs) response elements. So, we predicted the target miRNA of circ-007209 by online data, such as circBase, circinteractome, and circRNADb. Chen et al[14] have shown that circ-0072309 can regulate tumor progression through miR-100. The

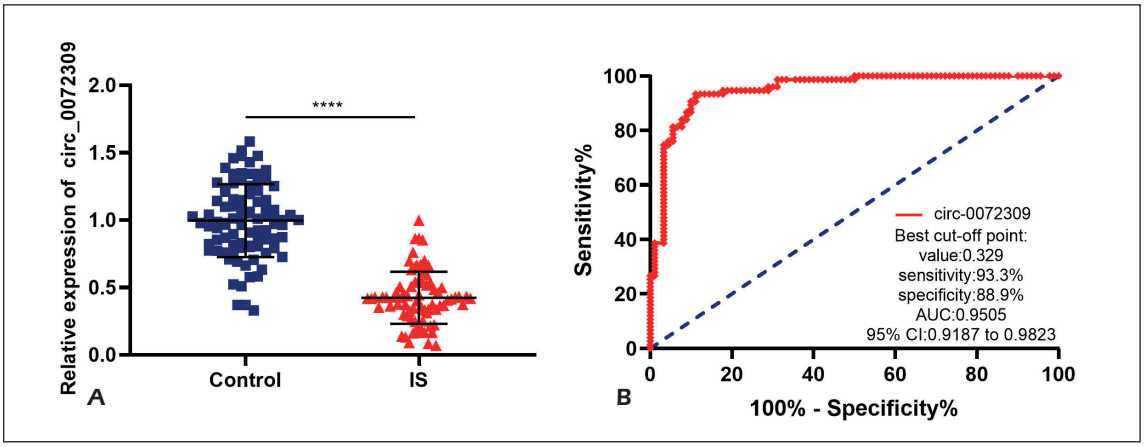

Figure 1. The expression of circ_0072309 in Serum of patients with IS. **A**, The relative expression of circ_0072309 in peripheral blood of male IS patients (n = 90) and in control group (n = 75). The relative expression levels were normalized to the mean value of control group. ****p<0.0001. **B**, Predictive value of circ_0072309 for IS risk evaluated by ROC curve.

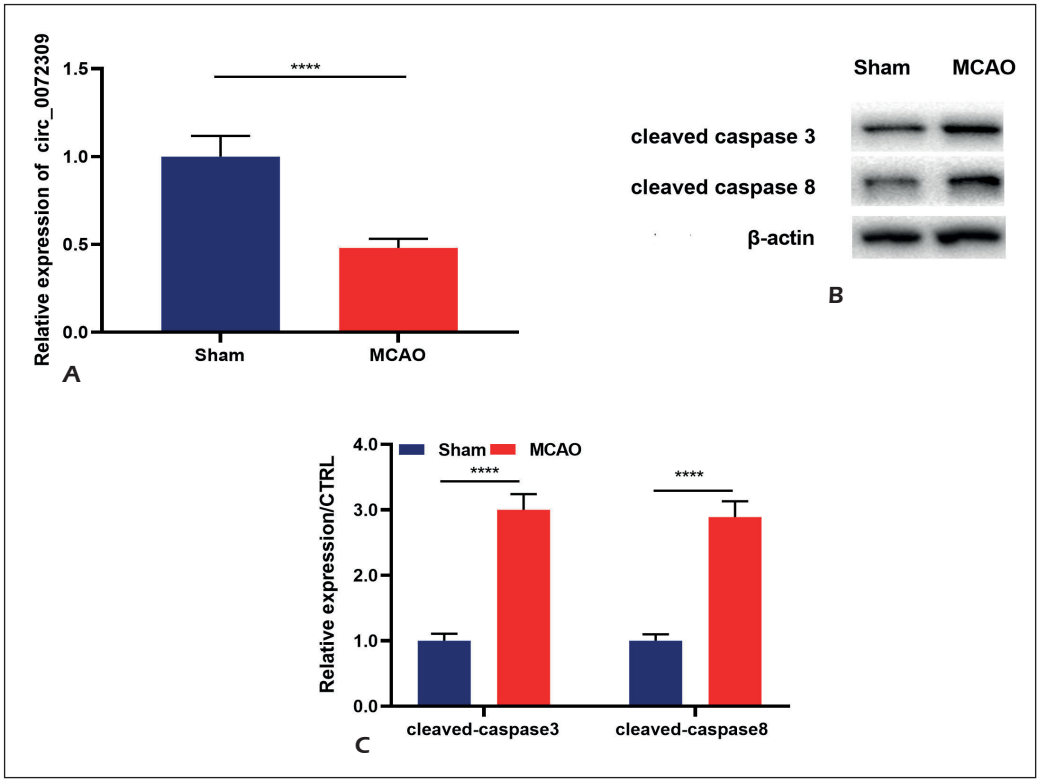

Figure 2. The circ_0072309 can regulate cell apoptosis in vivo of MCAO mice. The IS model of humanized were established by MCAO operation. Mice were divided into sham control (n=10) and MCAO groups (n=10). **A**, The relative expression of circ_0072309 in Sham (n=10) and MCAO (n=10). ****p<0.0001. **B-C**, The relative protein expression levels cleaved caspase-3 and cleaved caspase-8 in MCAO group were lower than that in Sham group. ****p<0.0001.

expressions of miR-100 in peripheral blood of IS group (n = 90) and in control group (n = 50) were detected by qRT-PCR. The expression of miR-100 in IS group was increased in comparison with that in the control group (Figure 3A). There was a significant negative correlation between the expression of circ-0072309 and miR-100 in patients with IS (Figure 3B). In addition, the expression of miR-100 in MCAO group was higher than that in Sham (Figure 3C). Those results imply that the expression of circ_0072309 was related with miR-100.

Circ-0072309 Can Promote Cell Apoptosis by MiR-100

To further study the role of circ_0072309 and miR-100 in IS, we used the mouse brain microvascular endothelial cell line bEnd.3 (ATCC, Manassas, VA, USA) to evaluate threaten of IS *in vitro*. Firstly, we predicted binding sites of circ-0072309 and miR-100 (Figure 4A). Then, we conducted a Luciferase reporter assay. Compared with other groups, co-transfection with miR-100 mimic and circ-0072309-WT significantly decreased the Luciferase activity in bEnd2 (Figure 4B). In addition, Oxygen-glucose deprivation (OGD) injuries were used to mimic post-stroke ischemic damage[16]. When bEnd2 treated with miR-100 mimic, the cell viability was lower than other groups (Figure 4C), and the ratio of cell apoptosis was higher than other groups (Figure 4D). The relative protein expression levels of cleaved caspase-3 and cleaved caspase-8 were increased in bEnd2 treated with miR-100-mimic (Figure 4E). The above results suggest that the increased expression of miR-100 would aggravate the ratio cell of apoptosis in the occurrence of IS.

MiR-100 Can Regulate the mTOR Signal

To investigate the regulation of apoptosis by miR-100, an online target prediction algorithm TargetScan bioinformatics analysis was performed to find the target mRNA. The result showed that miR-100 and mTOR had complementary binding sites (Figure 5A). So, we conducted a Luciferase reporter assay. Compared with other groups, co-transfection with miR-100 mimic and wt-

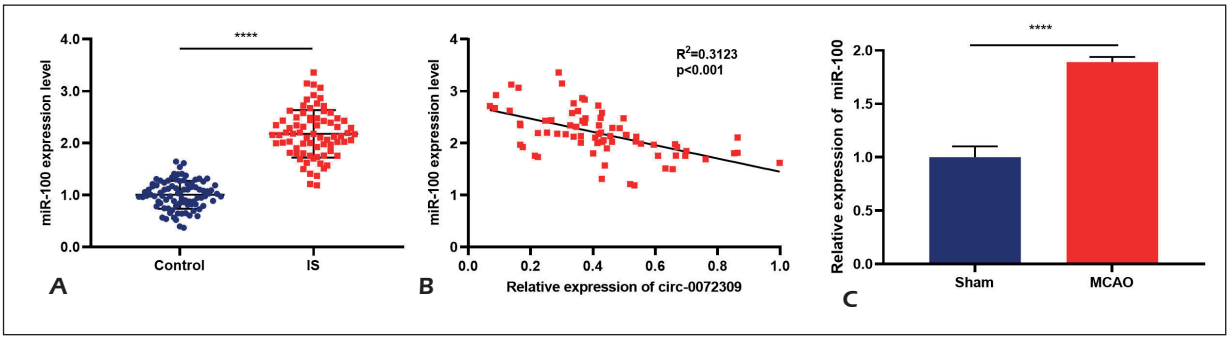

Figure 3. The expression of miR-100 in Serum of patients and mice. **A**, The relative expression of miR-100 in peripheral blood of male IS patients (n = 90) and in control group (n = 75). The relative expression levels were normalized to the mean value of control group. ****p<0.0001. **B**, The negative correlation between the relative expression between the miR-100 and the circ-0072309 in serum of IS patients. **C**, The relative expression of miR-100 in Sham group and MCAO group was detected by qRT-PCR (****p<0.0001).

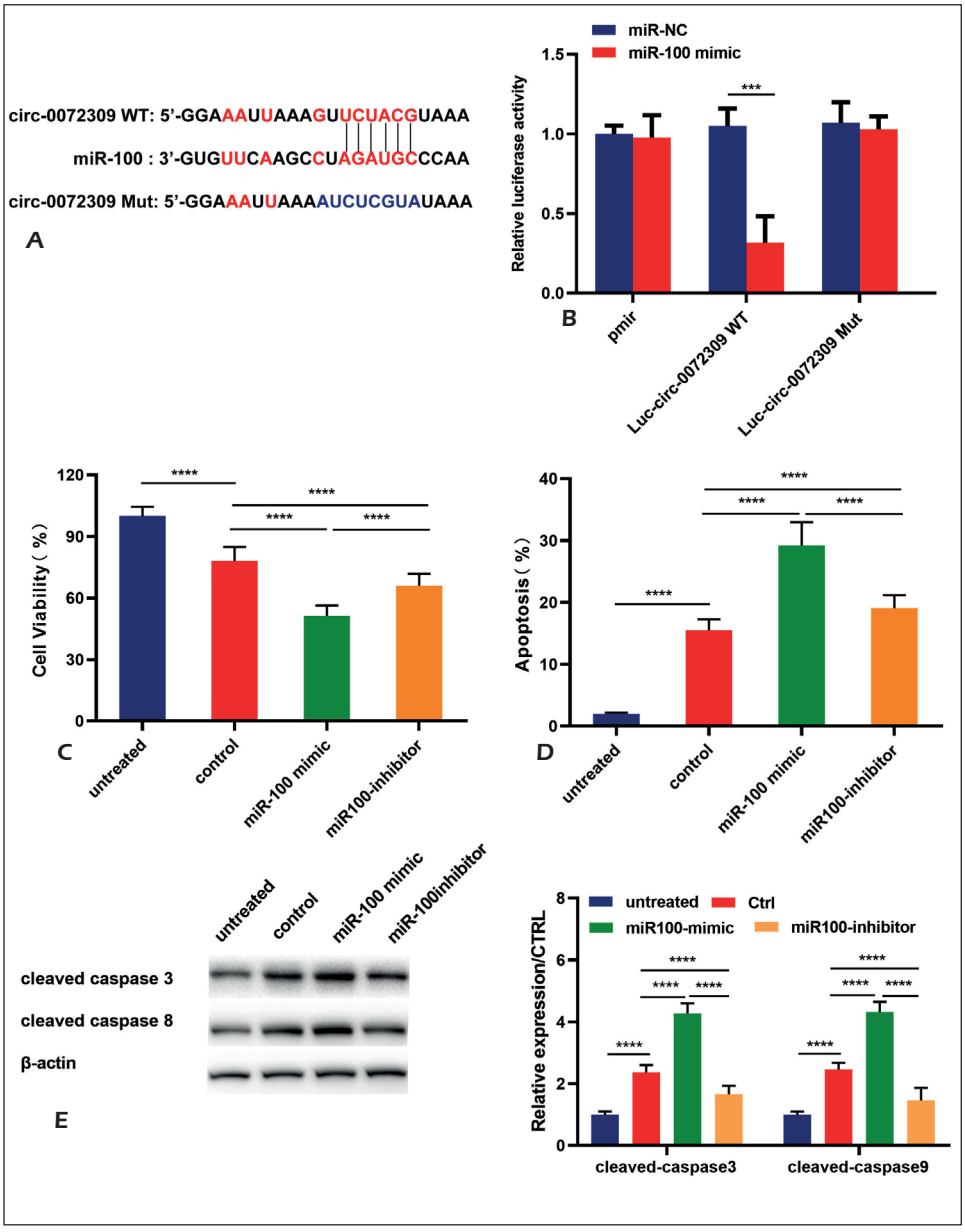

Figure 4. MiR-100 can promote cell apoptosis. **A**, The predicted binding sites between circ_0072309 and miRNA-100. **B**, The Luciferase reporter assay. Co-transfection with miR-100 mimic and circ-0072309-Wt significantly decreased the luciferase activity in bEnd2 cells compared with others group. ***p<0.001. **C**, The cell viability of bEnd2 treated with miR-100 mimic was lower than other groups. ****p<0.0001. **D**, The ratio of cell apoptosis of bEnd2 treated with miR-100 mimic was higher than other groups. ****p<0.0001. **E**, The relative protein expression levels cleaved caspase-3 and cleaved caspase-8 in bEnd2 with miR-100 mimic and miR-100 inhibitor. ***p<0.001, ****p<0.0001.

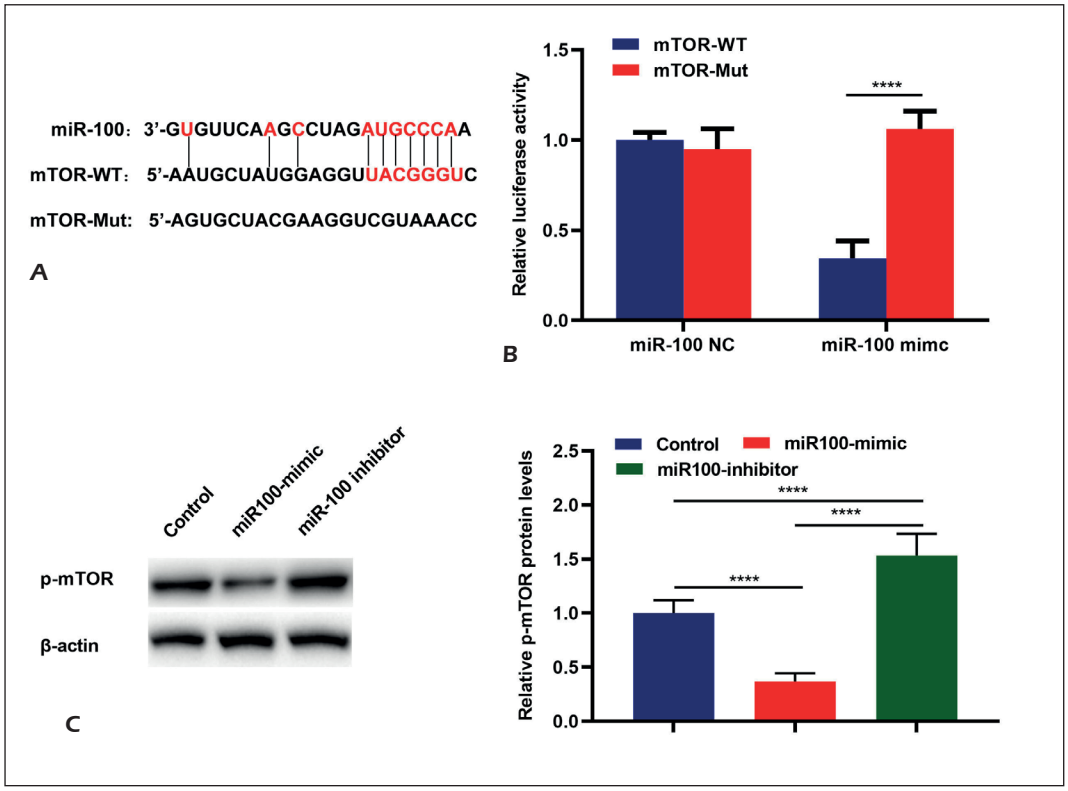

Figure 5. The miR-100 can regulate the mTOR signal. **A**, The predicted binding sites between mTOR and miRNA-100. **B**, The Luciferase reporter assay. Co-transfection with miR-100 mimic and mTOR-Wt significantly decreased the luciferase activity of bEnd2 cells compared with others group. ****p<0.0001. **C**, The relative protein expression levels p-mTOR in bEnd2 with miR-100 mimic and miR-100 inhibitor. ****p<0.0001.

mTOR significantly decreased the Luciferase activity in BMSCs (Figure 5B).

We also detected the relative protein expression levels of p-mTOR in bEnd2 treated with miR-100 mimic and miR-100 inhibitor and we found the miR-100 mimic can inhibit the expression of p-mTOR (Figure 5C).

Circ_0072309 Can Inhibit Cell Apoptosis by MiR-100/mTOR Axis

In order to further clarify the regulatory relationship between miR-100 and mTOR by circ-0072309, we transfected bEnd2 with circ-0072309, and then, added miR-100 mimic to observe the cell viability, the ratio of cell apoptosis and the protein expression of p-mTOR. The cell viability of bEnd2 treated with circ-0072309 was higher than control groups and miR-100 can alleviate the effect of the circ-0072309 (Figure 6A). The apoptosis of bEnd2 treated with circ-0072309 was lower than control groups and miR-100 can alleviate the effect of circ-0072309 (Figure 6B). The protein expression levels of p-mTOR were significantly increased treated with circ_0072309 and the tendency could be recovered by miR-100 mimic in bEnd2 cells. The protein expression of cleaved caspase-3 and cleaved caspase-8 had the opposed tendency (Figure 6C, 6D).

Discussion

Ischemic brain damage caused by sudden cardiac arrest or stroke remains an important cause of human morbidity as they are the leading cause of morbidity and mortality in the world. In order to better intervene and reduce the harm of stroke, it is very important to understand the mechanism of cell injury after stroke[21,22].

Apoptosis plays an important role in the pathogenesis of ischemic stroke. Between the two most common apoptosis pathways, mitochondrial-mediated apoptosis plays an important role in apoptosis after ischemic stroke[23,24]. Caspase-9 and caspase-3 can regulate by mTOR signal, which eventually leads to apoptosis. Many studies[25,26] have focused on the role of circRNA in cardio-cerebrovascular diseases.

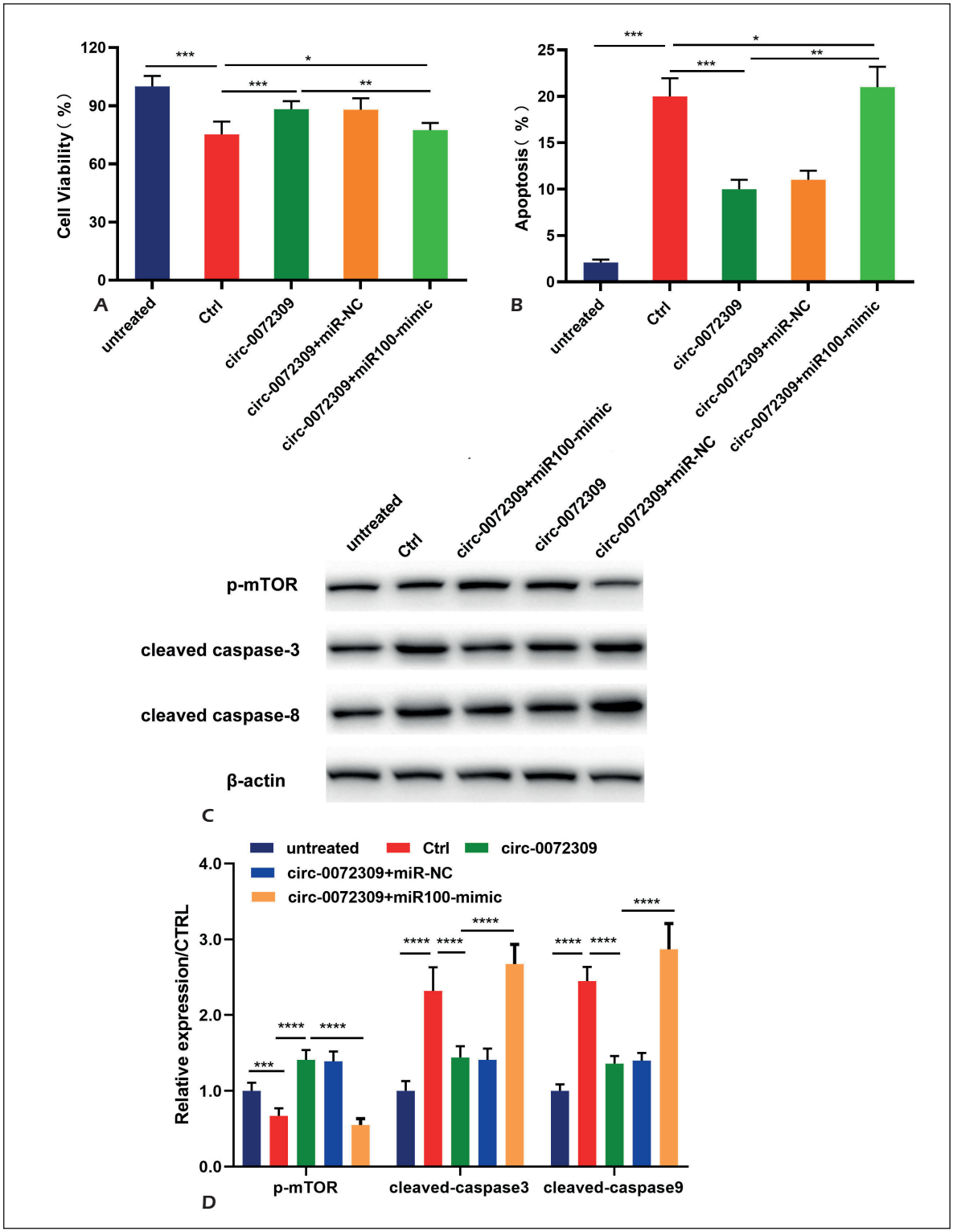

Figure 6. The circ_0072309 can inhibit cell apoptosis by miR-100/mTOR axis. **A**, The cell viability of bEnd2 treated with circ-0072309 was higher than control groups and the miR-100 can alleviate the effect of the circ-0072309. *p<0.05, **p<0.01, ***p<0.001. **B**, The apoptosis of bEnd2 treated with circ-0072309 was lower than control groups and the miR-100 can alleviate the effect of the circ-0072309. *p<0.05, **p<0.01, ***p<0.001. **C-D**, The protein expression levels of p-mTOR were significantly increased treated with circ_0072309 and the tendency could be recovered by miR-100 mimic in bEnd2 cells. The protein expression of cleaved caspase-3 and cleaved caspase-8 had the opposed tendency. ****p<0.0001.

The classical mechanism of circRNA is that sponge miRNA, prevents miRNA from binding to target genes, thus regulating the expression of target genes[27]. In this study, we investigated the changes of expression circ-0072309 in serum of IS and found out the target miRNA, which can regulate its expression. In addition, we verified the pathway that affects apoptosis during the progress of IS.

Previously, Yan et al[13] found that hsa_circ_0072309 regulated breast cancer cell proliferation, migration, and invasion by inhibiting miR-492. Chen et al[14] found hsa-circ-0072309 regulate the progress of renal cancer by targeting miR-100 to block the PI3K/AKT and mTOR signal. In addition, the lower expression of miR-100 is related to poorer OS in patients with solid tumor[28], and transfected four GBM lines (U87, U251, 22T, and 33T) with miR-100 can reduce cell proliferation[29].

In our research, firstly we examined the expression of circ-0072309 in peripheral blood of patients with IS by qRT-PCR and found that ROC curve suggested that circ-0072309 was a good indicator for diagnosis. Then, we used MCAO to treat humanized *LIFR* mice and detected the expression of circ-0072309 and apoptosis-related protein in the ischemic hemisphere. The results showed that the expression of cleaved caspase-3, caspase-8, and apoptosis-related protein increased while the expression of circ-0072309 decreased. After that, we predicted that miR-100 might regulate the expression of circ-0072309, and the results of Dual-Luciferase reporter genes indicated that miR-100 could directly bind to circ-0072309 and regulate cell survival and apoptosis. Finally, we found that miR-100 can regulate cell survival and apoptosis by affecting the expression of p-mTOR. So far, we found that when IS occurred, the expression of circ0072309, miR-100, and p-mTOR would decrease to cause upregulation of cleaved caspase-3 and cleaved caspase-8.

Conclusions

Our findings suggested that the expression of circ_0072309 was decreased in patients with IS. Moreover, our results firstly revealed that circ_0072309 could alleviate the risk of IS by inhibiting the expression miR-100. The present results elucidate a potential mechanism showing that circ_0072309 can affect cell apoptosis by sponging miR-100 to regulate the expression of p-mTOR in IS and indicate that circ_0072309 might be used as a promising prognostic marker and a potential target.

Conflict of Interests

The authors declare that they have no conflict of interest.

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