Int J Clin Exp Pathol 2018;11(12):5885-5892
www.ijcep.com /ISSN:1936-2625/IJCEP0084788

Original Article

Expression of miR-9 in the serum of patients with acute ischemic stroke and its effect on neuronal damage

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Received August 29, 2018; Accepted October 9, 2018; Epub December 1, 2018; Published December 15, 2018

Abstract: Background: This research was aimed to measure the expression of miR-9 in serum of acute ischemic stroke (AIS) patients and explore the role of miR-9 on OGD-induced neuronal damage. Methods: In the present study, we measured the expression of miR-9 in serum of 65 AIS patients by real-time quantitative PCR (RT-qPCR) and the effect of miR-9 on oxygen-glucose deprivation (OGD)-induced neuronal injury was detected by CCK-8 in vitro. Western blot was used to measure the expression of protein. Results: We found that the serum level of miR-9 in 65 AIS patients was significantly higher than that in control group (no-AIS), and was positively correlated with NIHSS score (r=0.627, P<0.001), infarct volume ((r=0.576, P<0.001), serum IL-8 (r=0.376, P=0.002), TNF-α (r=0.418, P<0.001), IL-6 (r=0.545, P<0.001), and IL-1β (r=0.592, P<0.001). miR-9 expression levels were upregulated in cultured neurons with OGD treatment. The downregulation of miR-9 significantly alleviated OGD-induced neuronal injury. Dual-luciferase reporter assay demonstrated that SIRT1 was a target gene of miR-9, and miR-9 negatively regulated SIRT 1 expression and positively regulated p65 expression. Conclusions: All in all, our data showed that downregulation of miR-9 protected neurons against OGD/R-induced injury by the SIRT1-mediated NF-kB pathway.

Keywords: miR-9, acute ischemic stroke, neuronal, inflammation

Introduction

Acute ischemic stroke (AIS) is an infarction of brain tissue caused by occlusion of cerebral arteries, accompanied by the injury of neurons, astrocytes, and oligodendrocytes. It is the most important central nervous system vascular event leading to lethality and disability in modern society. Unlike other organs in the human body, although the brain accounts for only 2 percent of human body weight, adult brain tissue oxygen consumption is 20 percent of the total systemic oxygen consumption. It can rely on little substance to provide energy, while the ability to store energy is poor. This results in poor tolerance to ischemic injury. Once ischemic injury occurs, the brain tissue will be devastated and cause great harm to the human body [1]. Therefore, it is very important to explore the pathologicprocess of cerebral ischemia.

MicroRNAs are endogenous non-coding RNAs with a length of about 20 base pairs, which can bind to the 3'UTR region of the downstream target gene to regulate downstream gene expression, thereby regulating the expression level of the corresponding protein and playing a corresponding biological role. It has been confirmed that miRNAs are widely present in humans and play a crucial regulatory role in the alteration of self-expression levels in a variety of diseases [2, 3]. At the same time, a variety of miRNAs were found in brain tissue [4, 5] and are involved in the regulation of the pathophysiological processes of central nervous system diseases [6, 7]. In recent years, with the development of biochip technology, more miRNAs and their downstream regulatory genes have been found to be involved in the development and progression of neurological diseases [6], especially ischemic stroke [7].

As one of the most widely studied microRNAs in humans, miR-9 has been shown to play an important regulatory role in Huntington's disease [8], cardiac hypertrophy [9], human neuronal differentiation [10, 11], neurogenesis [12],

Table 1. Basic clinical data in healthy group and AIS group

Variables	Control (n=55)	AIS (n=65)	T or χ^2	*P* value
Age (n/%)	62.3±7.4	65.2±6.7	1.324	0.281
Male (n/%)	28/50.9	40/61.5	1.371	0.242
Hypertension (n/%)	29/52.7	42/64.6	1.743	0.187
Diabetes (n/%)	10/18.2	16/24.6	0.727	0.394
Hyperlipidemia (n/%)	13/23.6	17/26.2	0.101	0.751
Coronary heart disease (n/%)	5/9.1	8/12.3	0.319	0.572
Atrial Fibrillation (n/%)	8/14.5	12/18.5	0.329	0.566
BMI (kg/m^2)	24.4±3.2	25.7±3.6	0.932	0.618

and various malignant tumor diseases [13]. Ji et al. [14] found that the brain-specific miR-9 was increased in the serum exosomes of acute ischemic stroke patients. However, the expression of miR-9 in serum of AIS patients and the role of miR-9 remains on OGD-induced neuronal damage remain unknown. In the present study, we found that miR-9 expression was upregulated in the serum of AIS patients, and downregulation of miR-9 protected neurons against OGD/R-induced injury via SIRT1-mediated NF-kB pathway in vitro.

Materials and methods

Patients and blood sample

65 AIS patients and 55 controls (no-AIS) in Taizhou First People's Hospital were selected for this research. AIS patients were diagnosed by brain magnetic resonance imaging or computed tomography. Two neurologists who were deputy director and above performed NIHSS scores on AIS patients within 24 hours of cerebral ischemia. Non-stroke controls were recruited from those who underwent an annual medical examination at our hospital. Clinical data are shown in **Table 1**. For the whole blood samples, they were centrifuged at 1,000×g for 10 min to collect serum, and Elisa kit was used to measure the content of IL-8 (H-EL-IL-8, ZYscience, USA), TNF-α (50R-E.1693H, BIOVALUE, AUS), IL-6 (H-EL-IL-6, ZYscience, USA), and IL-1β (50R-E.1095H, BIOVALUE, AUS).

All subjects (or their guardians) included in this study consented the research protocol and signed an informed consent form. The ethics committee of Taizhou First People's Hospital approved this research protocol.

Isolation, culture and identification of neuronal cells

The SD rats that were pregnant for two weeks were sacrificed and the suckling rats were taken out. The brain tissue of the suckling rat was taken out in pre-cooled D-Hank's solution, and the cortex on both sides was separated, and then cut into pieces, 0.25% trypsin was added, and it was digested in a cell culture incubator for 5 min. The tissue was gently dried with a pasteurized tube, and the cell suspension was prepared and allowed to stand for 3 minutes, and the supernatant cell suspension was transferred to another centrifuge tube (the above steps were repeated 3 times). The supernatant was discarded by centrifugation (800 rpm for 5 min), and the cells were resuspended in DMEM medium (12491-15, ThermoFisher, CA, USA) to which was added 10% of fetal bovine serum (10100-147, ThermoFisher, CA, USA), and cultured (37°C, 5% CO_2) in the cell culture incubator. After 4-8 hours of culture, the whole medium was changed to Neurobasal medium (21103-049, ThermoFisher, CA, USA), and then changed every half for 2 d. The neurons were cultured for 9-12 d for a later experiment.

Immunofluorescence staining with MAP2 was done to identify neuronal cells, and primary antibody was anti-MAP2 antibody (ab5392, 1:500, Abcam, UK), and secondary antibody was Goat Anti-Rabbit IgG H&L (Alexa Fluor® 488) (ab150077, 1:500, Abcam, UK). Hoechst (H3570, 1:5000, Abcam, UK) was used to stain the nucleus.

Oxygen glucose deprivation

1×10^7 neuronal cells were seeded into a 100 mm dish, and the culture medium was replaced with HEPES medium (15630106, ThermoFisher, CA, USA). Then the cells were placed in an oxygen-free incubator for 0, 0.5, 1.0, 1.5 and 2.0 hours.

Cell transfection

miR-9-NC, miR-9-mimic, and miR-9-inhibitor were synthesized by Shenggong Bioengineering Co., Ltd. (Shanghai, China), and were directly

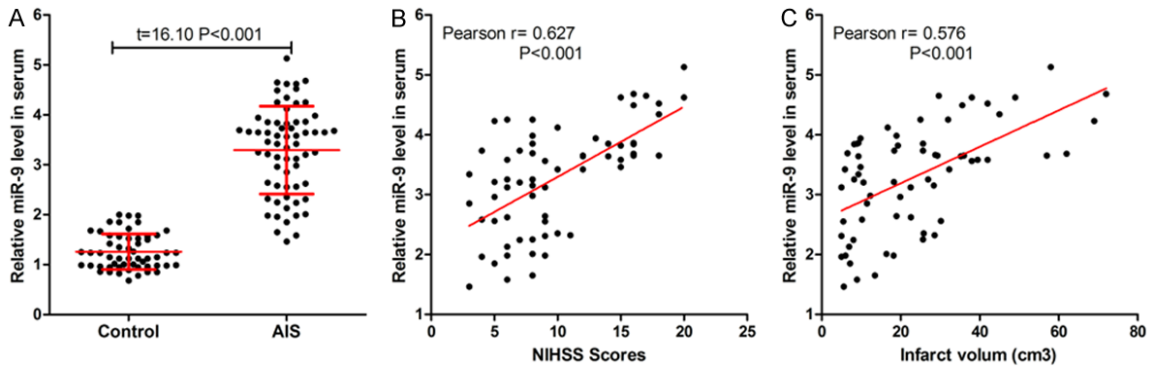

Figure 1. The expression of serum miR-9 in different groups. (A) Comparison of levels of serum miR-9 between AIS patients and non-stroke controls; (B, C) Correlations between the levels of serum miR-9 and the NIHSS score (B) or infarction volume (C) in 65 AIS patients.

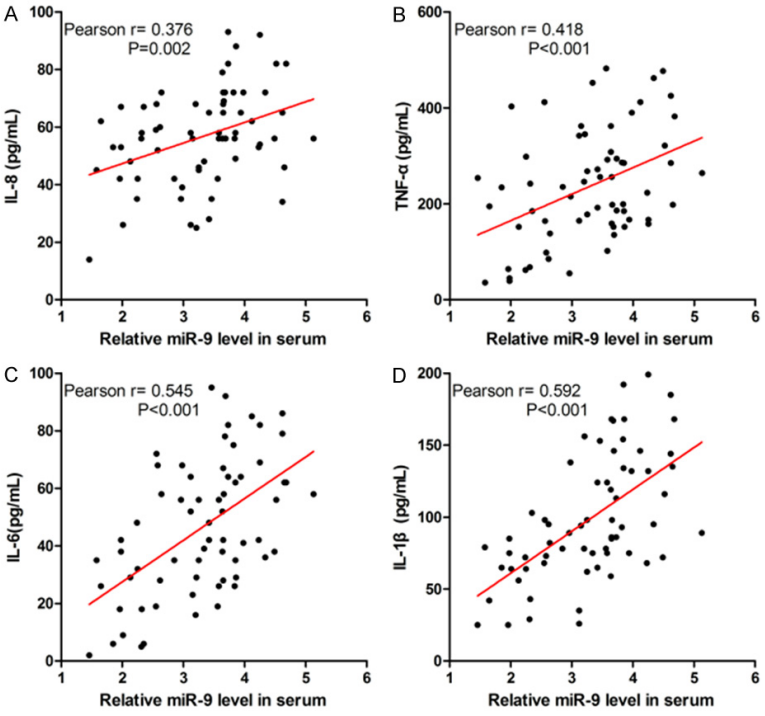

Figure 2. Correlation between serum level of miR-9 and TNF-α, IL-6, IL-8, and IL-10 in 65 AIS patients.

transferred into cells by Lipofectamine™ 2000 transfection reagent (11668019, Invitrogen, CA, USA). For wild type or mutation mRNA 3'-UTR of SIRT1, they were first connected to pisCHECK2 (Promega, WI, USA) and then transfected into cells as miRNA.

Real-time quantitative PCR

Real-time quantitative PCR (RT-qPCR) was used to measure the expression of miRNA and mRNA. For serum samples, total RNA was isolated with the miRNeasy Serum/Plasma Kit (5628, QIGEN, Dusseldorf, Germany). For cell samples, trizol was used to extract the total RNA of the tissue or cell. The extracted RNA was reverse transcribed into cDNA by using PrimeScript™RT Master Mix reverse transcription kit (RR-036B, PROMEGA, WI, USA). Then 20 μl RT-qPCR system was prepared according to the GoTaq qPCR Master Mix kit instructions (638320, TakaRa, Beijing, China) and amplified using ABI 7500 fluorescence quantitative PCR instrument (Applied Biosystems, Maryland, USA). RT-qPCR primers, miR-9-F: 5'-ACACTCCAGCTGGGTCTTTGGTTATCTAGCT-3'; miR-9-R: 5'-TGGTGTCGTGGAGTCG-3'; SIRT1-F: 5'-TGATTGGCACCGATCCTCG-3'; SIRT1-R: 5'-CCACAGCGTCATATCATCCAG-3'; GAPDH-F: 5'-TGGCCTTCCGTGTTCCTAC-3'; GAPDH-R: 5'-GAGTTGCTGTTGAAGTCGCA-3'; U6-F: 5'-CTCGCTTCGGCAGCACA-3'; U6-R: 5'-AACGCTTCACGAATTTGCGT-3'.

Western blot

Cell lysates were separated by SDS-page and then transferred to PVDF membrane. Primary antibody was selected as follows: anti-SIRT1 (ab110304, 1:1000), anti-p65 (ab16502, 1:1000), or anti-GAPDH (ab9484, 1:3000). Se-

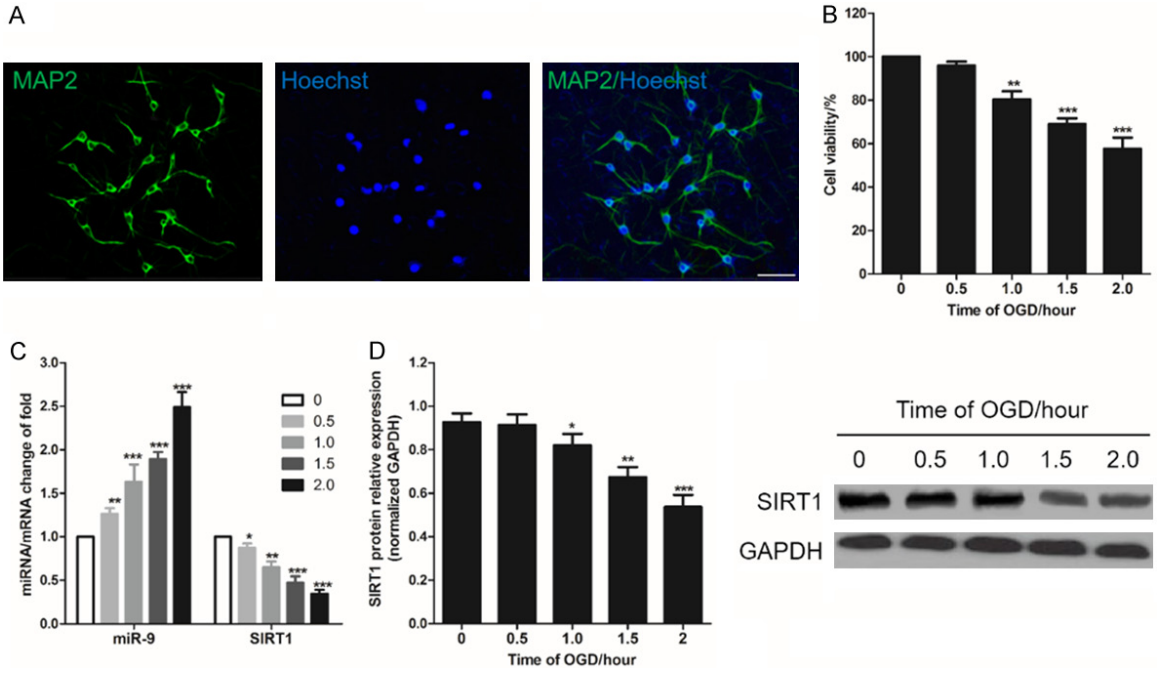

Figure 3. OGD induced miR-9 expression upregulation and SIRT1 expression downregulation in neurons in vitro. A. Immunofluorescence was used to identify primary neurons; B. Effects of different OGD time on nerve survival which was measured by CCK8 kit, bar =100 μm; C. The expression of miR-9 and SIRT1 mRNA were measured by RT-qPCR; D. Western blot was used to detect the expression of SIRT1 protein. Compared with 0 hours, *was P<0.05, **was P<0.01 and ***was P<0.001.

condary antibody was selected as follows: goat anti-mouse (ab6789, 1:3000) or goat anti-rabbit (ab150077, 1:3000). Primary antibody was incubated overnight at 4°C and secondary antibody was incubated for 1 hour at room temperature. All antibodies are purchased from Abcam unless otherwise stated.

CCK-8 assay

The cell culture medium was removed in the 96-well plates, and we added 10 μl of CCK-8 (40203ES60, Yeasen, Shanghai, China) to each well, and placed the 96-well plate back to the cell culture incubator at 37°C in the dark for 2 hours. Absorbance values of different treatment groups were read at 450 nm using a microplate reader (Bio-Rad 680, USA), and cell viability was calculated based on absorbance values.

Statistical analysis

The expression of miR-9, mRNA, protein and other serum indexes were represented as (Mean ± standard deviation), and Student's t test was used to compare differences between the two groups. Pearson's method was used to analyze the correlation between two types of clinical data. P<0.05 was considered significant in all the analyses.

Results

miR-9 was highly expressed in the serum of patients with AIS

The serum level of miR-9 in AIS group (n=135) and control group (n=45) were measured by RT-qPCR, and as shown in **Figure 1A**, the serum level of miR-9 in AIS group was significantly higher than that in control group. NIHSS score and infarct volume were used to assess the condition of patients with AIS, and their relationship with serum miR-9 levels was analyzed by Pearson correlation. As shown in **Figure 1B** and **1C**, the level of serum miR-9 in 65 AIS patients was positively correlated with NIHSS score (r=0.627, P<0.001) and infarct volume (r=0.576, P<0.001).

Based on the above results, it appears that serum miR-9 levels were related to the development of AIS.

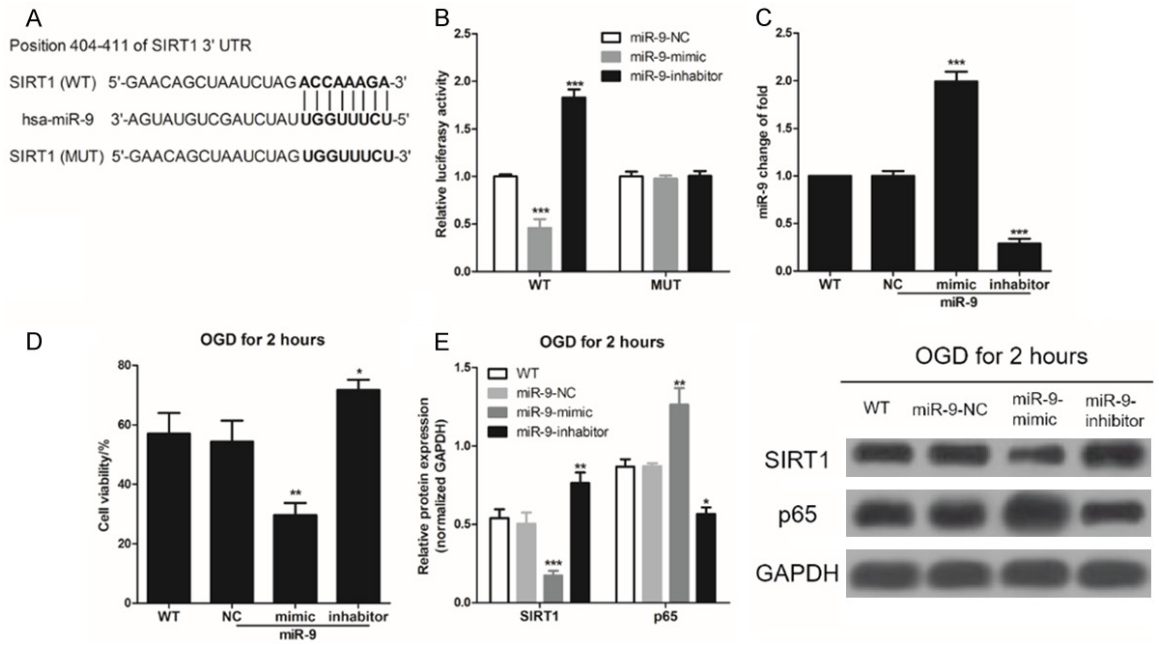

Figure 4. miR-9 regulated OGD-induced neuronal apoptosis via the SIRT1-mediated NF-kB pathway. A. WT-SIRT1 3'UTR luciferase reporter vector, and a MUT-SIRT1 3'UTR luciferase reporter vector with mutations on miR-9 binding sites of the SIRT1 3'UTR was constructed; B. miR-9-NC/miR-9-mimic/miR-9-inhibitor were transfected into primary neuronal cells and luciferase activity was detected; C. RT-qPCR was used to detect the expression of miR-9 after 48 hours transfecting; D. CCK8 kit was used to measure the cell viability of neurons after 2 hours of OGD. E. The expression of SIRT1 and p65 were detected. Compared with miR-9-NC group, *was P<0.05, **was P<0.01 and ***was P<0.001.

Correlation between serum miR-9 and inflammatory factors in AIS patients

The most important pathophysiologic mechanism of ischemic stroke is inflammation, and previous studies had shown that inflammatory factors were significantly elevated in ischemic stroke patients, and these inflammatory factors function and influence each other, which determines the transformation of ischemic stroke [15, 16]. In this study, we analyzed the correlation between serum inflammatory factors and miR-9 in AIS patients, and we found that the level of serum miR-9 in AIS patients had a positive correlation with serum IL-8 (r=0.376, P=0.002), TNF-α (r=0.418, P<0.001), IL-6 (r=0.545, P<0.001), and IL-1β (r=0.592, P<0.001). As shown in **Figure 2**. This suggested miR-9 might participate in the development of AIS by mediating the inflammatory response.

miR-9 and SIRT1 expression in primary neuronal cells after OGD

Neonatal rat neuronal cells were isolated and identified, and as shown in **Figure 3A**, MAP2-stained neurons were in a green fluorescent state, and their cell bodies and synapses grew well, forming a dense network of cells that could be used in subsequent experiments. CCK-8 kit was used to measure cell viability, and we defined normal cultured (OGD for 0 hours) neuronal cell activity as 100%. As shown in **Figure 3B-D**, the proportion of apoptotic cells in neurons increased, miR-9 expression was upregulated, and SIRT1 expression was downregulated with longer OGD time.

miR-9 regulated OGD-induced neuronal apoptosis by targeting SIRT1

miR-9 is a non-coding RNA that must play a biological role by regulating the expression of the encoded protein RNA. We predicted the target genes of miR-9 through bioinformatics, and found that there was a complementary sequence to miR-9 at the 3'-UTR end of SIRT1 mRNA (**Figure 4A**). To confirm that miR-9 regulated SIRT 1 expression by binding to SIRT1 3'-UTR, we validated the luciferase gene reporter system (**Figure 4B**), and found that transfection of miR-9-mimic significantly decreased WT type 3'-UTR luciferase activity (P<0.001) in neuronal cells, but did not work in MUT. miR-

9-inhibitor significantly increased WT type 3'-UTR luciferase activity (P<0.001) in neuronal cells, but did not work in MUT. This meant that miR-9 targeted the inhibition of SIRT1 expression.

To further verify the function of miR-9, we found that (**Figure 4C**, **4D**) miR-9-mimic could increase OGD-induced neuronal apoptosis, and miR-9-inhibitor could decrease OGD-induced neuronal apoptosis. Moreover, we also found miR-9 could regulate the expression of SIRT1 and p65 protein in neurons (**Figure 4E**). This suggests that miR-9 activates the NF-kB pathway by targeting SIRT1.

Discussion

In this study, we measured the expression of miR-9 in the serum of AIS patients, and found that the level of serum miR-9 in 65 AIS patients was not only significantly higher than that in control group, but also was positively correlated with NIHSS score and infarct volume. miR-9 is one of the most widely studied miRNAs, and previous studies confirmed that miR-9 was abnormally expressed in a variety of malignant solid tumor tissues, as it was up-regulated in glioma [17], laryngeal squamous cell carcinoma [18], breast cancer [19, 20], and down-regulated in colorectal cancer [21], colon cancer [22], and gastric cancer [23]. In recent years, researchers had found and confirmed that miR-9 was involved in the development and differentiation of nerve cells and the maintenance of traits, and played an indispensable role in nerve cells. Krichevsky et al. [24] indicated that inhibition of miR-124, miR-128 and miR-9 in neural stem cells could reduce the differentiation of neural stem cells into glial cells or neurons, and inhibition of miR-9 and miR-124 could maintain neural stem cell dryness by inhibiting phosphorylation of STAT3. In addition, overexpressed miR-9 could induce neuronal differentiation by inducing Hes1 protein expression to induce nerve cells to exit the cell cycle [25].

Uncontrolled inflammatory response caused by central nervous system ischemia was one of the main causes of secondary injury of cerebral ischemia [26]. Cerebral ischemia can lead to increased expression of a variety of inflammatory cytokines, which increase the vulnerability of neurons, leading to the destruction of the blood-brain barrier, causing inflammation to be further enhanced, and resulting in more cell damage and death [27]. At the same time, inflammatory mediators can stimulate the expression of cell adhesion factors, leading to the exudation of neutrophils and monocytes, triggering ischemic tissue inflammation [28].

In this study, we found that the level of serum miR-9 in AIS patients had a positive correlation with serum IL-8, TNF-α, IL-6 and IL-1β. Previous studies had confirmed that cytokines served as the main participants to mediate neurotoxicity after cerebral ischemia, and the main neurotoxic effects included IL-1β, TNF-α-mediated neuroedema formation, promotion of gliosis, increased Ca^{2+} in neurons, and released white blood cells [29]. All in all, inflammation that is abnormally increased after cerebral ischemia is one of the main causes of neuronal damage. Since serum miR-9 was positively correlated with inflammatory factors, miR-9 might participate in the development of AIS by mediating the inflammatory response.

To further investigate the role of miR-9 in the development of ischemic stroke, we established a neuronal OGD model, and found that miR-9 expression was gradually upregulated and SIRT1 (silent information regulator 1) expression was gradually downregulated with the prolongation of OGD time. Dual-luciferase reporter assay demonstrated that SIRT1 was a target gene of miR-9. SIRT1 is a histone deacetylase that is widely expressed in human cells, and carries out important biological functions by deacetylating multiple transcription factors, such as p53 [30], UCP2 [31], P300 [32] and NF-kB [33]. p65 is an important component of NF-kB, which only functions after it is acetylated. In inflammatory responses, SIRT1 deacetylates p65 thus inhibiting the transcription of TNF-α, IL-6 and other inflammatory genes downstream of NF-kB [33]. p65 is an important protein in the TLR/NF-kB signaling pathway, and its phosphorylation-mediated translocation (from cytoplasm to nucleus) is an important marker of the activation of NF-kB signaling. In this study, we found that miR-9 negatively regulated SIRT1 expression and positively regulated p65 expression, which meant miR-9 positively regulated the NF-kB pathway.

Many previous studies had also confirmed that miR-9 could activate the NF-kB signaling pathway, such as, Wang et al. [34] found that epi-

genetic inactivation of miR-9 family microRNAs in chronic lymphocytic leukemia-implications on constitutive activation of NFκB pathway. Gu et al. [35] found that miR-9 regulated the development of knee osteoarthritis through the Nf-kappa B1 pathway in chondrocytes. More importantly was that miR-9-mimic could increase OGD-induced neuronal apoptosis, and miR-9-inhabitor could decrease OGD-induced neuronal apoptosis. Combined with the above clinical data analysis, we it appears that upregulated miR-9 enhanced inflammation via the SIRT1-mediated NF-kB pathway, and then regulated neuronal damage.

Conclusion

The level of serum miR-9 in AIS patients was upregulated and had a positive correlation with NIHSS score, infarct volume, and serum inflammatory factor. Moreover, miR-9 could activate the NF-kB signaling pathway by targeting inhibition of SIRT1 expression, thereby enhancing OGD-induced neuronal damage in vitro.

Acknowledgements

This research was supported by the fund of Taizhou City Project Foundation of Taizhou First People's Hospital ([2015]43-15yw05).

Disclosure of conflict of interest

None.

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