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Original Article

The TNFα-miR-106b-5p-Caspase 9 axis functions as a key regulator in cerebral infarction via modulating endothelial cell apoptosis

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Abstract: Objective: Ischemic infarction is one of the leading causes of death and disability worldwide. Accumulating research findings have demonstrated that microRNAs (miRNAs) play a critical role in cerebral ischemic injury. In the present study, we aimed to investigate the role and underlying molecular mechanism of miR-106b-5p in ischemic stroke, which may provide an effective clue to develop potential therapeutic applications for ischemic stroke. Methods: The serum samples of 28 patients with cerebral infarction and 13 healthy volunteers were collected within 48 hours of admission. Thirty-six male C57BL/6 mice were randomly divided into sham-operated group (n=6) and operated groups (n=30, 6 mice for each time point, 1, 3, 7, 14 and 28 days). In the latter groups, the cerebral artery occlusion (MCAO) model was established. Quantitative RT-PCR was performed to evaluate the expression of circulating miR-106b-5p and enzyme linked immunosorbent assay (ELISA) was applied to detect TNFα in the sera of these samples. Furthermore, the effect of TNFα on miR-106b-5p was determined by quantitative RT-PCR in human umbilical vein endothelial cells (HUVECs). Bioinformatics analysis shows that miR-106b-5p may target Caspase 9. In addition, the regulatory role of miR-106b-5p in Caspase 9 expression and hypoxia-induced apoptosis was evaluated by western blot and apoptosis assay, respectively. Results: Compared with normal control, the expression of miR-106b-5p was significantly down-regulated in human specimens and mice samples ($P<0.05$), while TNFα was upregulated. Importantly, the expression levels between miR-106b-5p and TNFα present a negative correlation. Moreover, we found that TNFα suppressed miR-106b-5p expression in HUVECs. Bioinformatics analysis demonstrate that the apoptosis-associated gene Caspase 9 is a direct target of miR-106b-5p. Functionally, miR-106b-5p inhibited Caspase 9 expression and thus suppressed hypoxia-induced apoptosis in HUVECs. Conclusions: These findings suggest that suppression of miR-106b-5p by elevating TNFα in cerebral ischemia upregulates Caspase 9 expression and promotes apoptosis in HUVEC, which provides a potential therapeutic target for ischemic stroke.

Keywords: Cerebral infarction, miR-106b-5p, TNFα, vascular endothelial cells, caspase 9, apoptosis

Introduction

Ischemic infarction has been recognized as one of the leading causes of death and long-term disability worldwide. Although the molecular mechanisms underlying ischemic infarction remains unclear, a wide range of studies have shown that multiple genes and signaling pathways are involved in the formation and progression of ischemic infarction. Until now, the diagnosis of ischemic infarction mainly relies on clinical detection and Neuroimaging technology. Recent studies opened up the exciting prospect of utilizing circulating miRNAs as powerful, non-invasive diagnostic markers [1-6]. However, ischemic infarction is difficult to diagnose due to lack of reliable diagnostic markers.

MiRNAs are a class of small non-coding RNA with 19-24 nucleotides in length, which usually inhibits gene expression at post-transcriptional level via binding to its 3'-untranslated region (3'UTR) [7]. Growing evidence has revealed that miRNAs serve important functions in a wide range of physiological processes, including cell growth, development and differentiation [8]. Notably, deregulation of miRNAs plays an essential role in almost all the pathological processes, including cancer, cardiovascular diseases, metabolic disturbance and neurodegenerative disease [9-11].

The circulating miRNAs in serum are stable in serum and always exhibit consistent in individuals. Recent studies identified the involve-

ment of circulating miRNAs playing a key role in the prognosis and detection of cerebral infarction [12]. The antagomir of miR-106b-5p drastically reduced malondialdehyde (MDA) content, restored superoxide dismutase (SOD) activity, increased the expression of myeloid cell leukemia-1 (Mcl-1) and B cell lymphoma-2 (Bcl-2), and decreased the expression of Bax in the ischemic cortex [13]. However, the molecular mechanism underlying miR-106b-5p effect on cerebral infarction remains unknown.

On the other hand, blood vessel is the most important composition of blood-brain barrier (BBB), which is essential for brain to control blood perfusion. All blood vessels are lined with endothelial cells, which enhances the oxygen and nutrient supply to the ischemia-affected tissue and facilitates neurogenesis and synaptogenesis. Previous studies have demonstrated that aberrant expression of miR-106b-5p was found in the serum samples of patients with atherosclerosis [14], which probably induced by apoptotic endothelial cells. However, it remains unknown whether miR-106b-5p modulate endothelial cell apoptosis and whether this modulation is involved in ischemic infarction [14, 15].

In the present study, we found that the expression of miR-106b-5p was significantly downregulated in serum samples derived from patients with cerebral infarction and MCAO model mice. Meanwhile, the TNFα level was drastically upregulated, which was negatively correlated with miR-106b-5p. Moreover, administration of TNFα obviously suppressed the expression of miR-106b-5p in HUVECs. Importantly, ectopic expression of miR-106b-5p significantly suppressed caspase 9 expression and inhibited hypoxia/reoxygenation (H/R) induced cell apoptosis in HUVECs. These findings suggest that suppression of miR-106b-5p by elevating TNFα in cerebral ischemia upregulates Caspase 9 expression and promotes apoptosis in HUVEC, which provides a potential therapeutic target for ischemic stroke. Our findings suggest that suppression of miR-106b-5p by elevating TNFα in cerebral ischemia upregulates Caspase 9 expression and promotes apoptosis in HUVEC, which provides a potential therapeutic target for ischemic stroke.

Materials and methods

Sample collections

Twenty-eight patients were enrolled who had been diagnosed with ACI at the Department of Neurology, Chengde Medical college. A total of 13 healthy volunteers were recruited to serve as the controls. Five ml of Venous blood samples were drawn from all participants. The sera were isolated by centrifuging at 1600 rpm at 4°C for 15 min, and then collected and stored at -80°C until analysis. The present study was approved by the Ethics Committee of Chengde Medical college, and all participants agreed and signed informed consent forms. Patients were excluded if any other CNS disease or inflammation was identified.

RNA extraction

Circulating miRNAs in serum samples was isolated by miRcute miRNA isolation kit (Tiangen, Beijing China) following the manual's protocols.

Establishment of MCAO models in mice

MCAO models were established as previously described. Briefly, the mice were anaesthetized using 7% chloral hydrate. The right common carotid artery (CCA), right internal carotid artery (ICA), and right external carotid artery (ECA) were separated, ECA and ICA were ligated, and ICA was clipped. A small incision was made on ECA near CCA and inserted slowly with a nylon line to a depth of about 18 mm to generate MCAO. After 2 h of ischemia, the nylon line was slightly withdrawn to allow reperfusion for 1 d (n=6), 3 d (n=6), 7 d (n=6), 14 d (n=6) and 28 d (n=6). For sham group, ECA, CCA, and ICA were separated but not ligated (n=6).

Establishment of H/R model in vitro

H/R model was established when the cells were in good condition with 80% confluency. The cells were then cultured in hypoxic medium without FBS and glucose and the culture dish was put into a sterilized hypoxic box. 5% CO_2 and 95% N_2 was charged into the hypoxic box to decrease the concentration of oxygen (reached less than 1% after more than half an hour). Then the cells were cultured in 37°C incubator for 8 h. After that they were cultured in medium

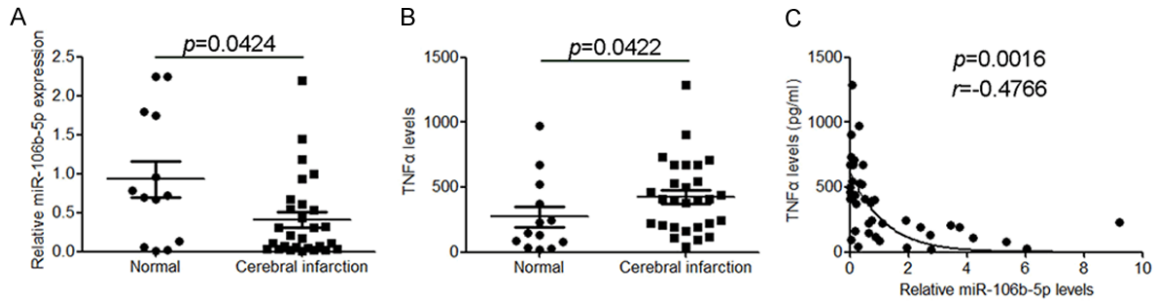

Figure 1. miR-106b-5p expression is downregulated in the serum samples derived from patients with ischemic infarction and its expression is negatively correlated with TNFα. A. The expression of circulating miR-106b-5p in patients with ischemic infarction compared with healthy control. B. TNFα expression in patients with ischemic infarction compared with healthy control. C. Correlation analysis of miR-106b-5p and TNFα expression in indicated samples.

with 20% FBS with high glucose and at 37°C incubator for reoxygenation for 4 h.

Quantitative RT-PCR

The SYBR green (Tiangen) method and Lightcycler Real-time PCR detection system (Bio-Rad, Hercules, CA) were used for real-time PCR to detect mRNA expression. Primer sequences were as follows: Caspase 8-forward: GGTCGAGAAGATTGTGAACATC, Caspase 8-reverse: CTGGGAAAGTAGAGTAGGACAC; β-ACTIN-forward: TCACCCACACGTGCCCATCTACGA, β-ACTIN-reverse: CAGCGGAACCGCTCATTGCCAATGG. The TaqMan Human miRNA Assays were used in this study for miRNA detection. Briefly, 10 ng of total RNA were reverse transcribed to cDNA with stem-loop primers and the TaqMan miRNA Reverse Transcription Kit (Ambion, Carlsbad, California, USA). Quantitative real-time PCR (qRT-PCR) was done by using an Applied Biosystems 7500 Real-time PCR System and a TaqMan Universal PCR Master Mix. All PCR primers were from the TaqMan miRNA Assays. miRNAs expression was quantified in relation to the expression of small nuclear U6 RNA as previously reported

ELISA assay

The ELISA kits used to analyze the serum levels were the Human and mouse TNF-alpha ELISA Kit. All the kits were purchased from Abcam Trading Company Ltd. (Shanghai, China).

Western blot

Western blot was performed as previously described [10]. The antibodies were as follows: Caspase 9: Abcam cat#202068; β-ACTIN: Santa Cruz cat#47778.

Apoptosis assay

100 μL HUVEC suspension (2×10^4 cells) was seeded into a 96-well plate. Each group had six wells. 20 μl of MTT with 5 mg/ml was added to each well. Then, the plates were incubated at 37°C for 4 h to allow the MTT to form formazan crystals by reacting with the viable cells. The MTT medium mixture was removed and the formazan crystals were dissolved in 150 μl DMSO at 37°C for 10 min. The absorbance was measured by a microplate reader at 570 nm.

Statistical analysis

The statistical significance between two groups was determined by unpaired Student's *t* test. The correlation of miR-106b-5p with TNFα was analyzed by Pearson method. A *p*-value smaller than 0.05 was considered statistically significant.

Results

Negative correlation of miR-106b-5p with TNFα in the serum samples derived from patients with ischemic infarction and MCAO model mice

Initially, to determine the effect of miR-106b-5p on ischemic infarction, we examined the expression levels of circulating miR-106b-5p in patients with ischemic infarction (n=28) and healthy controls (n=13) by quantitative RT-PCR. The results demonstrated that miR-106b-5p was significantly downregulated in the ischemic

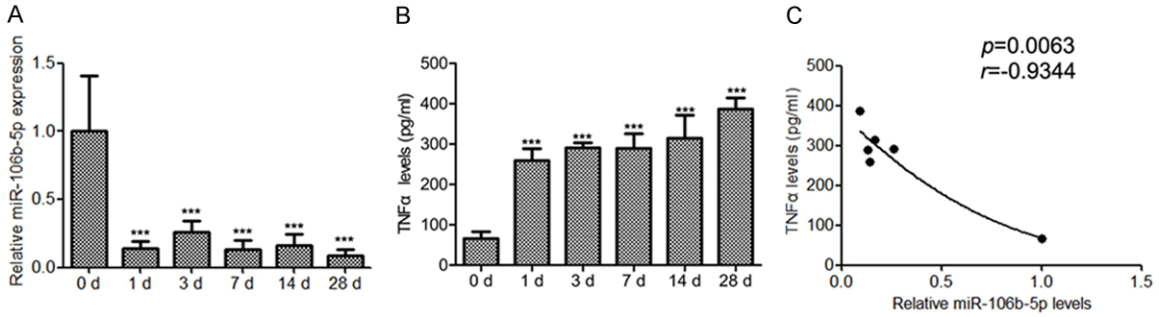

Figure 2. The expression levels of miR-106b-5p and TNFα and their correlation analysis in serum samples derived from MCAO model mice. A. The expression of miR-106b-5p in the serum samples of MCAO model mice compared with normal control. ***P<0.001. B. TNFα expression in the serum samples of MCAO model mice compared with normal control. C. Correlation analysis of miR-106b-5p and TNFα expression in indicated mice samples, which was determined by analyzed by Pearson method.

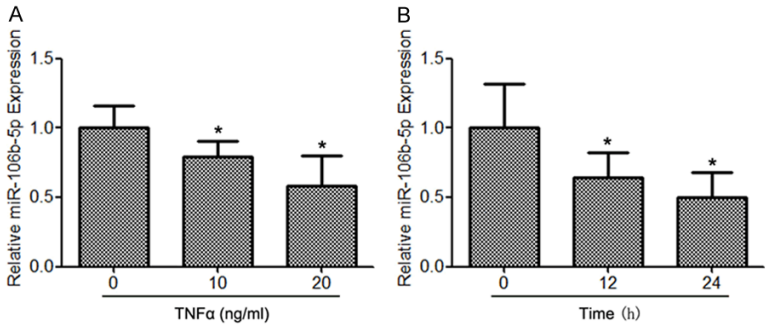

Figure 3. TNFα results in miR-106b-5p downregulation in a dose- and time-dependent manner. A. TNFα mediated miR-106b-5p downregulation is dose dependent. HUVECs were treated with TNFα for 24 h. B. TNFα mediated miR-106b-5p downregulation is time dependent. HUVECs were exposed to 20 ng/ml TNFα for variable treatment times as indicated. Data are expressed as mean ± SEM *P<0.05 versus untreated control.

infarction group compared with healthy control group (*P*=0.0424, **Figure 1A**). In addition, we also detected the levels of inflammatory factor TNFα in these samples by ELISA. Compared with control, the TNFα expression levels were drastically upregulated in patients with ischemic infarction (*P*=0.0422, **Figure 1B**). Next, we related the TNFα levels to the miR-106b-5p expression and observed that a negative correlation between miR-106b-5p and TNFα (*P*=0.0016, **Figure 1C**). We then established a MCAO model in mice and determined the expression of miR-106b-5p and TNFα in the serum samples. Consistent with the sham group, the expression of miR-106b-5p was significantly reduced in ischemic mice (**Figure 2A**), while the expression of TNFα was notably elevated (**Figure 2B**) and a negative correlation between miR-106b-5p and TNFα was observed (**Figure 2C**).

TNFα suppresses miR-106b-5p expression in HUVECs

To determine the relationship between TNFα and miR-106b-5p in endothelial cells, we treated HUVECs at indicated concentrations for 24 h (**Figure 3A**) or exposed to 20 ng/ml of TNFα for indicated times (**Figure 3B**) and found that TNFα suppressed miR-106b-5p expression at a dose- and time- dependent manner.

miR-106b-5p inhibits H/R-induced cell apoptosis by suppressing Caspase 9 expression in HUVECs

To further elucidate the effect of miR-106b-5p on endothelial cell apoptosis, we performed a bioinformatics analysis using Targetscan (http://www.targetscan.org/vert_71/) to predict the possible target genes of miR-106b-5p involved in cellular apoptosis. We found that miR-106b-5p may suppress Caspase 9 expression by its 3'UTR region at the 2388-2408 site (**Figure 4A**). Additionally, ectopic expression of miR-106b-5p (**Figure 4B**) significantly inhibited the protein level of Caspase 9 (**Figure 4C**) in HUVECs, determined by western blot. These results demonstrate that endogenous Caspase 9 expression in HUVECs is directly targeted by miR-106b-5p. To further delineate the effect of miR-106b-5p on H/R-induced cell death, we performed Annexin V/PI double staining and flow cytometry to determine the apoptosis rate

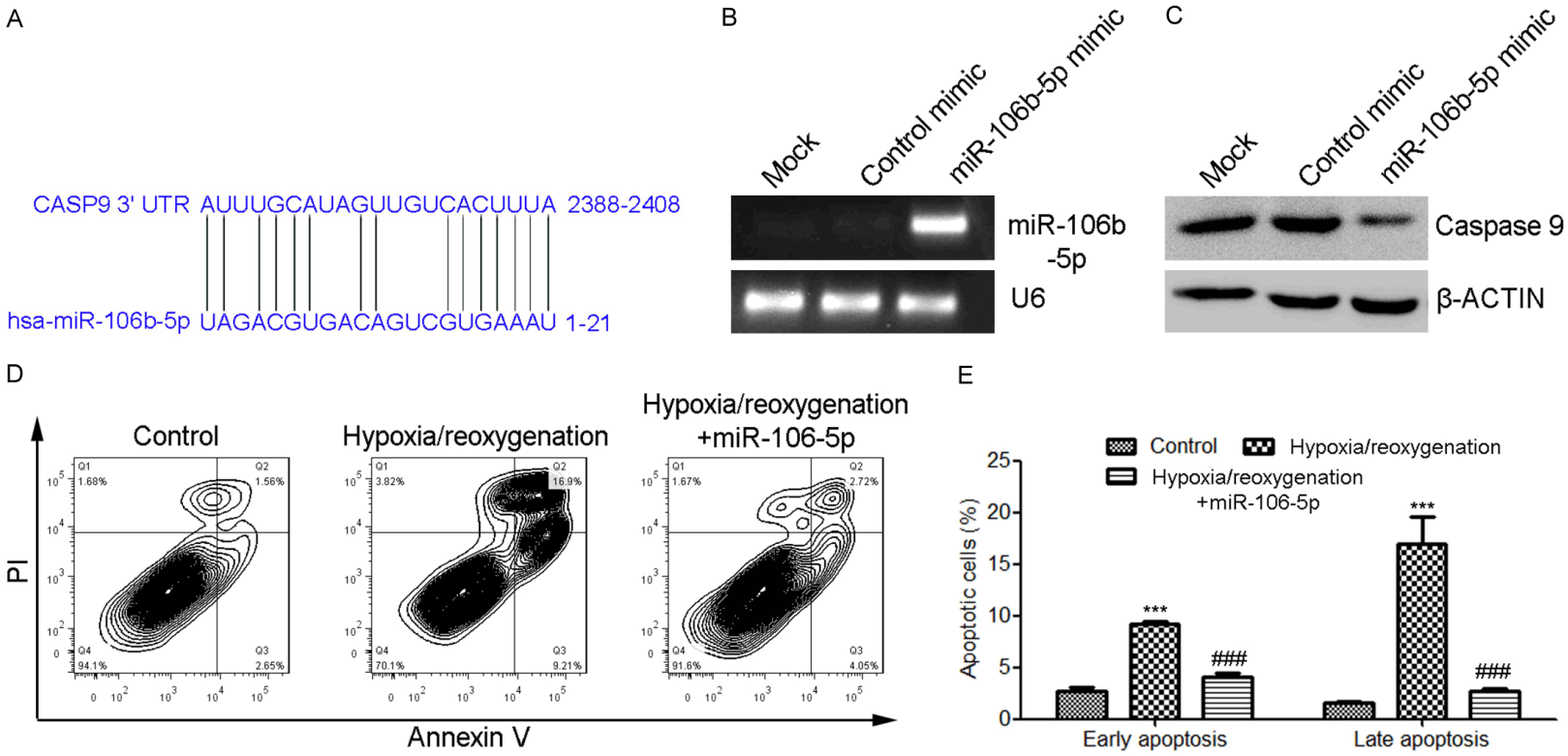

Figure 4. miR-106b-5p suppresses Caspase 9 expression and inhibits cellular apoptosis induced by H/R in HUVECs. A. Schematic of miR-106b-5p binding sites within the TNFα 3'UTR. B. 100 nM of miR-106b-5p mimics were transfected into the HUVECs and semi-quantitative RT-PCR was performed to detect the miR-106b-5p expression. C. Western blot analysis was performed the determine the protein levels of Caspase 9 in HUVECs transfected with 100 nM of miR-106b-5p mimics compared with control. D. Flow cytometry was performed to determine the effect of miR-106b-5p on apoptosis of HUVECs induced by H/R. E. Analysis of apoptotic cells in the early and late phase in the indicated groups. ***P<0.001 versus untreated control. ###P<0.001 versus H/R.

of the cells with indicated treatment. As shown in **Figure 4D** and **4E**, H/R induced significant apoptosis and miR-106b-5p, in contrast, markedly inhibited apoptosis induced by H/R.

Discussion

In the present study, we found that the expression of miR-106b-5p was drastically downregulated in patients with ischemic infarction and in MCAO model mice compare with control. Meanwhile, a significant correlation was observed between miR-106b-5p and TNFα. Herein, the downregulation of miR-106b-5p and upregulation of TNFα may be powerful circulating biomarkers for ischemic infarction. In further studies, we plan to explore the relationship among miR-106b-5p and ischemic infarction at different stages.

The miR-106 family contains two members, miR-106a and miR-106b, in which miR-106a has been reported to be deregulated in ischemic diseases. For instance, in renal ischemia, miR-106a is activated in the serum [16]. Li *et al.* has demonstrated that miR-106b-5p was upregulated in acute cerebral ischemic stroke, which is inconsistent our results. Therefore, we need to amplify the samples or make a meta-analysis to conclude the expression change of miR-106b-5p in ischemic infarction [13].

Accumulating studies have shown that a wide range of inflammatory factors and cytokines are involved in ischemic infarction, including TNFα, IL-1β, IL-6 and IFNγ [17, 18]. In our present study, we found that TNFα was upregulated in patients with ischemic infarction and MCAO mice. Meanwhile, a significant correlation between TNFα and miR-106b-5p was observed in these samples, which suggest a regulatory relationship may exist between miR-106b-5p and TNFα. To confirmed that, we determined the effect of TNFα on miR-106b-5p expression. The results indicated that TNFα suppressed miR-106b-5p expression at a time- and dose-dependent manner. This observation was also confirmed by other researches. For instance, Zhang et al. have reported that TNFα can reduce miR-106b-5p expression to activate Caspase 3 and DNA fragmentation. Moreover, overexpression of miR-106b-5p upregulates PTEN to inhibit TNFα-induced cell apoptosis [14].

Cerebral ischemic infarction can be induced by cytotoxicity, oxidative stress, inflammation, apoptosis, autophagy and various signaling pathways. Growing evidences have indicated that endothelial cell damage and apoptosis are involved in the formation and progression of ischemic infarction. In this study, we found that upregulation of miR-106b-5p suppressed endothelial cell apoptosis, which was consistent with previous findings that miR-106b-5p reduces cellular apoptosis during carcinogenesis. Importantly, we uncovered that the apoptosis related gene Caspase 9 could be a direct target of miR-106b-5p. Caspase 9 is preferentially activated in the apoptosis cascades and transmit the apoptotic signals to other caspase proteins via its proteinase activity. It is believed that miR-106b-5p controls the upstream of apoptotic signals in endothelial cells.

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Disclosure of conflict of interest

None.

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