Biochemical and Biophysical Research Communications 501 (2018) 501–506

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

miR-411 suppresses acute spinal cord injury via downregulation of Fas ligand in rats

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ARTICLE INFO

Article history:
Received 24 April 2018
Accepted 4 May 2018
Available online 16 May 2018

Keywords:
miR-411
Acute spinal cord injury (ASCI)
Rat
Fas ligand (FasL)
Basso
Beattie and Bresnahan (BBB) score
Cell apoptosis

ABSTRACT

Objective: To explore the role of miR-411/FasL in acute spinal cord injury (ASCI).
Methods: The ASCI rat model was established, and expression of miR-411 and Fas ligand (FasL) was examined. Basso, Beattie and Bresnahan (BBB) score was used to evaluate the rats' neurological function. PC12 oxygen-glucose deprivation (OGD) model was also established. Gene manipulation (including miR-411 mimic or inhibitor) was used to modulate gene expression. Luciferase reporter assay was conducted to confirm the targeting relationship between miR-411 and FasL. Flow cytometry was applied in the measurement of PC12 cell apoptosis. Finally, the miR-411 mimic was injected into the vertebral canal of ASCI rats to determine the effects of miR-411 *in vivo*.
Results: Compared with sham group, the expression of miR-411 and FasL was significantly decreased and increased in ASCI group, respectively ($P < 0.05$). Similarly, the expression of miR-411 and FasL was significantly lower and higher in OGD group than that in control group, respectively ($P < 0.05$). miR-411 directly controlled the FasL expression. miR-411 mimic can dramatically reduce the increased percentage of apoptosis cells caused by OGD when comparing to mimic control, which was greatly reversed by the overexpression of FasL ($P < 0.05$). Further, the BBB score was significantly elevated in the miR-411 mimic group when comparing to mimic control group, with decreased FasL expression ($P < 0.05$).
Conclusion: miR-411 mimic suppressed PC12 cell apoptosis via FasL, and relieved ASCI in rats.

1. Introduction

Acute spinal cord injury (ASCI) is a common and serious injury of the central nervous system (CNS), resulting in impaired sensory and motor functions, and thus severely affecting patients' quality of life. As estimated, there were about 2.5 million patients with ASCI worldwide, with 130,000 patients being newly found annually [1]. The underlying mechanisms of ASCI are usually divided into primary injury mechanism caused by initial mechanical change, and secondary injury mechanism induced by vascular change as well as biochemical change, finally leading to the impairments in neurologic functions of patients [2]. Cell apoptosis is an important hallmark of ASCI, which is observed in neurons, oligodendrocytes as well as white matter [3]. It has been reported that improvement of neuronal survival could greatly restore the functions of the spinal cord in ASCI patients [4].

miRNAs, an important class of small non-coding RNAs, participate in numerous pathophysiological processes. Studies have found that a variety of miRNAs expression was changed following ASCI, potentially affecting the progression of ASCI via regulating cell death or astrogliosis [5–8]. However, there have been limited studies about the direct role of miRNAs in ASCI. miR-411 was reported to be decreased in ASCI rats, potentially leading to increased inflammation [8,9]. However, the underlying mechanism of how miR-411 affects ASCI is not clear.

The Fas/Fas ligand (FasL) signaling pathway plays a critical role in cell apoptosis, inflammation and gliosis of various CNS diseases [10,11]. Studies have shown that Fas/FasL induced cell apoptosis of neurons and oligodendrocytes occurred in all ASCI cases [12,13]. Moreover, neuronal apoptosis was greatly reduced in Fas-deficient mice; and neutralization of Fas signaling was beneficial for the functional recovery following ASCI [13].

In this study, we found the targeting relationship between miR-411 and FasL using bioinformatics software (microrna.org). Therefore, we focused on investigating whether miR-411 affects neuronal apoptosis following ASCI via FasL in a rat model of ASCI.

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https://doi.org/10.1016/j.bbrc.2018.05.022
0006-291X/

2. Materials and methods

2.1. Establishment of acute spinal cord injury (ASCI) rat model

This research was approved by the ethics committee of the Changzhou first people's hospital, the Third Affiliated Hospital of Soochow University. Animal experiments were carefully performed in accordance with the Guide for the Care and Use of Laboratory Animals proposed by the Chinese National Institutes of Health. ASCI rat model was established as previously reported [14]. A total of 12 rats were randomly and equally allocated to two groups: sham group and ASCI group. After 4-h deprivation of water and food, intraperitoneal injection of 10% chloral hydrate was administered to rats (3 mL/kg) for general anesthesia. The rats in ASCI group were fixed in the prone position and the laminectomy was performed at the level of the T9-11 vertebral bodies. After the spinal cord was exposed, the rats were subjected to 20 s of spinal cord compression at T10 level by using tweezers to suppress the rope from both sides. The successful ASCI model resulted in paralysis of the lower extremities. The sham group received the same treatment, including contacts, laminectomy, and the placement of forceps around the spinal cord, without causing any comminuted injury. Twenty-four hours after spinal cord injury, the rats were injected with a large dose of pentobarbital (200 mg/kg; Sigma, Shanghai, China) and then sacrificed.

2.2. Basso, Beattie and Bresnahan (BBB) score

BBB score was used to evaluate the rats' neurological function [15]. The BBB score was classified into 3 categories: early phase (BBB score from 0 to 7), where rats showed little or no hindlimb movement; intermediate phase (8-13), where rats had uncoordinated steps; and late phase (14-21), where rats had the coordination of forelimb and hindlimb.

2.3. Isolation of spinal cord

After the rats were sacrificed, the spinal cord tissues were removed from spine carefully. The T10 vertebral body was cut out, and the scissor was opened horizontally to spread apart the spine. Then the spinal cord was exposed and put into a mortar, followed by the addition of 10 mL cold phosphate buffered saline (PBS). The spinal cord was crushed using a pestle. The PBS solution containing spinal cord cells were moved to a 50 mL tube and centrifuged at 1500 rpm for 5 min. The cell pellet was resuspended in appropriate medium.

2.4. RNA isolation and quantitative real-time PCR (qPCR)

Total cellular RNA was extracted using TRIzol reagent (Invitrogen). The first-strand cDNA was synthesized using the M-MLV Reverse Transcriptase Kit (Promega) with the guidance of manufacturer. The qPCR was conducted using SYBR Select Master Mix (Thermo Fisher) and analyzed on an ABI 7900-fast thermocycler (Applied Biosystems). The comparative Ct ($\Delta\Delta$Ct) method was used to determine the expression levels of miR-411 and Fas ligand (FasL). The primers used for qPCR were designed and synthesized by Sangon Biotech (Shanghai, China).

2.5. Western bolt

Total cellular proteins were extracted with the Radio Immunoprecipitation Assay (RIPA) lysis buffer (including a protease inhibitor cocktail) [16]. After the concentration of proteins measured with a BCA protein assay kit (Thermo Fisher), they were subjected to western blotting. Proteins were separated by SDS-PAGE with electrophoresis system and transferred into the polyvinylidene difluoride (PVDF) membrane (Bio-Rad). The membrane was blocked with 5% skimmed milk for 1 h at RT, and then incubated with primary antibodies including anti-FasL antibody (Abcam, 1:2000) and anti-β-actin antibody (Abcam, 1:500) at 4 °C for overnight. The membrane was incubated with HRP-bounded antibodies for 1 h and the target proteins were visualized by ECL Plus Western Blotting Substrate (Thermo Fisher). The β-actin protein served as a control to quantify protein level.

2.6. Establishment of PC12 oxygen-glucose deprivation (OGD) model

The cell medium was replaced by glucose-free medium, which was pretreated with 95% N_2 plus 5% CO_2 for 30 min. Then, the PC12 cells were immediately placed in the sealed chamber filled with 95% N_2 plus 5% CO_2, and cultured at 37 °C for 12 h. Afterwards, the cells were taken out and cultured in the normal medium at 37 °C for 24 h. Finally, the cells were collected.

2.7. Flow cytometry

Flow cytometry was used to measure the cell apoptosis of PC12 cells. Cells were collected in 10 mL tubes and centrifuged at 350 g, room temperature for 5 min. After removing supernatants, the cells were resuspended in 1 mL PBS, transferred to 1.5 mL tubes, and centrifuged again at 350 g, room temperature for 5 min. The supernatants were removed, and the cells were treated with 1 mL of 75% ethanol at −20 °C overnight for fixation. Afterward, the cells were centrifuged at 350 g, room temperature for 5 min, followed by one time of PBS washing. The 500 μL of PI solution (containing 50 μg/mL PI and 25 μg/mL RNase) was added to cells. Finally, the cells were incubated in darkness at room temperature for 30 min, immediately followed by the flow cytometry analysis.

2.8. Cell transfection

To modulate the miR-411 and FasL expression, the PC12 cells were transfected with miR-411 mimic or inhibitor, or FasL overexpression vector (pcDNA-FasL). PC12 cells were planted into 6-well plates for 24 h, and miR-411 mimic or inhibitor, pcDNA-FasL, or the matched control was transfected into the cells with Lipofectamine 2000 (Invitrogen, US) with the guidance of manufacturer. The PC12 cells were collected after 48-h transfection.

2.9. Luciferase reporter assay

The luciferase reporter plasmids, wild-type FasL 3′-UTR and mutant FasL 3′-UTR were constructed. The PC12 cells were planted into 12-well plates for 24 h, and co-transfected with wild-type FasL 3′-UTR and miR-411 mimic/inhibitor/control or mutant FasL 3′-UTR and miR-411 mimic/inhibitor/control using Lipofectamine 2000. After 48 h, the cells were collected and the luciferase activity was evaluated using the dual luciferase reporter assay system (Promega) with the guidance of manufacturer.

2.10. Injection of miR-411 mimic

The injection of miR-411 mimic to rats was performed as previously described [14]. Briefly, a total of 12 ASCI rats were randomly and equally allocated to 2 groups, including mimic control group and miR-411 mimic group. Twenty-four hours after the ASCI model establishment, the rats were anesthetized with the intraperitoneal injection of ketamine (80 mg/kg) plus xylazine (40 mg/kg). And