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miR-152 promotes spinal cord injury recovery via c-jun amino terminal kinase pathway

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Abstract. – OBJECTIVE: The aim of this research is to explore the possible role of miR-152 in spinal cord injury and its underlying mechanism.

MATERIALS AND METHODS: After a mouse model of spinal cord injury (SCI) was developed, Real Time-quantitative Polymerase Chain Reaction (RT-qPCR) was used to detect the expression of miR-152 and c-jun in the mouse. In addition, the expression levels of interleukin-1b (IL-1b), interleukin-18 (IL-18) and tumor necrosis factor-α (TNF-α) were detected by enzyme-linked immunosorbent assay (ELISA). Subsequently, miR-152 was overexpressed and the levels of inflammation and c-jun after spinal cord injury were detected by Western blot. Furthermore, the grip strength of double forelimb, left forelimb or right forelimb of the mice was detected using a grip force test after miR-152 was overexpressed in the injured area of each group.

RESULTS: By constructing a mouse model of spinal cord injury, we found that the expression of miR-152 in the injured area decreased with time; meanwhile, the inflammatory relative genes including IL-1b, IL18, TNF-α, and c-jun were significantly increased. However, miR-152 overexpression significantly reduced the levels of inflammation genes as well as the expression of c-jun. Besides, the strength of the forelimbs in the spinal cord injury mice was restored.

CONCLUSIONS: MiR-152 could inhibit inflammatory responses and promote the recovery of the spinal cord injury through the c-jun N-terminal kinase pathway and it can be a target molecular for treating spinal cord injury.

Key Words:
Spinal cord injury, MiR-152, C-jun, Inflammation.

Introduction

Spinal cord injury is a very common spinal injury with poor prognosis, which is often caused by high-altitude falling, traffic accidents, sports injuries and violent injuries[1]. In particular, cervical spinal cord injury accounts for 54% of all spinal cord injuries, which may induce upper limb dysfunction[2], causing great physical and psychological trauma as well as bringing economic burden to patients. The pathological process of spinal cord injury is a complex dynamic process involving multiple functional changes in the system. According to the time of onset, the spinal cord injury can be divided into primary spinal cord injury and secondary spinal cord injury and can last for several months. The recovery of neurological function of the spinal cord has long been a difficult point in basic research and clinical treatment.

MicroRNAs (miRNAs) are non-coding single-stranded RNAs that can regulate protein expression. The first miRNA was discovered in 1993 in the study of developmental disorders of C. elegans[3]. In the central nervous system such as the spinal cord of mammals, many microRNAs are highly expressed and have specific expression[4,5], and some miRNAs are specifically expressed in nerve cells[6]. MiRNAs play key parts in all aspects of the central nervous system. It not only participates in the development of nerve cells and synaptic formation, but also has an important relationship with myelination[7,8]. Also, numerous studies have confirmed the association of miRNA dysregulation with a variety of

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central nervous system disorders. For example, in central nervous system degenerative diseases such as Alzheimer's disease, down-regulation of the miR-29 gene cluster is related to an increase in BACE1 and amyloid levels, finally leading to disease occurrence. In many other diseases involved in central nervous system, miRNA also play key parts[9].

Studies have shown that miRNAs can regulate a variety of transcription factors that play important roles in spinal cord development and function establishment during spinal cord neuronal differentiation and cell type maintenance. For example, miR-9 regulates the expression of FoxP1, which is a transcription factor that controls the differentiation of lateral motor neurons. The overexpression of miR-9 can lead to an abnormal expression of FoxP1 in the spinal cord, thereby altering the motor neuron subtype of the developing spinal cord[10]. Researchers[11,12] have found that miRNA-9, miRNA-196, miRNA-124a, miRNA-124b may be involved in the regulation of the spinal cord development. Therefore, exploring the role and expression of microRNA in the development of the spinal cord can further provide new ideas for the targeted therapy of spinal cord injury.

Materials and Methods

SCI Modeling and Mouse Grouping

30 female C57bl/6J mice between 8-10 weeks old weighed 20-25 g were numbered by tail numbering and randomly divided into two groups using the Random number generator. This study was approved by the Animal Ethics Committee of Shanxi Medical University Animal Center.

C5 was recognized as the center and then a longitudinal incision was made, followed by an incision of the skin and fascia. The C4-6 spinous process was exposed and then the C5 laminar was cut off by ophthalmology scissors to reveal the dural sac. Then the position of the spinal cord hitter against the hammer was adjusted to combat the dural sac successfully, while the muscles and fascia as well as the skin incision were sutured. The mice in the sham operation group only received cervical blunt contusion.

Mice were sacrificed before the injury and 6 hours, 24 hours, 72 hours, and 128 hours after spinal cord injury. MiR-152 mimics were injected into the tail vein to achieve miR-152 overexpression.

Spinal Cord Acquisition and RNA Extraction

After the mice were sacrificed by CO_2 asphyxiation, the spinal cord tissue containing the upper and lower 4 mm was cut out from the lesion area and stored in a 1.5 ml centrifuge tube. During the experiment, the spinal cord tissue was placed into a pre-cooled glass mortar and triturated with TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The RNA was extracted by sequentially adding chloroform, isopropanol and ethanol, and the RNA concentration was measured using Nanodrop. Total RNA was reverse transcribed into complementary Deoxyribose Nucleic Acid (cDNA) using reverse transcription kit. Quantitative polymerase chain reaction (qPCR) was performed with the SYBR mix. The relative expression levels of miR-940, TLR4 and MPO were calculated by $2^{-\Delta\Delta CT}$ method. The primer sequences are as follows: c-jun (F: 5'-GTCCTCCATAAATGCCTGTTCC-3', R: 5'-GATGCAACCCACTGACCAGAT-3'), TNF-α (F: 5'-GGAACACGTCGTGGGATAATG-3', R: 5'- GGCAGACTTTGGATGCTTCTT-3'), interleukin-18 (IL-18) (F: 5'-CCTACTTCAGCATCCTCTACTGG-3', R: 5'-AGGGTTTCTTGAGAAGGGGAC-3'), interleukin-1b (IL-1b) (F: 5'-ATGATGGCTTATTACAGTGGCAA-3', R: 5'-GTCGGAGATTCGTAGCTGGA-3'). The miR-152 real-time fluorescent quantitative PCR detection kit of Guangzhou Ruibo Biotechnology Co., Ltd. (Guangzhou, China) was used for PCR amplification.

Enzyme-Linked Immunosorbent Assay (ELISA)

Serum levels of IL-1b, IL18 and TNF-α were detected by ELISA according to the optical density (OD) value measured at 450 nm.

Basso-Beattie-Bresnahan (BBB) Score

The mice were placed in an open basin, then the wall of the basin was tapped to make mice crawl. The animal's hip, knee, ankle joint, trunk movement and coordination were observed. The score is divided into three parts, the first part is 0-7 points and the second part of the joint activities of the hind limbs is 8-13. The gait and coordination function of the hind limbs are judged to be 14-21, and the fineness of the claws in the exercise is judged. The action is three points out of 21 points.

Western Blot Assay

Radioimmunoprecipitation assay (RIPA) (Beyotime, Shanghai, China) cell lysate containing the protease inhibitor was added to lyse the spinal cord tissue of the mice on ice to extract the total proteins. The protein concentration was measured by the bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA). The sample was electrophoresed in a 10% sodium dodecyl sulphate (SDS)-polyacrylamide gel for about 3 h and then transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). The membrane was blocked for 1 h in Tris-Buffered Saline and Tween 20 (TBST) containing 5% skim milk. Then the membranes were incubated with the primary antibodies at 4°C overnight. After being washed 3 times in TBST, horseradish peroxidase-labeled antibody (1:1000) was added for 30 min at room temperature. After being washed 3 times in TBST, protein bands were detected by electrochemiluminescence (ECL) method (Thermo Fisher Scientific, Waltham, MA, USA).

Grip Force Measurement

Mice were lightly lifted by the tail and when the two forelimbs were close to the crossbar, they could be induced to actively grasp the crossbar. After the mouse was able to complete the double front paw grip, the unilateral forelimb grip was trained by wrapping one side of the front paw with a small piece of paper tape and using the above method to induce it to grip the crossbar with the other front paw. The above grip strength measurements were recorded four times for each successful measurement. In one measurement, if the mouse was unable to grasp the crossbar due to severe forelimb movement disorder, then the grip strength was recorded as 0.

Statistics Analysis

Statistical analysis was performed using Statistical Product and Service Solutions (SPSS) 18.0 software (SPSS Inc., Chicago, IL, USA). GraphPad (La Jolla, CA, USA) was used for image editing. Data were expressed in terms of mean ± standard deviation. All experiments were repeated 3 times. The measurement data were compared using *t*-test. The difference was statistically significant at $p<0.05$; *$p<0.05$.

Results

miR-152 is Significantly Decreased in Spinal Cord Injury Mice While the Inflammatory Cytokines are Increased

A mouse model of spinal cord injury was constructed. RT-qPCR was performed to detect the expression of miR-152 on the spinal cord tissue of the injured group at 6 h, 24 h, 72 h and 128 h after operation. It was found that the expression of miR-152 gradually decreased from 6 h after operation, which reached the lowest at 128 h (Figure 1A). Since miR-152 interacts with c-jun, the expression of c-jun at different stages of spinal cord injury was also detected. In contrast to that of miR-152, the expression of c-jun gradually increased from 6 h after surgery and reached the highest at 128 h postoperatively (Figure 1B). As more and more studies have found that the immune inflammatory response after SCI plays an important part in the recovery of SCI and post-injury, we examined the protein levels of inflammatory cytokines such as IL-1b, IL-18 and TNF-α. Compared with those of the sham operation group, the levels of IL-1b, IL-18, and TNF-α in the spinal cord injury area increased (Figure 1C). Further mRNA expression assays showed that IL-1b and TNF-α mRNA expressions increased immediately during the first 6 h, which remained at this high level until 24 h, and then decreased rapidly, but still higher than that of the Sham operation group (Figure 1D). These results indicated that mir-152 expression was decreased while inflammatory cytokines were increased in mice with spinal cord injury.

Activation of c-jun N-terminal Kinase Pathway in Mice With Spinal Cord Injury

Since the expression of c-jun, IL-1b, IL-18, and TNF-α were significantly increased in mice after spinal cord injury, in order to further investigate the effect of miR-152 against c-jun, IL-1b, IL-18, and TNF-α after spinal cord injury, we overexpressed miR-152 in the mouse spinal cord injury area by vein injection. After 3 days, we found that the expression levels of c-jun, IL-1b, IL-18, and TNF-α mRNA levels were significantly decreased, although still higher than in the sham operation group (Figure 2A-2D). These results indicated that mir-152 could decrease the expression of c-jun expression.

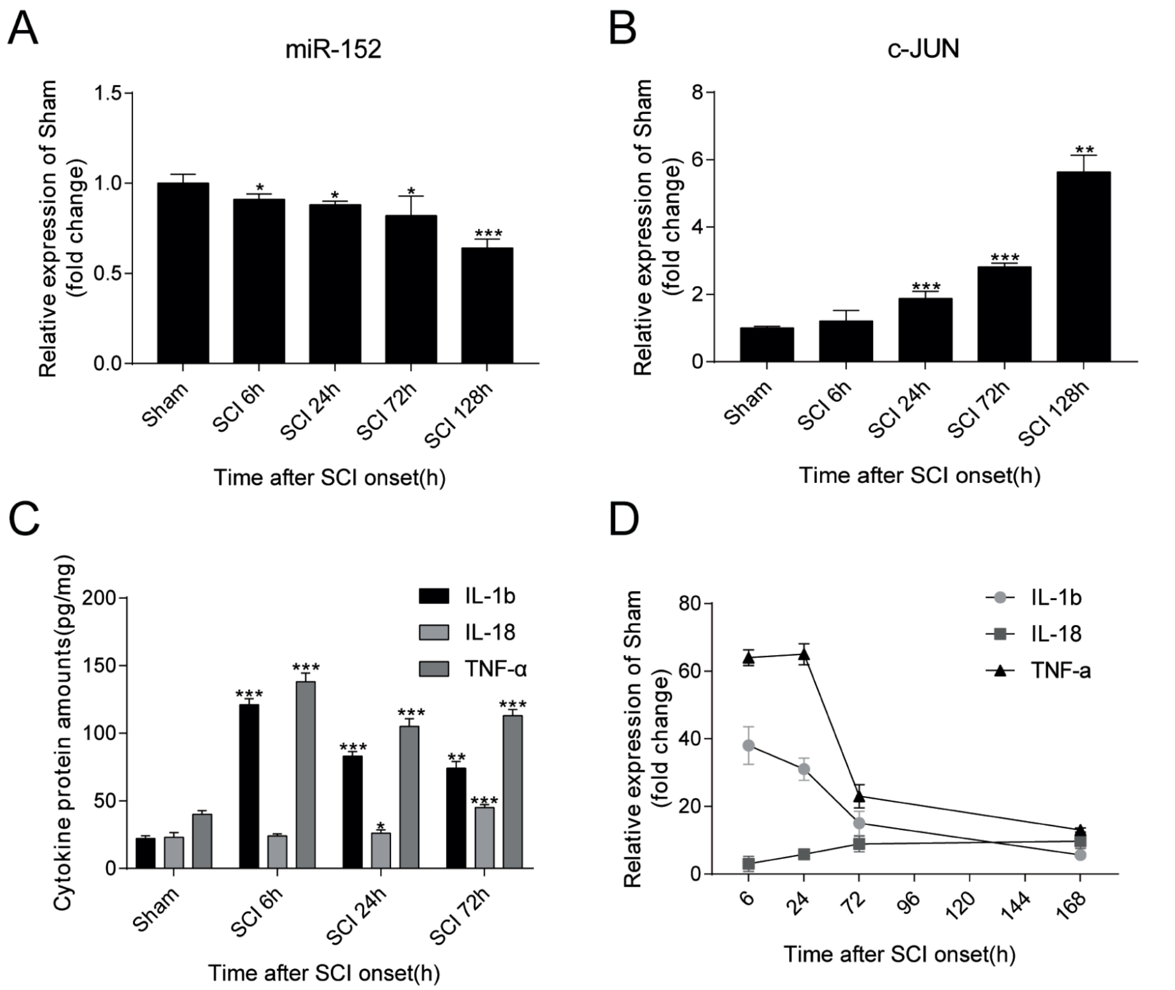

Figure 1. The expression of mir-152 in the spinal cord injury group (SCI group) decreased and inflammatory cytokines increased. ***A,*** The expression level of mir-152 was detected by RT-qPCR after SCI at 6 h, 24 h, 72 h, and 128 h. ***B,*** The expression level of c-jun was detected by RT-qPCR after SCI at 6 h, 24 h, 72 h and 128 h. ***C,*** IL-1b, IL-18, and TNF-α protein content were detected by Western blot after spinal cord injury in mice. ***D,*** IL-1b and TNF-α mRNA expressions were detected by RT-qPCR.

miR-152 Inhibits the Inflammatory Response Via the C-Jun Amino Terminal Kinase Pathway

To further demonstrate the role of miR-152 in the recovery of spinal cord injury, we overexpressed miR-152 in the spinal cord injury zone of SCI mice and found that the biological score of mice gradually increased after miR-152 overexpression (Figure 3A). miR-152 usually acts through c-jun, so the expression of c-jun was detected after miR-152 overexpression. Results showed that overexpression of miR-152 decreased c-jun expression as well as the protein levels of IL-1b, IL-18, and TNF-α (Figure 3B). These results suggested that miR-152 inhibited the inflammatory response *via* the c-jun amino-terminal kinase pathway.

miR-152 can Promote Spinal Cord Injury Recovery

After the operation, the mice in the spinal cord injury group suffered severe motor function damage in the first week, and could not use the forepaw to perform any movement. When the grip strength was measured from 7 d to 14 d, the mouse tried to grasp the measuring rod, but could not complete successful grasping or they could grasp in time, but failed to resist pulling and complete the measurement. From 21 d to 42 d, the grip strength of the forepaws and the grip strength of the two claws gradually improved slightly with time. Although the measurements were completed, the readings were significantly lower than those before surgery, indicating that there were serious motor dysfunctions. In the miR-152 treatment group, the mice had severe motor function damage after the spinal cord injury. However, on the 10th day after the injury, the forepaw function of some mice showed a preliminary recovery, and the grip strength gradually improved with time. At 21 d-42 d after injury, the left and right forepaws of the mice in the treatment group showed significant improvement in the grip strength of the two paws compared with the that of the simple injury group (Figure 4A-4C), suggesting that miR-152 can promote spinal cord injury recovery.

Discussion

As a common and serious surgical injury, traumatic spinal cord injury has a serious impact on

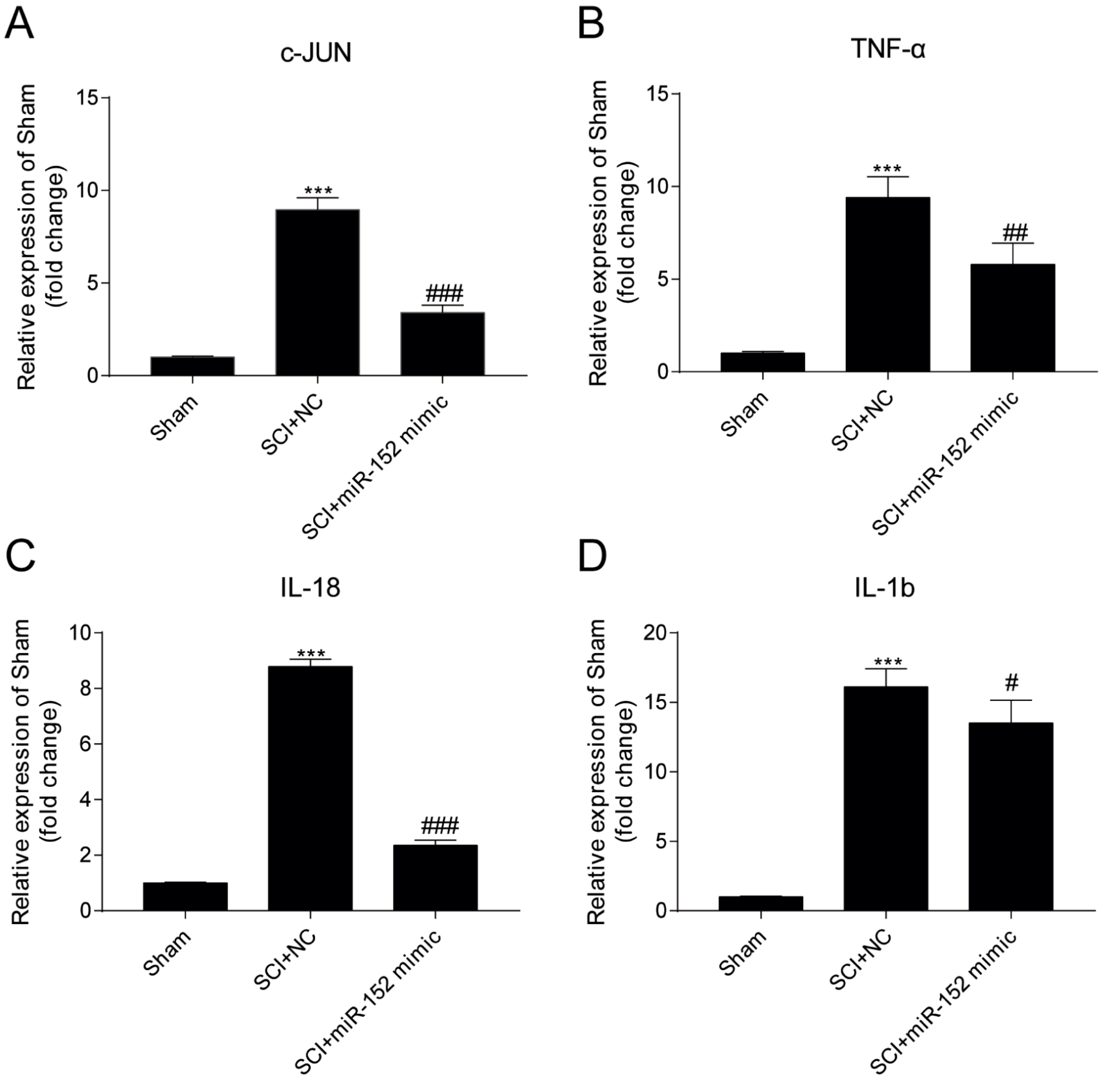

Figure 2. The activation of the c-jun N-terminal kinase pathway in the spinal cord injury group (SCI group). ***A-D,*** After overexpression of miR-152 in the spinal cord injury area of mice for 3 days, the expression levels of -jun, IL-1b, IL-18, and TNF-α mRNA levels were significantly decreased. (*Compared with Sham group, #Compatible with SCI+NC group).

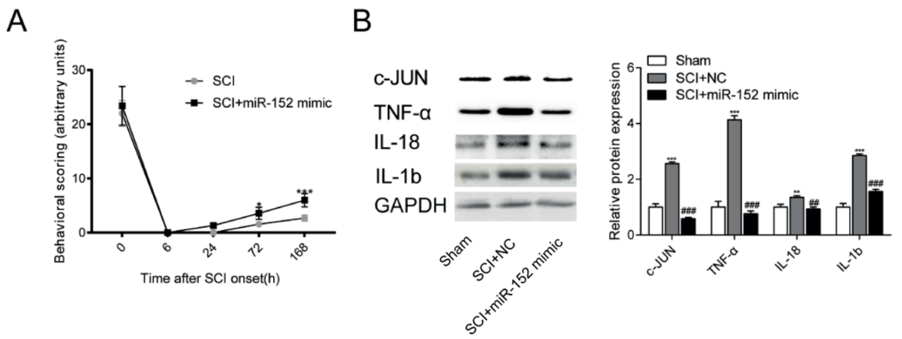

Figure 3. miR-152 inhibits the inflammatory response *via* the c-jun amino terminal kinase pathway. ***A,*** After miR-152 was overexpressed in the mouse spinal cord injury area, the biological score of the mouse was evaluated. ***B,*** After overexpression of miR-152 in the spinal cord injury area of each group, the expression levels of c-jun, IL-1b, IL-18, and TNF-α protein levels were significantly decreased. (*Compared with Sham group, #Compatible with SCI+NC group).

patients' self-care ability and quality of life. Cervical spinal cord injury accounts for more than half of all injuries. Patients with cervical spinal cord injury often have severe injuries along with limb and trunk dysfunction, especially involving the movement disorders of the upper limbs. SCI often brings a catastrophic blow to the quality of life of patients. Therefore, restoring the motor function of the upper limbs has become the biggest appeal of such patients[13].

The models currently used for the study of spinal cord injury recovery and regeneration are diverse. The advantage of the dorsal hemisection model of the spinal cord is that the corticospinal tract can be severely cut, which is a commonly used method for observing motor function changes. However, the disadvantage is that it is not a common mechanism of injury to clinical spinal cord injury, and cannot completely reflect the complex pathological changes after spinal cord injury[14]. The animal model used in this experiment was a modified C5 spinal cord blunt trauma model, using the Infinite Horizon spinal cord perforator to quantify the spinal cord accurately. The advantage of this model is that the hitting dose is precisely controllable, and the damaged property is close to the mechanism of spinal cord injury. It can cause secondary injuries such as spinal cord edema, ischemia and cavity formation, which is convenient for studying the pathophysiological changes of the spinal cord after injury and treatment[15].

Sequencing studies on changes in miRNA expression during spinal cord development suggest that differential expression of miRNA is associated with spinal cord development[12]. Studies have found a variety of highly differentially

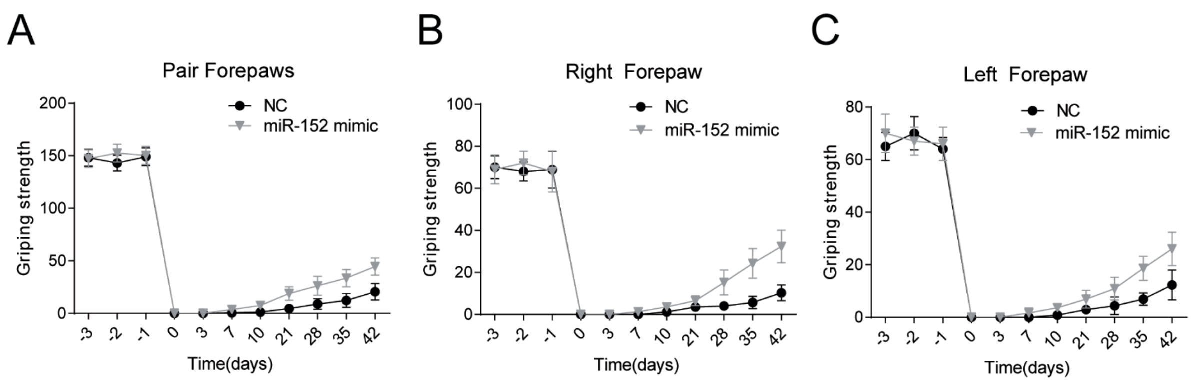

Figure 4. miR-152 can promote spinal cord injury repair. ***A-C,*** After overexpression of miR-152, the grip strength of the mice's forelimb, left forelimb and right forelimb was detected.

expressed miRNAs in the mouse spinal cord[16]. A large number of studies[17,18] on miRNA dysregulation after spinal cord injury have showed that a variety of miRNAs are involved in the regulation of various signaling pathways such as inflammatory response, apoptosis and axonal regeneration after spinal cord injury. The study of miRNA as a target for research and treatment has become a hot topic in recent years[19]. miR-152 has the function of regulating a variety of tissue repair regeneration. In the nervous system, miR-152 can be differentially expressed in the midbrain of human or mouse and can participate in the differentiation of dopaminergic neurons. A small amount of literature has suggested changes and effects of miR-152 in spinal cord injury. Our work detected the expression of miR-152 in the injured area of mice after spinal cord injury by qPCR, and found that its expression was significantly reduced.

The important regulatory role of miRNAs in the pathological process of spinal cord injury has also been gradually confirmed. Studies have shown that miRNAs play important parts in regulating the signaling pathways of inflammatory responses. After the occurrence of spinal cord injury, the expressions of many miRNAs are changed, which are thought to be related to immune responses such as regulation of inflammatory signaling pathways[20-23]. In this study, the expressions of IL-1b, IL-18, and TNF-α were increased after the spinal cord injury, which further decreased after miR-152 treatment.

C-jun is a cellular homologous sequence of v-jun. It is a transforming gene carried by avian sarcoma virus 17 (asv17), which normally has low levels of expression in most cells. Scholars[24] have shown that the expression of c-jun gene is related to the regeneration of nerve cells. In the peripheral nerve tissue or in the central nervous tissue, the expression of c-jun gene appears in the nerve cells after being damaged, which is beneficial to the repair of spinal cord injury. It is found that, after regeneration of central axons after transplantation, the regenerated neurons respond to the transplantation when the transcription factor c-jun gene is up-regulated[25]. More and more experiments have shown that c-jun predicts the regenerative capacity after axonal injury. The stronger the regeneration ability, the higher the expression of c-jun. In this study, the expression of c-jun was significantly increased after spinal cord injury, while miR-152 overexpression decreased the expression of c-jun.

Conclusions

We observed that the expression of mir-152 was decreased after the spinal cord injury while the inflammatory cytokines were increased and the c-jun amino-terminal kinase pathway was activated. miR-152 can be used as a potential target to inhibit inflammatory responses and promote spinal cord injury repair through the c-jun N-terminal kinase pathway.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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