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MicroRNA-448 inhibits the regeneration of spinal cord injury through PI3K/AKT/Bcl-2 axis

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Abstract. – OBJECTIVE: This study aims to elucidate the potential role of microRNA-448 in the recovery of spinal cord injury (SCI), and to explore the underlying mechanism.

MATERIALS AND METHODS: MicroRNA-448 expression was determined by microarray and the established SCI model in mice. The target gene of microRNA-448 was predicted using bioinformatics. The functional binding of the target gene to microRNA-448 was verified by Dual-Luciferase reporter gene assay. The regulatory effects of microRNA-448 and Bcl-2 on apoptosis, motor neuron number and grip strength were evaluated. After injection of microRNA-448 mimics, microRNA-448 inhibitor or Bcl-2 siRNA in mice, expression levels of PI3K/AKT and Caspase3 were detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) and Western blot.

RESULTS: Grip strength of SCI mice significantly decreased compared with mice in the sham group. The microRNA-448 expression gradually increased with the progression of SCI, whereas the Bcl-2 expression decreased. Dual-Luciferase reporter gene assay showed the binding condition between microRNA-448 and Bcl-2. Furthermore, the Bcl-2 expression was negatively regulated by microRNA-448 at both mRNA and protein levels. The injection of microRNA-448 inhibitor into the injured spinal cord of SCI mice significantly upregulated the expressions of p-PI3K, p-AKT and Caspase3, as well as motor neuron regeneration and grip strength. However, the promotive effects of microRNA-448 inhibitor were blocked by Bcl-2 siRNA transfection.

CONCLUSIONS: MicroRNA-448 is upregulated after SCI, which may be involved in the regenerative process of spinal motor nerves by regulating PI3K/AKT/Bcl-2 axis.

Key Words:
MicroRNA-448, PI3K/AKT, Bcl-2, Apoptosis, SCI recovery.

Introduction

Spinal cord injury (SCI) is a spinal trauma caused by falling or traffic accidents, sports and violent injuries[1]. The incidence of SCI is relatively high, which is about 54/1,000,000 in the United States and 23.7/1,000,000 in China per year[2,3]. Neurological function recovery after SCI is difficult in basic researches and clinical treatments[3]. So far, no effective measures have been discovered to cure SCI. Severe SCI may lead to paralysis and even death. Usually, the prognosis of SCI patients is poor, which brings a heavy economic burden to patients and their families. Sequelae at post-SCI seriously affect their life quality.

The pathological process of SCI is a complex dynamic process involving multiple stages of primary injury, secondary injury and chronic injury[4]. The degree of primary injury is determined by torsional force, compression force and nerve transection at the time of injury. Secondary injury resulted from late-stage blood-spinal barrier dysfunction, ischemic edema, inflammatory response, lipid peroxidation and impaired ion channels. These pathological changes can aggravate neuronal death and apoptosis in the lesion sites and adjacent tissues. Meanwhile, it is the major reason for persistent deterioration after SCI[5-7]. Studies have shown that neuronal apoptosis is crucial in SCI. It has been demonstrated that the PI3K/AKT pathway is responsible for the pathogenesis of SCI by promoting neuronal survival, inhibiting neuronal apoptosis and regulating neuronal differentiation and movement[8]. In addition, the Bcl-2 expression is significantly downregulated after SCI, suggesting its potential role in the regulation of neuronal apoptosis[9].

MicroRNA is a type of non-coding RNA containing about 22-24 nucleotides in length. It specifically recognizes and binds to the 3'UTR of target mRNAs. MicroRNA degrades or inhibits the translation of target mRNAs to regulate the gene expression at the post-transcriptional level, thus mediating cellular performances such as differentiation, proliferation and apoptosis[10]. Some investigations[10,11] have shown that a large number of microRNAs are differentially expressed after

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SCI. Genes regulated by these microRNAs are involved in pathological processes, including spinal cord ischemia, edema, inflammation and neuronal necrosis[12]. The expression of miR-199a-5p is upregulated after SCI, which in turn protects the spinal cord from ischemia/reperfusion-induced injury by downregulating ECE1 in rats[13]. MicroRNA-125b inhibits ischemia-reperfusion-induced neuro-inflammation and abnormal activation of p53 by targeting TP53INP1[14]. Furthermore, microRNA-448 has been widely studied in tumors, which serves as an important miRNA in inhibiting tumor cell growth[15,16]. However, its biological function in SCI has not been well analyzed. Therefore, explorations on the role and mechanism of microRNA-448 in SCI may provide new therapeutic targets and intervention strategies for the treatment and rehabilitation of SCI.

Materials and Methods

Establishment of SCI Model in Mice

Female C57bl/6J mice 8 to 10 weeks old were routinely housed. Mice were anesthetized with 3% phenobarbital (30 mg/kg, i.p.). After the skin was disinfected, they were placed on the surgical table with a thermostatic pad. Mice were cut open alongside the neck for dissecting the skin and fascia and removing paravertebral muscles around the C5 vertebral body. The sclera and lamina of C4-C6 were removed to fully expose the dura mater. The C5 spinous process was hit using the Infinite Horizon device (2 mm in diameter and 10 g in weight) from the height of 25 mm. After the surgical procedure, the incision was sutured layer by layer. Postoperative pain relief, fluid infusion and anti-inflammatory treatment were applied. The subdural injection was performed 5 minutes after SCI. A mixture of 10 μL microRNA-448 mimics (or miR-NgCtr or microRNA-448 inhibitor) and Lipofectamine 2000 was subcutaneously injected into the injured spinal cord tissue under a microscope using a Hamilton syringe at a final concentration of 40 μM. Bcl-2 siRNA was injected into mice one day after microRNA-448 inhibitor injection. Finally, mice were sacrificed at the appointed time points as required by the experiment. This study was approved by the Animal Ethics Committee of Jining No. 1 People's Hospital.

Grip Strength Measurement

Mice were gently held so that their tails were brought to the bar of gripping strength meter. They were quickly pulled back in the horizontal direction when their paws were grabbed in the bar. Subsequently, the grip strength of mouse forepaw was recorded when the grip was released. Grip strengths of left or right forepaw were recorded, respectively. Four successful records were taken, and the average grip strength was calculated. The grip strength that mice could not grab in the bar was recorded as 0. Before the experimental test, mice were practiced on the preoperative day 7, 3, and 1, respectively.

Sample Collection

Mice were sacrificed with CO_2 asphyxiation at day 1, 3, 5, 7, and 14. Cardiac perfusion was performed using 10% formaldehyde solution for fixation. The surrounding segmental lamina was removed for fully exposing the injured spinal cord tissues. The lesioned spinal cord tissues were cut 4 mm from the upper to the bottom of the lesion site, which was preserved in liquid nitrogen for subsequent use.

Spinal Motor Neuron Counting

Cardiac perfusion was performed using 10% formaldehyde solution for fixation. The surrounding segmental lamina was removed, and the injured spinal cord tissues were exposed. The lesioned spinal cord tissues were cut 4 mm from the upper to the bottom of the lesion site. The collected tissues were paraffin-embedded, sectioned in 6-μm slices and stained with hematoxylin and eosin. Normal motor neurons in ventricolumna were calculated using a microscope with three randomly selected fields in each sample (magnification × 200).

RNA Extraction and Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Total RNA in tissues (30-40 mg) was extracted by TRIzol method (Invitrogen, Carlsbad, CA, USA). The content of the RNA sample was determined by acid protease apparatus, and then diluted with diethyl pyrocarbonate (DEPC) water (Beyotime, Shanghai, China). Complementary Deoxyribose Nucleic Acid (cDNA) was synthesized according to the instructions of TaKaRa RNA PCR Kit (TaKaRa, Otsu, Shiga, Japan). QRT-PCR parameters were: 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 60 s. Primers used in this study were as follows: MicroRNA-448, F: 5′-TTCCCCTCAGCTCGGACTATA-3′, R: 5′-CGGGCTCAGGTCAAC-

GGG-3′; U6, F: 5’-CTCGCTTCGGCAGCAG-CACATATA-3’, R: universal primer; Bcl-2, F: 5′-GTCGCTACCGTCGTGACTTC-3′, R: 5′-CAGACATGCACCTACCCAGC-3′; GAPDH, F: 5′-AGGTCGGTGTGAACGGATTTG-3′, R: 5′-TGTAGACCATGTAGTTGAGGTCA-3′.

Western Blot

Homogenate was allocated and the supernatant was used to quantify the protein. Electrophoretic and transmembrane were carried out. 5% skim milk was used for blocking non-specific sites at room temperature for 90 min. Membranes were incubated with primary antibody at 4°C overnight. At the other day, membranes were incubated with peroxidase-labeled secondary antibody at room temperature for 2 h. Images were obtained by continuous exposures through the enhanced chemiluminescence (ECL) imaging system. The final results were semi-quantitative analyzed relative to the optical density of β-actin.

Cell Culture

Human nerve cell line PC12 was purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM; Thermo Fisher Scientific, Waltham, MA, USA) containing 10% fetal bovine serum (FBS; Hyclone, South Logan, UT, USA), 100 IU/mL penicillin and 100 μg/mL streptomycin (Invitrogen, Carlsbad, CA, USA). PC12 cells were placed in a 37°C, 5% CO_2 incubator.

Dual Luciferase Reporter Gene Assay

The possible binding site of microRNA-448 on the Bcl-2 sequence was predicted by TargetScan (http://www.targetscan.org/). The predicted sequence was inserted into the Luciferase reporter plasmid for constructing the reporter vector Bcl-2 WT. Meanwhile, predicted binding sequence in Bcl-2 was mutated to establish Bcl-2 MUT. Bcl-2 WT or Bcl-2 MUT (500 ng) and microRNA-448 mimics (100 nmol/L) were co-transfected in cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After 48 h of transfection, cells were harvested for the determination of the activity.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 (Chicago, IL, USA) was used for all statistical analysis. Experimental data were expressed by $\bar{x}\pm s$. The *t*-test was used to compare the differences between two different groups. $p<0.05$ was considered statistically significant.

Results

Behavioral Analysis and MicroRNA-448 Expression in SCI Mice

To assess the injury of SCI on mouse behaviors, we first observed grip strength changes in mice. Mice in the sham group could not grip at all in the early stage after surgery. Meanwhile, the recorded grip significantly decreased when compared with the preoperative level. One week after the surgery, the wound and grip strength gradually recovered. However, it was still markedly lower when compared with the preoperative grip strength. In the SCI group, the grip strength recorded was basically 0, suggesting that mouse forepaws completely lost the physical function. Until postoperative day 7 to day 14, SCI mice tended to grasp the measuring rod, but unfortunately failed. From postoperative day 14 to day 49, their grip strengths slightly improved, which were still far away from those of the sham group (Figure 1A-1C). To explore the possible mechanism of SCI, we analyzed miRNA microarrays relative to SCI. The results showed that microRNA-448 was significantly upregulated in injured spinal cord tissues (Figure 1D). Furthermore, microRNA-448 expression in injured spinal cord tissues of mice was determined at postoperative day 1, 3, 4, 7, and 14, respectively. The results indicated that microRNA-448 expression gradually increased with the progression of SCI (Figure 1E). Radulovic et al[17] have pointed out that the Bcl-2 expression decreases after SCI. Here, both the mRNA and protein levels of Bcl-2 in SCI mice gradually decreased in a time-dependent manner, which was consistent with the previous studies (Figure 1F). These results suggested that microRNA-448 might be involved in the recovery process of SCI.

Bcl-2 was the Target Gene of MicroRNA-448

To elucidate the potential mechanism of microRNA-448 in regulating the pathogenesis of SCI, its target gene was predicted by bioinformatics. We found that the Bcl-2 sequence contained a potential binding site with microRNA-448 (Figure 2A). Therefore, it was speculated whether microRNA-448 could regulate Bcl-2 and further mediate the recovery process of SCI. Subsequently, we constructed Bcl-2 WT and Bcl-2 MUT plasmids. Dual-Luciferase reporter gene assay revealed that the Luciferase activity in PC12 cells co-transfected with Bcl-2 WT and microRNA-448

mimics significantly decreased. However, no significant difference in Luciferase activity was observed in PC12 cells co-transfected with Bcl-2 MUT and microRNA-448 mimics, suggesting that Bcl-2 was the target gene of microRNA-448 (Figure 2B). Next, we evaluated the regulatory effect of microRNA-448 on Bcl-2 expression by injecting microRNA-448 mimics into injured spinal cord tissues of mice. Compared with controls, both the mRNA and protein levels of Bcl-2 were remarkably downregulated in SCI mice injected with microRNA-448 mimics (Figure 2C and 2D). Therefore, we proposed that microRNA-448 was involved in the recovery process of SCI by directly targeting Bcl-2 to inhibit its expression.

MicroRNA-448 Regulated Neuronal Apoptosis

Numerous studies have shown the vital role of neuronal apoptosis in secondary injury after SCI[18,19]. The PI3K/AKT pathway promotes neuronal survival and exerts anti-apoptotic effects[20,21]. Therefore, we examined the expression levels of p-AKT/AKT and p-PI3K/PI3K in SCI mice. The results showed that the expressions of p-AKT and p-PI3K in the SCI group remarkably decreased compared with the sham group. Notably, the injection of the microRNA-448 inhibitor into SCI mice markedly upregulated p-AKT and p-PI3K (Figure 3A). In addition, the expression level of Caspase3 was determined as well. The results demonstrated that this apoptosis indicator sharply increased in SCI mice but was inhibited by microRNA-448 inhibitor treatment (Figure 3B). We thereby believed that microRNA-448 regulated neuronal apoptosis in SCI mice through the PI3K/AKT pathway. The number of motor neurons in SCI mice was calculated, and the effect of the microRNA-448 inhibitor on spinal cord function was evaluated. SCI mice showed significantly fewer motor neurons than those of the sham group. However, the injection of the microRNA-448 inhibitor greatly enhanced its number (Figure 3C). In addition, the grip strength of SCI mice was observed after the injection of the microRNA-448 inhibitor. Pair forepaws, right and left forepaw of SCI mice injected with microRNA-448 inhibitor markedly improved in a time-dependent manner, especially at postoperative day 14 (Figure 3D-3F).

MicroRNA-448 Regulated SCI Recovery by Targeting Bcl-2

The above results showed that microRNA-448 inhibitor could promote SCI recovery. To verify the role of Bcl-2 in microRNA-448 inhibitor-induced SCI recovery, Bcl-2 siRNA was injected

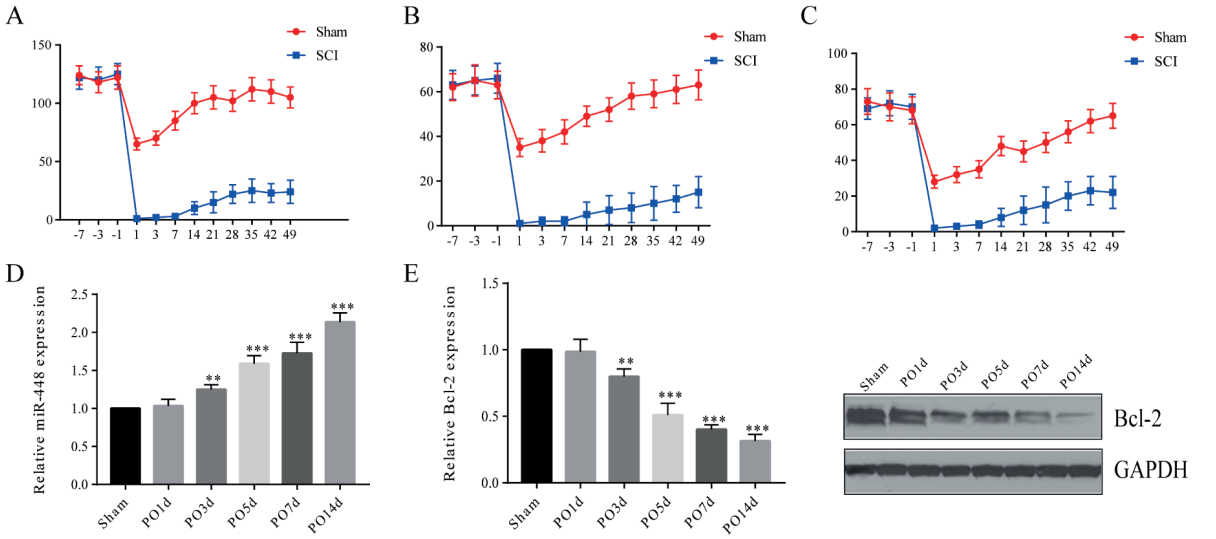

Figure 1. Behavioral analysis and microRNA-448 expression in SCI mice. Mice were divided into the sham group and the SCI group. ***A-C***, Grip strengths of pair forepaws, right and left forepaw in mice of the sham group and the SCI group. Data were calculated from four inde-pendent records. ***D,*** Heat map of differentially expressed genes in SCI. Red represented high-expression and green represented low expression. ***E,*** The mRNA level of microRNA-448 in-creased in a time-dependent manner after SCI. ***F,*** The mRNA and protein levels of Bcl-2 de-creased in a time-dependent manner after SCI. GAPHD was used as an internal control. **p*<0.05, ***p*<0.01, ****p*<0.001.

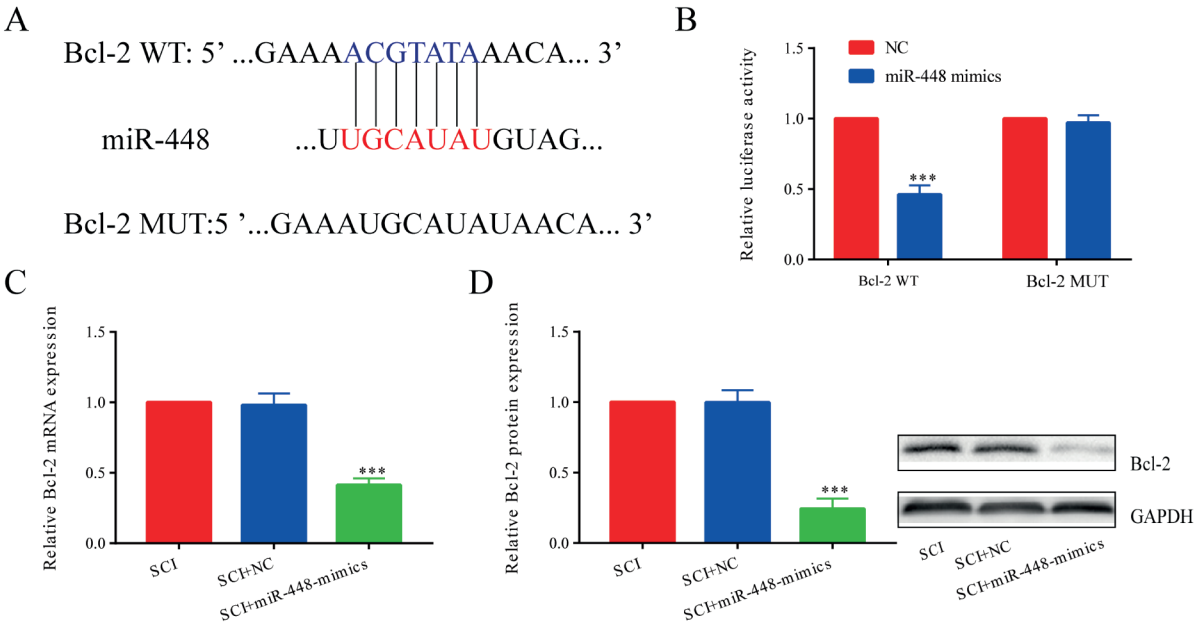

Figure 2. Bcl-2 was the target gene of microRNA-448. ***A,*** Bcl-2 sequence contained a potential binding site with microRNA-448. B, Dual-Luciferase reporter gene assay revealed the binding condition between Bcl-2 and microRNA-448. ***C,*** The mRNA level of Bcl-2 was significantly lower in the SCI+microRNA-448-mimics group than the SCI group and the SCI+NC group. ***D,*** The protein level of Bcl-2 was remarkably lower in the SCI+microRNA-448-mimics group than the SCI group and the SCI+NC group. *p<0.05, **p<0.01, ***p<0.001.

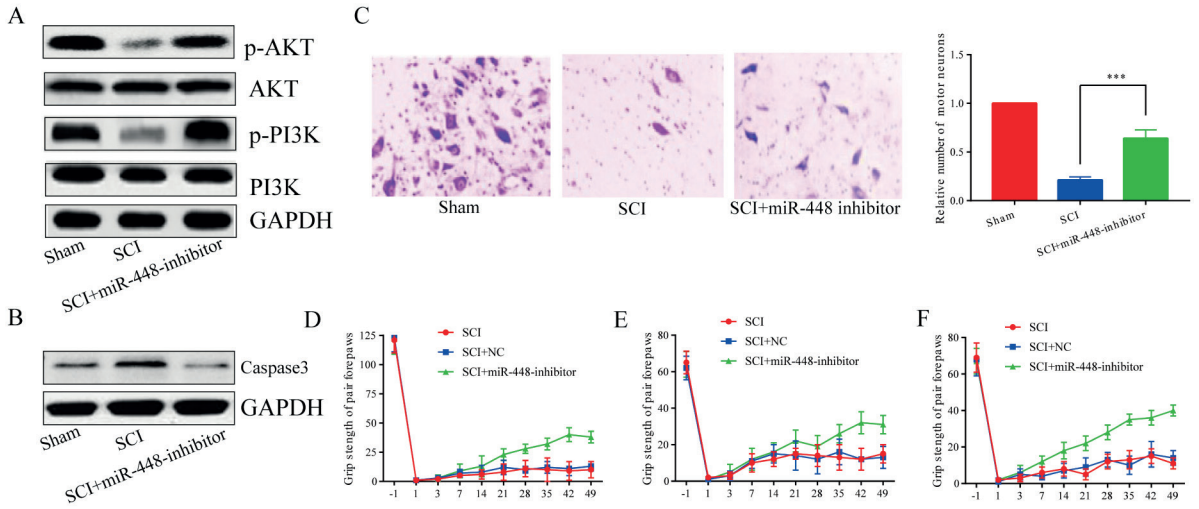

Figure 3. MicroRNA-448 regulated neuronal apoptosis. ***A,*** Western blot analysis of p-AKT, AKT, p-PI3K, PI3K and GAPDH in the sham group, SCI group and SCI+microRNA-448-inhibitor group. ***B,*** Western blot analysis of Caspase3 and GAPDH in the sham group, SCI group and SCI+microRNA-448-inhibitor group. ***C,*** Relative number of motor neurons in the sham group, the SCI group and the SCI+microRNA-448-inhibitor group (magnification x 40). D-F, Grip strengths of pair forepaws, right and left forepaw in mice of the sham group, SCI group and SCI+microRNA-448-inhibitor group. *p<0.05, **p<0.01, ***p<0.001.

into the injured spinal cord of SCI mice. The results indicated that the injection of Bcl-2 siRNA effectively downregulated the Bcl-2 expression at both mRNA and protein levels (Figure 4A and 4B). Furthermore, the promotive effect of microRNA-448 inhibitor on the regeneration of injured spinal motor neurons was reversed by Bcl-2 siRNA injection (Figure 4C). Besides, the improved

pair forepaws, right and left forepaw of SCI mice injected with microRNA-448 inhibitor were deteriorated after the injection of Bcl-2 siRNA (Figure 4D). These results indicated that microRNA-448 regulated SCI recovery by targeting Bcl-2.

Discussion

Traumatic SCI is a common and serious surgical injury, in which cervical SCI accounts for over 50% of all SCI cases[22]. Patients with cervical SCI are often accompanied by severe injuries and physical dysfunction, especially movement disorders of the upper limbs. Moreover, SCI is a catastrophic disease that seriously affects the life quality of the patients. Based on the pathological changes after SCI, it is divided into primary injury and secondary injury. Massive neuronal death caused by ischemia and hypoxia after SCI is the major reason for the deterioration of the disease. It is believed that the prevention of the secondary neuronal death is an important strategy to improve the prognosis of SCI[23].

The fundamental function of microRNAs is to control gene expressions by regulating specific mRNA translation. A variety of microRNAs is involved in regulating the development and recovery of SCI. For example, miR-219 is capable of regulating astrocyte proliferation, oxidative stress and inflammatory response by targeting downstream gene ELOVL7, thereafter leading to lipid accumulation in the cerebral white matter[24]. The upregulation of miR-21 can reduce cell hypertrophy, inhibit compact scars formation, promote axonal regeneration at the injury site and contribute to recovery after SCI[25]. MiR-486 inhibits the expression of neuro-differentiation factor NeuroD6. Meanwhile, low expression of miR-486 in SCI model can promote the functional recovery of hind limbs[26]. Therefore, it is particularly important to study the role of microRNAs in the development of SCI. Previous studies[27] have pointed out the crucial function of microRNA-448 in tumors. It is reported that microRNA-448 inhibits the proliferation and accelerates the apoptosis of glioma cells *via* downregulating CTTN.

To explore the role of microRNA-448 in SCI, microRNA-448 expression was determined by microarray and the established SCI model in mice. The results showed that microRNA-448 was highly expressed in injured spinal cord tissues, whereas Bcl-2 was lowly expressed. Previously scholars[28,29] have found that microRNA-448 can inhibit the Bcl-2 expression and regulate the apoptosis of bladder cancer and

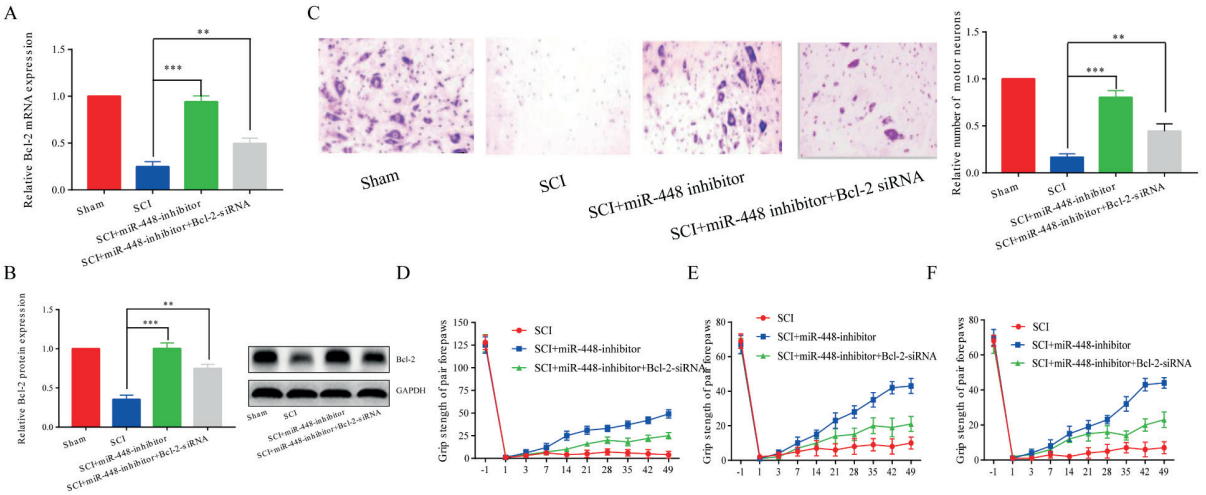

Figure 4. MicroRNA-448 regulated SCI recovery by targeting Bcl-2. ***A,*** The mRNA level of Bcl-2 in the sham group, SCI group, SCI+microRNA-448-inhibitor group and SCI+microRNA-448-inhibitor+Bcl-2-siRNA group. ***B,*** The protein level of Bcl-2 in the sham group, SCI group, SCI+microRNA-448-inhibitor group and SCI+microRNA-448-inhibitor+Bcl-2-siRNA group. ***C,*** Relative number of motor neurons in the sham group, SCI group, SCI+microRNA-448-inhibitor group and SCI+microRNA-448-inhibitor+Bcl-2-siRNA group (magnification x 40). ***D-F***, Grip strengths of pair forepaws, right and left forepaw in mice of the sham group, SCI group, SCI+microRNA-448-inhibitor group and the SCI+microRNA-448-inhibitor+Bcl-2-siRNA group. *p<0.05, **p<0.01, ***p<0.001.

lung adenocarcinoma. Subsequently, the Dual-Luciferase reporter gene assay verified that microRNA-448 could interact with Bcl-2 in neurons, which was further confirmed by qRT-PCR and Western blot results. The regulatory effect of microRNA-448 on Bcl-2 was consistent with previous conclusions[29]. PI3K/AKT is crucial in neuronal regeneration and inhibition of neuronal apoptosis. Subsequently, we injected microRNA-448 mimics into the injured spinal cord in mice. The results suggested that the knockdown of microRNA-448 significantly inhibited neuronal apoptosis by activating the PI3K/AKT pathway. For SCI patients, the recovery of upper limb function is of great significance for the improvement of self-care ability. Therefore, motor neuron regeneration ability and upper limb motor function were evaluated in SCI mice. The injection of microRNA-448 mimics markedly alleviated mouse grip strength and improved spinal motor neuron regeneration but was blocked by the injection of Bcl-2 siRNA. The above experimental results suggested that microRNA-448 was involved in the process of motor nerve recovery after SCI by regulating the PI3K/AKT/Bcl-2 axis.

Conclusions

We showed that microRNA-448 was upregulated after SCI, which may be involved in the regenerative process of spinal motor nerves by regulating the PI3K/AKT/Bcl-2 axis.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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