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# Overexpression of miRNA-433-5p protects acute spinal cord injury through activating MAPK1

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**Abstract. – OBJECTIVE: The aim of this study was to clarify the role of microRNA-433-5p (miRNA-433-5p) in influencing pathological lesions following acute spinal cord injury (SCI) by targeting mitogen-activated protein kinase 1 (MAPK1).**

**PATIENTS AND METHODS: SCI model was successfully established in mice by performing hitting injury procedures. Serum levels of miRNA-433-5p and MAPK1 in SCI patients and mice were determined. Grip strengths of both forelimbs in SCI mice and controls were determined. Dual-Luciferase reporter gene assay was applied to verify the binding relation between miRNA-433-5p and MAPK1. After overexpression of miRNA-433-5p and MAPK1 in vivo, the grip strength changes in SCI mice were assessed. Furthermore, the protein level of inflammatory factor iNOS in 293T cells influenced by miRNA-433-5p and MAPK1 was detected by Western blot.**

**RESULTS: MiRNA-433-5p was significantly downregulated in the serum of SCI patients and mice, whereas MAPK1 was up-regulated. Grip strengths of SCI mice were significantly lower than those of controls at different postoperative time points. However, this could be markedly reversed by the in vivo overexpression of miRNA-433-5p. Western blot indicated that the protein level of iNOS was remarkably downregulated in 293T cells overexpressing miRNA-433-5p. MAPK1 was confirmed as the target of miRNA-433-5p, whose expression level was negatively regulated by miRNA-433-5p. Importantly, MAPK1 partially reversed the protective role of miRNA-433-5p in grip strength of SCI mice and inflammatory response at post-SCI.**

**CONCLUSIONS: Overexpression of miRNA-433-5p protects SCI-induced motor dysfunction and inflammatory response by targeting MAPK1.**

*Key Words:*
Spinal cord injury (SCI), MiRNA-433-5p, MAPK1.

## Introduction

Spinal cord injury (SCI) is one of the serious traumas in the central nervous system. It is further deteriorated by various factors, such as ischemia, edema, oxygen-free radical formation, and inflammatory reaction. Primary and secondary injuries at post-SCI can eventually lead to the loss of limb movement and sensory ability[1]. Currently, there is still a lack of satisfactory therapy for SCI[2].

MicroRNAs (miRNAs) are a type of non-coding RNAs with about 22 nucleotides in length. They are capable of regulating gene expressions at the transcriptional level. As a transcriptional regulator, miRNA can also regulate protein synthesis, thus influencing disease progression[3]. Currently, plenty of miRNAs have been identified in mediating nervous system function under physiological conditions. These certain miRNAs have been observed to maintain the morphological structure of nerve cells, as well as axonal growth and synaptic plasticity[4]. During the deterioration of SCI, tissue-specific miRNA levels may reflect the severity of the disease[5]. MiRNAs are vital mediators in SCI-induced primary and secondary injuries of the central nervous system[6].

Mitogen-activated protein kinase 1 (MAPK1) is an important protein of MAPK signaling. It inactivates MAPK through dephosphorylating serine/threonine and tyrosine[7]. As a by-regulator of MAPK signaling, MAPK1 is mainly involved in the regulation of cell proliferation, growth, and differentiation. Previous studies[8] have found that it exerts a promoting effect during the development of cancer. In this paper, the SCI model was first successfully established in mice. The regulatory effects of miRNA-433-5p/MAPK1 on

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SCI-induced pathological lesions were further explored. Our findings might provide a theoretical basis for SCI treatment.

## Patients and Methods

### Patients

Blood serum samples were harvested from SCI patients, and healthy controls enrolled in the Second Affiliated Hospital of Harbin Medical University. SCI patients were pathologically confirmed by MRI, CT or during the surgery. No patient reported the history of nervous system diseases, cerebrovascular diseases, and spinal cord compression. Blood sample (5 mL) was collected under the fasting state. After centrifugation at 3000 rpm for 10 min, the supernatant serum was collected. Subsequently, serum samples were subpacked into cryotubes and preserved at –80°C for use. Informed consent was obtained from each patient before the study. Our resarch was approved by the Ethics Committee of the hospital.

### Establishment of SCI Model in Mice

A total of 12 female and 12 male adult mice weighing 18-23 g were housed in SPF-level experimental animal center (with room temperature of 25±3°C, humidity of 55±5%, and light/dark cycle of 12 h/12 h). All mice were given free access to food and water. Mice were anesthetized by intraperitoneal injection of 10% chloral hydrate (0.33 mL/kg). After skin disinfection on the surgical area, the mice were fixed on the table. A 2-cm longitudinal incision was made on the back to expose the T9-T11 spinous process. Muscles attaching on the processes were separated, followed by removal of T9-T10 laminas. Subsequently, the mice were fixed on the stereo positioner. SCI model was established by 5-g hitting from the height of 3 cm. Retracted hind legs and swaying tail indicated the successful establishment of SCI model in mice. Next, the incision was sutured layer by layer. Meanwhile, mice in control group only underwent removal of laminas and spinous processes.

SCI mice were further assigned into three groups, including SCI+NC group, SCI+miR-433-5p group, and SCI+MAPK1 group. Subdural injection of the corresponding lentivirus in the lessoned area was conducted at 5 min after spinal hitting.

### Cell Transfection

The cells were first cultured in antibiotics-free medium overnight. Until 80% of confluence, cell transfection was performed according to the instructions of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Briefly, 16 μg DNA and 24 μL lipofectamine were diluted in 800 μL of the medium, respectively, followed by incubation at room temperature for 5 min. After mixing, the mixture was maintained at room temperature for 20 min and added in cells. Transfected cells were harvested at 24-48 h for subsequent experiments.

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

Total RNA in cells was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Extracted RNA was reverse transcribed into complementary deoxyribose nucleic acid (cDNA). Subsequently, obtained cDNA was applied for PCR using the SYBR Green method (TaKaRa, Komatsu, Japan). U6 was used as the internal reference. Primer sequences were synthesized by Invitrogen Co., Ltd. (Carlsbad, CA, USA). Primer sequences used in this study were as follows: miRNA-433-5p, F: 5'-GCGTGCACTTCACCGTCCAA-3', R: 5'-GCCGGTATCTGTACAAGCC-3'; U6: F: 5'-CTCGCTTCGGCAGCACA-3', R: 5'- AACGCTTCACGAATTTGCGT-3'.

### Grip Strength Determination

The mice were gently held so that their tails were brought to the bar of GSM (grip strength meter). Subsequently, they were induced for grabbing the bar using their paws. The mice were pulled back quickly and gently in horizontal direction. Forepaw griping strength was recorded when the grip was released. Grip strength for pair, left and right forepaws were recorded four times. Grip strength that the mice could not grab in the bar was recorded as 0.

### Western Blot

Total protein was extracted from transfected cells by radioimmunoprecipitation assay (RIPA) solution (Beyotime, Shanghai, China). Protein samples were separated by electrophoresis and transferred onto polyvinylidene difluoride (PVDF) membranes (Roche, Basel, Switzerland). After blocking with skimmed milk, the membranes were incubated with primary antibodies overnight at 4°C. On the next day, the mem-

branes were incubated with the corresponding secondary antibody at room temperature for 1 h. Immuno-reactive bands were finally exposed by enhanced chemiluminescence (ECL) assay.

### Dual-Luciferase Reporter Gene Assay

The potential targets of miRNA-433-5p were predicted by TargetScan (http://www.targetscan.org/). MAPK1 WT and MUT luciferase vectors were constructed based on the binding sites in promoter regions of miRNA-433-5p and MAPK1, respectively. 293T cells were co-transfected with miRNA-433-5p mimics/NC and MAPK1 WT/MAPK1 MUT. After transfection of 48 h, the cells were lysed. Finally, relative luciferase activity was determined by relative kit (Promega, Madison, WI, USA).

### Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 statistical software (IBM Corp., Armonk, NY, USA) was used for all statistical analysis. Experimental data were expressed as mean ± SEM (Standard Error of Mean). The *t*-test was used to compare the differences between the two groups. $p<0.05$ was considered statistically significant.

## Results

### MiRNA-433-5p Was Lowly Expressed in SCI Patients and Mice

The serum level of miRNA-433-5p was markedly lower in SCI patients relative to healthy controls (Figure 1A). Similarly, miRNA-433-5p was lowly expressed in SCI mice, showing a time-dependent decrease (Figure 1B). Grip strengths of pair, right and left forepaw were determined in SCI mice and sham group, respectively. A remarkable decline was observed in grip strength of sham group due to postoperative pain. With the healing of the incision, their grip strengths gradually recovered to normal. Motor function in SCI mice was severely impaired within the first week of SCI procedures. SCI mice failed to move with their pair forepaws until the postoperative 10$^{th}$ day. At that time point, SCI mice were able to complete grip strength examination. However, their grip strengths were extremely lower than those of sham group (Figure 1C-1E).

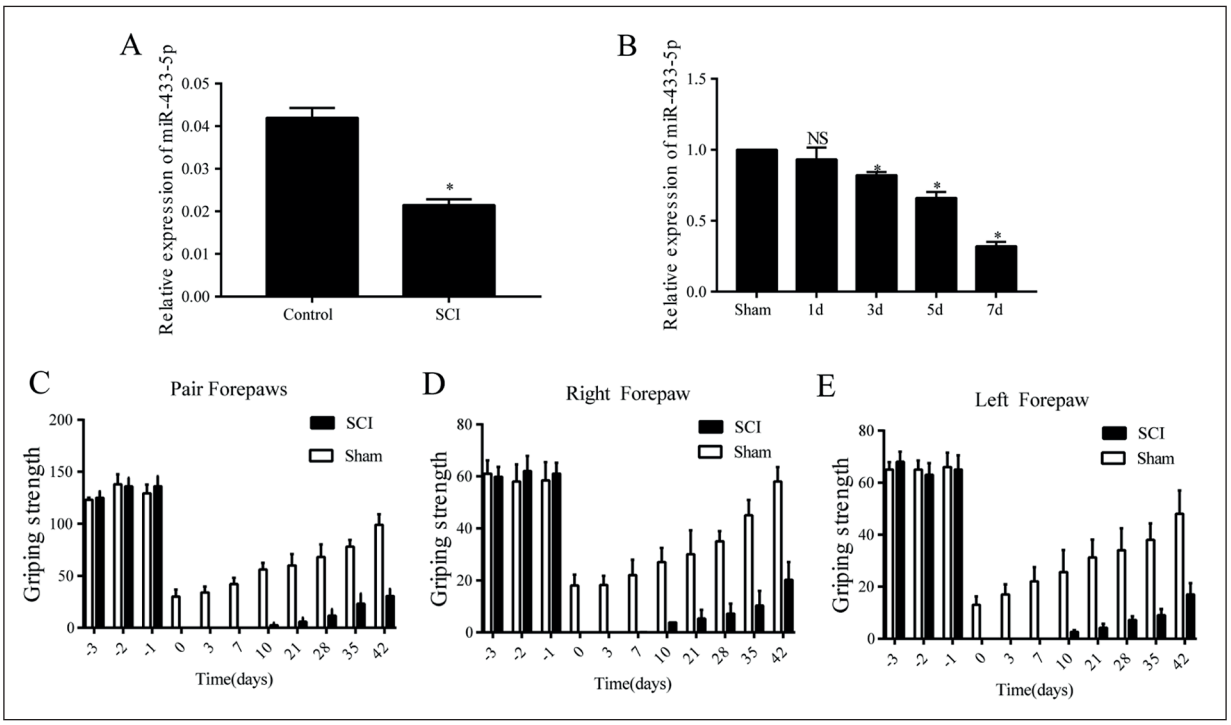


**Figure 1.** Low level of miRNA-433-5p in SCI patients and mice. **A,** Serum level of miRNA-433-5p in healthy controls and SCI patients. **B,** Serum level of miRNA-433-5p in mice of sham group and SCI mice at day 1, 3, 5, and 7. **C-E,** Griping strengths of pair forepaws (**C**), right forepaw (**D**) and left forepaw (**E**) at day -3, -2, -1, 0, 3, 7, 10, 21, 28, 35, and 42 in SCI mice or sham group.

### Overexpression of MiRNA-433-5p Protected SCI-Induced Pathological Lesions

To clarify the function of miRNA-433-5p *in vivo*, SCI mice were administrated with miRNA-433-5p overexpression lentivirus or negative control, respectively. From the postoperative 3rd day, the level of miRNA-433-5p increased in a time-dependent manner in SCI mice administrated with miRNA-433-5p overexpression lentivirus (Figure 2A). Grip strengths in SCI mice overexpressing miRNA-433-5p decreased significantly on the first day following SCI. However, they were still capable of completing the examination. Motor function of SCI mice administrated with NC gradually recovered at the 10th day. Meanwhile, their grip strengths were remarkably lower than those with miRNA-433-5p overexpression (Figure 2B-2D). Western blotting indicated that the protein level of inflammatory factor iNOS was significantly downregulated in 293T cells overexpressing miRNA-433-5p (Figure 2E). Hence, miRNA-433-5p overexpression protected motor function and alleviated inflammatory response following SCI.

### MAPK1 Was the Target Gene of MiRNA-433-5p

Online bioinformatics predicted the binding sites in the promoter region of miRNA-433-5p and MAPK1 (Figure 3A). MAPK1 was found significantly upregulated in the serum of SCI patients (Figure 3B). The serum level of MAPK1 was identically higher in SCI mice, which was time-dependently elevated at post-SCI (Figure 3C). In addition, the *in vivo* expression level of MAPK1 decreased gradually in SCI mice overexpressing miRNA-433-5p. This indicated that there was a negative correlation between miRNA-433-5p and MAPK1 (Figure 3D). In 293T cells, Dual-Luciferase reporter gene assay verified the binding relationship between miRNA-433-5p and MAPK1 (Figure 3E).

### MiRNA-433-5p Influenced SCI by Targeting MAPK1

In 293T cells transfected with pcDNA-MAPK1, MAPK1 level was significantly upregulated. This suggested pronounced transfection efficacy (Fig-

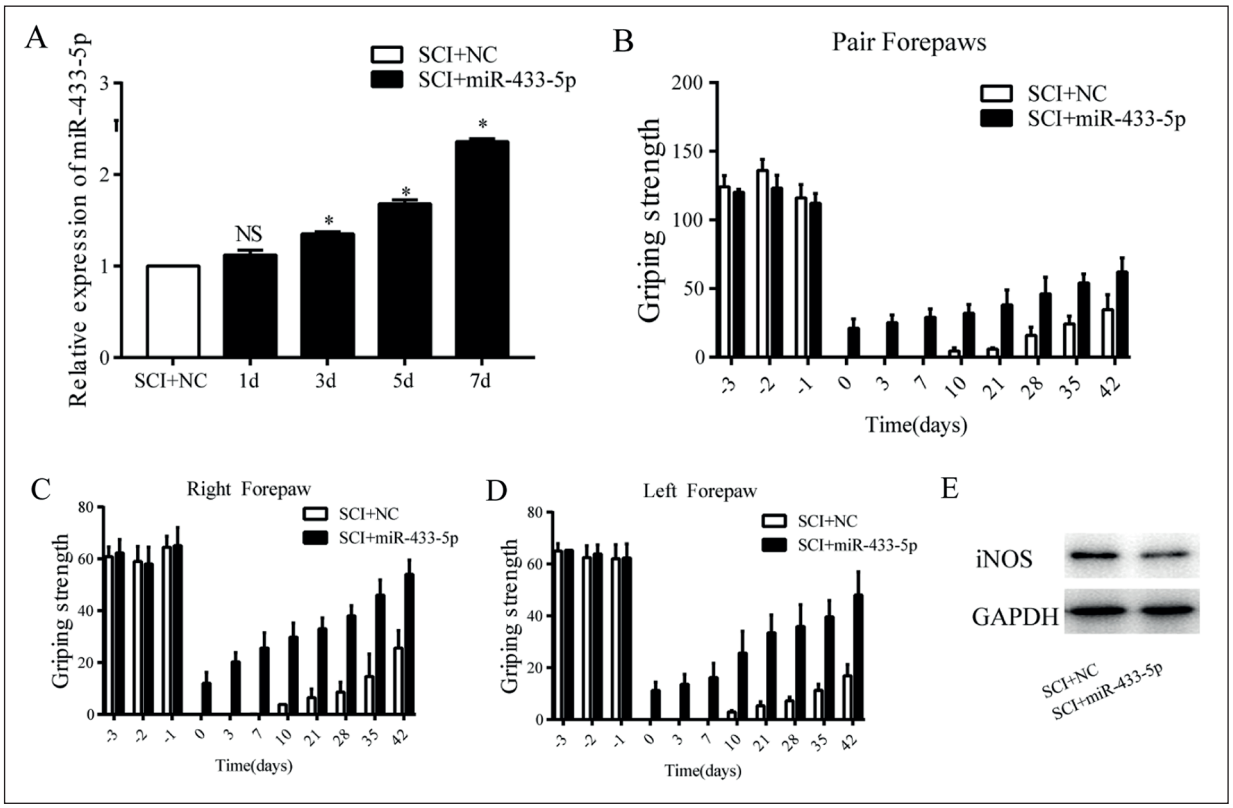


**Figure 2.** Overexpression of miRNA-433-5p protected SCI-induced pathological lesions. **A,** Serum level of miRNA-433-5p in SCI mice and those overexpressing miRNA-433-5p at day 1, 3, 5 and 7. **B-D,** Griping strengths of pair forepaws (**B**), right forepaw (**C**) and left forepaw (**D**) at day -3, -2, -1, 0, 3, 7, 10, 21, 28, 35, and 42 in SCI mice or those overexpressing miRNA-433-5p. **E,** Protein level of iNOS in SCI mice or those overexpressing miRNA-433-5p.

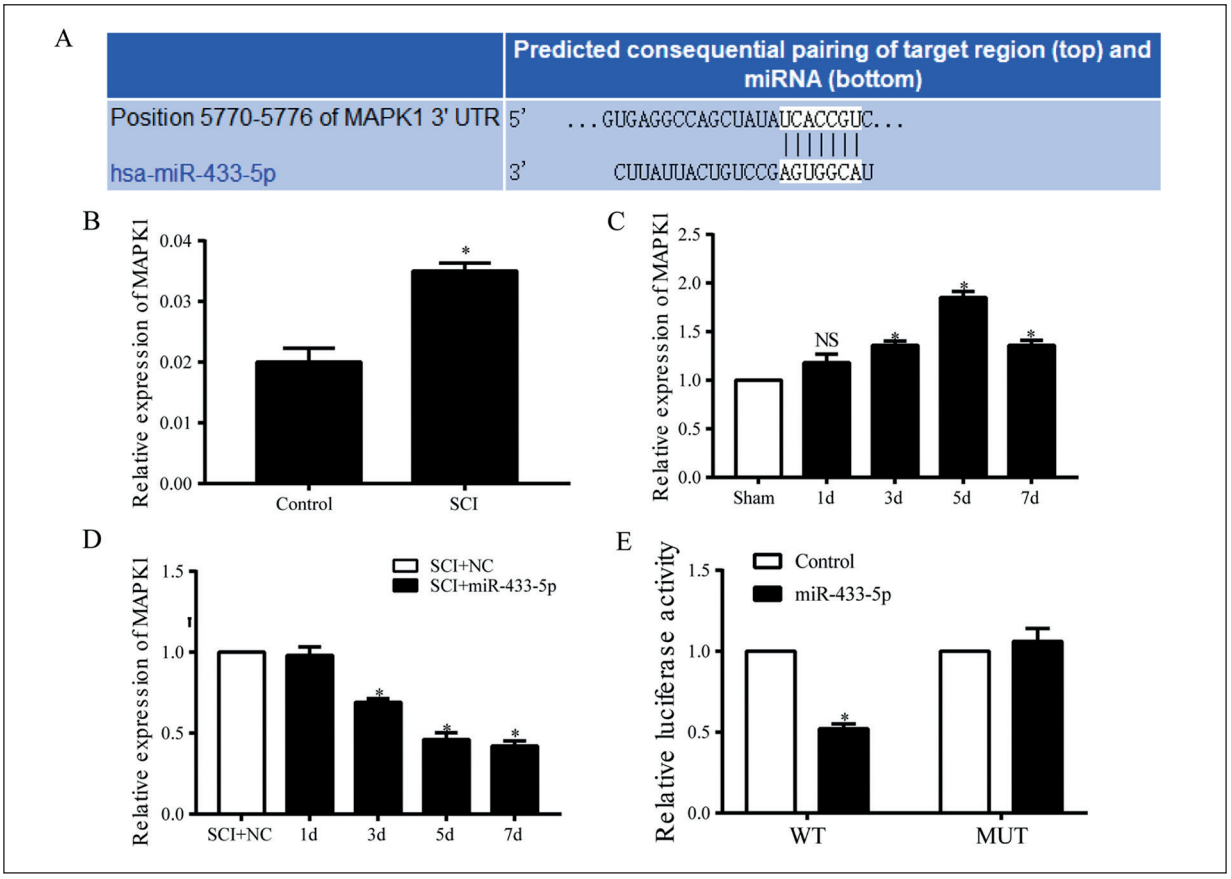


**Figure 3.** MAPK1 was the target gene of miRNA-433-5p. **A,** Binding sites between miRNA-433-5p and MAPK1. **B,** Serum level of MAPK1 in healthy controls and SCI patients. **C,** Serum level of MAPK1 in mice of sham group and SCI mice at day 1, 3, 5, and 7. **D,** Serum level of MAPK1 in SCI mice or those overexpressing miRNA-433-5p at day 1, 3, 5, and 7. **E,** Luciferase activity in 293T cells co-transfected with miRNA-433-5p mimics/NC and MAPK1 WT/MAPK1 MUT.

ure 4A). Later, the results demonstrated that grip strengths in SCI mice co-overexpressing miRNA-433-5p and MAPK1 were remarkably worse when compared with those only overexpressing miRNA-433-5p (Figure 4B-4D). Moreover, downregulation of iNOS in SCI mice overexpressing miRNA-433-5p could be reversed after co-overexpression of MAPK1 (Figure 4E). The above findings proved the involvement of MAPK1 in the protective role of miRNA-433-5p in SCI.

## Discussion

SCI is a severe trauma manifested as high incidence, mortality, and disability[9,10]. With the development of modern economic society, transportation, and construction, its incidence increases each year in the United States. It is reported that there are 10,000 new cases of SCI every year in the United States[11]. In our country, the onset number of SCI rises to 60,000. This poses a great economic burden to the patients and their families owing to high medical expense[12]. Hence, effective treatment of SCI should be well-studied[13,14].

Accumulating evidence has demonstrated miRNA level changes following SCI. Change trend in SCI-related miRNAs includes three circumstances: (1) upregulation; (2) downregulation; and (3) upregulation in the early phase (4 h) and downregulation in the late phase (1-7 d). Bioinformatics has uncovered that target downstream genes of SCI-related miRNAs are mainly involved in regulating the inflammatory response, oxidative stress, and cell apoptosis. All of these processes are major pathological steps at post-SCI[15-17]. Target genes of one miRNA are varied under different cell types or oxidation

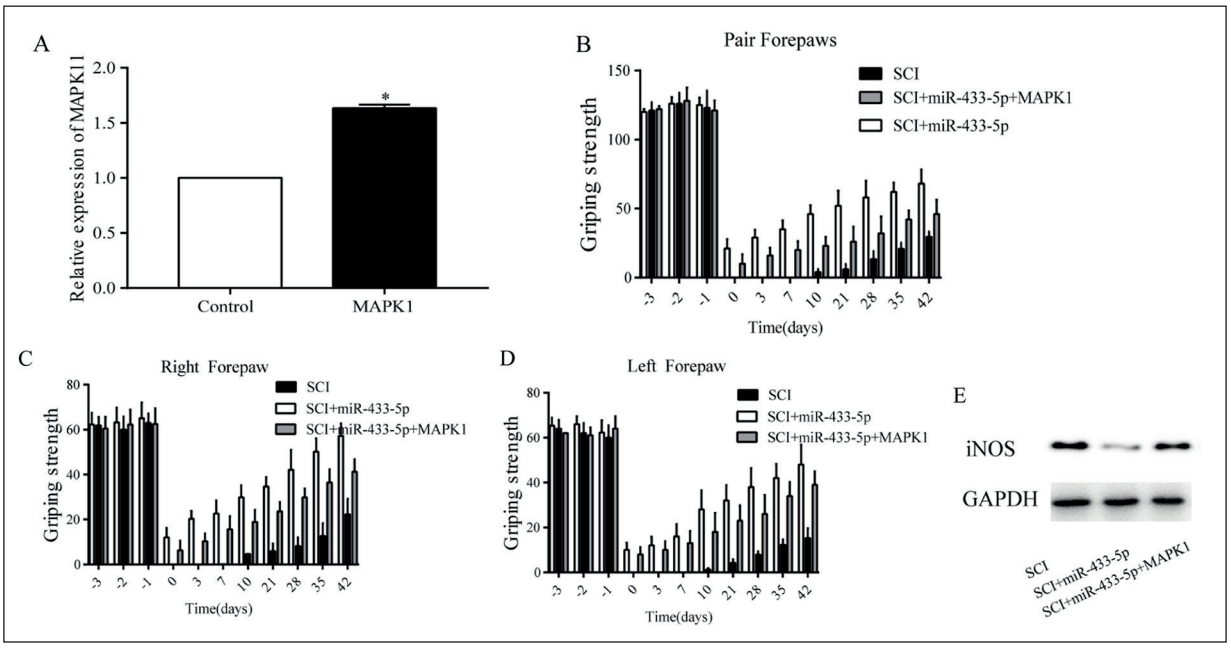


***Figure 4.*** MiRNA-433-5p influenced SCI by targeting MAPK1. **A,** Transfection efficacy of pcDNA-MAPK1 in 293T cells. **B-D,** Griping strengths of pair forepaws (**B**), right forepaw (**C**) and left forepaw (**D**) at day -3, -2, -1, 0, 3, 7, 10, 21, 28, 35, and 42 in SCI mice, those overexpressing miRNA-433-5p or those co-overexpressing miRNA-433-5p and MAPK1. **E,** Protein level of iNOS in SCI mice, those overexpressing miRNA-433-5p or those co-overexpressing miRNA-433-5p and MAPK1.

circumstances[18]. In this paper, miRNA-433-5p was lowly expressed in the serum of SCI patients and mice. *In vivo* overexpression of miRNA-433-5p significantly improved postoperative grip strengths of SCI mice. In addition, inflammatory response was alleviated by overexpression of miRNA-433-5p *in vivo*. All these findings suggested that overexpression of miRNA-433-5p was able to protect SCI-induced motor dysfunction and inflammatory response.

MAPK1-mediated signaling is responsible for cancer cell proliferation and apoptosis[19,20]. MAPK signaling mainly consists of JNK/SAPK and p38MAPK pathways. The former is involved in oxidative stress and cell apoptosis. Meanwhile, the latter is responsible for cell proliferation regulation. Many miRNAs have been confirmed to influence tumor progression by activating MAPK1[20]. Wang et al[21] have proposed that miR-335 suppresses the proliferation and invasion of bladder cancer cells by inactivating the MAPK1 signaling. By downregulating MAPK1, miR-217 inhibits growth and induces apoptosis in colorectal cancer[22]. Breast cancer progression and chemotherapy-resistance are influenced by miR-20a by downregulating its downstream gene MAPK1[23]. In prostate cancer, lncRNA SChLAP1/miR-198/MAPK1 regulatory loop accelerates tumor progression[24].

In our study, bioinformatics predicted the presence of binding sites in the promoter regions of miRNA-433-5p and MAPK1. The binding relationship between the two molecules further verified by Dual-Luciferase reporter gene assay. In particular, MAPK1 expression was negatively regulated by miRNA-433-5p. Notably, MAPK1 overexpression partially reversed the protective role of miRNA-433-5p in grip strength of SCI mice and inflammatory response at post-SCI. Therefore, we suggested that overexpression of miRNA-433-5p improved motor function and alleviated inflammation at post-SCI by targeting MAPK1.

## Conclusions

We showed that the overexpression of miRNA-433-5p protected SCI-induced motor dysfunction and inflammatory response by targeting MAPK1.

### Conflict of Interest

The Authors declare that they have no conflict of interests.

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