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MiR-543-3p promotes locomotor function recovery after spinal cord injury by inhibiting the expression of tumor necrosis factor superfamily member 15 in rats

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Abstract. – OBJECTIVE: To explore the effect of miR-543-3p on the recovery of locomotor function after spinal cord injury (SCI) by regulating tumor necrosis factor superfamily member 15 (TNFSF15) mediated inflammation and apoptosis.

MATERIALS AND METHODS: Macrophages were isolated from the abdominal cavity of 2-3 months old Sprague-Dawley (SD) rats and cultured. The levels of miR-543-3p, tumor necrosis factor superfamily member 15 (TNFSF15), TNF-like molecule 1A (TL1A) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) after transfection of miR-92b-5p into activated macrophages were detected by quantitative Real-time polymerase chain reaction (qRT-PCR). Moreover, the mRNA expressions of miR-543-3p, TNFSF15, TL1A and NF-κB after SCI in rats were detected by qRT-PCR. Meanwhile, the protein expressions of tumor necrosis factor (TNF-α), interleukin-1 β (IL-1β) and Caspase8 were detected by Western blot. After intrathecal injection of miR-543-3p mimics, the mRNA expressions of miR-543-3p, TNFSF15, TL1A and NF- κ B and the protein expressions of TNF-α, IL-1β and Caspase8 in spinal cord injured area of mice were measured by qRT-PCR and Western blot, respectively. Basso Beattie Bresnahan (BBB) locomotor rating scale was used to determine the recovery of locomotor function after spinal cord injury and injection of miR-543-3p mimics.

RESULTS: Compared with inactivated cells, the expression of miR-543-3p in activated macrophages was significantly declined. However, the levels of TNFSF15, TL1A and NF-κB were significantly elevated. The expressions of TNFSF15, TL1A and NF-κB were remarkably downregulated after transfection of miR-543-3p. In addition, the level of miR-543-3p was significantly downregulated, accompanied by an increase in TNFSF15, TL1A and NF-κB in SCI rats. Accordingly, the protein levels of TNF-α and IL-1β and Caspase8 were also significantly increased. However, the expressions of TNFSF15, TL1A and NF-κB were significantly down-regulated in rats injected with miR-543-3p mimics, whereas the protein levels of TNF-α and IL-1β and Caspase8 were significantly suppressed. Finally, compared with SCI group, the recovery of locomotor function in miR-543-3p mimics administration group was significantly improved.

CONCLUSIONS: After SCI, miR-543-3p can inhibit the activity of NF-κB by suppressing the inflammatory aggravation of TNFSF15 and decreasing its product TL1A. MiR-543-3p leads to the improvement of neuron protection and locomotor function via attenuating inflammatory reaction and cell apoptosis.

Key Words:
MiR-543-3p, Inflammation and apoptosis, Spinal cord injury (SCI), Locomotor function recovery.

Introduction

Spinal cord injury (SCI) refers to various motor, sensation and sphincter dysfunctions, as well as dystonia below the corresponding injured segment caused by direct or indirect external factors[1]. Usually, SCI can lead to severe neurological deficits and disability. This eventually results in the loss of sensory and motor capacities (paraplegia or quadriplegia). Furthermore, other common complications associated with SCI may occur, such as bladder, kidney and intestinal tract complications as well as heart and respiratory dysfunctions[2]. These complications greatly affect the physiological, psychological and social behaviors of SCI patients. The causes of SCI mainly include: 1) traffic

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accident, which is now the leading cause of SCI; 2) falling accident from high places, the falling due to building construction or suicide can cause cervical cord injury and thoracolumbar and lumbar fractures complicated by SCI; 3) sports or life accident, 4) penetrating gunshot wounds and other forms of violence[3]. Therefore, the development of treatment strategies for physiological, psychological and social behaviors of SCI patients is urgently needed. SCI, from the perspective of pathophysiology, can be divided into two types, including: (1) mechanical primary damage caused by external force; and (2) secondary damage induced by inflammation and apoptosis due to ischemia and hypoxia of cells[4]. Secondary damage is considered as a critical stage affecting the prognosis of SCI. Therefore, how to increase the number of survival neurons by improving the microenvironment of spinal cord cells during acute injury is a potential entry point for the improvement of SCI prognosis[5]. Tumor necrosis factor superfamily member 15 (TNFSF15), also known as vascular endothelial growth inhibitor (VEGI), is a TNF-like factor located at 9q32. Previous studies have indicated that TNFSF15 is close to CD30L[6,7]. The messenger ribonucleic acid (mRNA)[8] of TNFSF15 is composed of about 6,500 nucleotides, which has long untranslated regions at 5' and 3'. The reading frame of TNFSF15 consists of 522 nucleotides, including 4 exons and 3 introns. Its homology with the sequence of TNF-α gene is about 30%. Originally, TNFSF15 was thought to be related to rheumatic arthritis and arteriosclerosis. Nowadays, studies[9,10] have discovered that TNFSF15 plays a vital role in various diseases. In inflammatory bowel disease, TNFSF15 mRNA and its protein product TNF-like molecule 1A (TL1A) show significant abnormalities in macrophage and T lymphocytes of affected tissues[11,12]. TNFSF15-encoded protein includes three subtypes, namely VEGI2174, VEGI2192 and VEGI2251. They are active regions containing 151 amino acid residues at exon 4-encoded carboxyl terminus[13]. TL1A (namely VEGI2192), a type-II transmembrane protein, can be divided into intracellular region, transmembrane domain and extracellular region. Soluble molecules composed of 149 amino acid residues at extracellular C-terminus can inhibit endothelial cell proliferation and regulate cell apoptosis[14]. TL1A is capable of stimulating the secretion of IFN-γ and interleukin-2 (IL-2) by binding to DR3, which is a member of the TNF receptor superfamily. Meanwhile, the death domain of DR3 enables DR3 to induce apoptosis or activate nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)[15]. NF-κB is a kind of DNA-binding protein, which plays an important role in intracellular signal transduction[16]. On the one hand, it induces cell apoptosis by binding to its downstream signal transduction protein TNF receptor-associated factor 2 (TRAF2), ribosome inactivating protein (RIP), Fas-associated death domain protein (FADD) and Caspase 8 together with TNF receptor-1 associated death domain (TRADD)[17]. On the other hand, NF-κB binds to the enhancer regions of inflammatory cytokines, thereby activating their transcription[18]. The interaction between TL1A and DR3 can lead to the activation of intracellular NF-κB, eventually affecting the occurrence and development of inflammation[19]. MicroRNA (miRNA) is a kind of endogenous small RNA with about 20-24 nucleotides in length. Previous studies have indicated that miRNA has various important regulatory effects in cells[20]. Each miRNA may have several target genes, and several miRNAs can regulate a single gene. With this complex regulatory network, the expressions of many genes can be regulated by one miRNA. Meanwhile, the expression of a certain gene can be subtly regulated through the combination of several miRNAs[21]. It is speculated that miRNA regulates one-third of human genes. It is known to all that miRNA has many forms, the most primitive of which is primary miRNA (pri-miRNA) with about 300-1,000 bases in length[22]. After primary processing, pri-miRNA becomes a miRNA precursor (pre-miRNA) with a length of about 70-90 bases. Pre-miRNA is digested with Dicer to form a mature miRNA that is about 20-24 nt in length[23]. Since the expression levels of various miRNAs are changed after SCI, the intervention of miRNA may become an effective therapeutic target for SCI.

Materials and Methods

Extraction and Culture of Macrophage

Sprague-Dawley (SD) rats aged 2-3 months were obtained from Animal Center of Shandong University. All rats were sacrificed by cervical vertebra luxation and soaked with 75% alcohol for 7 min. Subsequently, the rat was lifted by its tail, followed by injection of 10 mL serum-free Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Rockville, MD, USA) from a lower part of the abdominal cavity on one side. Next, the rat was flat on its back, and its abdomen was gently massaged for 2-3 min. After standing for 5-7 min, the abdominal cavity of rats was opened under aseptic conditions. If

flattened intestinal tube and pale yellow peritoneal fluid were observed, peritoneal fluid (8-9 mL) was collected using a syringe. Then collected peritoneal fluid was centrifuged and added with conventional DMEM containing 10% fetal bovine serum (FBS, Gibco, Rockville, MD, USA), 100 U·mL penicillin and 100 μg·mL streptomycin. The density of cells was adjusted into desired concentration. Thereafter, the cells were inoculated into culture flasks and 6-well plates containing sterile coverslips, followed by culture in a 37°C and 5% CO_2 incubator. 12 h later, the medium was replaced, and unattached cells were removed. Cells cultured for 7 d were activated by lipopolysaccharide (LPS) (100 ng/mL) (Solarbio, Beijing, China) for 24 h, and used for subsequent experiments.

Animals

Totally 30 male SD rats (weighing about 280-320 g, aged 6-8 weeks old) were obtained from Animal Center of Shandong University. All rats were normally fed in a capacious place under normal environment (12 h light and 12 h dark /day). Subsequently, the rats were randomly divided into 3 experimental groups with 10 rats in each, including: 1) sham-operation group: only treated with laminectomy, 2) SCI group: moderate spinal contusion model was established, and an equal volume of normal saline was injected, and 3) miR-543-3p mimic group: intrathecal injection of exogenous miR-543-3p mimics at the injury site. This study was approved by the Animal Ethics Committee of Jining NO.1 People's Hospital Animal Center.

SCI Model in Rat

Establishment of SCI model in rat: moderate spinal contusion was caused through impact against T9-T11 spinal cord. Specific operation procedure was as follows: after the rats were anesthetized *via* intraperitoneal injection of 10% chloral hydrate, the hair at the surgical site was shaved off in the prone position. After disinfecting with iodophor, the skin was cut, followed by separation of the fascia and dorsal muscle to completely expose the vertebral plate. T9-T11 vertebral plates were removed *via* laminectomy, and the same segment of spinal cord was exposed. Exposed spinal cord was then impacted using a 10 g × 2 cm impactor. Spinal congestion, straight swinging of both lower limbs, tail flick reflex and delayed paralysis indicated successful modeling. After hemostasis, the muscle and dorsal skin were sutured. SCI mice were fed under suitable temperature (1 rat/cage) and normal diet.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) Detection

Cultured macrophage and spinal cord tissues (5 mm above and below the center of injury, taken from the corresponding spinal cord in sham-operation group) were extracted after euthanasia. Subsequently, collected tissues were ground in a mortar filled with pre-cooled TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Chloroform, isopropanol and ethanol were added to extract RNA. The concentration of extracted RNA was determined using the Nano-Drop method. Extracted RNA was then synthesized into complementary deoxyribonucleic acid (cDNA) using reverse transcription kit. QRT-PCR was then performed according to the instructions of SYBR mix. The relative expression levels of miR-543-3p, TNFSF15, TL1A and NF-κB were calculated by the $2^{-\Delta\Delta CT}$ method. The primer sequences were as follows: MiR-543-3p: 5′-CGGGGGTAATTTTATGTATAAGCTAGT-3′; TNFSF15 (forward): 5′-TACTCCCAGGTCACATTCCGT-3′; TNFSF15 (reverse): 5′-GGGTAGCTGTCTGTTACCTTGG-3′; TL1A (forward): 5′-GACTAACAAAGATGCCTGCCTGTGG-3′; TL1A (reverse): 5′-GCCATCCTTCTGCTGTCTTGGAGA-3′; NF-κB (forward): 5′-CCCCTGTACGATAGTCGG-3′; NF-Kb (reverse): 5′-AGGAGCGTTGCTTTGGAT-3′; Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (forward): 5′-GACTCATGACCACAGTCCATGC-3′; GAPDH (reverse): 5′-AGAGGCAGGGATGATG-TTCTG-3'.

Western Blotting

Cultured macrophage and fresh spinal cord tissue were split by whole cell lysis buffer containing protease inhibitor. The total protein was extracted on ice. The concentration of total protein was measured by the bicinchoninic acid (BCA) (Abcam, Cambridge, MA, USA). After separated by 10% sodium dodecyl sulfate and polyacrylamide gel, protein sample was transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After sealing with 5% skim emulsion at room temperature for 1 h, the membranes were rinsed with Tris-buffered saline with Tween-20 (TBST) three times (5 min per time). Then the membranes were incubated with TNF-α antibody of rabbit (1:500), IL-1β antibody of rabbit (1:200), Caspase8 antibody of rabbit (1:1000) and rabbit GAPDH at 4°C overnight. On the second day, the membranes were washed three times (10 min per time) with TBST, followed by incubation with goat anti-rabbit antibody (1:10000) at room

temperature for 1 hour. Finally, immunoreactive bands were detected by electrochemiluminescence (ECL) (Thermo Fisher Scientific, Waltham, MA, USA).

Behavioral Analysis

Behavioral function was evaluated by Basso Beattie Bresnahan (BBB) locomotor rating scale at 1, 3, 7, 14, 28 days after SCI, respectively. The movement ability of experimental rats included: hind limb movement, weight bearing, and coordinated movement of forelimbs and hind limbs. The rats were placed in an open field for 4 min. The experiment was scored by two researchers in a blind manner, respectively. Score ranged from 0 to 21 (0 for complete paralysis, 21 for normal activity).

Statistical Analysis

Experimental data were expressed as mean ±standard deviation (SD). *t*-test was used to compare the difference between two groups. Statistical Product and Service Solutions (SPSS) 18.0 (SPSS Inc., Chicago, IL, USA) was used for all statistical analysis. $p<0.05$ was considered statistically significant.

Results

The Decrease of miR-543-3p was Associated with the Increase of Inflammatory Activators In Activated Macrophages

TNFSF15 can produce TL1A protein by transcription and translation. TL1A protein activates the activity of nuclear factor NF-κB. Subsequently, NF-κB binds to the inflammatory cytokine enhancer region, thereby activating transcription process and inducing inflammatory response. After macrophage activation, the expression of miR-543-3p was significantly suppressed. However, the levels of TNFSF15, TL1A and NF-κB were significantly increased (Figure 1A and 1B). These results suggested that lowly expressed miR-543-3p might aggravate the activation of inflammatory.

The Activation of Inflammatory was Significantly Inhibited After Transfection of miR-543-3p

Then, we transfected miR-543-3p into macrophages to observe the change trend of inflammatory activators. After transfection of miR-543-3p, the levels of TNFSF15, TL1A and NF-κB were significantly inhibited with the increase of miR-543-3p (Figure 2A and 2B). The results indicated that up-regulation of miR-543-3p could effectively attenuate the role of inflammatory activators in activated macrophages.

Inhibition of miR-543-3p was Associated With Up-Regulation of Inflammatory Activator After SCI

One week after SCI, the level of miR-543-3p in SCI group was significantly lower than that of sham operation group. However, the levels of TNFSF15, TL1A and NF-κB were significantly increased (Figure 3A and 3B). Moreover, the protein levels of classical inflammatory factors TNF-α and IL-1β also increased significantly. The level of Caspase8 was remarkably elevated as a result (Figure 3C and 3D). The results suggested that the inhibition of miR-543-3p might be

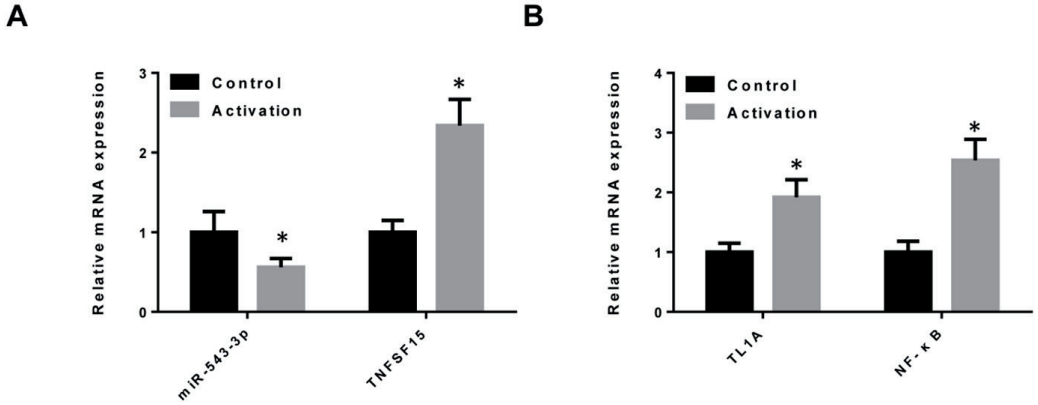

Figure 1. The decrease of miR-543-3p was associated with the increase of inflammatory activators in activated macrophages. ***A,*** The expression level of miR-543-3p was significantly decreased after macrophage activation. However, the expression of TNFSF15 increased significantly. ***B,*** The levels of TL1A and NF- κ B in activated cells were also significantly elevated.

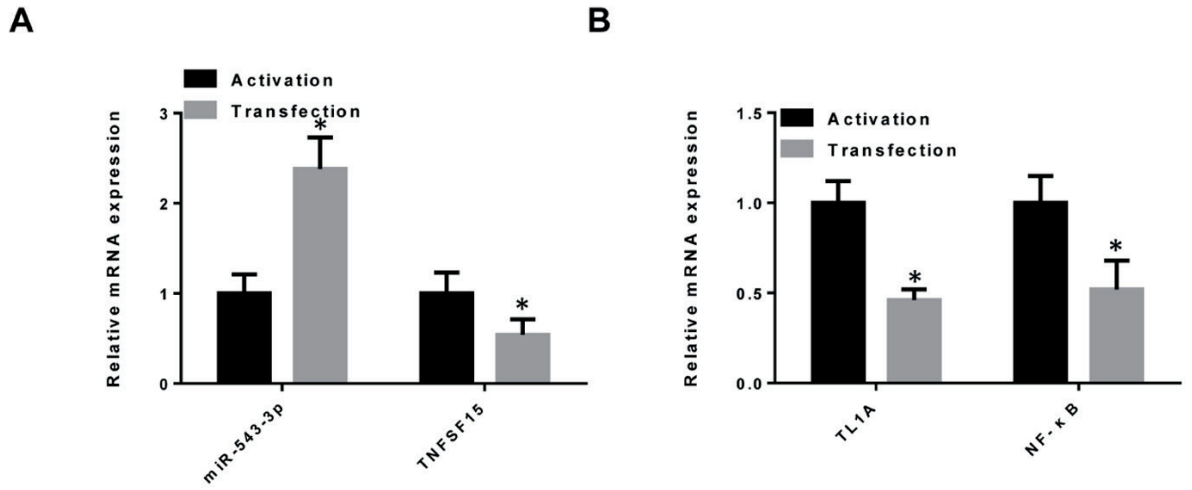

Figure 2. Inflammatory activation was significantly inhibited after transfection of miR-543-3p. ***A,*** The level of miR-543-3p was significantly increased, whereas the expression of TNFSF15 was inhibited after transfection of miR-543-3p into activated cells. ***B,*** The mRNA levels of TL1A and NF- κ B were significantly down-regulated after transfection.

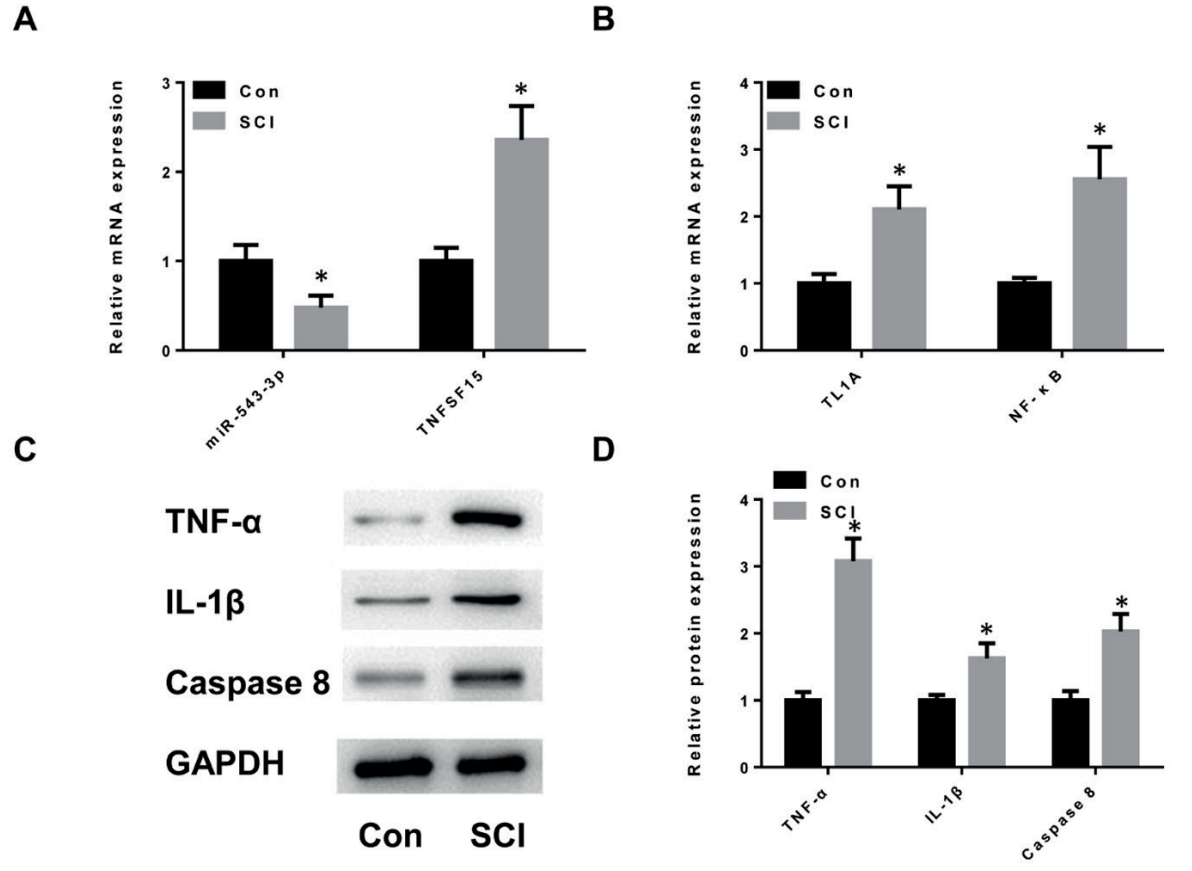

Figure 3. Inhibition of miR-543-3p expression was associated with up-regulation of inflammatory activators after SCI. ***A,*** The expression level of miR-543-3p decreased significantly, while the expression of TNFSF15 increased significantly after SCI. ***B,*** The levels of TL1A and NF-κB after SCI were also significantly elevated. ***C,*** The protein levels of TNF-α, IL-1 β and apoptosis-related enzyme Caspase 8 were remarkably up-regulated after SCI. ***D,*** According to Grey value analysis, the changes of protein levels were statistically significant after SCI.

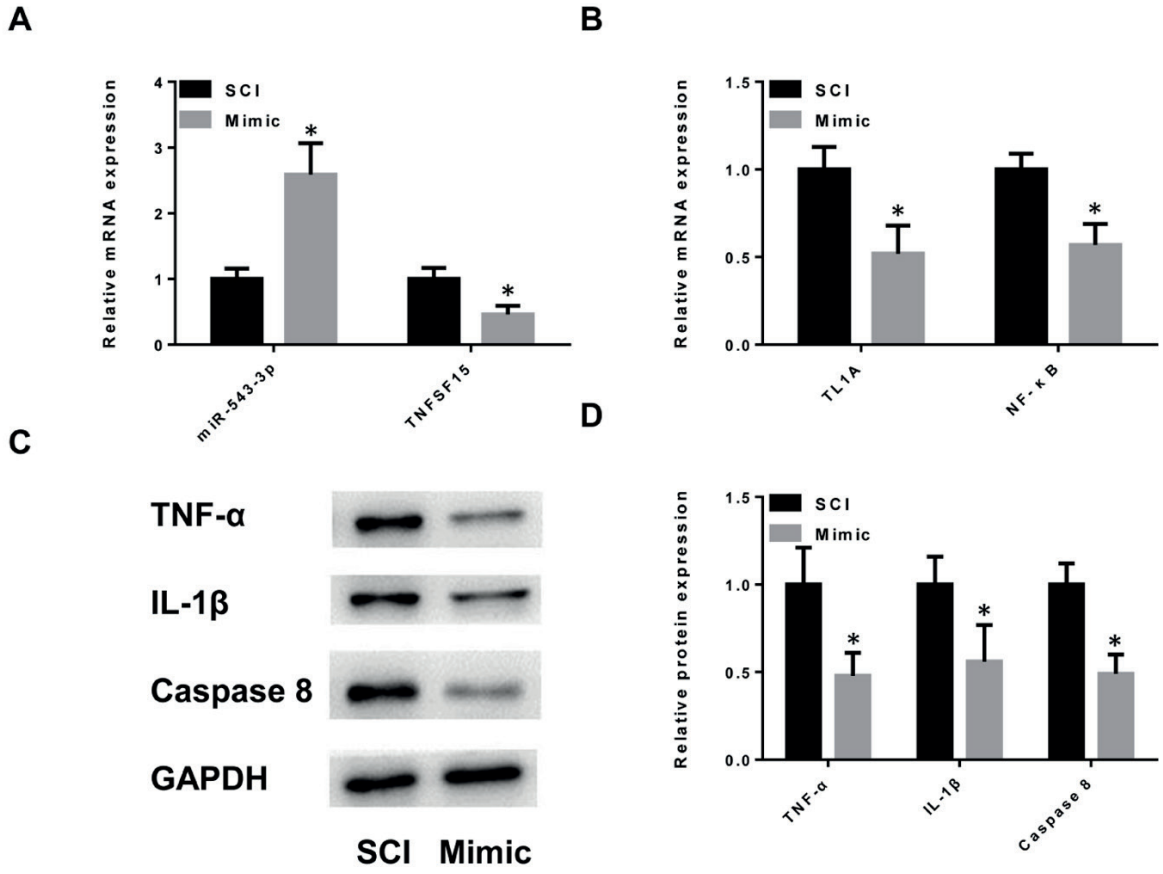

Figure 4. The increase of miR-543-3p after SCI could effectively attenuate the role of TNFSF15 in promoting inflammation and apoptosis. ***A,*** The level of miR-543-3p in SCI rats was significantly increased, while TNFSF15 level was suppressed after miR-543-3p mimics transfection at 3 day. ***B,*** The mRNA expressions of TL1A and NF-κB were significantly downregulated at 3 days after administration. ***C,*** The protein levels of TNF-α, IL-1β and Caspase 8 were also significantly inhibited at 3 days after administration. ***D,*** It was found that the changes of various proteins were statistically significant.

involved in the regulation of inflammatory activation and apoptosis after SCI.

The Increase of miR-543-3p After SCI Could Effectively Attenuate the Role of TNFSF15 in Promoting Inflammation and Apoptosis

Compared with SCI group, the level of miR-543-3p in SCI rats injected with miR-543-3p mimics was significantly increased within one week. However, the mRNA levels of TNFSF15, TL1A and NF-κB were decreased significantly (Figure 4A and 4B). In addition, Western blotting indicated that the protein levels of TNF- α, IL-1 β and Caspase8 were also significantly declined (Figure 4C and 4D). The above results showed that up-regulation of miR-543-3p after SCI could significantly inhibit the pro-inflammatory and apoptotic effect of TNFSF15 and protect neurons.

Highly Expressed miR-543-3p Could Effectively Promote the Recovery of Locomotor Capacity After SCI in Rats

The recovery of locomotor function in three groups was evaluated by BBB locomotor rating scale at 1 day, 3 days, 7 days, 14 days and 28 days after SCI, respectively. In sham operation group, the scores were 17.83±2.36, 18.32±3.45, 18.62±3.27, 18.71±4.63, 19.52±3.56. The scores of SCI group were 0.56±0.08, 1.78±0.14, 2.97±0.36, 3.25±0.88, 4.21±1.42. Meanwhile, the scores of miR-543-3p group were 0.46±0.11, 1.63±0.15, 4.27±0.34, 6.54±0.51, 7.69±0.62 (Figure 5). The results demonstrated that highly expressed miR-543-3p after SCI could improve locomotor function.

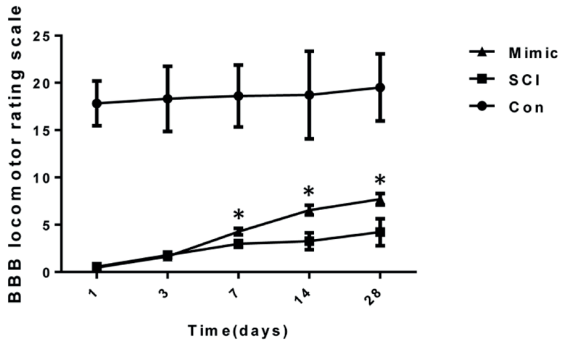

Figure 5. Highly expressed miR-543-3p could effectively promote the recovery of locomotor capacity after SCI in rats. The locomotor function of rats treated with miR-543-3p was significantly improved at 1 week after SCI.

Discussion

SCI is a neurological disorder below the affected segment, which is caused by direct or indirect violence. In severe cases, SCI can lead to the loss of sensory and motor functions [24]. Mechanical injury caused by external force is primary SCI. Meanwhile, damage caused by inflammation and apoptosis due to ischemia and hypoxia of tissues is secondary SCI[25]. Studies have manifested that immune and inflammatory responses play key roles in SCI. Therefore, the regulation of immune and inflammatory responses may be a useful treatment for improving the prognosis of SCI[26]. The expressions of multiple miRNAs are changed after SCI. MiRNA plays an extremely vital role in maintaining the normal function of the central nervous system (CNS)[27]. Moreover, many studies have proved that miRNA dysregulation is related to various diseases of CNS. Abnormal regulation of miRNAs after SCI may be associated with inflammatory response, apoptosis, glial scar formation and regenerative inhibition during injury[28]. The up-regulation of miR-486 after SCI may mediate the production of reactive oxygen species and oxidative damage[29]. After suffering from SCI, miR-21 plays an anti-apoptotic role in rats to ensure the number of survival neurons[30]. In the early stage of SCI, the expression of miR-223 contributes to neutrophil inflammation[31]. However, the role of miR-543-3p over-expression in regulating immune inflammatory response after SCI has not been elucidated. In this study, it was found that miR-543-3p was inhibited in activated macrophages. Meanwhile, the levels of inflammatory cytokine TNFSF15 and its downstream product TL1A in cells were significantly decreased after overexpressing miR-543-3p. Subsequently, the activation level of NF-κB was significantly downregulated. It was hypothesized in this work that overexpression of miR-543-3p might suppress NF-κB activity by blocking the transcription and translation of TNFSF15, eventually reducing the extent of apoptosis and inflammatory response after SCI. Subsequently, the effect of miR-543-3p overexpression on SCI was verified in a rat model. Our results showed that the mRNA levels of TNFSF15, TL1A and NF-κB were significantly decreased in SCI rats. At the same time, protein levels of classical inflammatory factors (TNF-α and IL-1β) and key enzyme (Caspase 8) were remarkably inhibited. In addition, overexpression of miR-543-3p improved motor function after SCI. All these results indicated that miR-543-3p had anti-inflammatory and anti-apoptotic effects. Moreover, it could improve the microenvironment of neurons after SCI, which was conductive to the recovery of neurological function. To sum up, miR-543-3p, as a potential SCI therapeutic target, could interfere with TNFSF15 expression to inhibit NF-κB activity, thus exerting its anti-inflammatory and anti-apoptotic effects.

Conclusions

We demonstrated that miR-543-3p effectively inhibited TL1A expression and NF- κ B activity, alleviated inflammatory effect and pro-apoptosis, and saved and protected the neurons *via* TNFSF15 interference after SCI.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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