Original Article Salvianolic acid B protects against spinal cord injuries via microRNA-21/PTEN/AKT pathways in rats

Jianzhong Sun, Ren Ding, Tai Ma, Xiaobing Shi, Jianhua Huang, Xiaojian Wu

Department of Orthopedics, Shanghai Baoshan Hospital of Integrated Traditional Chinese and Western Medicine, Shanghai, China

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Abstract: Recently, salvianolic acid B (Sal B), a compound extracted from Salvia miltiorrhiza, has been demonstrated to hold antioxidant, anti-inflammatory, and antiapoptotic properties. It has been widely used in diseases of the nervous system. However, the function and mechanisms of Sal B on spinal cord injuries (SCI) have not been well understood. The aim of this study was to investigate the therapeutic potential of Sal B in SCI and the associated mechanisms. Rat SCI models were induced using a weight drop device on the dorsal spinal cord via laminectomy. Subsequently, this study evaluated the therapeutic effects of Sal B against SCI, demonstrating that treatment with Sal B obviously improved motor function, reduced lesion size, and inhibited apoptotic cells in rats following SCI. To further explore the molecular mechanisms of Sal B treatment, this study verified that a large set of miRNAs expression was altered in SCI rats following SaI B treatment using miRNA microarray. miR-21 was one of the miRNAs most significantly upregulated. Moreover, it was found that downregulation of miR-21 by antagomir-21 suppressed the therapeutic effects of Sal B in rats following SCI. Furthermore, present data showed that phosphatase and tensin homolog (PTEN), a negative regulator of AKT/mTOR pathways, was proven to be a direct target of miR-21. Most importantly, this study demonstrated that Sal B inhibited expression of PTEN and promoted expression of phospho-AKT (p-AKT) and phospho-mTOR (p-mTOR), indicating that Sal B could reactivate AKT/mTOR pathways via inducing miR-21 in rats following SCI. Present findings showed that Sal B exerted protective effects against SCI by targeting miR-21/PTEN/AKT pathways in rats. Results indicated that Sal B may serve as an effective therapeutic agent for SCI.

Keywords: Salvianolic acid B, spinal cord injury, microRNA-21, PTEN/AKT pathways

Introduction

Spinal cord injuries (SCI) are serious and disabling. They can cause the loss of motor or sensory neurons, having serious socioeconomic impact [1]. Despite great effort that has been made regarding SCI, effective treatment for this condition remains limited. Therefore, the search for an effective treatment for SCI is urgent.

Salvianolic acid B (Sal B) is a natural polyphenolic compound, extracted from the Chinese medical herb Salvia miltiorrhiza (Danshen) [2], with a variety of pharmacological effects, such as antioxidation, anti-inflammation, and antitumor [3]. It has been widely used in treatment of various diseases, such as angina pectoris, myocardial infarction (MI), and strokes [4]. Importantly, recent observations have shown that Sal B also has neuroprotective effects on spinal cord injuries in rats [5, 6]. However, the underlying mechanisms have not been fully elucidated.

MicroRNAs (miRNAs) are a group of highly conserved short non-coding RNA molecules of ~22 nucleotides in length. They negatively modulate protein expression by binding to complementary target messenger RNAs [7, 8]. A vast amount of research has demonstrated that many miRNA have been implicated in several neurological diseases, such as traumatic brain injury and brain ischemia [9-12]. Many kinds of miR-NAs are involved in the pathogenesis of SCI in a rat contusion SCI model [13-15]. Zhu et al. found that miR-494 improved functional recovery by modulating PTEN/AKT/mTOR pathways in rats after SCI [15]. However, whether miRNAs participate in the neuroprotective effects of Sal B against SCI in rats remains unknown.

In the present study, miR-21 was investigated as a potential miRNA associated with neuroprotective effects of Sal B on SCI rats. Furthermore, it was demonstrated that the miR-21/PTEN/ AKT signaling pathway is essential for Sal B-presented neuroprotective effects, providing clinical insight for treatment of SCI using Sal B.

Materials and methods

Experimental animals and group

Female Sprague-Dawley (SD) rats (220-250 g) were purchased from the Animal Center of the Chinese Academy of Sciences (Shanghai, China). All animal procedures were conducted in accordance with guidelines reviewed and approved by the Institutional Animal Care and Use Committee of the Department of Orthopedics, Shanghai Baoshan Traditional Chinese Medicine-Integrated Hospital. All animals were housed in individual cages in a temperature-and light cycle-controlled environment with free access to food and water.

Establishment of contusion SCI model

Rats were intraperitoneally injected with 10% chloral hydrate (400 mg/kg). A rat model of contusion was established by heavy impact, according to the method described by Gruner et al. [16]. After sterilization, the skin was incised along the midline of the back and the vertebral column was exposed. A laminectomy was performed at the T9 level. A 10 g 2 cm-diameter metal rod was vertically dropped from a 25 mm height to impact the exposed spinal cord and to cause SCI. Sham groups received the same surgical procedure but sustained no impact injury.

Sal B (purity > 99%) was purchased from Sigma-Aldrich and dissolved using normal saline containing 10% vitamin C. Rats were randomized into seven groups: sham-operated group, SCI group, and SCI + Sal B groups-rats were treated (intraperitoneally (i.p.) with Sal B at doses of 1, 10, or 50 mg/kg once a day for 3 days, starting half an hour after SCI. SCI + Sal B + antagomir-miR-21 group and SCI + Sal B + antagomir negative group (NC) group-rats were injected intrathecally with antagomir-miR-21 and negative control antagomir (1 µl/h, 20 nmol/mL) at 2 hours before surgery, receiving the treatment of Sal B (10 mg/kg, i.p.).

Motor function test

The motor function of rats subjected to compressive injury was evaluated at 1, 3, 7, 14, 21, and 28 days after SCI. Recovery from motor dysfunction was estimated by the modified murine Basso, Beattie, and Bresnahan (BBB) hindlimb locomotor rating scale, as described previously [17]. The final score of each animal was obtained by averaging the values from both investigators.

Lesion identification by cresyl violet staining

At 28 days after surgery, the rats were deeply anesthetized with 10% chloral hydrate (chloral hydrate 400 mg/kg, i.p.) and fixed with 4% paraformaldehyde. A 1 cm segment of spinal cord T9 around the lesion epicenter was excised. After post-fixation for 24 hours at 4°C. the spinal cord sections were embedded in paraffin and then 10-µm-thick sections were prepared using a microtome. For histological examination, every 40th section of the lesion site sample was stained with cresyl-violet (0.1%, w/v) and imaged using a microscope (BH-2; Olympus, NY). Using Image-Pro Plus 6.0 (Media Cybernetics, USA) software, the lesion area and spared tissue area were outlined and quantified.

Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling (TUNEL) staining

After being deparaffinized with xylene, spinal cord sections were rehydrated with ethanol at graded concentrations of 100%-70% (v/v), followed by washing with water. Next, spinal cord segments were subjected to 100μ L proteinase K (20μ g/ml, Roche) for 15 minutes at RT, then washed three times with PBS. TUNEL solution preparation and staining were performed using TUNEL Apoptosis Detection Kit (Alexa Fluor 488) (Roche, Basel, Switzerland). Cell quantification was obtained using an inverted fluorescence microscope (DP73; Olympus) at 400 × magnification. TUNEL-positive cells were counted in three fields of view per section.

Immunohistochemistry

Spinal cord tissues of rats in the Shamoperated group, SCI group, and SCI + Sal B groups (1 mg/kg and 5 mg/kg), for 24 hours, were fixed by 4% paraformaldehyde. Sections were blocked in blocking buffer (3% BSA) for 20 minutes and incubated with the rabbit polyclonal antibody anti-cleaved Caspase-3 (diluted 1:100, Beyotime Institute of Biotechnology, China) at 4°C overnight. Subsequently, sections were incubated with secondary antibodies (anti-rabbit IgG antibodies). The reaction was stopped with 3, 3'-diaminobenzidine (DAB). Finally, staining was observed under a light microscope. Optical densities and positive neuron numbers of cleaved-caspase-3 were counted at 5 randomly selected fields per sample.

Microarray analysis

Total RNA was extracted from spinal cord tissues using the miRNeasy mini kit (Oiagen, West Sussex, UK). Purity and quantity of total RNA were assessed by NanoDrop ND-1000 Spectrophotometry (Thermo Scientific, USA) and Agilent's 2100 Bioanalyzer. Total RNA (200 ng) was labeled with fluorescence dye hy3 or hy5 using the miRCURY Hy3/Hy5 Power Labeling kit and hybridized on the miRCURY™ LNA Array (v.16.0) (Exigon, Copenhagen, Denmark), according to manufacturer instructions. Data was analyzed using Genespring software (Agilent Technologies, USA). Observations with adjusted *p*-values \geq 0.05 were removed and, thus, excluded from further analysis. The heat map of the 60 microRNAs most obvious differences was created using a method of hierarchical clustering by GeneSpring GX, version 7.3 (Agilent Technologies, California, United Stages).

Quantitative real-time polymerase chain reaction (qRT-PCR)

MicroRNA was prepared using the miRNeasy mini kit (Qiagen, West Sussex, UK) and total RNA was prepared using TRIzol Reagent (Life Technologies), according to manufacturer protocol. For miRNA reverse transcription, cDNA was synthesized using a miRNA reverse transcription kit (Qiagen, Valencia, CA, USA). For mRNA reverse transcription, cDNA was synthesized using the Takara PrimeScriptTM First Strand cDNA Synthesis (Takara Bio, Inc., Dalian, China). Real-time PCR for miRNA and mRNA were performed on an ABI PRISM 7300 sequence detection system in an SYBR Green I Real-Time PCR kit (Applied Biosystems; Thermo

Fisher Scientific, Inc.). Relative quantification was determined by normalization to U6 or GAPDH. Primers for qRT-PCR analysis were as follows: miR-140 forward primer 5'-TGCGGCA-GTGGTTTTACCCTATG-3' and reverse primer 5'-CCAGTGCAGGGTCCGAGGT-3'; miR-15a forward primer 5'-GGGGTAGCTTATCAGACTG-3' and reverse primer 5'-AGTGCGTGTCGTGGAGTC-3'; mi-R-21 forward primer: 5'-GCGCGTCGTGAAGCGT-TC-3': reverse primer: 5'-GTGCAGGGTCCGAG-GT-3'.U6forwardprimer:5'-TGCGGGTGCTCGCTTC-GCAGC-3'; U6 reverse primer: 5'-CCAGTGCAGG-GTCCGAGGT-3'; PTEN forward primer: 5'-ACCA-GGACCAGAGGAAACCT-3' and reverse primer: 5'-TTTGTCAGGGTGAGCACA-3'; GAPDH forward: 5'-AGGTCGGTG TGAACGGATTTG-3', reverse: 5'-TGTAGACCATGTAGTTGAGGTCA-3'; PCR amplification protocol was as follows: an initial 95°C for 5 minutes and 40 cycles of 94°C for 15 seconds, 55°C for 30 seconds, and 70°C for 30 seconds. qRT-PCR assays were performed in triplicate and changes in expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method.

Transfection

miR-21 mimics, mimics negative control (mimics NC), miR-21 inhibitor, and inhibitor NC were obtained from GenePharm (Shanghai, China). HEK293 cells $(1.0 \times 10^6$ per well) were seeded and grown overnight in six-well plates. The next day, transfection was performed using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), following manufacturer instructions.

Luciferase reporter assay

3'-UTR of PTEN and the mutated sequence were inserted into the pGL3 control vector (Promega Corporation, Madison, WI, USA) to construct wt PTEN-3'-UTR vector and mutant PTEN-3'-UTR vector, respectively. For luciferase reporter assay, HEK293 cells were transfected with the corresponding vectors. Forty-eight hours after transfection, the dual-luciferase reporter assay system (Promega, Shanghai, the People's Republic of China) was used to measure luciferase activity. All experiments were performed in triplicate.

Western blot

Total protein was extracted using radio immunoprecipitation assay (RIPA) lysis buffer



Figure 1. Sal B has neuroprotective effects against SCI in rats. The rats received treatment of Sal B at doses of 1, 10, or 50 mg/kg (i.p.) at 10 minutes and every 24 hours post-surgery. A. Hindlimb recovery was assessed from day 1 to day 28 after SCI using Basso, Beattie, and Bresnahan (BBB) Score. B. Cresyl violet stained sections were analyzed for lesion size 28 days post-injury. Spared tissue within the injury site and 1600 µm rostral and caudal to the epicenter were quantified at 28 days post-injury. C. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining was used to analyze neuronal apoptosis. D. Expression of cleaved-caspase 3 was measured by Immunohistochemistry. Data represents the mean \pm SD of three independent experiments. **P* < 0.05, ***P* < 0.01 vs. SCI group; #**P* < 0.05 vs. SCI group.

(Beyotime Institute of Biotechnology, China). Concentrations of total cellular protein were determined using a BCA assay kit (Pierce, Rockford, IL, USA). Total protein samples (40 µg) were analyzed by 8% SDS-PAGE gel and transferred to polyvinylidene difluoride (PVDF) membranes (GE Healthcare, Freiburg, DE) by electroblotting. Primary antibodies against PTEN (Santa Cruz Biotechnology, 1:1000 dilution), p-mTOR (Cell Signaling Technology, 1:1,000 dilution), mTOR (Cell Signaling Technology, 1:1,000 dilution), phospho-AKT (Cell Signaling Technology, 1:1,000 dilution), AKT (Cell Signaling Technology, 1:1,000 dilution), and β-actin (Santa Cruz Biotechnology, 1:2000 dilution) were probed with proteins on membranes at 4°C overnight. After incubating with secondary antibodies (1:10000, Cell Signaling Technology, Danvers, MA), bands were detected by enhanced chemiluminescence (ECL) kit (GE Healthcare, Freiburg, DE). Intensities of the bands of interest were analyzed by Image J software (Rawak Software, Inc. Munich, Germany).

Statistical analysis

Statistical analysis was performed using the SPSS program (version 18.0; SPSS, Chicago, IL, USA). Data are presented as mean \pm S.D. Student's t-test or one-way ANOVA was used to analyze differences among/between sample groups. P \leq 0.05 is considered statistically significant.

Results

Sal B has neuroprotective effects against SCI in rats

To evaluate the therapeutic role of Sal B in SCl, a classical SCl model was established, as previ-



Figure 2. Sal B upregulates expression of miR-21. In the SCI group, rats were subjected to SCI and treated intraperitoneally (i.p.) with saline (50 mg/kg). In the SCI + Sal B group, rats were subjected to SCI and treated i.p. with Sal B (50 mg/kg), once a day for 14 days, starting half an hour after SCI. A. Heatmap of normalized expression levels of miRNAs in SCI + Sal B and SCI group 14 days post-injury (n = 3/group). B. Expression of miR-140, -15a, -466g, -1945, -21, and -1951 were determined by qRT-PCR. C. Expression of miR-21 was assessed by qRT-PCR at 1, 3, 7, 14, 21, and 28 days in RNA samples isolated from SCI rats treated with Sal B. Data represents the mean \pm SD of three independent experiments. **P* < 0.05, ***P* < 0.01 vs. SCI group.

ously described [16]. After moderate spinal cord contusion injuries at T9, Sal B (1, 10, or 50 mg/kg) was immediately injected intraperitoneally for 28 days. As shown in Figure 1A. immediately after the surgery, both hindlimbs movements were abolished, consistent with that in previous studies [18, 19], suggesting the success of model establishment. After Sal B treatment, it was found that BBB scores of rats treated with Sal B (10 and 50 mg/kg) were significantly higher than that of SCI rats. In the following days, rats treated with Sal B (10 and 50 mg/kg) exhibited more rapid recovery of locomotor function, represented by higher BBB scores than the SCI rats (Figure 1A). However, low doses of Sal B (1 mg/kg) did not change the BBB score of SCI rats. Results indicated that medium and high doses of Sal B treatment could promote locomotor function recovery after SCI.

Histopathological alterations in the spinal cord after injury were examined using cresyl violet

staining. Spared tissues were larger in Sal B (10 and 50 mg/kg)-treated rats, not only at the injury epicenter (**Figure 1B**), but also in regions extending away from the epicenter, in both rostral and caudal directions. However, spared tissues in low dose of Sal B (1 mg/kg) group were like SCI rats.

Apoptosis is another prominent characteristic in the spinal cord after SCI. Thus, cell apoptosis in the spinal lesions were detected by TUNEL staining. It was we found that TUNEL positive cells in the spinal cord were significantly increased in SCI groups compared with the sham group. However, when Sal B (10 mg/kg and 50 mg/kg) was subjected to the SCI rats, TUNEL positive cells were significantly decreased (**Figure 1C**). The anti-apoptosis effects of 10 mg/kg and 50 mg/kg Sal B were better than that in the 1 mg/kg dosage. This study also examined changes in expression levels of apoptosis associated protein cleaved-caspase-3, through immunohistochemical stain-



Figure 3. miR-21 mediated the neuroprotective effects of Sal B against SCI in rats. Antagomir-miR-21 and antagomir NC were injected intrathecally into rats at 2 hours before surgery and received the treatment of Sal B (50 mg/kg, i.p.). A. Hindlimb recovery was assessed from day 1 to day 28 using Basso, Beattie, and Bresnahan (BBB) Score. B. Cresyl violet stained sections were analyzed for lesion size at 28 day in spinal cord tissues from antagomir-miR-21 and Sal B treated rats following SCI. C. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining was used to analyze neuronal apoptosis in spinal cord tissues from antagomir-miR-21 and Sal B treated rats following SCI. Data represents the mean \pm SD of three independent experiments. **P* < 0.05, ***P* < 0.01 vs. SCI + Sal B + antagomir NC group.

ing. Results showed that expression of cleavedcaspase-3 was markedly increased in the spinal cord of SCI rats, whereas it was reduced after Sal B treatment (**Figure 1D**). These results indicate that Sal B has protective effects against SCI in rats via alleviated apoptosis.

Sal B upregulates expression of miR-21

It has been reported that miRNAs play an important role in many types of injury models. A recent study showed that Sal B protected cardiac myocytes from ischemia/reperfusion injury through upregulation of miR-30a [20]. Thus, this study sought to determine whether Sal B exerts its neuroprotective effects on SCI through modulation of miRNAs. First, a microarray was performed to analyze the differentially expressed miRNAs in SCI rats treated with/ without Sal B (50 mg/kg). Based on hierarchical clustering analysis, 21 miRNAs were upregulated while 39 miRNAs were downregulated in the spinal cord tissues in SCI + Sal B group, compared with that in the SCI group (Figure 2A). Six of the miRNAs that were abnormally expressed (miR-140, miR-15a, miR-466g, miR-21, miR-1945, and miR-1951) were further confirmed by gRT-PCR (Figure 2B). Of these, miR-21 was shown to have a protective effect against SCI in rats [21, 22]. Therefore, it was hypothesized that miR-21 might be a downstream effector of Sal B. In performing qRT-PCR analysis, it was observed that miR-21 levels were increased after Sal B treatment, with its expression reaching the peak at 14 days and returning to near normal levels 28 days postinjury (**Figure 2C**). Results indicate that miR-21 may be involved in the protective effects of Sal B on SCI.

miR-21 mediated the neuroprotective effects of Sal B against SCI in rats

To confirm the role of miR-21 in the neuroprotective effects of Sal B against SCI in rats, antagomir-miR-21 and antagomir NC were injected intrathecally into rats at 2 hours before surgery, receiving the treatment of Sal B (50 mg/kg, i.p.) at 10 minutes and every 24 hours following SCI. Results of BBB scores showed that antagomir-miR-21 attenuated the function recovery induced by Sal B from 1 day to 28 days after SCI (Figure 3A). Moreover, compared with the SCI + Sal B + antagomir NC group, antagomir-21-treated rats had significantly larger lesion areas at multiple distances from the injury epicenter (Figure 3B). Cell apoptosis in the spinal cord was also evaluated in the antagomir-miR-21 treatment group using TUNEL staining. In Sal B-treated SCI rats, there was seldom TUNEL-positive cells in the spinal cord, whereas many TUNEL-positive cells were



Figure 4. PTEN was a direct target of miR-21. (A) The putative binding site of miR-21 and PTEN is shown. (B) Luciferase assay of HEK293 cells co-transfected with firefly luciferase constructs containing the PTEN wild-type or mutated 3'-UTRs and miR-21 mimics, mimics NC, miR-21 inhibitor or inhibitor NC, as indicated (n = 3). Data represents the mean \pm SD of three independent experiments. ***P* < 0.01 vs mimics NC, ##*P* < 0.01 vs inhibitor NC. (C, D) Expression of PTEN mRNA (C) and protein (D) after transfection with miR-21 mimic or miR-21 inhibitor was measured by Western Blot.

found in the lesion sites of spinal cords in Sal B + antagomir-21-treated SCI rats (**Figure 3C**). Data suggests that Sal B exerts its protective role in SCI through inducing miR-21 in rats.

PTEN was a direct target of miR-21

Previous studies have reported that PTEN plays a vital role in SCI [23, 24]. It is worth mentioning that PTEN has been validated as a target of miR-21 [21]. As a result, it was speculated that PTEN is involved in antagomir-21 mediated inhibition of the therapeutic effects of Sal B. First, bioinformatics tools were used to search for potential targets of miR-21, finding that PTEN contained a 3'-UTR sequence complementary to the seed sequence of miR-21 (Figure 4A). Next, luciferase reporter assay was used to test whether miR-21 directly targets PTEN. Results showed that overexpression of miR-21 significantly decreased the luciferase activity of wt-PTEN-3'UTR, whereas knockdown of miR-21 increased the luciferase activity. Likewise, cells co-transfected with miR-21 mimic, miR-21 inhibitor, and PTEN-mut-3'UTR showed no obvious changes in luciferase activity (Figure 4B). Furthermore, this study explored whether miR-21 can modulate expression of PTEN. As shown in Figure 4C and 4D, PTEN mRNA and protein expression levels were decreased after overexpression of miR-21, whereas they were increased after inhibition of miR-21. Present data indicates that miR-21/PTEN axis may play an important role in the neuroprotective effects of Sal B against SCI in rats.

Sal B activated AKT/mTOR signaling pathways in the spinal cords of SCI rats

It has been established that the tumor suppressor PTEN negatively regulates PI3K/AKT pathways, which are directly related to the recovery of motor function in SCI model [25, 26]. Interestingly, several studies have reported that Sal

B exerts its anti-tumor and pro-apoptotic activities through Akt/mTOR signaling pathways [27, 28]. Thus, it was speculated whether AKT/ mTOR signaling pathways were involved in the neuroprotective effects of Sal B against SCI in rats. Protein levels of PTEN, p-AKT, total AKT, p-mTOR, and total mTOR were assessed by Western Blot in the spinal cords of Sal B treated SCI rats. Results showed that protein levels of PTEN were increased, while p-AKT and p-mTOR were significantly decreased in SCI-rats compared with the sham group. However, protein levels of PTEN were deceased, while p-AKT and p-mTOR were increased after Sal B treatment in SCI rats (Figure 5). Results suggest that Sal B reactivated AKT/mTOR signaling pathways which were blocked by SCI.

Discussion

The present study found Sal B treatment has protective effects in SCI rats. Moreover, it was demonstrated that the neuroprotective effects of Sal B on SCI rats may be mediated by miR-



Figure 5. Sal B restored the activation of AKT/mTOR pathway. Rats were subjected to SCI and treated intraperitoneally (i.p.) with saline (50 mg/kg). In the SCI + Sal B group, rats were subjected to SCI and treated i.p. with Sal B (50 mg/kg), once a day for 3 days, starting half an hour after SCI. At 14 days, rats were deeply anesthetized with 10% chloral hydrate and spinal cords were harvested for Western Blot. A. Protein expression of PTEN, AKT, p-MTOR, and mTOR was detected by Western blot. B. The bands were semi-quantitatively analyzed by using Image J software, normalized to β -actin density. Data represents the mean \pm SD of three independent experiments. ***P* < 0.01 vs. SCI group.

21/PTEN/AKT signaling pathways. Therefore, Sal B may be a potential drug for SCI treatment.

Sal B has been demonstrated to be effective in diseases of the nervous system. For example, Zhu et al. found that Sal B protected rat brains against ischemia and reperfusion injury by targeting the JAK2/STAT3 pathway [29]. Kim et al. showed that Sal B could attenuate cognitive dysfunction induced by a cholinergic blockade and Aß 25-35 peptide in mice [30]. A study performed by Chen et al. demonstrated that Sal B attenuated brain damage and inflammation after traumatic brain injuries in mice [31]. In addition, Sal B has been confirmed as an antitumor agent in human glioma cells [32]. However, little attention has been paid to the effects of Sal B in SCI. Xun et al. demonstrated that the use of Sal B significantly improved locomotor function recovery in rats with induced spinal cord injuries [33]. In the present study, results reinforced these neuroprotective effects of Sal B, showing the ability of Sal B to improve locomotor function recovery, attenuate tissue damage, and suppress apoptosis in rats following SCI, suggesting that Sal B could be used for treatment of SCI. However, it remains unclear how Sal B works in amelioration of SCI rats.

Increasing evidence has demonstrated an important role for miRNAs and their target genes in regulating SCI, according to several models. For instance. Cao et al. showed that miR-99b-5p may attenuate SCI-induced harmful alterations in spinal cord neurons via regulation of mTOR expression [13]. Fu et al. found that miR-30a-5p ameliorated spinal cord injuryinduced inflammatory response and oxidative stress by targeting Neurod 1 through MAPK/ERK signaling [14]. Despite extensive research, the precise miRNA-mediated mechanisms involved in Sal B protection against SCI remain incompletely understood. Hence, to find the molecular mechanisms of Sal B against

SCI, this study paid attention to miRNAs regulation. Using miRNA microarray analyses, many miRNAs were found to be altered in Sal B treated rats following SCI, with miR-21 the most significantly upregulated. Notably, several studies have shown that miR-21 has protective effects against SCI. For example, Huang et al. found that tetramethylpyrazine (TMP) improved locomotor functional recovery of rats after contusion spinal cord injuries by upregulation of miR-21 [34]. Bhalala et al. found that miRNA-21 expression was increased in a time-dependent manner following SCI in mice, demonstrating miR-21 as a potential therapeutic target for enhancing functional outcomes [22]. A study performed by Hu et al. showed that miR-21 was one of the most upregulated miRs in rats after contusion SCI, with protective effects via its regulation on pro-apoptotic genes [21]. The present study found that knockdown of miR-21 by antagomir-21 suppressed the therapeutic effects of Sal B against SCI in rats, suggesting that Sal B exerts its neuroprotective effects on SCI though upregulation of miR-21.

PTEN, a classical tumor suppressor, has been found to play a key role in the pathogenesis of SCI. For example, several studies have demonstrated that PTEN deletion promotes neurogenesis and enhances neural survival after SCI [23, 24]. In this study, PTEN was confirmed to be a downstream target of miR-21. This finding is similar to the study reported by Hu et al. [21], demonstrating that, in hindlimb ischemia, miR-21 was upregulated in spinal cords after contusion SCI and inhibited neuron cell apoptosis and senescence via targeting PTEN. It has been established that PTEN is an important regulator of AKT/mTOR pathways. Present data found that PTEN/AKT/mTOR signaling pathways were suppressed in the lesion sites of spinal cords in the SCI group. Sal B treatment restored the activity of this signaling pathway, indicating that the neuroprotective effects of Sal B on SCI rats may be mediated by miR-21/PTEN/AKT/mTOR signaling pathways.

In conclusion, the present study provides evidence that Sal B has neuroprotective effects against SCI in rats. These effects may be associated with the activation of miR-21/PTEN/AKT signaling pathways. This suggests that administering the natural compound Sal B or modulating endogenous miR-21 expression may be effective treatment strategies for SCI.

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Disclosure of conflict of interest

None.

Address correspondence to: Jianzhong Sun and Xiaojian Wu, Department of Orthopedics, Shanghai Baoshan Hospital of Integrated Traditional Chinese and Western Medicine, 181 Shanghai Youyi Road, Shanghai 201900, China. Tel: +86-21-56601100-293; E-mail: sunjianzhongsjz@163.com (JZS); xiaojianwuxj@163.com (XJW)

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