Original Paper

Molecular Mechanism of MiR-136-5p Targeting NF- κ B/A20 in the IL-17-Mediated Inflammatory Response after Spinal Cord Injury

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Key Words

Rat Astrocyte • MicroRNAs • Viral Transfection • NF- κ B/A20 • Spinal Cord Injury

Abstract

Background/Aims: The pathophysiology of spinal cord injury (SCI) results in serious damage to the human body via an increase in the secondary biological processes imposed by activated astrocytes. Abnormal expression of microRNAs after SCI has become a potential research focus. However, the underlying mechanisms are poorly understood. **Methods:** SCI models were established in rats using Allen's method, and the BBB scoring method was employed to assess locomotor function. Lentivirus was used to infect rat astrocytes and SCI rats. Real-time PCR and antibody chip were used to measure gene expression and cytokine secretion. Western blot analysis was employed to detect protein expression. HE staining was used to assess the histological changes in SCI. The immunohistochemical staining of A20 and p-NF- κ B in SCI was also analyzed. **Results:** The *in vitro* experiment showed that miR-136-5p up-regulated the expression of p-NF- κ B by down-regulating the expression of A20 so that astrocytes produced inflammatory factors and chemokines. The *in vivo* experiment indicated that overexpressed miR-136-5p promoted the production of inflammatory factors, chemokines and p-NF- κ B in SCI rats, whereas it inhibited the expression of A20 protein and increased inflammatory cell infiltration and injuries in the spinal cord. **Conclusion:** The current findings indicate that silencing miR-136-5p effectively decreased inflammatory factors and chemokines and protected the spinal cord via NF- κ B/A20 signaling *in vivo* and *in vitro*. In contrast, overexpression of miR-136-5p had the opposite effect.

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Introduction

Brain and spinal cord injuries (SCIs) are among the most common injuries in traffic accidents, and they may lead to serious consequences. The main pathological changes include necrosis and apoptosis of neuronal and glial cells. When the brain and spinal cord are injured, neutrophils are initially activated. The activated neutrophils subsequently release a series of inflammatory factors that move to the injury sites to participate in the local inflammatory response. This response may lead to necrosis and apoptosis of nerve cells along with the infiltration of neutrophils and a substantial number of T lymphocytes into the SCI site. These cells participate in the immune inflammatory response after SCI, and excessive immune inflammation will accelerate the necrosis of nerve cells and the axon demyelination reaction. Simultaneously, macrophages and activated glial cells secrete various cytokines, such as IL-17, IL-6, and TNF- α , which may function synergistically with various inflammatory mediators and other cytokines to aggravate the SCI [1-3].

IL-17, which is a recently discovered proinflammatory cytokine, is involved in mediating the immuno-inflammatory response of various diseases. Acting as a powerful proinflammatory cytokine, IL-17 plays a part in inducing cells to release proinflammatory cytokines and mobilizing neutrophils to release a series of cytokines via TRAF6, NIK, IKK- α , NF- κ B, MAPK, and the JAK/STAT signal transduction pathway. Evidence indicates that IL-17 and IL-22 generated after Th17 cells were activated could aggravate neuronal apoptosis. Moreover, after the brain was injured, NF- κ B activation could induce the secretion of pro-inflammatory factors, such as IL-17, which may aggravate the immune response in the injured area. This cascade of immune responses increases neuronal apoptosis [4-6]. Therefore, proper adjustment of the IL-17 signal axis may contribute to the recovery of motor function after SCI.

By comparing the histology and behavioristics between IL-17 gene knockout rats and normal rats after SCI, the roles of IL-17 in functional recovery and glial scar formation after SCI were assessed [7]. The results showed that after SCI, the deficiency of IL-17 improved motor function, reduced the lesion area, and reduced the secondary injuries of SCI; however, there was no relationship with the post-injury glial cell reaction, and these factors only had a small role in forming a glial scar. It was also shown that IL-17 may be an important factor in the SCI inflammatory response because when IL-17 was deficient, inhibiting the expression of inflammatory factors (IL-6, TNF- α and IL-1 β) reduced inflammation, leukocyte aggregation, and tissue destruction and improved the function of IL-17 gene knockout rats after SCI. In an animal model of cerebral ischemic injury, the mRNA levels of IL-6, TNF- α and IL-1 β in IL-17 knockout rats were significantly lower compared to wild-type rats, which reduced the inflammatory response [8]. Results from clinical practice have shown that IL-1 β , IL-6 and TNF- α were present in the blood of an SCI caused by trauma, and they sharply increased 1 h after the injury [9].

The focus of SCI treatment is to reduce secondary damage and promote functional reconstruction. Nevertheless, the molecular mechanism of the pathological process after injury must be established to mitigate the secondary damage. Changes in miRNA may become a novel therapeutic target. The regulation of miRNA in spinal cord development and the manipulation of miRNA in spinal plasticity play important roles in promoting the development of functional regenerative therapy [10]. Currently, studies on miRNA after SCI are limited, and the current research is not sufficient. The links between molecular mechanisms are scarce, and detailed studies on specific miRNA molecular mechanisms are lacking. Both upstream and downstream molecules regulated by specific miRNA molecules and the signaling pathways involved have not been studied in depth; thus, the regulation pattern of the regulatory networks formed by various miRNAs in SCI cannot be further explored. Studies on miRNA in SCI must be conducted in depth, and the mechanisms and relationships of various miRNAs may be the main areas of focus for future research. In the previous study, we identified changes in the miRNA profiles in both the spinal tissue of SCI

rats (*in vivo*) and astrocytes stimulated with IL-17 (*in vitro*). We selected miR-136-5p, a co-upregulated miRNA *in vivo* and *in vitro*, to further explore its function and mechanism in regulating the production of inflammatory factors and chemokines after SCI. This study will improve awareness of miR-136-5p regulation after SCI and promote the development of SCI treatments based on miRNA regulation.

Materials and Methods

Ethics Statement

This project fully considered and protected the rights and interests of the study objects. The use of animals in the experiments was approved by the relevant authority (The Ethics Committee of the first affiliated hospital of Guangxi medical university). All experimental procedures were conducted in compliance with institutional guidelines for the care and use of laboratory animals in the first affiliated hospital of Guangxi medical university, China. The study meets the criteria of the Ethical Review Committee.

Materials

Fetal bovine serum (FBS) and DMEM/F12 medium were purchased from HyClone (Logan, UT, USA). Recombinant murine IL-17 was purchased from Peprotech (Rocky Hill, New Jersey, USA). Trypsin was purchased from Sigma-Aldrich (St. Louis, MO, USA). Quantibody® Rat Cytokine Array 3 was purchased from RayBiotech (Inc., Guangzhou, China). A20, p-NF-κB antibody and β-actin rabbit polyclonal antibody were purchased from Abcam (Cambridge, Massachusetts, USA). High throughput sequencing of miRNA expression profiles was conducted by Huada gene Biotechnology (Shenzhen) Co., Ltd. in China; miRNA mimics and the negative control of miRNA mimics (miR-NC) were synthesized (Guangzhou iGeneBio Co., Ltd. in China). The construction and identification of pGL3-Promoter/WT20 luciferase reporter plasmid were completed by Huada gene Biotechnology (Shenzhen) Co., Ltd. in China. Regarding the experimental animals and cells, astrocytes were isolated from the cerebral cortex of 0-2-day-old SD rats (Experimental Animal Center of Guangxi Medical University).

Extraction, Culture and Identification of Rat Astrocytes

The rat hippocampal astrocytes were cultured as previously described in the literature [11]. The brains of 1-day-old Sprague-Dawley (SD) rats were collected under sterile conditions, and the blood vessels, meninges and hippocampus were carefully separated and cut into small pieces under magnification. After 30 min of digestion with 0.125% trypsin, DMEM/F12 containing 10% fetal bovine serum was used to terminate the digestion. The cell suspension was subsequently transferred into a poly lysine-coated culture flask with a planting density of $2.0 \times 10^5/\text{cm}^2$ and was cultured in an incubator with 5% CO₂ under 37°C. The liquid was changed every 3 days. On the 10th day, the cells were fused. The supernatant was discarded after shaking (240 rpm) for 24 h in a 37°C constant temperature shaker. To ensure that microglial cells and oligodendrocytes were completely removed before subculturing, the shaking intervals were repeated 2 times after 1-2 days, and the supernatant was discarded. After digestion with 0.125% trypsin, the cells were transferred to a 24-well culture plate pre-coated with polylysine at a planting density of 1.0×10^5 cells/cm². Immunofluorescence was applied to stain glial fiber acid protein (GFAP, astrocyte marker), and the content of astrocytes should be greater than 90%.

Construction of Lentiviral Vector (miR-136-5p) and Grouping Transfection of Rat Astrocytes

Construction of the lentiviral vector, packaging and titer determination were completed by Guangzhou iGeneBio Co., Ltd. in China. The lentiviral solution was added to the culture system of rat astrocytes at different multiplicities of infection. The cells were divided into four groups: the DMEM group with no transfection of virus solution; the LV-ctrl control group with transfection of LV-ctrl virus solution; the LV-miR-136-5p group with transfection of LV-miR-136-5p; and the LV-sponge group with transfection of LV-sponge. The infection duration was 72 h, and puromycin screening was conducted for 15 days. The expression of green fluorescent protein was observed under a fluorescence microscope, and the infection efficiency of the three groups was subsequently calculated and compared.

Application of MTT to Detect the Proliferation of Astrocytes after Transfection

We seeded astrocytes of the third generation in a 96-well plate with a density of 10^4 cells/well (100 μ l per well). Once the astrocytes were adhered, we transfected the LV-ctrl, LV-miR-136-5p and LV-sponge virus solutions separately based on the best MOI values. No virus solution was added to the control group. These groups were incubated for 7 days. Every day, 20 μ l of MTT was added to 3 cell wells of each group. After 4 h, the solution in these wells was removed, and 150 μ l of DMSO was added. We then horizontally shook the shaker at a low speed for 10 min. The absorbance values of each hole were measured using a microplate reader at 490 nm (Thermo Multiskan GO, USA), and the proliferation curve was plotted by employing the mean value.

Fig. 1. Establishment of Allen's spinal cord injury model in rats.

Construction and Clinical Evaluation of the Rat SCI Model

Thirty-two SD rats were randomly divided into four groups with 8 rats per group, including the negative control (NC) group and spinal cord injury (SCI) groups (LV-ctrl+SCI group, LV-miR-136-5p+SCI group and LV-sponge+SCI group). Prior to the operation, the SD rats were weighed and intraperitoneally injected with 0.3 ml of 10% chloral hydrate per 100 g. After anesthesia, the rats were fixed in a prone position and sterilized via routine disinfection. The position of the T10 spinous process was ensured, the hair was removed, and iodophor was used to disinfect the skin. The skin was subsequently cut with a 3-cm incision, the subcutaneous fascia was separated, and the paravertebral muscle was dissected with a hemostatic clamp blunt along the edge of the spinous process to expose the spinous process. The spine and interspinous ligament were cut using an ophthalmic shear to fully reveal the T9-T11 spinous process; a vascular clamp was used to nip the T9 spinous process, and the T10 spinous process was cut away to expose the T10 lamina. The yellow ligament between the T9 and T10 lamina was gently removed, and bite forceps were used to remove the T10 lamina to expose the dura. After full hemostasis, a homemade Allen's device was used to lay 10-cm Kirschner wire freely from a 3 cm height to hit the dura sac, which resulted in an impingement of the spinal cord at T10. After the disruption, the spinal cord was washed with disinfectant saline, and the muscles, fascia and skin were sutured. A successful SCI model has occurred when the Allen's device impacts the spinal cord, the rat's body shakes and both its lower extremities rapidly rebound and bounce with the tail tilting along with quick falling and rapid blood stasis at the local surface where the spinal cord was impacted (Fig. 1). In the NC group, only the lamina was opened, and the injury was not treated. After the operations, the rats were individually housed and subcutaneously injected with penicillin at a dose of 300,000 units per day for 3 days. The bladder was squeezed regularly twice per day. The BBB (Basso, Beattie and Bresnahan) scoring method [12] was used to assess the behavior and hindlimb motor function of the functional evaluation group before and after the SCI, which was conducted before the operation and on the 1st day after the operation with 3 replicates of each indicator. The BBB scorers were not among the experimental personnel for this study, but they knew the grading standards well. All animals were examined with their bladders full before the scoring to prevent bladder filling, which may affect the activity.

Stimulating Rat Astrocytes with IL-17

IL-17 (100 ng/ml) was used to stimulate astrocytes at 0, 3, 6, 12, 24 and 48 h to determine the best stimulation time. Rat astrocytes were stimulated with different concentrations of IL-17 (0, 10, 25, 50, 100, and 200 ng/ml) at the optimal stimulation time. The various virus transfection groups were subsequently treated with IL-17 at the optimal stimulation time and concentration.

Infection of Each SCI Group with Lentiviruses

The corresponding virus suspension (2×10^8 transforming units/rat of recombinant lentivirus, 200 μ l) was immediately injected into the tail vein for all SCI groups 14 days and 7 days before the operation, as well as on the day of the operation; the same volume of sterile saline was injected into the NC group. The rats in all groups were euthanized 7 days after the operation (the operation day is considered day 0).

Detection of Inflammatory Cytokines and miR-136-5p Expression with Real-time PCR

Total RNA was extracted using TRIZOL reagent and was used to synthesize cDNA with an RT kit. According to the manufacturer's protocol, reverse-transcription PCR (RT-PCR) was performed using first-strand cDNA synthesized with 1 μ g of total RNA and oligo d(T) 18 primers. The primers for the real time-PCR assays for IL-1 β , IL-6, TNF- α , MIP-2, MCP-1 and miR-136-5p were designed using Primer Premier 5.0. β -actin and U6 were used to normalize each sample and each gene. All samples were assayed in duplicate.

Detection of Differential Expression of Inflammatory Cytokines with Antibody Chip

A QAR-CYT-3 inflammatory factor antibody chip and its detection kit were employed according to the kit instructions. First, the slides and chips were removed from the kit and dried for 1-2 h at room temperature for the standard operation. Then, 100 μ l of buffer solution was added to each hole of the chip, which was incubated on a shaker at room temperature. After completing a certain amount of antibody chips for 1 h, the buffer in each hole was absorbed, and 100 μ l of standard solution and sample were added to the well for incubation overnight at 4°C. The process using the Thermo Scientific Wellwash Versa chip washer to clean the slides was divided into two steps. The first step included cleaning the slides with 1 x lotion I. Then, 250 μ l of 1 x lotion I was added to each hole, which was washed 10 times and shaken for 10 s each time. Then, 1 X lotion II was used to wash each hole with 250 μ l of 1 x lotion II 6 times, which was shaken for 10 s each time. The antibody mixture was subsequently added to the chips at 80 μ l for each hole. They were placed on a shaker, incubated at room temperature for 2 h and washed again (the same method as previously described). Cy3-streptavidin was added to the chips at 80 μ l for each hole. These samples were placed on a shaker, incubated at room temperature for 1 h and washed again (the same method as previously described). Finally, fluorescence detection was performed using QAR-CYT-3 data analysis software for data analysis.

Western Blot Analysis

The total protein from the cells was extracted following the manufacturer's protocols. The protein concentration in the supernatant was subsequently determined using a BCA Protein Assay Reagent Kit. An amount equivalent to 40 μ g of total protein sample was dissolved in 20 μ l of 1x sodium dodecyl sulfate loading dye and boiled for 5 minutes. After an equivalent amount of the sample was added, the proteins were separated by 8 and 12% SDS-PAGE gel electrophoresis and transferred onto nitrocellulose membranes. The membranes were blocked for 2 h at room temperature with a blocking solution (5% nonfat milk in Tris buffered saline with Tween 20 (TBST)) and incubated overnight at 4°C with rabbit polyclonal antibody anti-A20 (1:1000), rabbit polyclonal antibody anti-p-NF- κ B (1:1000) and anti-beta actin (1:1500). After being washed 3 times for 10 min with TBST, the membranes were incubated with secondary antibodies (1:1500) for 1 h at room temperature. The membranes were subsequently washed with TBST (3 times $\times$ 10 min). Finally, the immunoreactive proteins were visualized using an ECL detection kit according to the manufacturer's instructions and were exposed to a radiograph film. The results of the graphs were analyzed using Digital Gel Imaging Analyst (Nikon 990-Doc 1000, USA). β -actin was used as a loading control for comparisons between samples.

Tissue Sampling and Slice Preparation

Blood samples were obtained from the eyeballs of the rats 7 days after the operation to collect serum. The spinal cord of the injured part of the SCI group was obtained at the same time that the corresponding part of the spinal cord tissue of the rats in the NC group was obtained. To anesthetize the animals, approximately 200 ml of heparin saline was perfused through the left ventricle until there was an outflow of clear fluid. Approximately 200 ml of 4% paraformaldehyde was subsequently used to lavage and fix the tissue. Then, 4% paraformaldehyde was used to fix approximately 5 mm of spinal cord tissue centered on the injury and the corresponding tissue for the NC group for 24 h. The fixed tissue was embedded in paraffin and continuously sectioned into 10 slices for each sample with 5 μ m per slice.

Immunohistochemical Staining and HE Staining

The spinal cord paraffin sections were immunohistochemically stained with p-NF- κ B and A20. The concentrations of p-NF- κ B were 1:100, 1:200 and 1:400, and the concentrations of A20 were 1:50, 1:100 and 1:200. The dyeing was conducted according to immunohistochemical staining conditions and steps, and the results were subsequently analyzed to determine the best primary resistance concentration. The spinal cord paraffin sections of each group were used for HE staining, p-NF- κ B and A20 immunohistochemical staining. HE staining was conducted according to hematoxylin-eosin staining conditions and procedures, and the results were observed with a microscope to determine the degree of tissue damage. The immunohistochemical staining of each spinal cord tissue was subsequently conducted based on the optimal primary resistance concentration previously described, and the distribution and content changes of p-NF- κ B and A20 were analyzed in the spinal cord tissue of each group. Finally, Image-Pro Plus software was used to analyze the images of each group, and the optical density IOD in the positive expression regions of p-NF- κ B and A20 were measured for each group.

Statistical Analysis

All experimental data were analyzed using the statistics software SPSS19.0. The data are presented as the mean and standard deviation (mean + S.D.) of each group; $P < 0.05$ was considered to be statistically significant. ANOVA was performed to compare data between groups, and an LSD t-test was also performed to compare groups.

Results

Identification of Rat Astrocytes by Immunofluorescence

A rabbit anti-GFAP polyclonal antibody was applied to identify immunofluorescence staining, which showed that the expression of GFAP in cultured astrocytes was localized to the cytoplasm. According to the positive cell rate (positive cells/total cell number) $\times$ 100%, 5 fields of vision were observed in each slice to count the number of positive cells and the total number of cells. The calculated rate could reach greater than 90% (Fig. 2).

High Throughput Sequencing of miRNA Expression Profiles in the Spinal Tissue of SCI Rats and Astrocytes Stimulated with IL-17

In the previous study, high-throughput sequencing of whole genome miRNA expression profiles was conducted for the primary rat astrocytes stimulated 6 h or 24 h by IL-17, as well as the miRNAs of the spinal cord tissue 6 h and 24 h after SCI. Compared to the NC group, there were 138 significantly expressed miRNAs in the 6 h group after the SCI, among which 87 miRNAs were up-regulated and 51 miRNAs were down-regulated; moreover, there were 143 significantly expressed miRNAs in the 24 h group after the SCI, among which 92 miRNAs were up-regulated and 51 miRNAs were down-regulated. Following stimulation by IL-17, there were 108 significantly expressed miRNAs in the 6 h group, among which 65 miRNAs

Fig. 2. Immunofluorescence staining of rat astrocytes. (a) $\times 200$ Immunofluorescent staining of glial fibrillary acidic protein (GFAP) in rat astrocytes; (b) $\times 200$ DAPI staining.

Fig. 3. (a-b) Real-time PCR analysis for three miRNAs showed that these miRNAs occurred at higher levels both *in vivo* and *in vitro* compared to the DMEM or NC groups. (c) The protein level of A20 was significantly reduced in astrocytes transfected with miRNA mimics (miR-29b-3p, miR-136-5p and miR-153-3p) compared to the control miRNA mimics (miR-NC), particularly miR-136-5p. (d) The luciferase activity of pGL3-Promoter/WT A20 was significantly suppressed in the miR-29b-3p, miR-136-5p and miR-153-3p groups compared to the miR-NC groups. * $P < 0.05$, ** $p < 0.01$ vs DMEM group or NC group; # $p < 0.05$, ## $p < 0.01$ vs miR-NC.

were up-regulated and 43 miRNAs were down-regulated; as for the 24 h group, there were 123 significantly expressed miRNAs, among which 58 miRNAs were up-regulated and 65 miRNAs were down-regulated. Furthermore, 36 miRNAs were up-regulated both *in vivo* and *in vitro*, from which we selected miR-13b-3p, miR-136-5p and miR-153-3p to perform qRT-PCR. The results were consistent with the high-throughput sequencing results (Fig. 3a-b).

MiR-136-5p Binds to 3'UTR of A20 and Inhibits Protein Translation of A20

Based on the miRBase, PICTAR and Target scan, the computational prediction indicated that the miRNAs (miR-29b-3p, miR-136-5p, and miR-153-3p) could bind to A20 3'-UTR. Using a Neon TM electroporation system, we employed miRNA mimics (miR-29b-3p, miR-136-5p, and miR-153-3p) and NC mimics (negative control) to transfect rat astrocytes. After 48 h, the protein was extracted and analyzed via Western blot. The results are shown in Fig. 3c, which indicates that the protein expression of A20 was significantly decreased compared to that of the miR-NC group after miR-29b-3p, miR-136-5p and miR-153-3p were used to transfect the cells. This finding indicated that these three types of miRNAs in astrocytes may regulate the translation of A20 after transcription, and the protein expression of A20 in the miR-136-5p group decreased most significantly. We subsequently applied pGL3-Promoter/WT A20, the recombinant reporter plasmid, together with the miR-negative control, miR-29b-3p, miR-136-5p or miR-153-3p to co-transfect rat astrocytes. The results of the luciferase activity detection after 48 h showed that the luciferase activity of pGL3-Promoter/WT A20 was significantly reduced when the transfections of miR-29b-3p, miR-136-5p and miR-153-3p were compared with that of the miR-negative control; moreover, miR-136-5p had the most significant inhibitory effect (Fig. 3d).

Lentivirus Transfection and Its Efficiency Evaluation

Seventy-two hours after the transfection of astrocytes and 15 days after puromycin was added to the screen, the number of fluorescent cells was counted under different views in the LV-ctrl group, LV-miR-136-5p group and LV-sponge group; the transfection efficiency was also calculated. The results showed (Fig. 4a, Tab. 1) that there was an approximately 90% transfection efficiency (greater than 70%), which indicated that the vectors could be used in the subsequent experiment. Table 1 shows that the differences in the transfection efficiency among various groups were not statistically significant. Moreover, according to the MTT results, the cells in the transfection group and the control group all started to grow logarithmically at the 3rd day and reached a stabilized growth after the 6th day. There was no significant difference in the cell proliferation rate among the four groups ($P > 0.05$) (Fig. 4b).

BBB (Basso, Beattie and Bresnahan) Results

The rats were placed on a flat floor of 2 m×2 m to observe the movement of the body and limbs below the injured spinal cord segments for 5 min per rat. Behavioral scores were obtained before the operation and on the 1st day after the operation. The results (Tab. 2) showed that there were no significant differences between the BBB scores in each group before the operation ($P > 0.05$). After the operation, the function of both hind limbs of the rats in the SCI model group was completely lost with a score of 0 for muscle strength. The animals could walk in a prone position, and all animals had urinary retention and spinal cord

Table 1. Evaluation of transfection efficiency of lentivirus. Note: * $p < 0.05$, ** $p < 0.01$ vs LV-ctrl group

	Field 1	Field 2	Field 3	Transfection efficiency (%)
LV-ctrl	87	89	92	89.33±2.51
LV-miR-136-5p	95	91	90	92.00±2.64
LV-sponge	92	96	92	93.33±2.30

Table 2. Four groups rats of average BBB score. Note: * $p < 0.05$, ** $p < 0.01$ vs NC group

Group	NC	LV-ctrl+SCI	LV-sponge+SCI	LV-miR-136-5p+SCI
Preoperative	21±0.0	21±0.0	20.8±0.6	21±0.0
First day after operation	19.47±0.7	0±0.0**	0±0.0**	0±0.0**

Fig. 4. (a) Transfection of lentivirus in the LV-ctrl+ group, LV-miR-136-5p group and LV-sponge group. (b) The growth curve of astrocytes in each group (Group A: astrocytes; Group B: astrocytes transfected with LV-ctrl; Group C: astrocytes transfected with LV-miR-136-5p; Group D: astrocytes transfected with LV-sponge).

Fig. 5. Relative mRNA and protein expression levels of inflammatory cytokines and chemokines. (a,c) Cells were stimulated with IL-17 (100 ng/ml), and the relative mRNA (heatmap) and protein expression levels of IL-1 β , IL-6, TNF- α , MIP-2 and MCP-1 were detected at different time points (0 h, 3 h, 6 h, 12 h, 24 h and 48 h). * p <0.05, ** p <0.01 compared to the 0 h group. (b,d) The determination of IL-17 concentrations. Cells were stimulated with different concentrations of IL-17 (0, 10, 25, 50, 100 and 200 ng/ml). The relative mRNA (heatmap) and protein expression levels of IL-1 β , IL-6, TNF- α , MIP-2 and MCP-1 were determined at 6 h. * p <0.05, ** p <0.01 vs 0 ng/ml group.

Fig. 6. Real-time PCR detection of relative inflammatory cytokines and chemokines and protein expression levels of miR-136-5p. (a-b) Real-time PCR detection of the miR-136-5p expression. (c) The effects of miR-136-5p on the mRNA of certain inflammatory cytokines and chemokines produced by IL-17-induced astrocytes. (d) The effects of miR-

The effects of miR-136-5p on the mRNA of certain inflammatory cytokines and chemokines produced by SCI rats. * P <0.05, ** p <0.01 vs DMEM group or NC group; # p <0.05, ## p <0.01 vs LV-ctrl group or LV-ctrl +SCI group.

shock. The BBB score was lower than that for the NC group, and there was a statistically significant difference (P <0.01). Thus, the postoperative BBB behavior score of the rats in the SCI group was 0, which is an important index for indicating a successful operation.

Fig. 7. Anti-body microarray detection of the expression of inflammatory factors and chemokines. (a) The effects of miR-136-5p on certain inflammatory cytokines and chemokines produced by IL-

17-induced astrocytes. (b) The effects of miR-136-5p on certain inflammatory cytokines and chemokines produced by SCI rats. * $P < 0.05$, ** $p < 0.01$ vs NC group; # $p < 0.05$, ## $p < 0.01$ vs LV-ctrl group or LV-ctrl+SCI group.

Expression of Inflammatory Cytokines, Chemokines and miR-136-5p in Rat Astrocytes

To investigate the effects of IL-17 on the production of certain inflammatory cytokines in rat astrocytes, we stimulated astrocytes that were cultured *in vitro* with 100 ng/ml IL-17, collected the total RNA of cells at different times (0 h, 3 h, 6 h, 12 h, 24 h and 48 h) and detected the mRNA expression of IL-1β, IL-6, TNF-α, MIP-2 and MCP-1. The results (Fig. 5a) showed that compared with the DMEM group, the mRNA expression of these inflammatory factors began to increase at 3 h, significantly increased and peaked at 6 h, and subsequently decreased at 12 h; moreover, the amount of expression remained significantly greater than that in the DMEM group at 48 h. Therefore, 6 h was considered to be the best stimulation time.

To determine the optimal stimulation concentration of IL-17, cells were stimulated with different concentrations of IL-17 (10 ng/ml, 25 ng/ml, 50 ng/ml, 100 ng/ml and 200 ng/ml). The results (Fig. 5b) showed that the mRNA expression of IL-1β, IL-6, TNF-α, MIP-2 and MCP-1 began to increase with 6 h of IL-17 (10 ng/ml) stimulation. The expression of these inflammatory factors significantly increased when the concentration increased to 25 ng/ml, and it continued to increase when the dose increased to 200 ng/ml. However, there was no significant difference ($P > 0.05$) in expression between 50 ng/ml and 200 ng/ml. Thus, 50 ng/ml IL-17 was selected as the best stimulus concentration in the experiment.

To verify the effective expression of LV-miR-136-5p and LV-sponge in rat astrocytes after lentiviral transfection, we stimulated cells with IL-17 for 6 h and examined the expression of miR-136-5p. The results (Fig. 6a) showed that the mRNA levels of miR-136-5p were significantly higher in the LV-miR-136-5p virus-infected astrocytes compared to the LV-ctrl group; however, the expression decreased in the LV-sponge group. The LV-ctrl, LV-miR-136-5p and LV-sponge groups were subsequently stimulated by IL-17 (50 ng/ml). After 6 h, real-time PCR was used to detect the mRNA expression of IL-1β, IL-6, TNF-α, MIP-2 and MCP-1. As shown in Fig. 6c, the results indicated that the expression of IL-1β, IL-6, TNF-α, MIP-2 and MCP-1 in the LV-miR-136-5p group was substantially higher than that in the LV-ctrl group, whereas the expression in the LV-sponge group was significantly reduced.

Expression of Inflammatory Cytokines, Chemokines and miR-136-5p in the Spinal Cord of SCI Rats

To verify whether miR-136-5p could be overexpressed or silenced, total RNA was extracted from rat spinal cord tissue, and the mRNA expression of miR-136-5p was examined by real-time PCR 7 days after the rats were infected by lentivirus suspensions of LV-ctrl, LV-miR-136-5p and LV-sponge. The results (Fig. 6b) showed that the expression of miR-136-

Fig. 8. Western blot analysis of A20 and p-NF- κ B. (a-c) The changes in the A20 and p-NF- κ B protein expression levels in rat astrocytes at different time points after IL-17 stimulation. (d-f) The effects of miR-136-5p on A20 and p-NF- κ B proteins produced by IL-17-induced astrocytes. (g-k) The effects of miR-136-5p on A20 and p-NF- κ B proteins produced by SCI rats. B-actin was used as the internal control. * $P < 0.05$, ** $p < 0.01$ vs 6 h group, LV-ctrl group or LV-ctrl+SCI group.

5p in all SCI model groups was significantly higher than that in the negative control group (NC group), which indicated that the expression of miR-136-5p increased after SCI. At the same time, the mRNA abundance of miR-136-5p in the LV-miR-136-5p-infected SCI rats was significantly higher than that in the LV-ctrl+SCI group, and the miR-136-5p level in the LV-sponge-infected SCI rats sharply decreased. These findings indicated that LV-miR-136-5p recombinant lentivirus could be overexpressed in rats, whereas LV-sponge could inhibit the expression of miR-136-5p.

Seven days after SCI rats were infected by a lentivirus of LV-miR-136-5p, LV-sponge and LV-ctrl, the total RNA was extracted from the injured rat spinal cord tissue, and the mRNA expression of IL-1 β , IL-6, TNF- α , MIP-2 and MCP-1 was examined via real-time PCR. The results (Fig. 6d) showed that the mRNA expression of these inflammatory cytokines and chemokines was substantially higher than that in the NC group, which indicated that the expression of inflammatory cytokines and chemokines increased after SCI. The mRNA expression of inflammatory cytokines and chemokines in the LV-miR-136-5p+SCI group was significantly higher than that in the LV-ctrl+SCI group, whereas the expression in the LV-sponge+SCI group sharply decreased ($P < 0.05$), which indicated that the overexpression of miR-136-5p could increase the production of inflammatory cytokines and chemokines in rats, as well as inhibit miR-136-5p expression.

Antibody Chip Detection of the Expression Differences of Inflammatory Cytokines and Chemokines in Rat Astrocytes

After astrocyte cultures were stimulated by 100 ng/ml IL-17 *in vitro*, the supernatants were collected at different times (0 h, 3 h, 6 h, 12 h, 24 h and 48 h), and the proteins for IL-1 β , IL-6, TNF- α , MIP-2 and MCP-1 were detected by an antibody chip. The QAR-CYT-3 analysis software results (Fig. 5c) showed that compared to the DMEM group, the protein expression of IL-1 β , IL-6, TNF- α , MIP-2 and MCP-1 significantly increased 6 h after IL-17 stimulation and peaked at 12 h ($P < 0.05$). Similarly, after stimulating cells with different concentrations of IL-17 (10 ng/ml, 25 ng/ml, 50 ng/ml, 100 ng/ml and 200 ng/ml), the supernatants were collected, and the protein expression of IL-1 β , IL-6, TNF- α , MIP-2 and

Fig. 9. Pathological HE staining. a, b, c and d indicate the HE stain levels in the spinal cord tissues of the NC group, LV-ctrl+SCI group, LV-sponge+SCI group and LV-miR-136-5p+SCI group, respectively. (a) Spinal gray matter and white matter exhibit a normal structure; no swelling, necrosis or gliocyte proliferation was identified in nerve cells. (b) Spinal white matter and gray matter exhibit disorder and a loose structure with a large number of vacuoles, inflammatory cell infiltration and gliocyte proliferation. (c) Spinal white matter and gray matter exhibit a normal structure, a partially damaged neuronal structure and a very small amount of gliocyte proliferation. (d) Spinal white matter and gray matter exhibit disorder and a loose structure with a large number of vacuoles, inflammatory cell infiltration and gliocyte proliferation; parenchymal cells exhibit degeneration or necrosis.

MCP-1 was detected with an antibody chip. The QAR-CYT-3 analysis software showed the results (Fig. 5d) compared to the DMEM group, and the expression of IL-1 β , IL-6, TNF- α , MIP-2 and MCP-1 significantly increased after stimulation with 10 ng/ml IL-17. The expression of proteins was significantly increased with an increase in the stimulation concentration (10 ng/ml-50 ng/ml). The difference was statistically significant ($P < 0.05$). There was no significant difference in the expression of proteins when the IL-17 stimulation concentration varied at 50 ng/ml-200 ng/ml ($P > 0.05$).

Six hours after the stimulation of rat astrocytes with IL-17 (50 ng/ml) in three groups (LV-ctrl, LV-miR-136-5p and LV-sponge groups), the supernatants were collected, and an antibody chip was used to detect the protein expression of IL-1 β , IL-6, TNF- α , MIP-2 and MCP-1. The QAR-CYT-3 analysis software indicated (Fig. 7a) that compared to the LV-ctrl group, the expression of inflammatory cytokines and chemokines in the LV-miR-136-5p group significantly increased, whereas the expression of the LV-sponge group decreased. The difference was statistically significant ($P < 0.05$).

Antibody Chip Detection of the Expression Differences for Inflammatory Cytokines and Chemokines in Serum of SCI Rat Models

Seven days after infection of three groups of rats with a lentivirus suspension, blood samples were collected from the eyeballs of the rats, and the serum was collected. The proteins of IL-1 β , IL-6, TNF- α , MIP-2 and MCP-1 were detected using an antibody chip. The QAR-CYT-3 analysis software showed (Fig. 7b) that the expression of inflammatory factors in different groups after SCI increased compared to the NC group, and the difference was statistically significant ($P < 0.05$). This result indicated that when the spinal cord was injured, the production of inflammatory cytokines and chemokines improved. Compared to the LV-ctrl + SCI group, the expression of inflammatory cytokines and chemokines in the LV-miR-136-5p+SCI group significantly increased ($P < 0.05$), whereas the expression in the LV-sponge+SCI group significantly decreased ($P < 0.05$).

Fig. 10. Immunohistochemical staining of the expression levels of p-NF- κ B. (A) a, b, c and d indicate the expression levels of p-NF- κ B in the spinal cord tissues of the NC group, LV-ctrl+SCI group, LV-sponge+SCI group and LV-miR-136-5p+SCI group, respectively. The expression of p-NF-

ly increased in the LV-ctrl+SCI and LV-miR-136-5p+SCI groups and decreased in the LV-sponge+SCI and NC groups (IHC 100 $\times$). (B) The changes in the p-NF- κ B contents in spinal cord tissues in each group. * $P < 0.05$, ** $p < 0.01$ vs LV-ctrl+SCI group.

Western Blot Detection of the Protein Expression of A20 and p-NF- κ B

We measured the expression of A20 and p-NF- κ B in astrocytes at different times (0 h, 3 h, 6 h, 12 h, 24 h and 48 h) after IL-17 stimulation. Western blot analysis (Fig. 8a-c) showed that the expression of A20 protein substantially decreased and reached its lowest point compared to the DMEM group after 6 h of IL-17 stimulation, whereas the mRNA and protein expression of inflammatory cytokines and chemokines significantly increased ($P < 0.05$). The protein expression of A20 increased after 12 h of stimulation, whereas the expression of p-NF- κ B began to increase at 3 h, peaked at 6 h and gradually decreased at 24 h, which reflects nearly the same trends as inflammatory cytokines and chemokines.

After lentiviral transfection, IL-17 was used to stimulate rat astrocytes in the LV-ctrl, LV-miR-136-5p and LV-sponge groups for 6 h. Western blot (Fig. 8d-f) showed that the protein expression of A20 substantially decreased after LV-miR-136-5p virus infection, and it significantly increased in the LV-sponge infection group. The expression of p-NF- κ B substantially increased in the LV-miR-136-5p virus infection group, whereas it showed a clear decrease in the LV-sponge group. The results showed that the lentiviral vectors LV-miR-136-5p and LV-sponge could be efficiently expressed in rat astrocytes and regulated the gene expression or activation of A20 and p-NF- κ B.

After establishing the SCI model, three lentiviral vectors (LV-ctrl, LV-miR-136-5p and LV-sponge) were transfected into rats to overexpress or silence miR-136-5p, and the expressions of A20 and p-NF- κ B in SCI were subsequently observed. The Western blot results (Fig. 8g-k) showed that the A20 protein expression in the spinal cord of the LV-miR-136-5p-infected rats was significantly lower compared to the LV-ctrl+SCI group ($P < 0.05$), whereas it substantially increased in the SCI rats after LV-sponge endogenous inhibition ($P < 0.05$). The expression of p-NF- κ B in the spinal cord tissue of the SCI rats treated with LV-miR-136-5p was significantly higher compared to the LV-ctrl+SCI group, whereas the expression in the LV-sponge+SCI group clearly decreased ($P < 0.05$).

HE and Immunohistochemical Staining

The results of the HE (Fig. 9) and immunohistochemical staining (Fig. 10 and Fig. 11) showed that the infiltration of inflammatory cells into the spinal cord and the expression of p-NF- κ B protein significantly increased compared to the negative control group (NC group), whereas the expression of A20 sharply decreased. The difference was statistically significant ($P < 0.05$). Compared to the LV-ctrl+SCI group, the infiltration of inflammatory cells in the

Fig. 11. Immunohistochemical staining of the expression levels of A20. (A) a, b, c and d indicate the expression of A20 in the spinal cord tissues of the NC group, LV-ctrl+SCI group, LV-sponge+SCI group and LV-miR-136-5p+SCI group, respectively. The expression of A20 was high in the NC group and LV-sponge+SCI group and lower in the LV-ctrl+SCI group and LV-miR-136-5p+SCI group (IHC 100×). (B) The changes in the A20 contents in spinal cord tissue in each group. * $P < 0.05$, ** $p < 0.01$ vs LV-ctrl+SCI group.

miR-136-5p-infected SCI rats significantly increased, and the A20 protein expression in the cytoplasm of spinal glial cells significantly decreased ($P < 0.05$). For the LV-sponge-infected SCI rats, the infiltration of inflammatory cells and the p-NF- κ B expression significantly decreased, and the A-20 expression significantly increased ($P < 0.05$). The results suggested that miR-136-5p could down-regulate the expression of A20 protein in the spinal cord of SCI rats, up-regulate the level of p-NF- κ B and aggravate the infiltration of inflammatory cells.

Discussion

In the central nervous system (CNS), the number of astrocytes is the largest at more than 50%. Astrocytes are the support bridge for various types of cells in the CNS. Activated astrocytes (AS) may reflect the metabolic activity of neurons, as they regulate the neurotransmitter and ion concentrations around synapses. Under physiological conditions, astrocytes often play an indispensable role. Under pathological conditions, the recruitment of monocytes, neutrophils and lymphocytes depends on the early proinflammatory factor release of astrocytes and microglial cells [13]. By generating neurotrophic and protective factors, astrocytes are not only involved in the physiological and energy metabolism of neurons but also participate in and maintain the repair process for the blood-brain barrier and CNS, as well as serve as antigen-presenting cells (APCs) [14-15]. Excessive release of proinflammatory cytokines and chemokines in the CNS may lead to neuronal and glial cell apoptosis, as well as immunopathological damage, and this release may affect peripheral immune system function and induce a systemic response. In addition, astrocytes, as the primary effector of central nervous system injury, may release a substantial number of pro-inflammatory cytokines and chemokines to regulate the pathological processes in MS, Alzheimer's disease (AD) and dementia or cerebral ischemia/trauma related to human immunodeficiency virus-1 [16-18].

After SCI, AS cells in the injury site promote their self-proliferation through autocrine and paracrine cytokines, and they form glial scars. Once astrocytes are activated, a substantial number of inflammatory mediators, such as NO, PGE2, excitatory amino acids, ATP and cytokines, such as IL-1 β , TNF- α , and IL-6, will be released. These mediators of inflammation

further activate glial cells, as well as the corresponding receptors in neurons, to enhance neuronal activity [19-21]. In the early stages of SCI, isolating the injury to stimulate and regulate the inflammatory response to maintain the stability of the local environment works as a protective barrier; however, when it develops in the later stages of an SCI, AS proliferates and leads to hypertrophy, which results in a substantial number of overlapping cells. This overlap not only prevents axonal growth by acting as a physical barrier but also secretes chemical constituents that inhibit axonal growth [22]. In the later stages of SCI, the glial scar formed by AS activation is an important physical and chemical barrier that prevents SCI repair [23]. Astrocytes after SCI repair the blood-brain barrier and limit the spread of inflammation to reduce the secondary injury caused by SCI. In addition to direct physical damage, SCI is followed by inevitable secondary damage, which occurs in most cases within several minutes and may continue for several weeks. The inflammatory response is the core process of secondary damage; thus, astrocytes play an important role in SCI, and inhibiting astrocytes from producing an inflammatory response may represent a novel treatment approach.

MicroRNAs (miRNAs) are a category of 18-25 nt non-coding single-stranded RNAs present in eukaryotic cells. By specifically recognizing the 3' untranslated region of the target gene mRNA, they inhibit the degradation or translation of mRNA, which results in the down-regulated expression of target genes and involves various biological processes, such as cell proliferation, differentiation and apoptosis and disease development [24-25]. miRNA has a complicated role in astrocytes. The role of miRNA in malignant glioma has primarily been investigated. The results showed that miRNA-145 was significantly down-regulated in gliomas, and the expression of miRNA-145 was significantly reduced in glioma cell lines compared to normal human astrocytes. miRNA-145, which binds to the target gene CTGF (connective tissue growth factor) 3' non-variable coding region, inhibits the migration of malignant glial cells. Researchers have also determined that a high expression of miRNA-145 predicted good patient tolerance [26]. Moreover, one study showed that miRNA-221/222 plays a role in the initiation and invasion of glioblastoma [27]. SCI triggers a series of pathophysiological changes that are regulated by special gene expression. miRNAs are involved in changes in the inflammation response, oxidative stress and apoptosis after spinal cord contusion in rats. The change is divided into three types: (1) up-regulation, (2) down-regulation and (3) up-regulation within 4 h after trauma and down-regulation 1-7 days after SCI [28]. In the crush injury model in rats, a microarray expression profile identified 101 differentially expressed miRNAs 12 h after the trauma, and the subsequent qRT-PCR demonstrated that at 6 h, 12 h and 3 days, the miRNA-223 expression increased and the miRNA-124 expression decreased 1-7 days after injury. The results of in situ hybridization confirmed that miRNA-223 was specifically expressed in the spinal cord. Recent literature has also indicated that miRNA abnormal regulation occurred after traumatic SCI, particularly 7 days after the injury. A subsequent bioinformatic analysis showed that changes in the miRNA expression affected the pathophysiological processes of the SCI, including the secondary injury inflammatory response and apoptosis [29].

Research has shown that NF- κ B is present in various tissues and cells; however, it has different states and activity levels in different types of cells and tissues. In the nervous system, it may be present in neurons, astrocytes, small glial cells and oligodendrocytes [30]. NF- κ B is a multidirectional transcription factor, and it is the converging point of many signal transduction pathways. It plays an important role in immunity, inflammation, cell cycle regulation, cell proliferation and differentiation, as well as apoptosis [31]. IKK is a complex that contains many types of protein components. It facilitates activation of the key kinase in the channel for NF- κ B and consists of the active subunits IKK α and IKK β and the regulatory subunits IKK γ and IKKAP. The homology of IKK α and IKK β is very high, and both are catalytic subunits in the IKK complex. In a resting state, NF- κ B binds to its inhibitory monomer (I κ B) in an inactive state and subsequently remains in the cytoplasm [32]. Many extracellular stimulatory signals (such as the inflammatory cytokines TNF- α and IL-1) may activate the NF- κ B signaling pathway and ultimately activate the IKK complex. NF- κ B has been shown

to promote neuronal apoptosis in the central nervous system. Activation of NF- κ B has been identified in apoptotic neurons arising from trauma, cerebral ischemia, Alzheimer's disease and Parkinson's disease. A20 (TNFAIP3) is an important negative regulatory factor of NF- κ B that negatively regulates the anti-apoptotic effects of NF- κ B. A20 is also involved in many pathophysiological processes of inflammation and the immune system with immune response changes and autoimmune disease [33-34]. A20-deficient rats are likely to have chronic inflammation and inflammatory damage in multiple organs, and they are very sensitive to TNF- α [35]. However, the overexpression of A20 may be attenuated and has a protective effect in the inflammatory response in mouse models of allergic asthma and rheumatoid arthritis and other inflammatory pathological autoimmune diseases. These results suggest that A20 is an ideal target molecule for slowing the inflammatory response [36-37].

In this paper, after miR-136-5p was transfected into rat astrocytes, stimulating the cells with IL-17 showed that miR-136-5p promotes the mRNA and protein expression of inflammatory cytokines and chemokines (IL-1 β , IL-6, TNF- α , MIP-2 and MCP-1), as well as affects the expression of p-NF- κ B protein. After LV-sponge was applied to inhibit the function of endogenous miR-136-5p, the mRNA and protein levels of these inflammatory factors were significantly reduced. The outcome suggested that miR-136-5p promotes IL-17-induced astrocytes to produce inflammatory chemokines.

The factors that influence *in vitro* experiments are simple because an artificial quality control treatment is more convenient; therefore, the results of the *in vitro* experiments must be confirmed with *in vivo* experiments. Therefore, to further determine the role of miR-136-5p in IL-17 stimulation of astrocytes to produce certain inflammatory cytokines and chemokines, we established an SCI model and injected a lentiviral vector (LV-ctrl, LV-miR-136-5p and LV-sponge) into the rats to observe its impact on SCI. The results showed that the contents of IL-1 β , IL-6, TNF- α , MIP-2 and MCP-1 in the serum of SCI rats infected by LV-miR-136-5p were significantly higher compared to the LV-ctrl+SCI group. For LV-sponge-treated SCI rats, the secretion of these inflammatory cytokines and chemokines was significantly reduced. The results suggested that miR-136-5p promotes the synthesis and secretion of these inflammatory cytokines and chemokines during the course of SCI. In addition, Western blot and immunohistochemistry showed that the protein expression of A20 substantially decreased in SCI rats after LV-miR-136-5p infection, whereas after the inhibition of the endogenous expression of miR-136-5p in LV-sponge, the A20 expression substantially increased. However, the expression of p-NF- κ B was opposite that of A20. In summary, miR-136-5p in SCI rats not only inhibits A20 protein expression but also up-regulates the expression of p-NF- κ B. Regarding the effects of miR-136-5p on spinal cord lesions of SCI rats, HE staining showed that the infiltration of inflammatory cells prevailed in the spinal cords of SCI rats with LV-miR-136-5p infection, whereas it was significantly reduced in the LV-sponge group. These experiments indicate that the overexpression of miR-136-5p may aggravate the infiltration of inflammatory cells in the spinal cords of SCI rats, whereas silencing miR-136-5p may have the opposite effect. Thus, miR-136-5p may represent a key molecule for the regulation of SCI lesions in rats and may become a novel method for treating this disease.

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