Original Paper

Effects of MicroRNA-494 on Astrocyte Proliferation and Synaptic Remodeling in the Spinal Cord of a Rat Model of Chronic Compressive Spinal Cord Injury by Regulating the Nogo/NgR Signaling Pathway

Yuan Wang Jing-Chuan Sun Hai-Bo Wang Xi-Ming Yang Yong Yang
Qing-Jie Kong Jian-Gang Shi

Department of Orthopedics, Changzheng Hospital, Second Military Medical University, Shanghai, China

Key Words

Microrna-494 • Astrocyte proliferation • Synaptic remodeling • Nogo/NgR signaling pathway • Chronic spinal cord injury • Spinal cord injury

Abstract

Background/Aims: Chronic compression of the spinal cord causes the loss of motor neurons in the anterior horn, but the precise and extensive mechanism for the loss is not completely determined. Therefore, this study aims to explore the role of microRNA-494 (miR-494) in the proliferation of astrocytes and in the synaptic remodeling in the spinal cord of a rat model of chronic spinal cord injury (SCI) by regulating the Nogo/NgR signaling pathway. **Methods:** A rat model of chronic compressive SCI was established, and the spinal cord state, blood supply changes, astrocyte apoptosis were observed. Reverse transcription quantitative polymerase chain reaction (RT-qPCR) and Western blotting were used to detect expression of miR-494 and Nogo/NgR signaling pathway-related genes. Fluorescence in situ hybridization (FISH) was used to detect miR-494 expression and distribution. **Results:** Higher miR-494 expression was accompanied by the inhibition of astrocyte proliferation and synaptic remodeling. In addition, CDK6 could be regulated by miR-494 and was shown to be one of the target genes of miR-494. Positive expression of miR-494 detected by FISH was consistent with the results of RT-qPCR that miR-494 could downregulate CDK6 gene expression. Moreover, the direct miR-494 target CDK6 plays important inhibitory roles in chronic SCI by suppressing the Nogo/NgR signaling pathway. **Conclusions:** The results demonstrated that miR-494 inhibition can promote astrocyte proliferation and synaptic remodeling by suppressing the Nogo/NgR signaling pathway in a rat model of chronic SCI.

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Y. Wang, J.-C. Sun and H.-B. Wang contributed equally to this work.

Dr. Jian-Gang Shi

Dept. of Orthopedics, Dept. of Orthopedics, Changzheng Hospital, Second Military Medical University
No. 415, Fengyang Road, Huangpu District, Shanghai 200003 (China)
Tel. +86-021-81885631, Fax +86-021-81885631, E-Mail jiangang_sjg@163.com

Introduction

Spinal cord injury (SCI) is a type of traumatic event that contributes to the interference of normal brain function, such as sensory, motor, and autonomic functions, and subsequently impacts a patient's physical, psychological, and social well-being [1-3]. Chronic compression of the spinal cord may result in the loss of motor neurons in the anterior horn [4], but the detailed mechanism of this type of neuronal loss is not fully understood [5]. Excessive mortality in chronic SCI is associated with several treatable factors, and mortality in chronic SCI was significantly decreased by the treatment of lung disease, diabetes, cardiovascular disease and smoking cessation [6]. Traumatic SCI leads not only to motor impairment but also to chronic central pain, making traumatic SCI difficult to treat [7, 8]. A rat model of compression-induced SCI was selected to test potential therapies that may weaken the progression of disorganization and dyskinesia after spinal injury [9]. Moreover, activated microglia may contribute to chronic pain after SCI [7]. In addition, the study of microRNAs (miRNAs) can provide a new perspective for the study of molecular mechanisms of SCI and novel potential therapeutic approaches [10].

miRNAs, a type of small, noncoding RNA molecules, have a profound impact on various biological processes, including cell growth, proliferation, differentiation and neural activity, along with the pathogenic processes of SCI, such as inflammation, demyelination and apoptosis [11, 12]. A previous study indicated that miR-494 (miR-494) can suppress cell proliferation and colony-forming activity and induce senescence [13]. The overexpression of some miRNAs, such as miR-9 and miR-133, plays important roles in the repair of SCI by promoting neural stem cell differentiation [14, 15]. miR-494 has been proven to inhibit osteosarcoma cell proliferation and metastasis via targeting of CDK6 [16]. In addition, CDK1 and CDK2 were found to be the kinases that phosphorylate Nogo B *in vitro* [17]. Moreover, there are neurite outgrowth-inhibitory domains in Nogo-A, and these inhibitory domains are exposed on the surface of myelinating oligodendrocytes, located within its amino-terminal region (amino Nogo-66) and C-terminal region (Nogo-66) [18]. In addition, Nogo-66 receptor (Nogo-66R) may be a potential strategy to promote both structural and functional recovery after SCI [19]. Although structurally different, Nogo-66, myelin-associated glycoprotein (MAG) and oligodendrocyte-myelin glycoprotein (OMgp) play major roles in inhibition via binding the cell-surface-linked neuronal NgR that transduces the inhibitory signal into the cell interior by the transmembrane co-receptors LINGO-1 and p75(NTR) or TROY [18]. A previous study has shown that nerve cell regeneration may be promoted by regulating Nogo-66R, the NgR gene expression, and the subsequently activation of the Nogo-NgR signaling pathway after acute SCI [20]. However, the role of the Nogo/NgR signaling pathway in chronic compressive SCI has not yet been reported. Therefore, our study was conducted to investigate the role of miR-494 in the proliferation of astrocytes and synaptic remodeling in the spinal cord in a rat model of chronic compressive SCI by regulating the Nogo/NgR signaling pathway.

Materials and Methods

Experimental animals

A total of 56 specific-pathogen-free (SPF) male SD rats (Hunan SJA Laboratory Animal Co., Ltd., Hunan, China) with a weight of 190 - 210 g were selected as experimental animals. All animal experiments were approved by the Animal Ethics Committee. All animals were raised in the SPF-class experimental animal room with a temperature of 25~26°C and relative humidity of 60~70%. The rats were raised with free access to bulk food and water. The bedding was replaced twice a week. The environmental conditions were in accordance with the relevant standards of the Chinese national standard "Environment Control for Experimental Animal Facilities" (GB14925-2001) on barrier animal facilities.

Establishment of the rat model with chronic spinal cord compression

Rats were anesthetized with an injection of 3% pentobarbital sodium (Merck KGaA, Darmstadt, Germany) intraperitoneally (60 mg/kg). After fixation, the skin was prepared and disinfected with iodophor, and subcutaneous tissues and muscle were incised in turn, exposing the T7 to T9 spinous process. The T8 spinous process was separated with the rongeur carefully, and the interspinous ligament, supraspinous ligament and surrounding muscle tissue from T7 to T9 were stripped. After a steel plate was clicked into the upper and lower spinous process, moderate pressure was placed on the upper and lower steel plate with the rongeur. A specialized tapping clamp was used to tap the center of the T8 vertebral plate carefully, and the plate was slowly screwed until it just penetrated the T8 vertebral plate layer, and the screw was placed carefully to avoid causing an acute SCI. An X-ray was used to confirm whether the screw was placed at the proper length. The model was successfully established if the screw did not penetrate the vertebral canal. Hemostatic suture was performed layer by layer, the end of the screw was placed *in vivo* and an X-ray was then applied for disinfection. Three days after the operation, the rats received an intramuscular injection of 1 g gentamicin (Beijing Biotype Co., Ltd., Beijing, China) to prevent infection. The suture was taken out after one week. In the sham group, only the vertebral plate drilling was performed without placement of the head screw. After the operation, the screw was moved one further rotation every week, and the screw pitch was 0.3 mm. The entire process lasted for 4 weeks, and in the fourth week, the length of the screw was approximately 1.8 mm. After the screw placement was complete, the changes in histology and nerve function were observed. In the meantime, animals with acute subcutaneous infection during the operation were discarded. Successive animal models were included to ensure a sufficient number in each group for further detection and analysis.

Animal grouping and treatment

Eight rats out of the total 56 SPF male SD rats were placed into the normal group and eight into the sham group. The remaining 40 rats were established in the rat model with chronic compressive SCI and were randomly divided into the blank model (blank model), negative control (NC), model + miR-494 mimic (miR-494 mimic), model + NgR siRNA (si-Rg) and model + miR-494 mimic + NgR siRNA (mimic + si-Rg) groups, with 8 rats in each group. The miR-494 mimic group, miR-494 (20 nmol/L, 1 µl/h) was injected intrathecally for 3 consecutive days every day after the operation for an intervention. An equal volume of saline was injected intrathecally in the blank group at the same time and speed. An equal volume of the negative control sequence, NgR siRNA sequence and miR-494 mimic sequence plus the NgR siRNA sequence was given to rats in the NC, si-Rg and mimic + si-Rg groups at the same time, dose and speed.

Basso, Beattie & Bresnahan (BBB) locomotor rating scale (BBB scale)

After the establishment of animal model, the BBB scale [21] was used to evaluate rats in each group on the 7th, 14th, 21st and 28th days after modeling, and their scores were recorded at different time periods. The rats in each group were placed on a circular smooth surface with a diameter of 1 m for free movement, and the behavior of the trunk, tail and hind limbs of each rat were observed and recorded for BBB scoring. The observation and score were evaluated by two individuals from another laboratory who were familiar with the BBB method but unfamiliar with the experimental animal grouping.

Spinal cord specimen preparation

After the BBB scale was completed 28 days postoperatively in each group, 2 rats were randomly selected, anaesthetized deeply with a high dose of pentobarbital sodium (60 mg/kg) and euthanized, and the phenyltetrazolium chloride (TTC) staining was used to observe the blood supply changes in the spinal cord. The remaining 6 rats in each group underwent cardiac perfusion after they were anaesthetized and euthanized. A left ventricular puncture was made with a smooth coarse needle for access into the ascending aorta. Hemostatic forceps were used to fix the needle and the heart, and a small puncture was made on the right auricle and cardiac perfusion was performed with 200 mL saline. After clear effluent was obtained by continuous perfusion of saline, paraformaldehyde (400 mL) was perfused to fix the rats until the limbs were stretched and stiff. Approximately 1.0 cm spinal cord in the compression section was obtained, and the corresponding spinal cord specimens at the same position were obtained from rats in the normal and sham groups. Half of the samples were fixed in 4% paraformaldehyde at 4°C overnight, which was later used for electron microscope observation, Hematoxylin-Eosin (HE) staining, terminal deoxynucleotidyl transferase

(TdT)-mediated dUTP nick end labeling (TUNEL) staining and immunohistochemistry detection. The spinal cord caudal and longitudinal of the remaining samples was cut equally, placed in liquid nitrogen, and then used for triphenyltetrazolium chloride (TTC) staining, reverse transcription quantitative polymerase chain reaction (RT-qPCR) and Western blot detection.

TTC staining

TTC staining was used to stain the ischemic area of the spinal cord in rats. TTC (2, 3, 5-triphenyltetrazolium chloride), a fat-soluble light-sensitive complex, has been used as a dye to detect the ischemic infarction in mammalian tissues since 1958. Cross-sections of 2.0 mm from frozen samples were colored in 15 mL 2% TTC solution and incubated to avoid exposure to light at 37°C for 30 min. The sections were shaken from time to time for even dyeing, fixed with 4% paraformaldehyde for 2 h, and then photographed.

HE staining

The spinal cord samples were fixed in 10% neutral formalin for 2 days, and the gradient dehydration was performed. Wax immersion was done after clearing with xylene. The samples were paraffin-embedded, sliced into 5 µm slices and dried at 60°C for 2 h. The slices were dewaxed 2 times in xylene, for 10 min each time, immersed successively in 100%~70% gradient ethanol to remove xylene, and then washed with distilled water for 3 min to remove the ethanol. After staining with hematoxylin for 10~15 min, tap water was used to rinse the extra staining fluid. The 0.5% fast green acid alcohol was used to differentiate the cells, and tap water was used to rinse for 1 min after the cell nucleus was blue. Eosin staining was performed for 20~30 s, and unfixed dyes were removed with tap water after the cytoplasm appeared red. Dehydration with a gradient ethanol of 80%~100% was performed with each gradient for 3 min. The slices were sealed with neutral balsam after clearing in xylene; the slices were observed in the microscope and pictures were taken for preservation.

TUNEL staining

After the slices were dewaxed with xylene, the gradient ethanol was used to remove the xylene. The slices were incubated with proteinase K (20 mg/mL) at room temperature for 20 min after washing with phosphate buffer saline (PBS), and then 0.3% hydrogen peroxide was added to the slices for a 30 min incubation. The slices were processed with 0.1% Triton X-100 for 10 min after washing with PBS. The slices were then dried in the air. After water usage, they were rinsed with PBS. A 10 µL volume of reaction liquid was dropped onto the specimen slides for incubation at 37°C for 1 h. Buffer A was used to rinse the specimen 2 times for 2 min, and 50 µL goat serum was added and blocked at 37°C for 30 min. The serum was removed, and 50 µL staining solution was added, followed by an incubation at 37°C for 1 h. Buffer B was then used to rinse the specimen 2 times for approximately 2 min. The slices were placed in 50 µL 5-bromo-4-chloro-3-indolyl phosphate (BCIP)/nitroblue tetrazolium (NBT) color liquid in the dark for 20 min. In addition, Buffer C was used to rinse the specimen 2 times. Nuclear fast red was used to redye the specimen for 5~20 s, followed by dehydration with gradient ethanol after washing with distilled water. The slices were sealed and observed with a microscope. Photos (n = 6) were taken to calculate the positive rate, and a statistical analysis was conducted to determine the mean ± standard deviation.

Immunohistochemical double staining and image analysis

Immunohistochemical double staining was used to detect the expression of glial fibrillary acidic protein (GFAP) and synaptic vesicle protein synaptophysin (SYP) in spinal cord tissues. After the spinal cord tissue was removed and embedded in paraffin, 4 µm coronal slices were cut continuously for use. Paraffin slices were dewaxed, incubated with 3% hydrogen peroxide in methanol solution at room temperature for 20 min, and then washed with water. Slice antigen repair treatment was performed 2 times in the microwave at 80°C and with 80% power for 5 min each time. At room temperature, the slices were sealed separately with 100 µL serum for 20 min, and then SYP and GFAP staining were performed. The primary GFAP antibody (1: 1000, ab4674) and SYP antibody (1:400, ab53166) were added and incubated at room temperature overnight. The secondary antibody was added after the stained tissues were washed by PBS 3 times and were incubated at room temperature for 2 h. The incubation with the avidin-biotin-peroxidase complex (ABC) was performed for 2 h, and then the slices were washed with PBS. After diaminobenzidine (DAB)

coloration and gradient dehydration, the slices were cleared and then sealed. A light microscope was used for observation and images were taken. All antibodies used in the experiment were purchased from the Abcam Company, USA. The HPIAS-1000 (Wuhan Champion Image Technology Co Ltd., Wuhan, China) pathological color image analysis was adopted to detect the optic density (OD) values of SYP immunoreactive product and GFAP-positive product. The measured position was the II layer of the dorsal horn of the spinal cord, and the average value of three visual fields was taken. All the OD value measurements were performed under the same optical conditions, and statistical analysis was used to compute the mean $\pm$ standard deviation.

Fluorescence in situ hybridization (FISH)

The paraffin sections were dewaxed and hydrated at room temperature. The sliced sections were fixed in the air, fixed with 4% PFA for 10 min, and washed with PBS for 5 min, and this procedure was repeated. Enzymatic hydrolysis of the proteins in the RNA in order to expose the targeted RNA in need of hybridization. The preheated 50 mL protease buffer solution was diluted to 10 μ L/mL by adding 100 μ L protease K in the liquid (5 mg/mL). After the solution was fully mixed, sections were placed into it and incubated at 37°C for 20 min. Glycine solution was added stop the protease reaction with the fixed slide. Then, formaline (13.2 mL) was added to 980 mL DEPC-treated water for adjusting the pH to 8.0, and 2.5 mL concentrated hydrochloric acid, and was fully mixed. Next, 4.5 mL acetic anhydride was added and quickly put onto the sections after mixing for 10 min. Sections were dehydrated and placed in a lunch box that contained absolute ethyl alcohol on the bottom to keep the sections strictly in a dehydrated state. The filled lunch box was sealed with parafilm and incubated at 4°C for 3~4 h. Hybridization solution A (hybridization solution A: 80 μ L; 10 $\times$ in situ salts; deionized formamide: 40 μ L; 50% dextran sulfate: 20 μ L; 50 $\times$ Denhardt's: 2 μ L; 100 mg/mL RNA: 1 μ L; DEPC-treated H₂O: 7 μ L) and hybridization solution B (hybridization solution B: 20 μ L; deionized formamide: 10 μ L; RNA probe: 2 μ L; DEPC-treated H₂O: 8 μ L) were prepared ahead of the experiment. An appropriate amount of hybridization solution was removed, heated at 80°C for 2 min, placed on ice, centrifuged and later placed on ice. Hybridization solution A was added to hybridization solution B and mixed carefully to avoid air bubbles. Each slide was added to 100 mL mixed solution with parafilm or coverglass carefully placed on top of the slide sections were added, no bubbles were produced because air bubbles would strongly influence the hybridization signal. The slides were laid in the wet box with an appropriate amount of 2 $\times$ SSC and 5% formamide on the bottom, blocked and hybridized at 52°C overnight, after which the slide was washed (with preheated 0.2 $\times$ SSC to 55°C and preheated NTE buffer (0.1 M NaCl, 0.01 M Tris, 0.001 M EDTA, pH 8.0) at 37°C). The procedure for slide washing was as follows: primarily, the slide was washed with 0.2 $\times$ SSC at 55°C for 60 min, washed with NTE at 37°C for 5 min, and then rinsed in RNase at 37°C for 30 min; then, the slide was washed with NTE at 37°C for 5 min, rinsed with 0.2 $\times$ SSC at 55°C for 60 min, and finally washed with PBS. Before each washing step, the solution was preheated, and during washing, the slide was gently rocked. Finally, the slide was placed at 4°C to stop the experiment. The slides were immediately blocked, and antibody was added for the reaction by adding 1% (W/V) bovine serum albumin in 100 mM Tris (pH = 7.5) and 150 mM NaCl, and the slide was placed in a shaker at room temperature for 30 min. Then, the original solution was replaced with 1% BSA in 100 mM Tris (pH = 7.5), 150 mM NaCl and 0.3% Triton X-100 and rocked for another 45 min. In the BSA/Tris/Triton solution (the same as the second step), the digoxin antibody was diluted to a concentration of 1:1000 by adding 1 μ L of digoxin antibody (1:300). Later, the sample solution was incubated for 30 min avoiding exposure to light, washed 3 times with PBS for 5 min each, blocked by adding 10% serum, and incubated at room temperature for 30 min. The primary antibodies rabbit-anti-neuN (1: 500), rabbit-anti-GFAP (1: 600) and rabbit anti-Ibal (1: 1000) were added and incubated at 4°C overnight and again washed 3 times with PBS. Finally, the secondary antibody was added to the sample solution, incubated at room temperature for 2 h avoiding exposure to light, washed 3 times with PBS, mounted by glycerol, observed under fluorescence microscope and imaged.

RT-qPCR

Total RNA was extracted from tissue samples by the TRIzol method (Invitrogen Inc., Carlsbad, CA, USA). Complementary DNA (cDNA) was synthesized by reverse transcription according to the TaqMan MicroRNA Assays Reverse Transcription Primer kit instructions (Applied Biosystems, USA). Primers of miR-494, Nogo A, NgR1, NgR2, Rho, ROCKII and β -actin were designed and then synthesized by Biolog (Biolog, Shanghai, China) (Table 1). The reverse transcription conditions were 16°C for 30 min, 42°C for 30 min, 85°C for 5 min, and 4°C for 25 s. Quantitative detection of miR-494, Nogo A, NgR1, NgR2, Rho, ROCKII and β -actin

was achieved in the ABI7500 according to the TaqMan Assays TaqMan Assays kit instructions from ABI. The PCR conditions were one cycle of initial denaturation at 95°C for 10 min, 45 cycles of denaturation at 95°C for 15 s, and 55°C for 30 s. U6 was used as internal reference for miR-494, and β -actin was used as an internal reference for the other genes. Relative expression of miR-494, Nogo A, NgR1, NgR2, Rho, and ROCKII were calculated by the $2^{-\Delta\Delta Ct}$ method.

Western blot

The protein extraction kit (No. C0481, Sigma, USA) was used to extract the total protein from tissue samples according to instructions. The protein concentration was measured according to the bovine carbonic anhydrase (BCA) protein assay kit instructions (BCA1-1KT; Sigma, USA). The 10% sodium dodecyl sulfate (SDS) separation gel and stacking gel were prepared. The sample was mixed with sample buffer and boiled at 100°C for 5 min. Electrophoresis was used after ice bath and centrifugation, and then the protein on the gel was transferred to a nitrocellulose membrane. The nitrocellulose membrane was sealed with 5% skimmed milk at 4°C overnight. The primary antibodies CDK6 (1: 1000, ab151247), Nogo A (1: 1000, ab62024), NgR1 (1: 200, ab2629), NgR2 (1: 500, ab136338), Rho (1: 200, ab98887), ROCKII (1: 200, ab71200), and β -actin (1: 200, ab5694) were added for incubation overnight and washed by PBS 3 times at room temperature for 5 min each time. The second antibody (1: 1000, Wuhan Boster Biological Engineering Co., Ltd., Hubei, China) was added with shaking incubation at 37°C for 1 h. The membrane was washed by PBS 3 times at room temperature for 5 min each time, and then immersed in the electrochemiluminescence (ECL) solution (Pierce Biotechnology, USA) at room temperature for 1 min. The membrane was exposed to X-ray in the darkroom and then was developed and fixed. β -actin was recognized as an internal reference, and the gray value ratio of target bands and internal reference bands was recognized as the relative expression of proteins.

Separation, purification and identification of astrocytes in the rat spinal cord

Five SPF neonatal rats aged 2–3 days were selected; the exposed skin was disinfected, and the spinal canal was opened to extract spinal cord tissues. After washing, the membranes surrounding the spinal cord was stripped and the spinal cord tissues were removed and taken for further treatment with 0.125% trypsin, and centrifugation for cell collection. The collected cells were incubated in a culture medium consisting of Dulbecco's modified Eagle's medium/Ham's F-12 medium (DMEM/F12) with 10% calf serum in a 5% CO₂ incubator. The culture medium replaced once every 3 days. Cells aged 7–9 days were chosen, and after the original culture medium was replaced, cells underwent vibration incubation overnight at constant temperature for 100 r/min. The supernatant of the cell suspension (mainly oligodendrocyte and microglia) was discarded; cells were washed one time to adhere astrocytes strongly to the wall and were detached by trypsin and then inoculated into a culture bottle or a 6-well plate with the cell density. Cells of the third generation were used for the dual luciferase reporter assay, RT-qPCR, Western blotting, and GFAP immunofluorescence identification (labeled by TRITC) using routine methods.

Dual luciferase reporter assay

The biological website mircorna.org was used to identify the binding site of miR-494 and CDK6-3'UTR, and dual luciferase reporter was used to verify the targeting relationship between miR-494 and CDK6. Gene fragments of CDK6-3'UTR were artificially synthesized by amplifying wild-type and mutant CDK6-3'UTR, which were introduced into the pMIR-reporter with the adoption of Xba I and pGL3 to obtain fragments of CDK6-WT and CDK6-MUT. Plasmids of the dual luciferase reporter with the correct sequencing (WT and MUT) were co-transfected with the miR-494 mimic or scramble into the rat astrocytes. After 24 h transfection, whether miR-494 was able to change the activity of luciferase was detected by the dual luciferase detection kit (Stratagene, La Jolla, CA, USA). The dual luciferase reporter gene system (Promega, Madison, WI, USA) was used for detecting the fluorescence activity. The whole experiment was repeated 3 times with 6 duplicate wells for each experiment.

Table 1. Primer sequences for RT-qPCR. Note: RT-qPCR, reverse transcription quantitative polymerase chain reaction; F, Forward; R, Reverse

Name	Sequence
miR-494	F: 5'-GGCGCGCTGAAACATAC-3' R: 5'-GGGTCCGAGGTATTCGCACT-3'
U6	F: 5'-CTCGCTTCGGCAGCACATA-3' R: 5'-ACGCTTCACCAATTTGCGT-3'
CDK6	F: 5'-TGCACACACGAACTGAA-3' R: 5'-ACCTCGGACACGTGA-3'
Nogo A	F: 5'TAGCACCTGTCACAGAG-3' R: 5'TAGCTCTAGCACTACACA-3'
NgR1	F: 5'-AGCAGCGGCACTGCG-3' R: 5'-AGAGGTGCACTGCT-3'
NgR2	F: 5'-CGGCTTCCAAAG-3' R: 5'-GGTCTGCTCTGCT-3'
Rho	F: 5'-TACATCTGAGCACTAGCA-3' R: 5'-AGATGAGGCTGCGACT-3'
ROCKII	F: 5'-GGCGCGCTGAAACATAC-3' R: 5'-GGGTCCGAGGTATTCGCACT-3'
β -actin	F: 5'-CATCCCCCAAGTT-3' R: 5'-GGAAGGCTCATCATT-3'

Statistical analysis

Data were analyzed by SPSS 21.0 statistical software (IBM Corp. Armonk, NY, USA). The measurement data were expressed as the mean $\pm$ standard deviation. The comparison among multiple groups was conducted by one-way analysis of variance, the comparison between two groups was examined by *t*-test, and *p* < 0.05 was considered statistically significant.

Results

Evaluation of the spinal cord state with the BBB scale

The BBB scale of rats in each group is shown in Table 2. The hind limb movement ability was good, the BBB scale was high at 7, 14, 21, 28 days for the normal and sham groups, and there was no marked difference between the two groups at each time period (*p* > 0.05). The score was slightly lower at 7 days for the si-NgR group, which was obviously lower than that in the normal and sham groups (all *p* < 0.05), but there was no marked difference compared to the other groups (model, NC, miR-494 mimic and mimic + si-NgR groups). The hind limb movement ability gradually recovered at 14, 21 and 48 days and compared with the normal and sham groups, there were no evident differences at the 3 time periods for the si-NgR group, but the ability was higher than the other groups (all *p* < 0.05). The hind limb movement ability of rats deteriorated with time gradually following the operation in the model, NC, miR-494 mimic and mimic + si-NgR groups, and the score at each time period was significantly decreased compared with the normal group (all *p* < 0.05). The score of miR-494 mimic group decreased most significantly with time, which showed a significant difference compared with the other groups (all *p* < 0.05). Compared with the model group, there were no marked differences in the score at each time period in the NC and miR-494 mimic + si-NgR groups (all *p* > 0.05).

Establishment of the chronic compressive spinal cord injury model and the observation of blood supply changes by TTC staining

The chronic compressive spinal cord models are shown in Figure 1A. Rats were fixed and their skin was removed by

Table 2. BBB scale in each group at different timepoints. Note: * *p* < 0.05 compared with the normal group; #, *p* < 0.05 compared with the model group; BBB scale, Beattie & Bresnahan locomotor rating scale; NC, negative control

Groups	7 days	14 days	21 days	28 days
Normal	20.13 $\pm$ 0.83	19.88 $\pm$ 0.64	20.13 $\pm$ 0.64	21.00 $\pm$ 0.64
Sham	20.25 $\pm$ 0.71	20.13 $\pm$ 0.64	20.00 $\pm$ 0.76	20.63 $\pm$ 0.52
Model	14.63 $\pm$ 0.52 *	12.25 $\pm$ 0.46 *	10.63 $\pm$ 0.52 *	8.75 $\pm$ 0.46 *
NC	14.13 $\pm$ 0.35 *	12.5 $\pm$ 0.53 *	10.50 $\pm$ 0.53 *	8.50 $\pm$ 0.53 *
miR-494 mimic	12.63 $\pm$ 0.52 *#	10.13 $\pm$ 0.35 *#	8.38 $\pm$ 0.52 *#	6.50 $\pm$ 0.53 *#
si-NgR	14.38 $\pm$ 0.52 *#	19.88 $\pm$ 0.83 *#	19.88 $\pm$ 0.64 *#	20.38 $\pm$ 0.52 *#
miR-494 mimic + si-NgR	14.63 $\pm$ 0.52 *	12.25 $\pm$ 0.46 *	10.63 $\pm$ 0.52 *	8.38 $\pm$ 0.52 *

Fig. 1. Establishment of the chronic compressive spinal cord injury model and the analysis of blood supply by TTC staining. Note: A, A diagrammatic sketch of the chronic compression model with chronic compression of the spinal cord. Rats were fixed and their skin was removed by disinfectant iodophor for preparation. Skin, subcutaneous tissue and muscle were cut open to expose the T7 - T9 spinous process. The T8 spinous process was separated with the rongeur carefully, and interspinous ligament, interspinous ligament and surrounding muscle tissue from T7 to T9 were stripped. b, After the steel plate was clicked into the upper and lower spinous process, moderate pressure was placed on the upper and lower steel plate with the rongeur. A specialized tapping clamp was used to tap the T8 vertebral plate center carefully, and it was slowly screwed until the plate just penetrated the T8 vertebral plate layer. B, The X-ray image of the spinal lateral position. C, Analysis of the blood supply in each group revealed by TTC staining; the black arrow indicates the pale area of the spinal cord. TTC, triphenyltetrazolium chloride; NC, negative control.

iodophor for preparation. Skin, subcutaneous tissue and muscle were cut open successively to expose the T7 - T9 spinous process. The T8 spinous process was separated with the rongeur carefully, and interspinous ligament, supraspinous ligament and surrounding muscle tissue from T7 to T9 were stripped. After the steel plate was clicked into the upper and lower spinous process, moderate pressure was placed on the upper and lower steel plate with the rongeur. A specialized tapping clamp was used to tap the T8 vertebral plate center carefully, and it was slowly screwed until the plate just penetrated the T8 vertebral plate layer. An X-ray was used to confirm whether the screw was positioned at the proper length (Fig. 1B). The model was successfully established if the screw did not penetrate the spinal canal. The TTC staining method was used to observe the blood supply changes in the spinal cords. The pale color indicated that the area was ischemic and the red color indicated that the area was not ischemic. Through TTC staining analysis on the spinal cord sections from rats in each group (Fig. 1C), it was revealed that the cross sections of the spinal cord in the normal group were red and the blood supply was good, and they were not significantly changed. There was no visible white matter in the sham group, and no marked difference was observed compared with the normal group. In the model group, there were many massive pale areas in the cross sections of the spinal cord compression area. In the miR-494 mimic + si-NgR groups, sporadic pale areas were observed in the white matter of the spinal cord compression section. Compared with the model group, the pale area of white matter in the spinal cord compression section increased significantly in the miR-494 mimic group; several pale areas were observed in the gray matter of the spinal cord, with a developing trend to the center. There was an involution area in the spinal cord compression section of the si-NgR group, which was close to the normal level.

Spinal cord pathological changes by HE staining

HE pathological staining results showed (Fig. 2) that in the normal and sham groups, the boundary of the section of normal spinal cord between the white matter and gray matter was clear, and white matter nerve fibers were arranged closely and neatly. The structure of axons and myelin was clear and symmetric, and glial cells were the main part of the white matter. Nerve cells were present in the gray matter, and the Nissl body and neurites were clearly visible. In the model group, a large number of inflammatory cells were found in the spinal cord, and spinal cord deformation appeared in the compression position. Compared with the NC group, the degree of spinal cord deformation was aggravated, the number of nerve cells were reduced in the compressed spinal cord, part of the cellular Nissl body disappeared, and astrocytes proliferated clearly in the model group. In comparison to the NC group, the miR-494 mimic group revealed neuronal loss, smaller sized neurons, and vacuolation formation from neuronal loss; in addition, leukoencephalopathy appeared in the gray matter of the compressed spinal cord with the appearance of lamellar irregular demyelination, myelin destruction, glial cell proliferation and frequent vacuolation formation. A spongy type could be seen, and the density of nerve cells clearly decreased. Compared with the model group, the scope and extent of the degeneration of SCI were markedly reduced in the si-NgR group. The morphology of the spinal cord was complete and the structure

Fig. 2. HE pathological staining in the spinal cord tissue from each group. Note: A, HE staining of the entire spinal cord tissue ($\times 100$). B, Amplified HE staining of spinal cord tissue ($\times 400$). HE, Hematoxylin-Eosin; NC, negative control.

was clear. There were more normal neurons and nerve fibers were arranged more closely and neatly. The proliferation of glial cells, the pyknosis, dissolution and necrosis of neurons were reduced. The inflammatory reaction was slighter. Compared with the si-NgR group, the miR-494 mimic + si-NgR group displayed loose spinal cord tissue, disordered structure, and seriously proliferated glial cells, with the observation of the obvious blank area structure. The astrocyte structure was incomplete.

Astrocyte apoptosis identification by TUNEL staining

TUNEL staining was used to detect astrocyte apoptosis in the spinal cord samples in each group (Fig. 3). Yellow particles in the nucleus of the TUNEL-positive cells could be observed. Except for the normal and sham groups, TUNEL-positive cells were few in the gray matter and white matter of the spinal cord in the other groups. The counting of positive cells in the visual field showed that the number of TUNEL-positive cells in the normal and sham groups was very small and the difference was not significant (all $p > 0.05$). Compared with the normal group, the number of positive cells in the other groups increased significantly (all $p < 0.05$). There was no marked difference in the number of positive cells among the model, NC and miR-494 mimic + si-NgR groups (all $p > 0.05$). Compared with the model group, the number of positive cells in the miR-494 mimic group significantly increased, and the number of positive cells in the si-NgR group significantly decreased (all $p < 0.05$).

SYP and GFAP expression by immunohistochemical double staining

In the GFAP-negative cells, a GFAP signal was observed in the cytoplasm, presenting as general graininess. In astrocytes, the positive expression of GFAP was significantly enhanced with a small amount of branches and protrusions. Significant reactive gelatinization appeared and the astrocytes became larger. The cell cytoplasm was thin and prominences were increased and extended. Cells began to proliferate and some cells showed a star shape, and staining was more obvious (Fig. 4A). SYP-positive staining was purple and granular (Fig. 4B). The absorption of immunohistochemical double staining (Fig. 4C) revealed that the expression of GFAP and SYP in each group tended to be mostly consistent, and the expression in the normal and sham groups was lower, with no significant difference (all $p > 0.05$). Compared with the normal group, the expression of GFAP and SYP in the other groups increased significantly (all $p < 0.05$). There were no marked differences among the model, NC and miR-494 mimic + si-NgR groups (all $p > 0.05$). Compared with the model group, the expression of GFAP and SYP in the miR-494 mimic group significantly increased, while expression in the

Fig. 3. TUNEL-positive cells were increased by transfection with miR-494 mimic and decreased by si-NgR. A, Cell morphology in each group revealed by TUNEL staining ($\times 200$). B, Number of TUNEL-positive cells in each group. *, $p < 0.05$ compared with the normal group; #, $p < 0.05$ compared with the model group. TUNEL, Terminal-deoxynucleotidyl Transferase Mediated Nick End Labeling; NC, negative control.

si-NgR group significantly decreased (all $p < 0.05$).

Expression of miR-494, Nogo A, NgR1, NgR2, Rho, ROCKII and CDK6 mRNA detected by RT-qPCR

There were no marked differences in the mRNA expression of miR-494, CDK6, Nogo A, NgR1, NgR2, Rho, and ROCKII between the normal group and the sham group (all $p < 0.05$). Compared with the normal group, the mRNA expression of miR-494, Nogo A, NgR1, NgR2, Rho, and ROCKII increased significantly, while that of CDK6 decreased in the model, NC, miR-494 mimic, si-NgR and miR-494 mimic + si-NgR groups (all $p < 0.05$). There were no marked differences in the mRNA expression of miR-494, Nogo A, NgR1, NgR2, Rho, and ROCKII between the model group and the NC group (all $p > 0.05$). Compared with the model group, the mRNA expression of miR-494, Nogo A, NgR1, NgR2, Rho, and ROCKII increased significantly, and that of CDK6 decreased notably in the miR-494 mimic group (all $p < 0.05$). In the si-NgR group, no marked differences in the mRNA expression of miR-494, Nogo A, NgR1, NgR2, Rho, and ROCKII were observed (all $p > 0.05$), and the mRNA expression of NgR1, NgR2, Rho, and ROCKII significantly increased (all $p < 0.05$). In the miR-494 mimic + si-NgR group, the mRNA expression of miR-494 and Nogo A increased significantly and that of CDK6 decreased significantly (all $p < 0.05$), and there were no marked differences in the mRNA expression of NgR1, NgR2, Rho, and ROCKII (all $p > 0.05$) (Fig. 5).

Protein expression of Nogo A, NgR1, NgR2, Rho, ROCKII and CDK6 revealed by Western blotting

The protein expression of CDK6, Nogo, NgR1, NgR2, Rho and ROCKII were detected by Western blotting in each group (Fig. 6), and the results were consistent with the tendency of the corresponding mRNA expression in the other groups. There were no marked differences in the protein expression of CDK6, Nogo A, NgR1, NgR2, Rho, and ROCKII between the normal group and the sham group (all $p > 0.05$). Compared with the normal group, the protein expression of Nogo A, NgR1, NgR2, Rho and ROCKII increased significantly and CDK6 decreased remarkably in the model, NC, miR-494 mimic, si-NgR and miR-494 mimic + si-NgR groups (all $p < 0.05$). There were no marked differences in the protein expression of CDK6, Nogo A, NgR1, NgR2, Rho and ROCKII between the model group and the NC group

Fig. 4. miR-494 mimics promoted the expression of GFAP and SYP demonstrated by immunohistochemistry. Note: A and C, The cell morphology of GFAP and SYP immunohistochemical staining ($\times 200$). B and D, Statistical analyses of the positive expression of GFAP and SYP in cells; *, $p < 0.05$ compared with the normal group; #, $p < 0.05$ compared with the model group. SYP, synaptophysin; GFAP, glial fibrillary acidic protein; NC, negative control.

Fig. 5. RT-qPCR revealed that mRNA expression of miR-494, Nogo A increased and expression of CDK6 decreased in the miR-494 mimic + si-NgR group. Note: A and B, mRNA expression of miR-494, CDK6, Nogo A, NgR1, NgR2, Rho, and ROCKII in each group revealed by RT-qPCR results; *, $p < 0.05$ compared with the normal group; #, $p < 0.05$ compared with the model group. RT-qPCR, reverse transcription quantitative polymerase chain reaction; NC, negative control.

Fig. 6. Protein expression of Nogo A increased and CDK6 decreased significantly by transfection of miR-494 mimic + si-NgR, as shown by Western blotting. Note: A, Western blot bands of spinal cord tissues from each group. B, Protein expression of Nogo A, NgR1, NgR2, Rho and ROCKII; *, $p < 0.05$ compared with the normal group; #, $p < 0.05$ compared with the model group; NC, negative control.

Fig. 7. Expression of miR-494-positive cells increased in the miR-494 mimic + si-NgR group, as shown by FISH (× 400). Note: A and B, Analysis of miR-494 expression in rat spinal cord tissues from each group revealed by FISH; *, $p < 0.05$ compared with the normal group; #, $p < 0.05$ compared with the model group. FISH, Fluorescence in situ hybridization; NC, negative control.

(all $p < 0.05$). Compared with the model group, the protein expression of Nogo A, NgR1, NgR2, Rho and ROCKII increased significantly and that of CDK6 decreased notably in the miR-494 mimic group (all $p < 0.05$). In the si-NgR group, differences in the expression of Nogo A protein and CDK6 were not obvious ($p > 0.05$), and the protein expression of NgR1, NgR2, Rho and ROCKII decreased significantly (all $p < 0.05$). In the miR-494 mimic + si-NgR group, the expression of Nogo A protein increased but that of CDK6 decreased significantly ($p < 0.05$), and the differences in the protein expression of NgR1, NgR2, Rho and ROCKII were not obvious (all $p > 0.05$).

Expression and distribution of miR-494 by FISH detection

The miR-494 expression detected by FISH is shown in Fig. 7. MiR-494-positive cells were mainly expressed in the ventral gray and white matter of the spinal cord. Expression of miR-494-positive cells was not significantly different between the normal and sham groups

Fig. 8. Observation and identification of the morphology of astrocytes. Note: A, Observation of astrocytes with the inverted microscope after culturing for 4 days ($\times 200$). B, Identification of star-shaped astrocytes by GFAP immunofluorescence ($\times 200$).

Fig. 9. CDK6 was the target gene of miR-494. Note: A, Verification of the targeting relationship between miR-494 and CDK6 by a biological prediction website. B, Verification of the targeting relationship between miR-494 and CDK6 by a dual luciferase reporter assay. C, RT-qPCR was applied to detect CDK6 mRNA expression in the scramble and miR-494 mimic groups. D, Western blot analysis was applied to detect CDK6 protein expression in the scramble and miR-494 mimic groups; *, $p < 0.05$ compared with the scramble group.

($p > 0.05$). Compared with the normal group, the expression of miR-494-positive cells was elevated in the model, NC, miR-494, miR-494 mimic + si-NgR, and miR-494 mimic + si-NgR groups (all $p > 0.05$). The expression of miR-494-positive cells in the model group was not different from the NC group ($p > 0.05$). Compared with the model group, expression of miR-494-positive cells was significantly increased in the miR-494 mimic group, which was not meaningfully different compared to the si-NgR group and was notably increased compared to the miR-494 mimic + si-NgR group ($p < 0.05$). All results presented above were consistent with the results of RT-qPCR.

Observation and identification of first-generation astrocytes in the spinal cord

After purification, astrocytes were placed in a culture dish, and 1 h later, they began to attach in the shape of an ellipsoid and polygon. With time, the protuberances of the astrocytes stretched out and grew larger, and touched the protuberances of nearby astrocytes to form attachment. The second-generation astrocytes grew in a flake shape and formed an astrocyte net. Under an inverted microscope, the astrocytes displayed a spindle, star, or irregular shape with rich cytoplasm, large nuclei, clear borders and light but even chromatin. There were 1 - 2 nucleoli in the center of nuclei, and the astrocytes were surrounded by 2 - 3 thick and large protuberances (Fig. 8A). After being cultured *in vitro* for 7 - 9 h, most of the astrocytes spread together to spread throughout the bottom of the culture bottle. GFAP was characteristically expressed in astrocytes, and the positive expression of GFAP was mainly present in the astrocyte cytoplasm and protuberances when visualized in red fluorescence (Fig. 8B).

CDK6 was targeted and regulated by miR-494

The biological prediction website showed that miR-494 could target CDK6. To verify that CDK6 was a direct target gene of miR-494, a dual luciferase reporter assay was conducted for identifying whether miR-494 targets CDK6, and the results demonstrated that compared with the NC group, the luciferase activity of CDK6-3'UTR-WT was significantly inhibited by miR-494 ($p < 0.05$), while that of CDK6-3'UTR-MUT was not suppressed (Fig. 9A & B). Furthermore, RT-qPCR and Western blot analysis were used to detect the mRNA and protein expression of CDK6, and the results indicated that after overexpression of miR-494, the mRNA and protein expression of CDK6 was significantly decreased (Fig. 9C & D). The above results showed that miR-494 could specifically bind to CDK6-3'UTR and downregulate CDK6 expression after being transcribed.

Discussion

SCI is medically and socioeconomically enervating and lacking in effective therapies. Improvements in the neurosciences have drawn more attention to SCI research, and until now, no available treatments for SCI could effectively decrease or stop the disease progression [22, 23]. The elucidation of the pathophysiological mechanisms underlying SCI is necessary for studying effective therapies for SCI [10]. The research on miRNAs may provide new insights for the study of the molecular mechanisms of SCI [10]. In this study, we explored the effects of miR-494 on the proliferation of astrocytes and the synaptic remodeling in the spinal cord in a rat model of chronic compressive SCI through regulation of the Nogo/NgR signaling pathway. The results have indicated that the inhibition of miR-494 can promote the proliferation of astrocytes and synaptic remodeling in the spinal cord of a chronic SCI model by inactivating the Nogo/NgR signaling pathway.

The open field BBB scoring system contributes to the analysis of motor dysfunction after SCI [24]. According to the results in the present study, the BBB scores in the miR-494 mimic group were significantly decreased as time progressed, and miR-494, CDK6, Nogo A, NgR1, NgR2, Rho and ROCKII expression was observed. In addition, compared with the model group, miR-494 and Nogo A expression increased in the miR-494 mimic + si-NgR group. There were no significant differences in the BBB scores at each time period in the NC and miR-494 mimic + si-NgR groups, indicating that miR-494 can promote the recovery of hind limb movement through activation of the Nogo/NgR signaling pathway. It has been reported by previous studies that the overexpression of some miRNAs, such as miR-124 and miR-133b, plays a significant role in the repair of SCI [16, 17]. The targeting relationship between miR-494 and CDK6 was confirmed by the online prediction website, and the regulation of miR-494 on CDK6 was shown by the dual luciferase reporter assay. Wang et al. found that Nogo receptor intervention may significantly improve hind limb kinematics, open field locomotion, and caudal raphe spinal axon density [24]. In addition, Nogo signaling may contribute to axon growth and the remyelination of central nervous system myelin during the process of neurodevelopment and myelination in the mature brain [25]. Nogo, NgR, Rho, EphA4 (Ephrine), and GFAP seem to preserve or reconstruct the connectivity between morphology and function across the lesion site [26]. Moreover, as has been determined by previous studies, miR-494 assesses the G1/S transition in dMSCs via the targeting of CDK6 and CCND1, which reassessed the relationship between miR-494 and CDK6 [27]. Therefore, we speculated that miR-494 can facilitate the recovery of hind limb movement via activating the Nogo/NgR signaling pathway.

Additionally, in comparison with the model group, increased cell injury and apoptosis, and positive expression of GFAP and SYP were found in the miR-494 mimic + si-NgR group. The opposite results were observed in the si-NgR group, however, indicating that the miR-494 can suppress the proliferation of astrocytes and synaptic remodeling in the spinal cord of rats with SCI by activating the Nogo/NgR signaling pathway. Reactive astrocytes are present in the cellular response to SCI [28]. A previous study has shown that miRNA, such as miR-181, may play a significant role in the molecular responses of astrocytes in the inflammatory response [29]. Moreover, miRNAs, as a type of small, non-protein-coding RNAs, play significant roles in many cellular processes, including cell proliferation, differentiation and apoptosis [30]. In addition, the overexpression of some miRNA, such as miR-2, in cultured astrocytes may cause a decreased cell size [31]. Further, epigenetic upregulation of miR-494 may significantly inhibit cell proliferation, colony formation, migration and invasion *in vitro*, as well as inhibit tumor growth in a nude mouse model [32]. In terms of synaptic remodeling, there is a remodeling of synaptic structures in the motor cortex following SCI [33]. A previous study has shown that miRNAs may function locally, such as at synapses modulating synaptic activity [34]. Synaptic remodeling was associated with LKB1 and AMPK [35], which can be regulated by miRNA, such as miR-451 [36]. Therefore, miR-494 can inhibit astrocyte proliferation and synaptic remodeling in the spinal cord of rats with chronic SCI,

and the inhibition of miR-494 could possibly promote astrocyte proliferation and synaptic remodeling, thus benefiting the spinal cord in chronic SCI.

All in all, these studies demonstrated that miR-494 can facilitate the recovery of hind limb movement as well as play an important role in inhibiting astrocyte proliferation and synaptic remodeling in the spinal cord of rats with chronic SCI. However, the trial size in this study was relatively small. Further studies are expected to address the abovementioned problem, and a more detailed mechanism of the effects of miR-494 on astrocyte proliferation and synaptic remodeling by regulating the Nogo/NgR signaling pathway in the spinal cord of rat models of chronic compressive SCI will be determined.

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Disclosure Statement

The authors declare to have no competing interests.

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