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LncRNA SNHG1 attenuates neuropathic pain following spinal cord injury by regulating CDK4 level

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Abstract. – OBJECTIVE: Neuropathic pain (NP) is one of the most intractable complications of spinal cord injury (SCI). This study aims to explore the role of long non-coding RNA (lncRNA) SNHG1 in influencing SCI-induced NP.

MATERIALS AND METHODS: After establishment of the spinal nerve ligation (SNL) model in rats, spinal tissues were extracted. SNHG1 level in rat spinal tissues was determined by quantitative real-time polymerase chain reaction (qRT-PCR). The role of SNHG1 in the development of NP was explored by assessing paw withdrawal threshold (PWT) and paw withdrawal latency (PWL) in model rats. The interaction between SNHG1 and CDK4 was explored by Luciferase assay and RIP (RNA-Binding Protein Immunoprecipitation). Enzyme-linked immunosorbent assay (ELISA) and qRT-PCR were conducted to determine inflammatory factor levels in rat spinal tissues.

RESULTS: SNHG1 was upregulated in rats undergoing SNL. Knockdown of SNHG1 alleviated the development of NP and overexpression of SNHG1 was capable of inducing NP symptoms in uninjured rats. SNHG1 induced NP by directly regulating CDK4 level.

CONCLUSIONS: SNHG1 is a novel target in the treatment of NP associated with neuroinflammation.

Key Words:
Neuropathic pain, LncRNA SNHG1, Spinal cord injury, CDK4.

Introduction

Spinal cord injury (SCI) is a serious disabling disease with a high mortality, which is often accompanied by severe neurological impairment and even leads to permanent motor dysfunction. Currently, great efforts have been made to improve clinical outcomes of SCI, including rehabilitation training for secondary SCI. Nevertheless, neuronal apoptosis following acute neurological injury is unavoidable and hardly to be cured[1,2]. NP is one of the most severe complications of SCI, manifesting as various forms of chronic pain below the injured level[3]. The incidence of NP at post-SCI has increased each year[4]. Clinical therapeutic efficacy of NP is unsatisfactory, which greatly restricts life quality of affected people.

The release of activated astrocytes and microglial inflammatory cytokines is closely related to the development of NP[5]. It is reported that inactivation of astrocytes and microglia can suppress neuroinflammation and improve pain-associated behaviors[6]. Nociception is a key component of host defense. However, persistent nociception can activate the inflammatory response and induce functional changes in nerve cells[7]. SCI is able to activate nociceptor-mediated host defense responses through neuroinflammation signals, thereafter, resulting in chronic pain[8].

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Long non-coding RNAs (lncRNAs) are non-coding RNAs longer than 200 nucleotides. They are vital in regulating gene expressions and information transmission. LncRNAs may be more powerful than proteins in disease diagnosis and treatment, displaying higher sensitivity and specificity[9]. Wan et al[10] have shown that lncRNAs are extensively involved in neurological diseases. Notably, overexpression of lncRNA MEG3 remarkably inhibits neovascularization after injury[11]. LncRNA Ptprj-as1 facilitates the release of inflammatory factors through activating the NF-κB pathway in microglia[12]. Inactivation of LncOL1 leads to axonal myelin formation disorders and remyelination defects after nerve injury[13]. Also, lncRNAs are also related to the development of NP[14].

LncRNA SNHG1 locates on human chromosome 11, which contains 8 small nucleolar RNAs. It is abnormally expressed in many types of diseases[15]. The role of SNHG1 in NP, however, remains largely unknown. In this paper, we performed SNL in rats to establish an *in vivo* NP model. Potential functions of SNHG1 in the development of NP were mainly explored, and our findings may provide a new target in clinical treatment of SCI-induced NP.

Materials and Methods

Establishment of NP Model in Rats

Male SD rats were provided by Luoyang Orthopedic Hospital of Henan Province Experimental Animal Center. Animal procedures were approved by Luoyang Orthopedic Hospital of Henan Province Ethics Committee. As previously described[16], NP model in rats was constructed by performing SNL. Briefly, rats in SNL group (n=8) were anesthetized by 1% pentobarbital sodium (40 mg/kg i.p.). The fifth lumbar spinal nerve (L5) was isolated and ligated using 6-0 suture. Rats in control group (n=8) were similarly treated without ligating the fifth lumbar spinal nerve. PWT and PWL were assessed to verify the success of the established NP model.

Lentivirus Transfection

Lentiviruses used in this study were provided by Genechem (Shanghai, China). On the 3rd day postoperatively, intrathecal injection of sh-SNHG1 or LV-CDK6 (MOI=100) was performed in rats of SNL group. On the 5th day postoperatively, intrathecal injection of LV-SNHG1 (MOI=100) was performed. Transfection efficacy was tested after rat sacrifice.

NP Assessment

Mechanical sensitivity was assessed by determining PWT. Each rat was placed in a transparent plastic box (22 × 12 × 22 cm) with a metal mesh bottom. The calibrated von Frey filaments (IITC, Woodland Hills, CA, USA) were used to load pressure on the plantar surface of rat hind paw. The filament diameter was recorded once rat paws were pulled out. PWL was recorded to evaluate thermohyperalgesia. The duration from the beginning of heat stimulation to the elevation of the hind paws was recorded. PWL was recorded with a 5 min interval, and each assessment lasted within 30 s.

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

Total RNAs in cells and tissues were extracted using TRIzol (Invitrogen, Carlsbad, CA, USA). A 20 μL qRT-PCR system was prepared, including 10 μL of SYBR GreenMaster Mix, 1 μL of forward primer, 1 μL of reverse primer, 0.4 μL of 50×Rox Dye, 2 μL of complementary deoxyribose nucleic acid (cDNA) and 5.6 μL of diethyl pyrocarbonate (DEPC) water (Beyotime, Shanghai, China). The relative gene expression was calculated using $2^{-\Delta\Delta Ct}$ method. CDK4: Forward 5'-ATGGCTACCTCTCGATATGAGC-3', Reverse 5'-CATTGGGGACTCTCACACTCT-3'; SNHG1: Forward 5'-ACGTTGGAACCGAAGAGAGC-3', Reverse 5'-GCAGCTGAATTCCCCAGGAT-3'; IL-6: Forward 5'-CTCTGGCTTTGTCTTTCTTGTTATCTTT-3', Reverse 5'-AGTTGTGCAATGGCAATTCTGA-3'; IL-1β: Forward 5'-GAAATGCCACCTTTTGACAGTG-3', Reverse 5'-TGGATGCTCTCATCAGGACAG-3'; TNF-α: Forward 5'-AGGCGGTGCCTATGTCTCAG-3', Reverse 5'-GCTCCTCCACTTGGTGGTTT-3'; glyceraldehyde 3-phosphate dehydrogenase (GAPDH): Forward 5'-TCAAGATCATCAGCAATGCC-3', Reverse 5'-CGATACCAAAGTTGTCATGGA-3'.

Cell Culture

PC12 cells were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), 100 U/mL penicillin and 100 μg/mL streptomycin in a 5% CO_2 incubator at 37°C.

Cell Transfection

Cells were cultured to 70-80% confluence and transfected with lentiviruses in DMEM containing 5 mg/mL polybrene (Sigma-Aldrich, St. Louis, MO, USA) for 48 h.

Luciferase Assay

Wild-type and mutant-type CDK4 vectors were constructed using the Fusion™ kit. PC12 cells were co-transfected with control/LV-SNHG1 and wild-type CDK4/mutant-type CDK4 vector, respectively. Cells were lysed for determining relative Luciferase activity 48 h later (Promega, Madison, WI, USA).

ELISA (Enzyme Linked Immunosorbent Assay)

Rat L5 segments were collected and lysed. Relative levels of IL-1β, IL-6 and TNF-α in tissue lysate were determined using the commercial ELISA kit (Cw Biotech, Beijing, China).

Determination of Subcellular Distribution

Cytoplasmic and nuclear RNAs were extracted using the PARIS kit (Invitrogen, Carlsbad, CA, USA) and subjected to qRT-PCR. U6 was the internal reference of nucleus and GAPDH was that of cytoplasm.

RIP (RNA-Binding Protein Immunoprecipitation) Assay

RIP assay was performed following the procedures of Millipore Magna RIP Kit (Millipore, Billerica, MA, USA). Cells were incubated with anti-Ago2 or anti-IgG at 4°C overnight. A protein-RNA complex was obtained after capturing intracellular specific proteins by the antibody. Subsequently, proteins were digested by proteinase K and the RNAs were extracted. During the experiment, the magnetic beads were repeatedly washed with RIP washing buffer to remove non-specific adsorption as much as possible. The immunoprecipitant RNAs were finally quantified by qRT-PCR.

Western Blot

Cells were lysed for isolating cellular protein and electrophoresed. Protein samples were loaded on polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Subsequently, non-specific antigens were blocked in 5% skim milk for 2 hours. Membranes were reacted with primary and secondary antibodies for indicated time. Band exposure and analyses were finally conducted.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 19.0 (IBM Corp., Armonk, NY, USA) was used for data analyses. Data were expressed as mean ± standard deviation. Differences between two groups were analyzed by the *t*-test. $p<0.05$ was considered as statistically significant.

Results

SNHG1 was Upregulated in Rats Undergoing SNL

After establishment of NP model in rats, we detected PWL and PWT in each rat. Decreased levels of PWL (Figure 1A) and PWT (Figure 1B) in SNL group verified the success of the established animal model. In addition, SNHG1 level was time-dependently upregulated in SNL group than that of control group (Figure 1C).

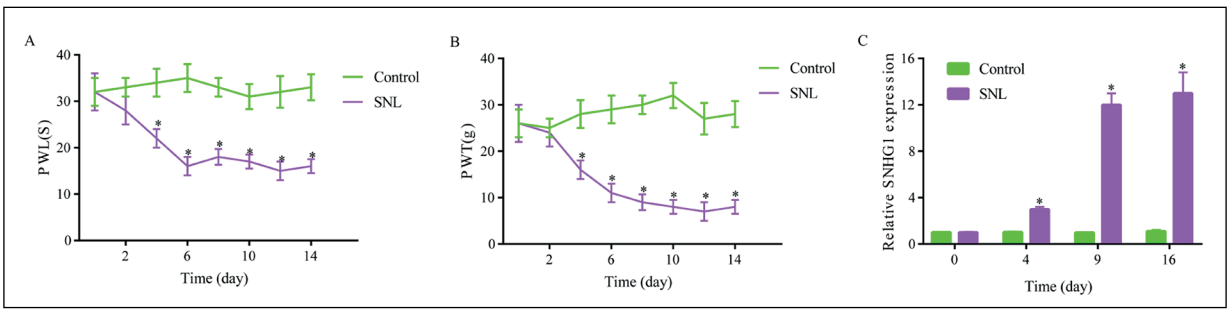

Figure 1. SNHG1 was upregulated in rats undergoing SNL. **A, B,** PWL (**A**) and PWT (**B**) in rats of control group (n=8) and SNL group (n=8). **C,** SNHG1 expression in rats of control group and SNL group at day 0, 4, 9 and 16.PWL: paw withdrawal latency; PWT: paw withdrawal threshold; SNL: spinal nerve ligation. *$p<0.05$.

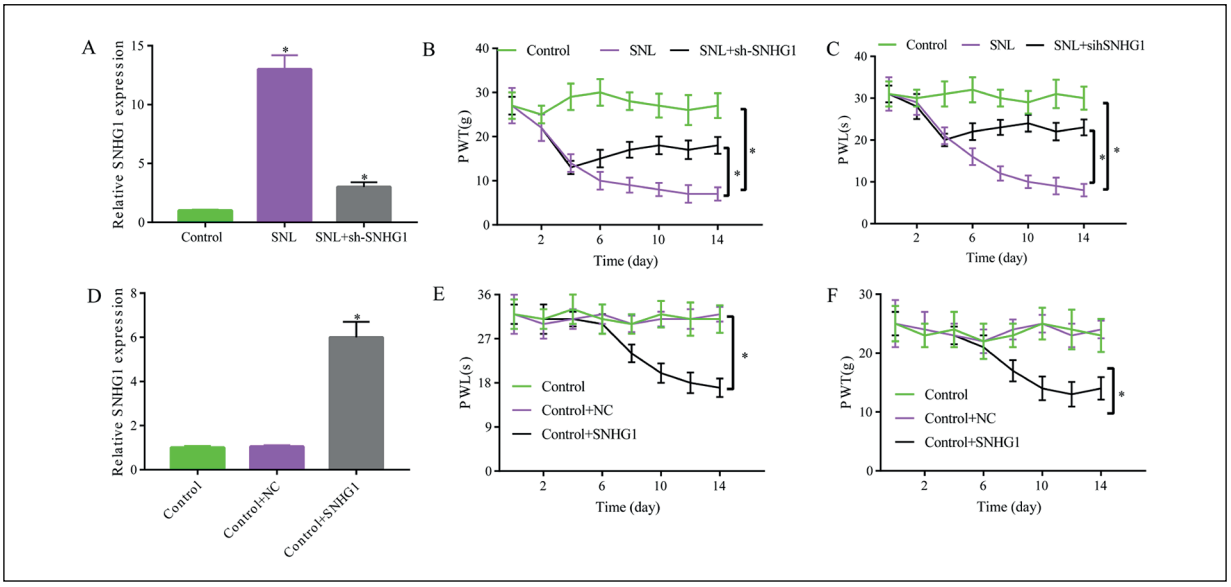

Figure 2. SNHG1 induced NP in rats undergoing SNL. **A,** SNHG1 expression in rats of control group, SNL group and those in SNL group administrated with sh-SNHG1. **B, C,** PWT (**B**) and PWL (**C**) in rats of the three groups. **D,** SNHG1 expression in control rats, or those administrated with NC or LV-SNHG1. **E, F,** PWT (**E**) and PWL (**F**) in rats of the three groups. **p*<0.05.

SNHG1 Induced NP in Rats Undergoing SNL

Rats administrated with sh-SNHG1 showed markedly lower level of SNHG1 than those administrated with sh-NC (Figure 2A). *In vivo* knockdown of SNHG1 in rats undergoing SNL increased PWT (Figure 2B) and PWL (Figure 2C). Subsequently, transfection efficacy of LV-SNHG1 in rats was tested (Figure 2D). Of note, overexpression of SNHG1 did induce NP in uninjured rats (Figure 2E, 2F). It is demonstrated that SNHG1 displayed a vital role in the development of NP.

SNHG1 Activated Neuroinflammation

The development of NP is closely linked to astrocyte activation and release of microglial inflammatory cytokines. Here, the marker of astrocyte activation, GFAP, was upregulated in SNL group. Protein level of GFAP was downregulated by silence of SNHG1 (Figure 3A, 3B). Moreover, lower levels of IL-6, IL-β and TNF-α were seen in rats undergoing SNL who were administrated with sh-SNHG1 than those administrated with sh-NC (Figure 3C). ELISA results showed similar trends (Figure 3D). We believed that SNHG1 was capable of influencing NP through regulating neuroinflammation.

SNHG1 Directly Targeted CDK4

The CDK family is a vital regulator during SCI. Here, we detected CDK4 level in PC12 cells transfected with control or LV-SNHG1. Both mRNA and protein levels of CDK4 were upregulated in PC12 cells overexpressing SNHG1 (Figure 4A, 4B). Luciferase assay indicated that SNHG1 markedly facilitated the activity in CDK4 promoter (Figure 4C). SNHG1 was identified to be mainly distributed in the cytoplasm (Figure 4D). Furthermore, RIP assay verified the close interaction between SNHG1 and CDK4 (Figure 4E). Collectively, SNHG1 was identified to bind CDK4 and thus regulated its biological functions.

SNHG1 Regulated the Development of NP by Targeting CDK4

Based on the above findings, we speculated that CDK4 was involved in SNHG1-regulated NP development. Increased PWT (Figure 5A) and PWL (Figure 5B) in rats undergoing SNL and administrated with sh-SNHG1 were partially abolished by overexpression of CDK4. Similarly, overexpression of CDK4 reversed the enhanced levels of inflammatory factors in injured rats with SNHG1 knockdown (Figure 5C, 5D).

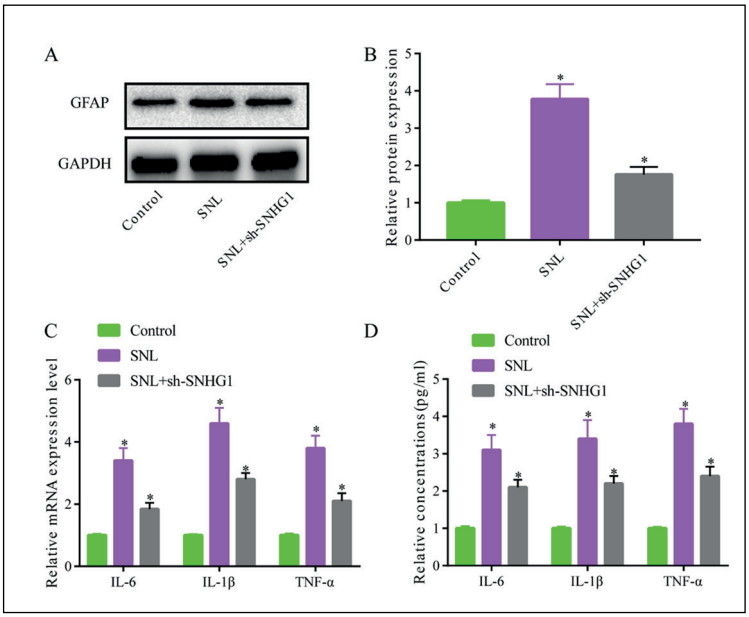

Figure 3. SNHG1 activated neuroinflammation. **A, B,** Protein level of GFAP in rats of control group, SNL group and those in SNL group administrated with sh-SNHG1. **C,** Relative mRNA levels of IL-6, IL-1β and TNF-α in rats of control group, SNL group and those in SNL group administrated with sh-SNHG1. **D,** Protein levels of IL-6, IL-1β and TNF-α in rats of control group, SNL group and those in SNL group administrated with sh-SNHG1 detected by ELISA. *p<0.05.

Discussion

NP is a severe public health problem and its pathology remains largely unknown. Peripheral inflammation and nerve damage result in expression changes of genes associated with pain cascade signaling, including lncRNAs[17]. Abundant abnormally expressed lncRNAs have been discovered in dorsal root ganglia and spinal cord[18]. Dou et al[19] reported that lncRNA CCAT1 is downregulated in spinal tissues of patients with chronic sciatica, and overexpression of CCAT1 greatly relieves the pain. Our findings uncovered that SNHG1 was highly expressed in rats undergoing SNL. SNHG1 level was time-dependently upregulated in rats with NP. By measuring PWT and PWL, we found that knockdown of SNHG1 could alleviate NP.

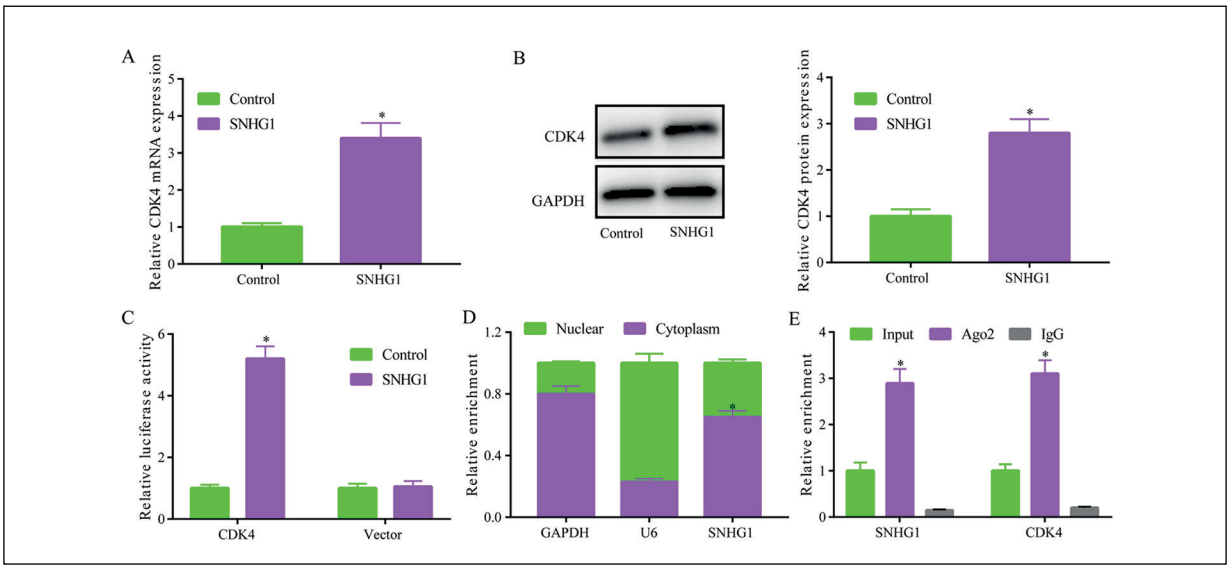

Figure 4.SNHG1 directly targeted CDK4. **A, B,** Relative mRNA level (**A**) and protein level of CDK4 (**B**) in PC12 cells transfected with control or LV-SNHG1. **C,** Luciferase activity in PC12 cells co-transfected with control/LV-SNHG1 and wild-type/mutant-type CDK4. **D,** Subcellular distribution of SNHG1. GAPDH and U6 were internal references of cytoplasm and nuclei, respectively. **E,** RIP showed enrichments of SNHG1 and CDK4 in anti-Ago2 and anti-IgG. *p<0.05.

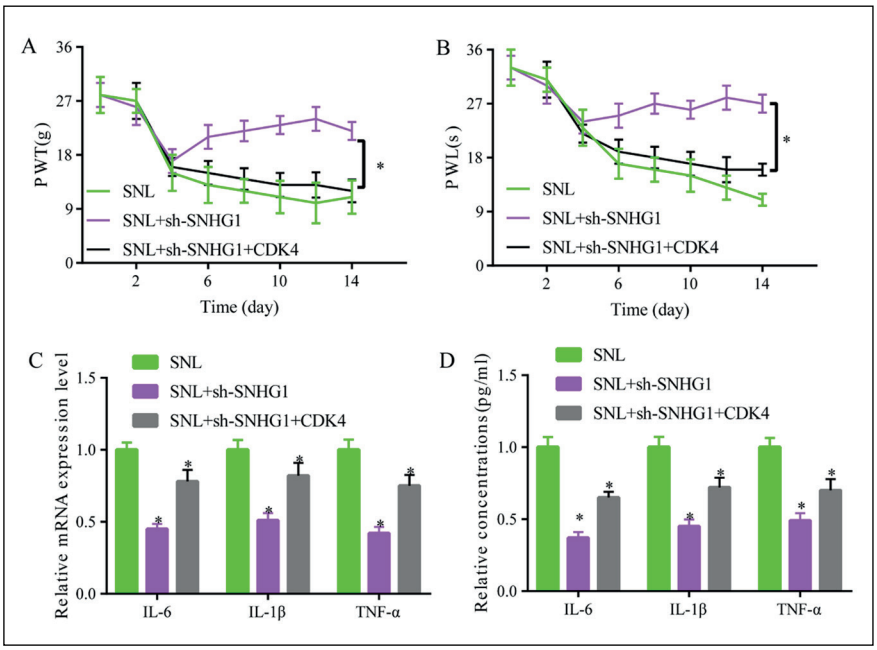

Figure 5. SNHG1 regulated the development of NP by targeting CDK4. **A, B,** PWT (**A**) and PWL (**B**) in rats with NP influenced by SNHG1 and CDK4. **C,** Relative mRNA levels of IL-6, IL-1β and TNF-α in rats with NP influenced by SNHG1 and CDK4. **D,** Protein levels of IL-6, IL-1β and TNF-α in rats with NP influenced by SNHG1 and CDK4. *p<0.05.

Notably, overexpression of SNHG1 even induced NP in uninjured rats. Relative levels of inflammatory factors were found to be positively regulated by SNHG1, suggesting that SNHG1 may influence the development of NP by regulating neuroinflammation.

Through bioinformatics prediction, CDK4 was considered to be the downstream gene binding SNHG1. The following Luciferase assay verified that SNHG1 was able to bind the promoter region of CDK4 and stimulated its expression. CDKs participate in life activities by regulating cell cycle progression and relative expressions of cell cycle genes[20]. CDKs stimulate expressions of pro-inflammatory factors at the transcriptional level in G1 phase. Recently, Gwak and Hulsebosch[21] showed that deficiency of cell cycle proteins is associated with pain behaviors. As a member of the CDKs family, CDK4 contributes to tumor cell survival *via* regulating lysosomal functions and activating mTORCh1 activity[22]. In chordoma, the upregulated CDK4 serves as a promising hallmark[23]. Overexpression of CDK4 induces apoptosis cascade in PC12 cells[24].

Conclusions

In this study, CDK4 was able to abolish the regulatory effects of SNHG1 on pain behaviors and inflammatory factor release in rats with NP. SNHG1, as a novel hallmark of SCI-induced NP, may be utilized in the clinical intervention of NP.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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