Int J Clin Exp Med 2016;9(2):2542-2548
www.ijcem.com /ISSN:1940-5901/IJCEM0018148

Original Article

Intrathecal siRNA against Ski-interacting protein attenuates nociception in a rat model of neuropathic pain

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Received October 19, 2015; Accepted January 10, 2016; Epub February 15, 2016; Published February 29, 2016

Abstract: Neuropathic pain is characterized by hyperalgesia, allodynia and spontaneous pain. Previous studies have shown that Ski-interacting protein (SKIP) played an important role in neuronal survival and neuroprotection. However, its expression and function in neuropathic pain remains unknown. We found that the level of SKIP was increased in the spinal cord in a time dependent manner after chronic constriction injury (CCI). Intrathecal injection of siRNA-SKIP inhibited the expression of SKIP and pro-inflammatory factors (TNF-α, IL-1β and IL-6), and alleviated mechanical allodynia and thermal hyperalgesia in CCI model of rats. In addition, siRNA-SKIP markedly suppressed the upregulation of β-catenin induced by the neuropathic pain. Taken together, our findings suggest that siRNA against SKIP can alleviate the chronic neuropathic pain through Wnt/β-catenin signaling pathway. Targeting SKIP may be a potential treatment for chronic neuropathic pain.

Keywords: Ski-interacting protein (SKIP), neuropathic pain, Wnt/β-catenin signaling pathway

Introduction

Neuropathic pain is defined as the pain caused by a series of lesions or diseases affecting the somatosensory system [1]. It has become a remarkable public health problem affecting approximate 10%-40% of the general population [2]. Although numerous treatment options are available for relieving neuropathic pain, the effective treatment of patients suffering from neuropathic pain remains a prevalent and persistent clinical challenge [3, 4]. Therefore, there is still a considerable need to explore novel treatment modalities for neuropathic pain management.

Ski-interacting protein (SKIP), an evolutionally conserved 60-80-kDa nuclear protein, was initially identified as a binding partner of the viral oncogene v-Ski [5]. It has been reported that SKIP interacts with Smad 2, 3 to enhance transforming growth factor β (TGFβ)-dependent transcription [6], and interacts with poly(A)-binding protein 2 to synergistically activate E-box-mediated transcription through MyoD [7]. SKIP is also considered as an retinoblastoma protein (Rb) binding protein that inhibits the Rb growth-suppressing function and induces cell cycle activation [8]. In addition, SKIP functions as an oncogene in various tumors [9-11]. For example, Liu et al. reported that high SKIP expression was detected in clinical hepatocellular carcinoma (HCC) samples, and SKIP depletion by small interfering RNA inhibited cell proliferation and blocked S phase entry in HepG2 cells [12]. Recent study implied that SKIP was significantly up-regulated in the adult rat brain cortex, may play an important role in regulating neuronal apoptosis after traumatic brain injury [13]. However, under neuropathic pain conditions, the role of SKIP is still unknown. In this report, we investigated the expression and role of SKIP in the rat model of chronic constriction injury (CCI)-induced neuropathic pain.

Materials and methods

Experimental animals

Male Sprague-Dawley rats (180 ± 20 g) were obtained from the Animal Experimental Center, Linyi People's Hospital, China. They were housed at 20-24°C under 12 h light-dark cycles and were allowed free access to water and commercial pellet. The protocol and procedure of the experiment were approved by the Institutional Animal Care and Use Committee of Linyi People's Hospital.

CCI-induced model of neuropathic pain

Neuropathic pain was induced in experimental animals by CCI of the sciatic nerve which was performed as previously described [14]. Briefly, mice were anesthetized with sodium pentobarbital. Four ligatures (silk 4-0) were tied loosely around proximal bifurcation part of the nerve with 1 mm spacing each ligature until a brisk twitch of the right hind limb was observed, respectively. In sham groups, an identical surgical procedure was performed, except that the sciatic nerve was not ligated.

SiRNA knockdown of SKIP expression

The siRNA against SKIP was commercially synthesized (Genepharma, Shanghai, China). For controls, negative control siRNA is an irrelevant siRNA with random nucleotides and no known specificity. Then, intrathecal injection of siRNA-SKIP (0.2 nmol, 20 μL), spinal cord puncture was made with a 30-gauge needle between the L5 and L6 levels to deliver the reagents to the cerebral spinal fluid [15]. We then closed the incision with silk sutures.

Evaluation of mechanical allodynia and thermal hyperalgesia

Mechanical allodynia was assessed as described by Chaplan et al. [16]. Calibrated nylon filaments (Von Frey hairs), in terms of different bending forces, were applied to the midplantar surface of left hind paw. The filaments were applied 10 times, starting with the softest and continuing in ascending order of stiffness. A brisk withdrawal of the left hind limb was considered a positive response. The criterion for the threshold value, in grams, was equal to the filament evoking a withdrawal threshold of the left hind paw five times out of 10 trials, that is, a 50% response.

Thermal hyperalgesia was tested according to the method as previously described [17]. After acclimation, the heat source was positioned under the glass floor directly beneath the hind paw. The intensity of the thermal stimulus was adjusted to achieve an average baseline paw withdrawal latency of approximately 9-11 s. A digital timer automatically recorded the duration between the start of stimuli and the paw withdrawal thermal latency (PWTL). Each paw was measured alternatively after more than 5 min. The cut-off was set at 20 seconds to avoid tissue damage.

Real-time quantitative PCR (RT-qPCR)

The total RNA of the spinal cord was extracted using Trizol reagent (Invitrogen, Carlsbad, CA). Two μg of total RNA was reverse transcribed using an oligo (dT) primer according to the manufacturer's protocol (Takara, Shiga, Japan). qPCR analysis was performed in the Real-time Detection System (Rotor-Gene 6000, Hamburg, Germany) by SYBR green I dye detection (Takara). The following primers were used: SKIP forward, 5'-CGGAATTCCGATGGCGCTCACCAGCA-3'; SKIP reverse, 5'-CGCGGATCCTTCCTTCCTCCTCTTT-3'; GAPDH forward, 5'-TGTTCCTACCCCCAATGTG-3'; GAPDH reverse, 5'-GTGTAGCCCAAGATGCCCT-3'. The PCR amplifications were performed at 95°C for 30 s, followed by 35 cycles of thermal cycling at 95°C for 5 s and 60°C for 45 s. GAPDH was used as endogenous control to normalize differences for mRNA detection. Melt curves were performed on completion of the cycles to ensure that nonspecific products were absent.

Western blot analysis

At the 7th day, rats were sacrificed by spinal dislocation. Then, the spinal cord (L4/5) was rapidly removed. Total proteins of the spinal cord were extracted by using protein extraction kits following the manufacturer's instructions. Protein concentrations of the cell supernatants were measured by BCA Protein Assay kit. Equal amounts of protein (30 μg) were separated by SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Whatman Schleicher & Schuell, Middlesex, UK). The membranes were incubated with rabbit polyclonal antibody

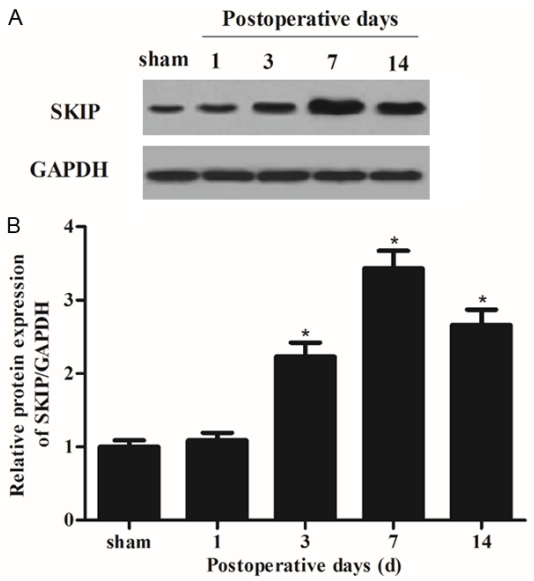

Figure 1. CCI induced SKIP protein upregulation in the spinal cord. (A) Western blot results showed that SKIP protein expression was increased in the spinal cord at 3 days and peaked at 7 days after CCI compared to naïve. (B) Quantification of (A). Data were presented as means ± SD, N = 5, *P < 0.05 versus sham.

against SKIP, β-catenin, glycogen synthase kinase-3β (GSK-3β) and GAPDH at 4°C overnight and washed with PBST containing 20% Tween-20. The membranes were then incubated with the secondary antibodies goat of anti-rabbit at room temperature for 1 h. After washing with TBST buffer, immunoreactivity was detected with enhanced chemiluminescence (ECL) and quantified by the Quantity ONE (Bio-Rad, Hercules, CA, USA) software.

Enzyme linked immunosorbent assay (ELISA)

At the 7th day, rats were sacrificed by spinal dislocation. Then, the spinal cord (L4/5) was rapidly removed and homogenized in lysis buffer. After centrifugation, the supernatants were measured by the TNF-α, IL-1β and IL-6 ELISA kits (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Statistical analysis

All data were expressed as mean ± SEM. The behavioral data were compared using the non-parametrical Friedman test, followed by individual comparisons between groups using the Mann-Whitney U-test. The data from the biochemical tests and Western blot were analyzed with one-way ANOVA followed by LSD post hoc test. Values of $P < 0.05$ were considered as statistically significant.

Results

CCI increased SKIP expression in the spinal cord of CCI rats

To explore the function of SKIP in neuropathic pain, we evaluated SKIP protein expression in the spinal cord from 1 to 14 days after CCI surgery by Western blot. The results showed that compared to naïve group, the expression of SKIP protein was significantly increased at 3 days, maximal at 7 days, and declined at 14 days after CCI operation (**Figure 1**).

Intrathecal injection of siRNA-SKIP alleviated mechanical allodynia and thermal hyperalgesia in CCI model of rats

Then, we determined the SKIP expression after intrathecal injection of siRNA-SKIP. The results demonstrated that siRNA-SKIP significantly inhibited CCI-induced SKIP mRNA and protein expression in the rat spinal cord tissues, as compared with nonspecific siRNA (**Figure 2A**). In addition, the PWT and PWL were tested. Compared with the non-specific siRNA, intrathecal injection of siRNA-SKIP significantly attenuated CCI-induced PWT decline after CCI operation (**Figure 2B**). Similarly, injection of siRNA-SKIP significantly raised PWL following CCI operation (**Figure 2C**).

Intrathecal injection of siRNA-SKIP inhibited the levels of pro-inflammatory factors in CCI model of rats

The effect of siRNA-SKIP on pro-inflammatory factors expression was evaluated using ELISA kits. As shown in **Figure 3**, CCI significantly elevated the levels of TNF-α, IL-1β and IL-6 in the rat spinal cord tissues on day 7 as compared to that in sham control rats. Whereas, intrathecal injection of siRNA-SKIP significantly CCI-induced increase in the levels of TNF-α, IL-1β and IL-6.

SiRNA-SKIP reduces β-catenin expression in spinal cord after CCI

Wnt/β-catenin signaling played a role in the development of neuropathic pain. So, to investigate the mechanism of SKIP in CCI-induced

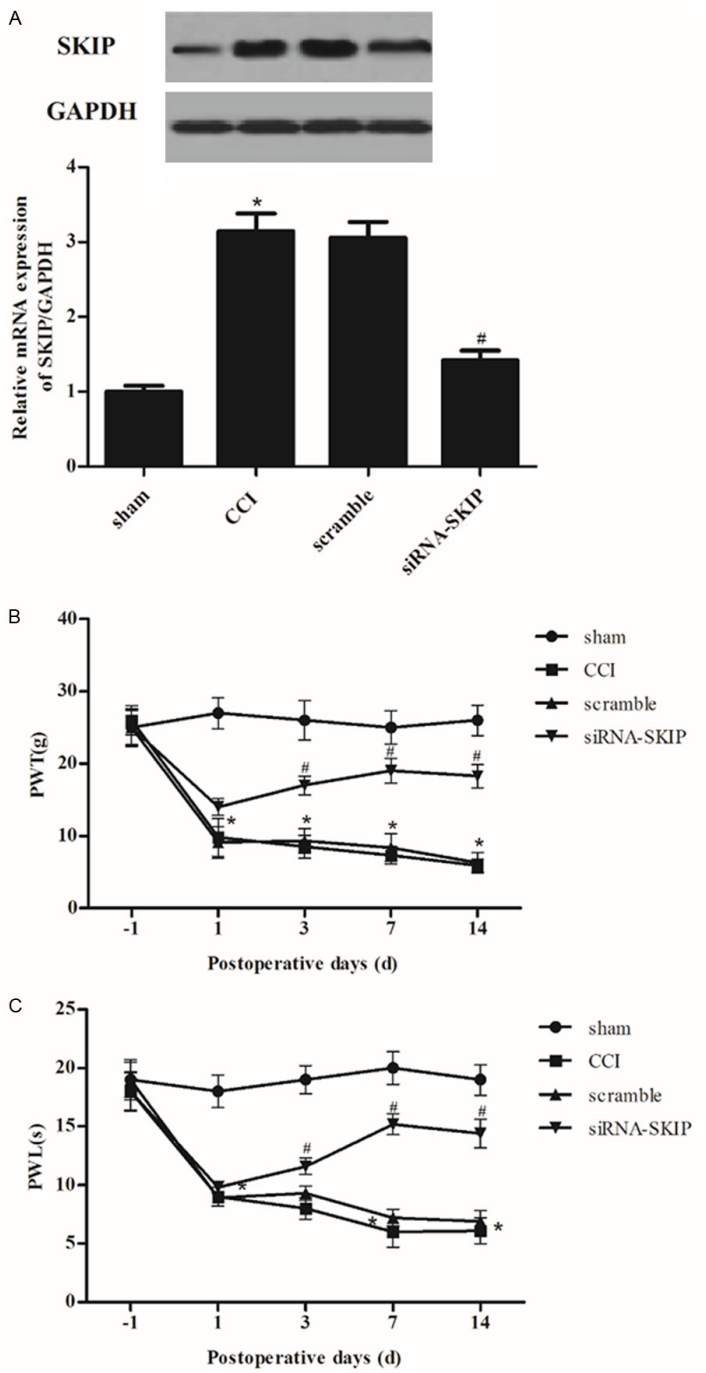

Figure 2. Intrathecal injection of siRNA-SKIP attenuated CCI-induced pain hypersensitivity. At the 7th day, rats were sacrificed by spinal dislocation. Then, the spinal cord (L4/5) was rapidly removed. (A) SiRNA-SKIP decreased SKIP mRNA and protein expression in the spinal cord. Intrathecal injection of siRNA-SKIP attenuated mechanical allodynia (B) and heat hyperalgesia (C) after CCI. Data were presented as means ± SD, N = 5, *P < 0.05 versus sham, #P < 0.05 versus CCI.

neuropathic pain, we evaluated the expression of β-catenin and GSK-3β in spinal cord of CCI rats. Western blot analysis showed that CCI significantly increased the expression of β-catenin and GSK-3β. However, knockdown of SKIP decreased the CCI-induced β-catenin and GSK-3β expression in spinal cord of CCI rats, as compared with the non-specific siRNA (**Figure 4**).

Discussion

The present study demonstrated the following novel findings: (1) CCI increased SKIP expression in the spinal cord of CCI rats. (2) Intrathecal injection of siRNA-SKIP alleviated mechanical allodynia and thermal hyperalgesia in CCI model of rats. (3) Intrathecal injection of siRNA-SKIP inhibited the levels of pro-inflammatory factors in CCI model of rats. (4) siRNA-SKIP reduced β-catenin expression in spinal cord after CCI.

It has been reported that up-regulation of SKIP was present in retina at 5 days after optic nerve crush (ONC) model [18]. Another study showed that the expression of SKIP was significantly up-regulated in rat brain cortex after traumatic brain injury [13]. Similarly, in the present study, we found that CCI increased SKIP expression in the spinal cord of CCI rats. These results suggest that SKIP may play an important role in the development of neuropathic pain.

It has been suggested that inflammatory process is strongly associated with neuropathic pain. Pro-inflammatory cytokines including TNF-α, IL-1β and IL-6 have been increasingly in the development of pain after nerve lesion [19-22]. For example, one study showed that the changes of mechanical and thermal pain thresholds and spinal TNF-α and IL-1β mRNA expression were isochronous, and inhibition on CCI-induced up-regulation of TNF-β mRNA and IL-1α mRNA expression might reduce mechanical allodynia and thermal hyperalgesia in neuropathic pain rats [23]. Herein, we found that CCI significantly elevated

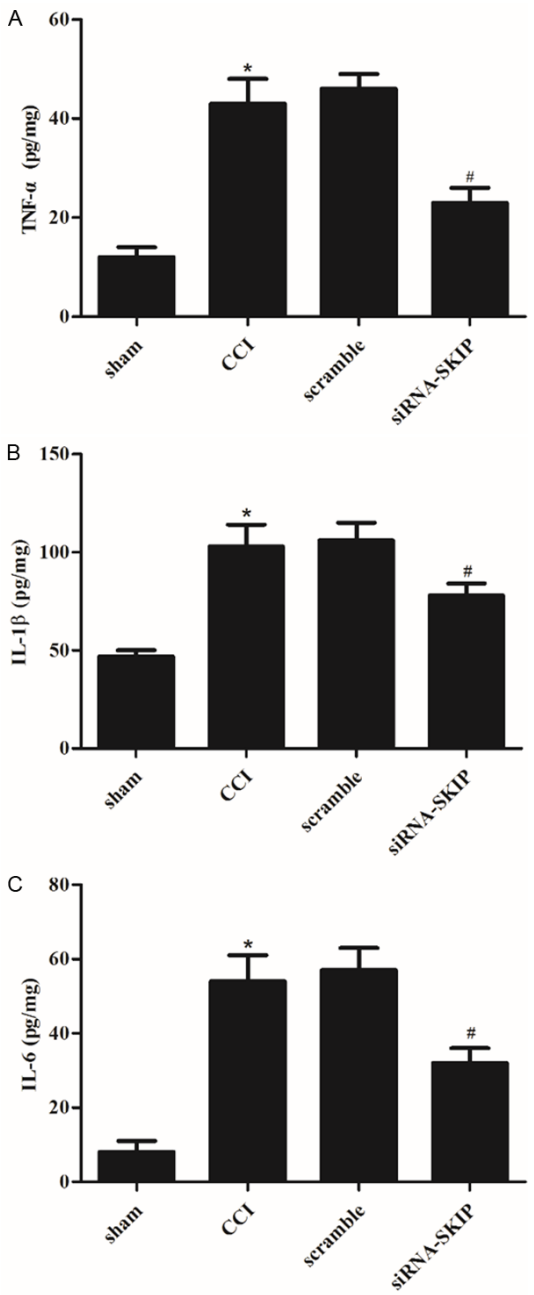

Figure 3. Intrathecal injection of siRNA-SKIP attenuated CCI-induced TNF-α, IL-1β and IL-6 expression in the spinal cord of CCI rats. At the 7th day, rats were sacrificed by spinal dislocation. Then, the spinal cord (L4/5) was rapidly removed and homogenized in lysis buffer. After centrifugation, the supernatants were measured by the TNF-α, IL-1β and IL-6 ELISA kits. Data were presented as means ± SD, N = 5, *P < 0.05 versus sham, #P < 0.05 versus CCI.

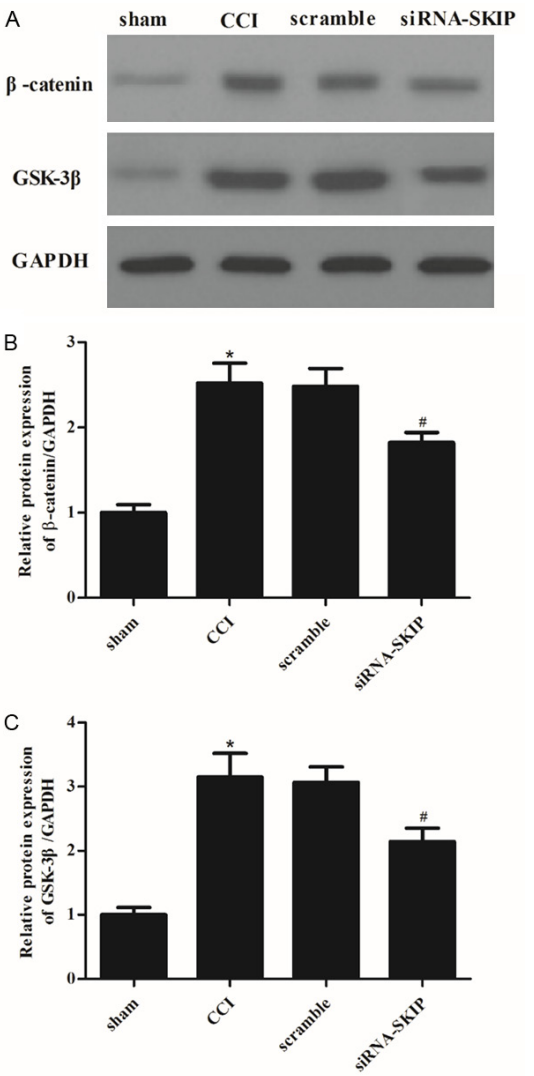

Figure 4. Intrathecal injection of siRNA-SKIP decreased the activation of β-catenin induced by CCI. At the 7th day, rats were sacrificed by spinal dislocation. Then, the spinal cord (L4/5) was rapidly removed. A showed the expression levels of β-catenin and GSK-3β target proteins in the spinal cord of neuropathic pain rats by using Western blot analysis. (B and C) Quantification of (A). Data were presented as means ± SD, N = 3, *P < 0.05 versus sham, #P < 0.05 versus CCI.

the levels of TNF-α, IL-1β and IL-6 in the rat spinal cord tissues. Whereas, intrathecal injection of siRNA-SKIP significantly CCI-induced increase in the levels of TNF-α, IL-1β and IL-6. We conclude that the analgesic effect of siRNA-SKIP might be mediated, at least partly, through the inhibition of TNF-α, IL-1β and IL-6 expression.

Emerging studies have indicated that the Wnt/β-catenin signaling pathway was involved in the development of inflammatory response and neuropathic pain [24-26]. Previous studies demonstrated that active β-catenin was upreg-

ulated in the dorsal horn in different neuropathic pain models, including partial sciatic nerve ligation (PSNL) model, spinal nerve ligation (SNL) model and CCI model. Furthermore, GSK3β regulates the signaling pathways of pro-inflammatory cytokine TNF-α and IL-1β [27]. It was reported that intraperitoneal treatment with a specific inhibitor of glycogen synthase kinase 3, AR-A014418, significantly inhibited mechanical hyperalgesia induced by PSNL, it also significantly prevented the increase of TNF-α and IL-1β in the spinal cord [28]. In line with these results, herein, we found that CCI significantly increased the expression of β-catenin and GSK-3β in spinal cord of CCI rats. Interestingly, one study showed that knockdown of endogenous SKIP caused down-regulation of Wnt/β-catenin signaling in *Xenopus* embryos [29], suggesting that SKIP is a necessary component of the Wnt/β-catenin pathway. In this study, we found that knockdown of SKIP decreased the CCI-induced β-catenin and GSK-3β expression. These results suggest that SKIP could mediate the maintenance of CCI-induced neuropathic pain through Wnt/β-catenin signaling pathway.

In conclusion, our results demonstrate that siRNA-SKIP may attenuate neuropathic pain partially through suppressing the Wnt/β-catenin signaling pathway in spinal cord. Thus, targeting SKIP might represent a new strategy for treating neuropathic pain.

Disclosure of conflict of interest

None.

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