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Original Article

MiR-19a targets suppressor of cytokine signaling 1 to modulate the progression of neuropathic pain

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Abstract: Purpose: we aimed to investigate whether miR-19a is associated with neuropathic pain and elucidate the underlying regulatory mechanism. Methods: We established a neuropathic pain model of bilateral chronic constriction injury (bCCI). Then bCCI rats were injected with mo-miR-19a, siR-SOCS1 or blank expression vector through a microinjection syringe via an intrathecal catheter on 3 day before surgery and after surgery. Behavioral tests, such as mechanical allodynia, thermal hyperalgesia and acetone induced cold allodynia, were performed to evaluate the pain threshold. Besides, quantitative real-time polymerase chain reaction (qRT-PCR) was performed to determine the expression of miR-19a and western blotting was carried out to measure the expression of SOCS1. Results: miR-19a expression levels were markedly increased in neuropathic pain models. Moreover, miR-19a significantly attenuated mechanical allodynia and thermal hyperalgesia, and similar results were obtained after knockdown of SOCS1 expression. However, miR-19a markedly increased the times that the rats appeared a sign of cold allodynia, and knockdown of SOCS1 expression had similar effects. Besides, the results of bioinformatics analysis and western blotting analysis were all confirmed that SOCS1 was a direct target of miR-19a in neuropathic pain models. Conclusions: Our finding indicate that SOCS1 is a direct target of miR-19a in neuropathic pain rats and miR-19a may play a critical role in regulating of neuropathic pain via targeting SOCS1.

Keywords: Neuropathic pain, miR-19a, suppressor of cytokine signaling 1, mechanical allodynia, thermal hyperalgesia, cold allodynia

Introduction

Neuropathic pain is considered as a direct consequence caused by a lesion or disease affecting the somatosensory system [1]. It is generally chronic and disabling, and treatment of it remains a great challenge due to the specificities of its complex mechanism [2, 3]. Considerable advances have been made [4], however, the molecular mechanism underlying neuropathic pain remain elusive, thereby hampering the development of effective therapeutics.

Recently, several reports have demonstrated that there is strong correlation between neuroinflammation (caused by chemokine and cytokine) and neuropathic pain [5, 6]. Jams kinase/signal transducers and activators of transcription 3 (JAK/STAT3) pathway which is activated by cytokines is shown to be crucial for regulating astrocyte proliferation and maintenance of tactile allodynia in neuropathic pain [7]. Suppressor of cytokine signaling 1 (SOCS1) is a key regulator of cytokine signals that can negatively regulate the feedback of inflammation [8]. Moreover, SOCS1 is an intrinsic JAK tyrosine kinase inhibitor and has been shown to suppress the cytokine-induced JAK-STAT3 pathway [9, 10]. Epigenetic modification of SOCS1 is also reported to negative regulate the activation of JAK-STAT3 in response to interleukin-6 receptor and epidermal growth factor receptor signaling in head and neck squamous cell carcinomas [11]. Despite these, the significance and regulatory mechanism of SOCS1 during the development of neuropathic pain are largely unknown.

miRNAs are small non-coding RNAs that can regulate expression of protein-coding genes posttranscriptionally and intracellular pathways of numerous inflammatory mediators [12, 13]. Since microRNAs (miRNAs) has been emerged

as a key player in numerous pathophysiological processes, a contribution of miRNAs to the development of neuropathic pain is possible [14, 15]. Moreover, miR-19a has been shown to augment JAK-STAT3 signal transduction via an effective regulation of SOCS3 expression [16]. MiR-19a also can regulate SOCS1 expression during multiple myeloma and be induced by cytokines [17], implying a role in the regulation of the JAK-STAT3 pathway. Besides, miR-19a is reported to promote cell growth and tumorigenesis through directly repressing the expression of SOCS1 in gastric cancer [18]. miR-19a is demonstrated to directly target SOCS1 and is involved in JAK-STAT3 pathway, however, the regulatory mechanisms of miR-19a in the development of neuropathic pain have not been fully explored.

In the current study, we established rat model of chronic constriction injury (CCI) to determine the effect of miR-19a on neuropathic pain and to investigate whether miR-19a play a crucial role in neuropathic pain via targeting SOCS1. These findings may be useful for the therapeutic intervention of neuropathic pain development in a clinical application.

Materials and methods

Animals

The experiments were approved and reviewed by our Institute's Animal Care and Use Committee in accordance with the guidelines of the International Association for the Study of Pain. Adult Sprague-Dawley rats (250-350 g) used in this experiment were obtained from the Laboratory Animal Center and housed at a temperature 22°C under a 12/12 h light/dark cycle with free access to food and water.

Lentivirus production and intrathecal injections

The primers of miR-19a were purchased from AuGCT, Inc. (Beijing, China), and the sequences were 5'-CGCGAATTCGCAGTCCTCTGTTAGTTTTGCATAGTT-3' (forward) and 5'-GCGGGATCCCAGGCCACCATCAGTTTTGCATAGATT-3' (reverse). SOCS1 shRNA was purchased from GeneChem (Shanghai, China). These sequences were cloned into the lentivirus-based expression vector (System Biosciences, California, USA). Rats with an intrathecal catheter were randomly assigned into three groups: mo-miR-19a, siR-SOCS1 and normal control (NC) group. Then bCCI rats were injected with either 10 μl mo-miR-19a, siR-SOCS1 or blank expression vector through a microinjection syringe via an intrathecal catheter on 3 day before surgery and after surgery.

Neuropathic pain model

The rat model of bilateral chronic constriction injury (bCCI) was used for neuropathic pain [19]. Rats were anesthetized by intraperitoneal injection of sodium pentobarbital (40-50 mg/kg). The rat model of bCCI was then established as described previously [19]. Briefly, at the level of the middle of the thigh, the sciatic nerve was exposed by blunt dissection through biceps femoris. Proximal to the sciatic's trifurcation (above 2 mm distal to sciatic nerve), sciatic nerves were tied loosely using 4 ligatures (4.0 chromic gut) with about 1 mm spacing and the length of nerve thus was 4-5 mm long. The artery on the surface of the sciatic nerve was just barely constricted and the degree of its circulation through the superficial epineurial vasculature was best to be retarded but not arrested when was observed using dissecting microscope (40× magnification). The desired degree of constriction sometimes produced a small, brief twitch in the muscle around the exposure. After washed with physiological saline, the incision at muscle fascia, subcutaneous tissue and skin were discontinuous closed. An identical dissection was performed on the opposite side in every rat. Sham procedures comprised equal treatment but without ligation of the sciatic nerve were performed to prepare some rats as sham group. Notably, all surgical operation was performed by the same person. The animals were housed postoperatively in clear plastic cages with solid floors instead of wire mesh floors to avoid exacerbate discomfort arising from the affected hind paw.

Evaluation of the pain threshold

The behavioral measurements were carried out between 8:00 and 14:00. All behavioral tests were performed by an investigator blinded to randomization schedule.

Mechanical allodynia: Mechanical allodynia was measured by the sharp withdrawal threshold after mechanical stimuli (MWT) in

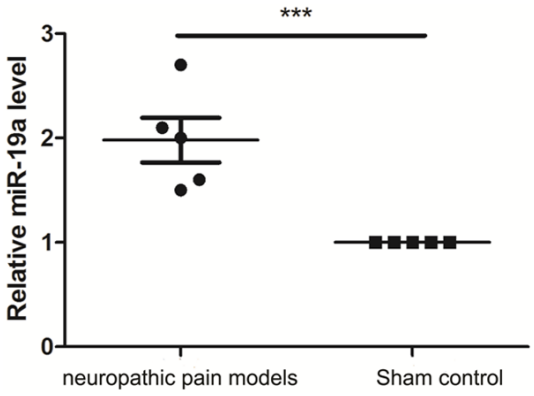

Figure 1. The relative expression level of miR-19a determined by quantitative real-time polymerase chain reaction (qRT-PCR). The expression level of miR-19a in rat model of bilateral chronic constriction injury (bCCI) significantly increased compared with shame group.

response to von Frey filaments as previously described [20]. In brief, the rats were acclimated in transparent plastic cages with wire mesh floor for 30 min. The plantar surface of each hind paw was applied pressure from below with the calibrated Electronic von Frey filament (Electronic von Frey 2393: IITC, USA) and held for approximately 5 s. The force applied at the time of sharp withdrawal was recorded. The three measurements of MWT were averaged.

Thermal hyperalgesia: Heat sensitivity was assessed by paw withdrawal threshold latency (PMTL) in response to radiant heat, which was in accordance with the Hargreaves method [21]. Briefly, rats were placed in perspex boxes. A radiant heat source BME-410A beneath a glassfloor was focused on the centre of the plantar surface of the hind paw. The heat intensity was approximately set up to 10 s to produce PWTL in normal animals and the cutoff time was set at 20 s to avoid tissue damage. The hind paws were given heat stimuli three times with greater than 3 min intervals between consecutive tests. Three measurements of PMTL were taken for each hind paw and averaged.

Acetone induced cold allodynia: Rats were acclimated in cages for 15 min and a drop (0.1 ml) of acetone was gently dropped to each hind paw through 1 ml syringe with a hose connection. A rapid withdrawal, licking, shaking or lifting of the hind paw after the spread of the acetone over the planter surface of the hind paw was defined as a sign of cold allodynia. The test was performed three times for each hind paw with an interval of approximately 2 min between consecutive tests. The times that the rats appeared a sign of cold allodynia were recorded.

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

qRT-PCR was performed to determine the expression level of miR-19a in the tissue samples. Large and small RNAs were isolated from tissues with the mirVana miRNA Isolation Kit (Ambion, Austin, TX, USA) following the manufacturer's protocol and were reverse transcribed to cDNA using MMLV reverse transcriptase (Promega, Madison, WI). The cDNA was used for the amplification of miR-19a and qRT-PCR was then performed to detect the relative transcript levels of it. The PCR was performed under the following conditions: 94°C for 3 min followed by 40 cycles of 94°C for 40 s, 56°C for 30 s and 72°C for 1 min. The relative expression level of miR-19a was normalized to β-actin and was calculated using he comparative threshold (Ct) cycle ($2^{-\Delta\Delta Ct}$) method [22]. All primers were purchased from AuGCT, Inc. (Beijing, China), and the primers of β-actin were: 5'-CGTGACATTAAGGAGAAGCTG-3' (forward) and 5'-CTAGAAGCATTTGCGGTGGAC-3' (reverse).

Western blotting

Protein was extracted from rat spinal cord tissues using RIPA lysis buffer with proteinase inhibitor. Total protein concentrations were determined using a BCA assay kit (Beyotime, China). Subsequently, equal concentrations (20 μg) of protein mixed with standard loading buffer was separated by 10% SDS-polyacrylamide gel electrophoresis (PAGE) and then transferred onto the nitrocellulose membrane (Bio-Rad, Munich, Germany). After blocking with nonfat dry milk (2.5%), the membranes were incubated with primary antibodies overnight at 4°C to detect the protein bands. Primary anti-SOCS1 (sc-9021) and anti-GAPDH (sc-48166) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The membranes were then incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies. The target protein was finally visualized using an enhanced chemiluminescence (ECL) system

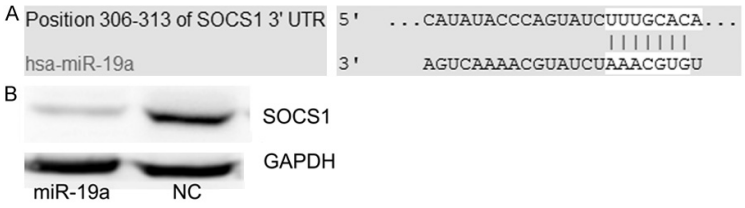

Figure 2. SOCS1 is a direct target of miR-19a in neuropathic pain models determined by bioinformatics analysis (A) and western blotting analysis (B), respectively.

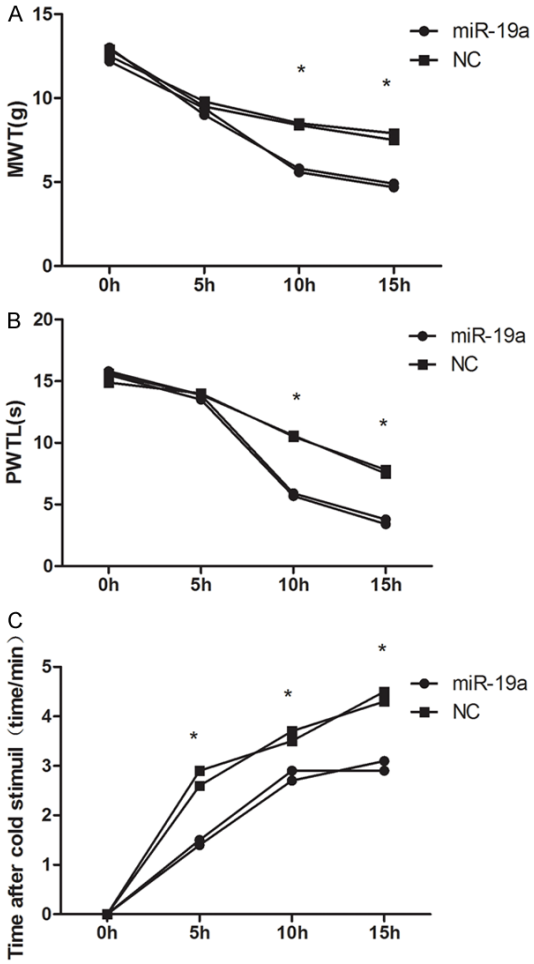

Figure 3. The effect of miR-19a on the withdrawal threshold after mechanical stimuli (MWL, A), paw withdrawal threshold latency (PMTL, B) and time after cold stimuli (C).

(Amersham, Little Chalfont, UK) according to the instructions of the manufacturer.

Statistical analysis

The data were expressed as the mean ± standard deviation (SD). The significance of differences between groups was analyzed by a Student's test or one-way ANOVA. Differences were considered statistically significant at a p value < 0.05.

Results

Analysis of the relative expression level of miR-19a in neuropathic pain models

The results of qRT-PCR analysis displayed that bCCI significantly increased the expression level of miR-19a at mRNA level ($P < 0.05$) compared with that in sham group (**Figure 1**), suggesting enhanced expression of miR-19a in neuropathic pain models.

SOCS1 was a direct target of miR-19a in neuropathic pain models

To explore the function and mechanism of miR-19a, we used bioinformatics tools to predict the targets of miR-19a. The results showed that SOCS1 was a directly target gene of miR-19a (**Figure 2A**). We next investigated whether miR-19a could directly down-regulate SOCS1 expression in neuropathic pain models by western blotting analysis. Positive results were obtained that ectopic expression of miR-19a in neuropathic pain models substantially decreased the levels of SOCS1 protein (**Figure 2B**).

Effect of miR-19a on the pain threshold

To test whether miR-19a affects neuropathic pain development, miR-19a was administered by intrathecal injection after bCCI. **Figure 3** illustrated the effect of miR-19a on the MWT (**Figure 3A**), PMTL (**Figure 3B**) and time after cold stimuli (**Figure 3C**). The results showed that MWT and PMTL significantly decreased after transfected with miR-19a compared with NC, while time after cold stimuli obviously increased. Moreover, after transfected with miR-19a, MWT and PMTL gradually decreased with the increase of treated time, while time after cold stimuli increased.

Effect of SOCS1 on the pain threshold

To further determine whether miR-19a could target SOCS1, the effect of SOCS1 on the pain

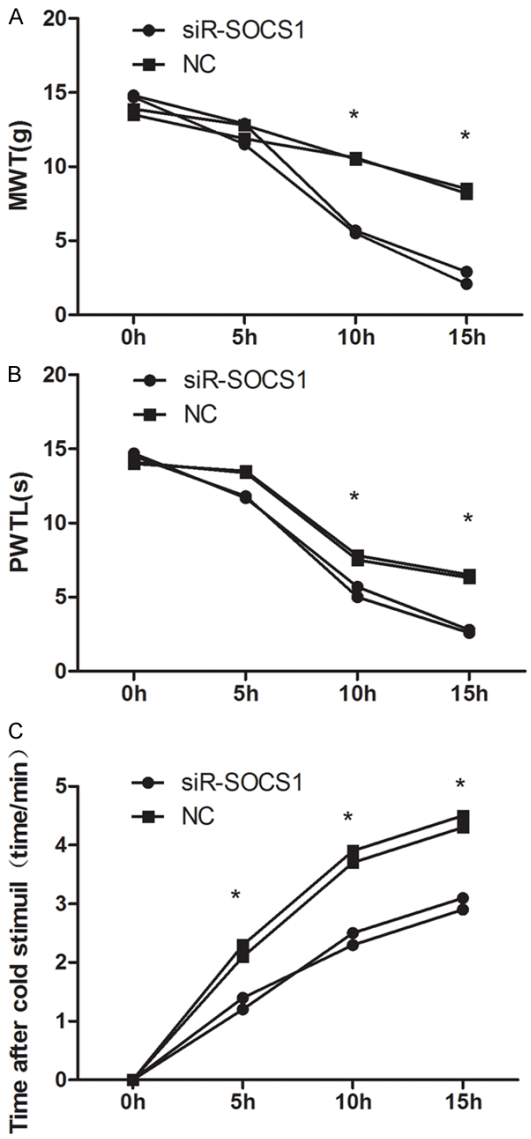

Figure 4. The effect of SOCS1 on the withdrawal threshold after mechanical stimuli (MWL, A), paw withdrawal threshold latency (PMTL, B) and time after cold stimuli (C).

threshold was investigated. **Figure 4** displayed the effect of SOCS1 on the MWT (**Figure 4A**), PMTL (**Figure 4B**) and time after cold stimuli (**Figure 4C**). After transfected with siR-SOCS1, similar results were obtained that MWT and PMTL significantly decreased compared with NC, while time after cold stimuli obviously increased. Meanwhile, MWT and PMTL gradually decreased with the increase of treated time, while time after cold stimuli increased. All these results implied that miR-19a might suppress SOCS1 in neuropathic pain models.

Discussion

This study showed that miR-19a was involved in the regulation of neuropathic pain in a rat model of bCCI. In briefly, miR-19a expression levels were markedly increased in neuropathic pain models. Moreover miR-19a significantly attenuated MWT and PMTL, while markedly increased the times that the rats appeared a sign of cold allodynia. Notably, knockdown of SOCS1 expression had similar effects. Besides, the results confirmed that SOCS1 was a direct target of miR-19a in neuropathic pain models. All these findings suggest that miR-19a may play key roles in the development of neuropathic pain via targeting SOCS1.

miR-19a is one of miR-17-92 cluster located on chromosome 13 [23]. miR-19a is reported to be induced by the interferon-alpha (IFN-α), antiviral JAK-STAT signaling cytokine [24]. The roles of JAK-STAT signaling pathway in neuropathic pain have been discussed before. Moreover, tumor necrosis factor-α (TNF-α) is up-regulated in esophageal squamous cell carcinoma cells when transfected with miR-19a [25], which is confirmed to be a neuropathic pain-related cytokine [26]. Besides, miRNAs generally exert their regulatory effects through regulating the expression of their target genes. Several miRNAs are shown to be involved in the nervous system development and neural plasticity via regulation of related genes [27, 28]. Altered gene regulation and protein expression is demonstrated to be responsible for a series of changes in pain sensitivity induced by peripheral nerve injury [29]. SOCS1 has been reported to as a target of miR-19 and its expression can be reduced, which may be linked to enhanced IL-6 signaling in multiple myeloma [30]. Zhang et al. confirmed that miR-19a might inhibit SOCS1 expression and consequently relieved the inhibition of inflammation by SOCS1 [31]. It can therefore be hypothesized here that miR-19 may be involved in neuropathic pain via reducing the expression of SOCS1.

To further verify our hypothesis, a decreased expression of SOCS1 in rats treated with miR-19 we observed by western blotting in the present study, implying SOCS1 was a direct target of miR-19a. SOCS1 is thought to have important effects in neuron survival following nerve injury [32]. Nerve injury is always associated with

neuro-inflammation and neuro-immune activation in nervous system and is consequently likely to lead to chronic neuropathic pain [33, 34]. Thus, SOCS1 may have inhibitory effects on the neuropathic pain development. Moreover, SOCS1 can attenuate the activation of cytokines-induced nuclear factor (NF)-κB which is a critical regulator of the inflammatory process and has been demonstrated to be activated in neuropathic pain [35-37]. Besides, SOCS1 deficiency is found to be associated with a wide range of acute and chronic inflammatory disorders [38]. The inflammatory process is shown to be responsible for the development of neuropathic pain [39]. Therefore, our results are in line with previous findings that knockdown of SOCS1 by miR-19a may contribute to the development of neuropathic pain. Moreover, knockdown of SOCS1 expression had similar effects on MWL, PMTL and time after cold stimuli with miR-19a. It is thus intriguing to speculate that miR-19a may promote the development of neuropathic pain via targeting SOCS1.

In conclusion, our findings indicate that SOCS1 is a direct target of miR-19a in neuropathic pain rats, and miR-19a may play a critical role in regulating neuropathic pain via targeting SOCS1. Inhibition of miR-19a may be a potential therapeutic strategy for neuropathic pain. However, further validations are still needed to explore the precise role of miR-19a in regulating neuropathic pain.

Disclosure of conflict of interest

None.

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