Overexpression of miR-30a attenuates neuropathic pain by targeting SOCS1 in rats with chronic constriction injury

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Abstract: Objectives: To explore the impact of miR-30a on neuropathic pain and associated mechanism. Methods: Sprague-Dawley (SD) rats were randomly assigned to bilateral chronic constriction injury (bCCI) group, sham group, and control group (ten in each group). Expression of miR-30a and suppressors of cytokine signaling (SOCS)1 were determined by quantitative real time RT-PCR (qRT-PCR). Then another 40 rats were induced model of bCCI and were randomly assigned to overexpression of miR-30a group and its control group, silencing of SOCS1 group and its control group using recombinant lentivirus vectors. The expression of miR-30a and SOCS1 after lentivirus transduction was evaluated by qRT-PCR and Western blotting, respectively. The rats in the four groups were assessed by neurobehavioral tests (mechanical hyperalgesia testing, thermal preference testing (TPT), and acetone testing) before surgery and at days 3, 7, and 14 after surgery. Results: The level of miR-30a was significantly decreased in the bCCI group than that in the sham group and control group, but the level of SOCS1 was significantly increased (P < 0.05). Besides, SOCS1 was a target gene of miR-30a and was negatively regulated by miR-30a. The mechanical shrinkage threshold (MWT) and paw withdrawal thermal latencies (PWTL) were significantly increased, while the frequency was significantly decreased in the overexpression of miR-30a group and knockout of SOCS1 group than those in their corresponding control groups (P < 0.05). Conclusion: Overexpression of miR-30a attenuates neuropathic pain maybe by targeting SOCS1.

Keywords: Neuropathic pain, miR-30a, SOCS1

Introduction

Neuropathic pain, defined as “pain arising as a direct consequence of a lesion or disease affecting the somatosensory system” according to International Association for the Study of Pain (IASP) [1, 2], is a frequent sequela originating from a primary injury of the nervous system or some diseases, such as diabetes, cancer, or trauma [3, 4]. It brings great pain intensity to the patients and has a major negative impact on sleep, work, mood, functionality, or other aspects of quality of life [5, 6]. The therapeutic recommendations for neuropathic pain include antidepressants, antiepileptics, opioids and neuro-stimulation methods [7]. However, these drugs produce side effects or often present drug resistance [8, 9]. Therefore, a better and a deeper understanding of the molecular mechanisms underlying neuropathic pain is benefit to develop novel and tailored therapeutic strategies.

Recently, the functional roles of microRNA (miRNA) have been gained considerable attention in various diseases [10]. MiRNAs are a class of 19-22 nucleotides, non-protein encoding RNA molecules that play a critical role in the regulation of gene expression by binding to the 3'-untranslated region (UTR) of target messenger RNA (mRNA), leading to decreased proteins expression encoded by such target RNAs [11]. A previous study identified a series of miRNAs, such as hsa-miR-221, hsa-miR-34a, hsa-miR-30d and hsa-miR-30a-3p, that may be associated with the development and maintenance of neuropathic pain using a miRNA expression profiling in a spinal nerve ligation (SNL) model of neuropathic pain [12]. In addition, pro-inflammatory chemokines and cytokines have been
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reported to be involved with peripheral nerve injury [13, 14]. The suppressors of cytokine signaling (SOCS) is a family of proteins that negatively regulates the inflammatory response by limiting cytokine signaling, for example, Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway [15-17]. SOCS1 and SOCS3 proteins have been reported to play different roles in Wallerian degeneration (WD) in the mouse sciatic nerve after cut/ligation and crush injuries [18].

However, rare studies are available regarding the impact of miR-30a on neuropathic pain, and whether its associated mechanism responding to regulate the expression of SOCS1 still remain unclear. Therefore, we aimed to investigate the effects of miR-30a on neuropathic pain, as well as the associated mechanism.

Materials and methods

Animals

Adult male Sprague-Dawley (SD) rats weighing 180-200 g that were maintained on 12:12 h light-dark cycle with controlled temperature (20-22°C) and humidity (50%) were used in the experiment. All the rats were single housed in individually ventilated cages and were provided standard diet and tap water ad libitum. The rats were acclimatized for one week before the experiment. The study was approved by the Animal Care and Use Committee of our hospital.

Experimental designs

SD rats were randomly assigned to bilateral chronic constriction injury (bCCI) group, sham group, and control group (ten in each group). The rats in the bCCI group and sham group were induced model of neuropathic pain and sham-operated, respectively. The rats in the control group received no intervention. L4-L6 dorsal spinal cord in the three groups was taken for further analysis. Then, another 40 SD rats were successfully induced model of neuropathic pain using bCCI and were randomly and equally (10 in each group) assigned to overexpression of miR-30a group and its corresponding control group, silencing of SOCS1 group and its corresponding control group using recombinant lentivirus vectors. The rats in the four groups were assessed by neurobehavioral tests before surgery and at days 3, 7, and 14 after surgery. After the neurobehavioral tests, the 40 animals were sacrificed and the sciatic nerves were taken for further analysis.

Bioinformatics prediction of miRNAs targeting SOCS1

TargetScan6.2 and microRNA.org were used to identify possible miRNAs targeting SOCS1.

bCCI of sciatic nerve

The bCCI surgery was performed according to the previously described method [19]. In brief, 48 rats were anesthetized with 40-50 mg/kg sodium pentobarbital by intraperitoneal injection. Approximately 1.5 cm incision was made along the dorsal to the pelvis. The common sciatic nerve was exposed and bluntly dissected from surrounding tissues. Four ligatures of 4.0 chromic catguts were placed circumferentially around the sciatic nerve with 1 mm intervals. When there was a brief twitch occurring in the rats’ hind limb, the ligatures were tied. The incision was then closed layers by layers. The other side of the sciatic nerve was operated with the same manipulations. For the sham-operated rats, the same surgical procedure was carried out but without ligatures. For the control group, the rats received no intervention. All the surgical procedure was performed by the same operator. After operation, both bCCI and sham rats were kept under standard conditions as the same as the control group.

Vector construction, production and transduction

Lentivirus vectors were produced as described previously [20]. The miR-30a lentivirus expression vector pWPXL-miR-30a was constructed by replacing the green fluorescence protein (GFP) fragment of the pWPXL vector with the pri-miR-30a sequence amplified from normal genomic DNA. The 3'-UTR sequence of SOCS1 was amplified from the genomic DNA and then subcloned into the region directly downstream of the stop codon of luciferase. The recombinant lentivirus of small interference RNA (siRNA) targeting SOCS1 (SOCS1-RNAi-lentivirus) and the control lentivirus (GFP-lentivirus) were prepared, and the gene transfection process was carried out based on the company’s instructions.
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provided by the company. All constructs were verified by sequencing. Lentivirus expression plasmids were co-transfected into 293T cells using the calcium phosphate method to construct viral stocks. The titers of lentivirus vector stocks ranging from 0.4×10⁹ to 2.0×10⁹ particles/mL were used.

The lentivirus vectors were delivered by intraparenchymal injection as described before [21]. In brief, under anesthetized with chloral hydrate (400 mg/kg), the rats' spine was kept with two individual bars placed around the L3 vertebra to avoid movements induced by breathing. Then the thoracic T13 vertebra was drilled to access the left side of the lumbar spinal cord with an operation microscope (Olympus OME 8000; Olympus, Tokyo, Japan). Approximately 1-2 cm incision in the intact dura mater and arachnoid mater was exposed, 2 μL of lentivirus vectors (LV-EGFP or LV-SOCS1t) were delivered with the help of an automatic microinjection device (KDS 310; KD Scientific, Holliston, USA). After the muscles and skin were sutured, the animals were then housed in individual cages to recover.

Mechanical hyperalgesia testing, thermal preference testing (TPT) and acetone testing

All animals were assessed before operation and at days 3, 7, and 14 after operation. Pretesting was performed for at least three consecutive days to acclimate the testing procedure. Animals were accommodated in a wire mesh bottom cages (50×30×20 cm, pore diameter 0.5×0.5 cm) for 10-30 minutes. The rats were assured quiet, and not presentation with seeking, exploratory defecation, urination movements, or resting over the paws before the tests. For the mechanical hyperalgesia testing, an electronic von Frey filament (Stoelting, Wood Dale, IL, USA) was performed. The force was alternated to hind paws, and the force presented paw withdrawal was recorded. The mechanical shrinkage threshold (MWT) was recorded as the minimal force that could initiate a withdrawal response. For the TPT, a radiant heat (BME-410A, biomedical Engineering Institute of the Chinese Academy of Medical Sciences) was used to test the paw withdrawal thermal latencies (PWTL). Each hind paw was repeated 3 times, and the least interval was 3 min. For the acetone testing, 0.1 ml of acetone was gently applied to each hindpaw. A brisk paw withdraw-
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(HRP)-conjugated secondary antibodies (Santa Cruz Biotechnology). Anti-GAPDH antibody (Santa, Cruz Biotechnology) was recorded as a reference. After that, the membranes were performed to enhanced chemiluminescence and densitometric analysis.

Statistical analysis

All data were expressed as the mean ± standard deviation (SD). Statistical analysis was performed with statistical package for the social sciences (SPSS) software (version 18.0; SPSS Inc., Chicago, IL). A Student’s t test was used to statistical comparisons. Differences were considered statistically significant when \( P < 0.05 \).

Results

Expression of miR-30a and SOCS1

To explore the expression level and significance of miR-30a and SOCS1 in neuropathic pain, we first determined the expression levels of miR-30a and SOCS1 in the bCCI group, sham group, and control group using qRT-PCR. As shown in Figure 1A, the expression level of miR-30a was significantly decreased in the bCCI group than that in the sham group and control group (\( P < 0.05 \)). However, there was no significant difference between the sham group and control group. The expression level of SOCS1 was significantly increased in the bCCI group compared with that of the other two groups (\( P < 0.05 \)) (Figure 1B). The results suggested that miR-30a and SOCS1 may play important roles in the neuropathic pain.

Effect of overexpression of miR-30a on neurobehavioral testing

To better understand the biological functions of miR-30a in the peripheral neuropathic pain, we first constructed a lentivirus vector expressing miR-30a and established the expression mRNA level of miR-30a in tissues using qRT-PCR after lentivirus transduction. The results showed that relative miR-30a mRNA level was significantly up-regulated in the lentivirus transduction with miR-30a that in the lentivirus transduction with empty vector (\( P < 0.05 \)) (Figure 2A). Then the effects of overexpression of miR-30a on behavioral tests included hindpaw withdrawal responses to mechanical hyperalgesia testing, TPT, and acetone testing were determined. These results showed that the MWT and PWTL in the overexpression of miR-30a group were significantly increased than that in the control group (\( P < 0.05 \)), while the frequency after cold acetone stimuli was significantly decreased in the overexpression of miR-30a group than that in the control group (\( P < 0.05 \)) (Figure 2B-D).

SOCS1 gene 3’-UTR carried a putative miR-30a binding site and was negatively regulated by miR-30a

To investigate the underlying molecular mechanism through which miR-30a prevents peripheral neuropathic pain, we explored the expres-
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The impact of miR-30a on neuropathic pain was investigated through several experiments. The expression level of SOCS1 was determined using Western blotting after overexpression of miR-30a. The results showed that the protein level of SOCS1 was significantly lower in the overexpression of miR-30a group than that in the control group (P < 0.05) (Figure 3A), indicating that SOCS1 might be negatively regulated by miR-30a.

To predict that SOCS1 gene mRNA 3'-UTR might contain a miR-30a binding site, the TargetScan6.2 and microRNA.org was used to confirm the prediction. The bioinformatic algorithms results showed that the 3'-UTR of SOCS1 mRNA comprises of several predicted miR-30a-binding sites (Figure 3B).

Effect of knockout of SOCS1 on neurobehavioral testing

To explore the role of SOCS1 in peripheral neuropathic pain, we knocked out SOCS1 using recombinant lentivirus of siRNA targeting SOCS1. Then the expression levels of SOCS1 in the both siSOCS1 group and control group were determined using qRT-PCR. As shown in Figure 2, the effect of overexpression of miR-30a on neurobehavioral testing. A. Relative miR-30a mRNA level in the lentivirus transduction with miR-30a and with control lentivirus; B-D. The results of mechanical hyperalgesia testing, thermal preference testing and acetone testing in the overexpression of miR-30a group and control group at different time points (before surgery and at days 3, 7, and 14 after surgery), respectively. MWT, mechanical shrinkage threshold; PWTL, paw withdrawal thermal latencies **P< 0.05.

Figure 2. The effect of overexpression of miR-30a on neurobehavioral testing. A. Relative miR-30a mRNA level in the lentivirus transduction with miR-30a and with control lentivirus; B-D. The results of mechanical hyperalgesia testing, thermal preference testing and acetone testing in the overexpression of miR-30a group and control group at different time points (before surgery and at days 3, 7, and 14 after surgery), respectively. MWT, mechanical shrinkage threshold; PWTL, paw withdrawal thermal latencies **P< 0.05.

Figure 3. SOCS1 gene 3'-UTR carried a putative miR-30a binding site and is negatively regulated by miR-30a. A. The expression level of SOCS1 using Western blotting after overexpression of miR-30a; B. SOCS1 gene 3'-UTR carries a putative miR-30a binding site. SOCS, suppressors of cytokine signaling; UTR, untranslated region.

Group A

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Figure 3. SOCS1 gene 3'-UTR carried a putative miR-30a binding site and is negatively regulated by miR-30a. A. The expression level of SOCS1 using Western blotting after overexpression of miR-30a; B. SOCS1 gene 3'-UTR carries a putative miR-30a binding site. SOCS, suppressors of cytokine signaling; UTR, untranslated region.

Group B

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Figure 3. SOCS1 gene 3'-UTR carried a putative miR-30a binding site and is negatively regulated by miR-30a. A. The expression level of SOCS1 using Western blotting after overexpression of miR-30a; B. SOCS1 gene 3'-UTR carries a putative miR-30a binding site. SOCS, suppressors of cytokine signaling; UTR, untranslated region.
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The expression level of SOCS1 was significantly decreased in the siSOCS1 group than that in control group ($P < 0.05$). We further investigated the effect of knockout of SOCS1 on neurobehavioral testing. We found that the MWT and PWTL in the knockout of SOCS1 group were significantly increased than that in the control group ($P < 0.05$), while the frequency after cold acetone stimuli was significantly decreased in the knockout of SOCS1 group than that in the control group ($P < 0.05$) (Figure 4B-D).

Discussion

In the present study, we investigated the functional role of miR-30a in neuropathic pain and explored its associated mechanism. The results suggested that miR-30a is significantly decreased; while SOCS1 is increased in model of neuropathic pain (bCCI). Both miR-30a and SOCS1 were involved in neuropathic pain. Besides, we demonstrated that SOCS1 is a target gene of miR-30a and is negatively regulated by miR-30a. Overexpression of miR-30a or knockout of SOCS1 could attenuate neuropathic pain. Taken together, these results indicate that miR-30a attenuates neuropathic pain maybe by targeting SOCS1.

It has been well demonstrated that gene expression patterns are significantly alternated in somatosensory nervous system [22, 23]. The neuropathic pain induced by peripheral nerve injury is characterized by changes of gene regulation and protein expression [24], which may be accompanied by alteration in miRNAs expression involved in nociceptive pathways. Previous studies have confirmed many miRNAs, such as miR-200b and miR-429, are expressed in spinal cord in a model of chronic neuropathic pain [12, 25, 26]. A study conducted by Von et

Figure 4. The effect of knockout of SOCS1 on neurobehavioral testing. A. Relative SOCS1 mRNA level in the lentivirus transduction with siRNA targeting SOCS1 and with control lentivirus; B-D. The results of mechanical hyperalgesia testing, thermal preference testing and acetone testing in the knockout of SOCS1 group and control group at different times points (before surgery and at days 3, 7, and 14 after surgery), respectively. MWT, mechanical shrinkage threshold; PWTL, paw withdrawal thermal latencies; siRNA, small interference RNA $^{**}P < 0.05$. 

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al. [12] suggested a series of miRNAs are differentially expressed in model of neuropathic pain, including has-miR30a-5p and has-miR30d, however, the exact mechanism and functional role were not explored. In addition, SOCS1, an important member of SOCS family, has been reported to be involved in the neuropathic pain by suppressing JAK/STAT pathway [18]. Then we proposed that there might be some correlations between miR-30a and SOCS1 playing important roles in neuropathic pain.

In order to prove the hypothesis, we first investigated the expression level of miR-30a and SOCS1 in the model of neuropathic pain, sham, and control rats. The results showed that expression level of miR-30a was significantly decreased in the model group, but the level of SOCS1 was markedly increased compared with the sham group and control group. These results were in line with previous studies [12, 18], suggesting that miR-30a and SOCS1 are involved in neuropathic pain. Subsequently, we predicted that SOCS1 gene mRNA 3'-UTR might contain a miR-30a binding site, the TargetScan6.2 and microRNA.org was used to assessed the prediction. The bioinformatic algorithms results showed that the 3'-UTR of SOCS1 mRNA comprises of several predicted miR-30a-binding sites, indicating that SOCS1 is one of target gene of miR-30a. To further the relationship between the miR-30a and SOCS1, we investigated the expression protein level of SOCS1 when the level of miR-30a had been changed using lentiviral vector. The results demonstrated that the level of SOCS1 was inversely correlated with miR-30a, suggesting that SOCS1 is negatively regulated by miR-30a.

Recently, JAK2/STAT3 signaling pathway has been paid attention to the neuropathic pain. The phosphorylation states of JAK2/STAT3 could induce neuropathic pain [27-29]. AG490, an inhibitor JAK2, could relieve the pain states induced by SNL injury [30]. In addition, Xue et al. [31] found the expression mRNA levels of JAK2, STAT3, and SOCS3 were significantly increased in a model of neuropathic pain induced by bCCI at each postoperative time point. But the pain-related behavioral changes were significantly relieved by administration of WP1066, a small molecule inhibitor of JAK2 and STAT3. Besides, Dominguez et al. selectively inactivated JAK/STAT3 signaling in a preclinical model of neuropathic pain by transduction with local, lentiviral-mediated production of SOCS3, and found that mechanical hypersensitivity induced by CCI was attenuated by preventing the abnormal expression of interleukin (IL) 6, CC chemokine ligand CCL2, and activating transcription factor (ATF) 3 [21]. Moreover, Wand et al. [32] showed that mechanical allodynia was clearly reduced by blockade of JAK2/STAT3 signaling with the JAK2 inhibitor AG490 or the STAT3 inhibitor S3I-201 or aspirin-triggered Lipoxin (ATL) in CCI rats. Interestingly, the expression of SOCS3 mRNA level in the spinal cord was further promoted after inhibition of JAK2/STAT3 signaling. Similarly, we also selectively inactivated JAK2/STAT3 signaling by transduction with local, lentiviral-mediated production of SOCS1, and analyzed its impacts on a model of neuropathic pain. Our results found that MWT and PWTL was significantly increased, while the frequency after cold acetone stimuli was significantly decreased, indicating that neuropathic pain was effectively relieved, which was in line with the study conducted by Dominguez et al. [21]. Additionally, we analyzed the consequences of overexpression of miR-30a by lentivirus transfection in the model of neuropathic pain. Similar results were obtained when enhanced the expression of miR-30a. Overexpression of miR-30a could similarly relieve neuropathic pain induced by bCCI. This phenomenon may be associated with the down-expression of SOCS1, which negatively regulated the JAK2/STAT3 signaling pathway and subsequently control expression of some pro-inflammatory chemokines and cytokines.

Taken together, our results suggest that overexpression of miR-30a attenuates neuropathic pain maybe by targeting SOCS1.

Disclosure of conflict of interest

None.

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