Microglia inflammatory response contributes to chronic constriction injury-induced neuropathic pain via miR-339/PFKFB3 axis

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Abstract

Purpose: To investigate the effect of miR-339 on neuropathic pain.

Methods: A rat neuropathic pain model was established through chronic constriction injury (CCI). Expression of miR-339 in spinal cord was determined 14 days later. Microglial inflammatory response was evaluated using immunofluorescence analysis of ionized calcium binding adaptor molecule 1 (Iba1), while IL-6 and TNF-α were assessed by enzyme-linked immunosorbent assay (ELISA). Pain-associated behavioral effects and microglia-related inflammation were investigated after intrathecal administration of miR-339 agomir into rats post-CCI. The target gene of miR-339 involved in neuropathic pain was evaluated by a luciferase reporter assay. Microglia cells were isolated from rats, then treated with lipopolysaccharide (LPS). The LPS-induced inflammatory response in microglia cells was determined using quantitative reverse transcription PCR analysis of IL-6 and TNF-α.

Results: CCI decreased mechanical allodynia and thermal hyperalgesia thresholds, but increased Iba1, IL-6, and TNF-α in rats. MiR-339 was reduced in rat spinal cord after CCI induction while intrathecal injection of miR-339 agomir alleviated CCI-induced changes in mechanical and thermal hyperalgesia in rats, and reversed expression of Iba1, IL-6, and TNF-α. Furthermore, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3 (PFKFB3) was identified as a miR-339 target gene, and overexpression of miR-339 decreased the expression of PFKFB3, IL-6, and TNF-α in LPS-induced microglia cells.

Conclusion: The miR-339/PFKFB3 axis ameliorates CCI-induced neuropathic pain by suppression of microglia inflammatory response, suggesting a novel strategy for neuropathic pain management.

Keywords: Neuropathic pain, miR-339, Chronic constriction injury, PFKFB3, Microglia cells

INTRODUCTION

Neuropathic pain is a pain syndrome caused by primary lesions or dysfunction of the central or peripheral nervous system [1]. Tactile pain is a major feature of neuropathic pain, mainly manifested as harmless tactile stimulation that induces a pain response [1]. Due to the
complicated pathogenesis of neuropathic pain, current effective treatment strategies are very limited [2]. Therefore, development of optimal strategies to manage neuropathic pain is an urgent need.

Microglia cells, with the ability to regulate synaptic remodeling and immune surveillance in the central nervous system, underlie neuropathic pain [3]. Drugs targeting microglia might offer potential therapeutic strategies for neuropathic pain [3]. In response to noxious stimuli, microglia cells release inflammatory factors, such as TNF-\(\alpha\) and IL-1\(\beta\), and contribute to pain hypersensitivity during neuroimmune activation of neuropathic pain [4]. Administration of microglia inhibitor attenuates pain hypersensitivity in neuropathic pain [4]. Therefore, regulators of the microglial inflammatory response could be effective as anti-nociceptive agents in neuropathic pain management.

MicroRNA (miRNA) plays key roles in immune cell differentiation and activation [5]. Dysregulated miRNA expression has been implicated in the microglia-mediated inflammatory response, thus participating in the pathogenesis of neuropathic pain [5]. A recent study showed that the expression of miRNAs in the nervous system regulates neuroimmunity and neuropathic pain [6]. MiR-339 was found to be reduced in neuropathic pain in rats [7], and demonstrated anti-inflammatory activity in alcohol-exposed brain tissues and microglia cells [8]. However, no relevant studies of mechanisms involved in miR-339-mediated neuropathic pain have yet been reported. Therefore, the present study established a rat model of neuropathic pain through chronic constriction injury (CCI), and performed functional assays to evaluate effects of miR-339 on neuropathic pain. Microglia cells were isolated, and the role and mechanism of miR-339 on the microglial inflammatory response were determined.

EXPERIMENTAL

Rat model of neuropathic pain

The study was approved by the Scientific Research Ethics Committee of First Affiliated Hospital of Gannan Medical University (approval no. LLSC-2020061901), and conducted in accordance with the National Institutes of Health Laboratory Animal Care and Use Guidelines [9]. Eighteen male Wistar rats (13 weeks old, 300 - 350 g), acquired from Shanghai Animal Laboratory Center (Shanghai, China), were divided into three groups: sham, CCI + NC agomir, and CCI + miR-339 agomir. For the CCI model, rats were anesthetized using intraperitoneal injection of 60 mg/kg sodium pentobarbital. The left sciatic nerve was then exposed and ligated with 4/0 silk, proximal to the trifurcation and 1 mm from the four ligations. Ethicon silk sutures were then used to suture the muscle and skin layers. Rats in the sham group underwent the same procedure without ligation of the left sciatic nerve. Pain-associated behavioral activities were investigated 1, 4, 7, and 14 days after the surgery.

For drug infusion, rats with CCI induction were anesthetized, and lumbar laminectomy of the L5 vertebra was performed. Following cutting of the dura, a polyethylene-10 catheter was inserted into the subarachnoid space at L4/5 of the spinal cord. After lumbar implantation of the intrathecal catheter, the rats were allowed to recover for 3 days before drug delivery. Four days after CCI surgery, 5 nM miR-339 agomir or NC agomir (20 \(\mu\)L; Ribobio, Beijing, China) were intrathecally administered into the catheter through a connected microinjection syringe. The drugs were administered once a day from day 4 to day 8. Pain-associated behavioral activities were also investigated 1, 4, 7, and 14 days after the drug delivery.

Behavioral test

Rats were habituated in a wire mesh, and an automated dynamic plantar aesthesiometer (Ugo Basile, Varese, Italy) was used to measure mechanical allodynia. The mechanical withdrawal threshold (MWT) was calculated as the lowest force (g) leading to rapid withdrawal of the right hind paw. Each measurement was repeated three times to determine the average force. For thermal hyperalgesia, rats were habituated in a clear glass plate at 30 °C. A heat source was then positioned beneath the hind paw. Paw withdrawal thermal latency (PWTL) was calculated as the duration (s) between the start of heat application and withdrawal of the paw, measured with a digital timer. Each measurement was also repeated three times to determine the average time.

Enzyme-linked immunosorbent assay (ELISA)

Fourteen days after CCI surgery and drug delivery, spinal cord segments L4-L6 were harvested, incubated with RIPA cell lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China), and the supernatants were collected after centrifugation. Levels of TNF-\(\alpha\) and IL-6 were determined using ELISA kits (Abcam, Cambridge, MA, USA).
Immunofluorescence

Fourteen days after CCI surgery and drug delivery, the transverse spinal cords were collected and cut into 25 μm-thick sections. After fixation with 4% paraformaldehyde and blocking with horse serum (Jackson ImmunoResearch, West Grove, PA, USA), the sections were incubated with anti-Iba1 antibody (1:200, Abcam) at 4 °C overnight. Following staining with Alexa Fluor® 488-conjugated secondary antibody (1:500, Abcam), images were acquired with an FV1000 fluorescence microscope (Olympus, Tokyo, Japan).

Microglia cell isolation

Brain tissues were collected from rats, and then were dissociated to generate mixed primary glia cell culture. Microglia cells were isolated from the mixture by mild trypsinization using 0.05% trypsin, and then cultured in DMEM (Dulbecco’s Modified Eagle Medium) containing 10% fetal bovine serum. HEK293T cells (ATCC, Manassas, VA, USA) were also grown in DMEM at 37 °C in a humidified incubator.

Cell transfection and treatment

Microglia cells were transfected with miR-339 mimic or NC mimic (Ribobio) via Lipofectamine™ 2000 (Invitrogen, Waltham, MA, USA). HEK293T cells were also transfected with miR-339 mimic or inhibitor, or with NC mimic or inhibitor. For LPS treatment, microglia cells, with or without transfection, were incubated with DMEM containing 5 μg/mL LPS for 24 h.

Luciferase reporter assay

Sequences of wild type or mutant PFKFB3 3′ UTR were cloned into pMIR-GLO™ Luciferase vector (Promega, Madison, WI, USA). HEK293T cells were co-transfected with PFKFB3-WT or PFKFB3-MUT luciferase vector and miR-339 mimic or NC mimic. Forty-eight hours later, Dual-Luciferase Reporter Assay system (Promega) was used to measure luciferase activity.

Quantitative reverse transcription PCR (qRT-PCR)

RNA was extracted from HEK293T cells, microglia cells, or dorsal horn of rat spinal cord via TRizol reagent (Invitrogen), and reverse-transcribed into cDNA with the MMLV RT kit (Promega). Relative expression of miR-339 and PFKFB3 were determined using TaqMan microRNA assays (Thermo Fisher), using the primers indicated in Table 1.

Western blot assay

Proteins were extracted from HEK293T and microglia cells via RIPA lysis buffer, and quantified by bicinchoninic acid assay (Beyotime). Protein samples (30 μg) were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis, then transferred onto a polyvinylidene difluoride membrane (EMD Millipore, Bedford, MA, USA). After blocking in 5% non-fat dry milk, membranes were incubated with primary antibodies against PFKFB3 (1:2000; Abcam) or β-actin (1:3000; Abcam). Following incubation with horseradish peroxidase-conjugated secondary antibodies (1:5000), protein signals were visualized using the Super Signal West Pico Chemiluminescent Substrate kit (Thermo Fisher; Waltham, MA, USA).

Statistical analysis

Data are expressed as mean ± standard deviation. GraphPad Prism 6.0 was used to conduct statistical analyses using Student’s t-test or one-way analysis of variance. Values of $p < 0.05$ were considered statistically significant.

RESULTS

MiR-339 was reduced in dorsal horn of spinal cord in CCI rats

To establish the neuropathic pain model, rats were subjected to CCI induction. Analysis of data for mechanical allodynia and thermal hyperalgesia showed that CCI could decrease MWT (Figure 1 A) and PWTL (Figure 1 B) measured 1, 4, 7, or 14 days post-CCI, suggesting successful establishment of the neuropathic pain model. Expression of miR-339 was reduced in the dorsal horn of the spinal cord.

### Table 1: Primer sequences

<table>
<thead>
<tr>
<th>ID</th>
<th>Sequence (5′-3′)</th>
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<tbody>
<tr>
<td>MiR-339 F</td>
<td>CGACGCGTCCGCGCCATTGCCACGGCACCAT</td>
</tr>
<tr>
<td>MiR-339 R</td>
<td>CCGCATACGAGTCATCGATGGGGCAAGAAGGAGTGCT AAG</td>
</tr>
<tr>
<td>PFKFB3 F</td>
<td>CCTCTGCTTTTGAACACAGGC</td>
</tr>
<tr>
<td>PFKFB3 R</td>
<td>ATCCAGACAGACACTGCAAGC</td>
</tr>
<tr>
<td>IL-6 F</td>
<td>TGGAGTCCAGAGGAGAAGATGTC CAC</td>
</tr>
<tr>
<td>IL-6 R</td>
<td>TCTGACCACAGTGAAGAATGTC CAC</td>
</tr>
<tr>
<td>TNF-α F</td>
<td>CCCCCTCAGCAACACCAAGT</td>
</tr>
<tr>
<td>TNF-α R</td>
<td>CTTGGCCAGATTGACCTCAAGC</td>
</tr>
<tr>
<td>β-actin F</td>
<td>CCACACCGCCACCAAGCTTCG</td>
</tr>
<tr>
<td>β-actin R</td>
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<tr>
<td>U6 F</td>
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</tr>
<tr>
<td>U6 R</td>
<td>GAGGTGTTGCCACAGAGA</td>
</tr>
</tbody>
</table>
in CCI rats compared to the sham group (Figure 1 C). Higher levels of IL-6 and TNF-α demonstrated that CCI induced neuroinflammatory processes (Figure 1 D). Expression of Iba1, a marker of activated microglial cells, was also up-regulated in rats post-CCI (Figure 1 E), suggesting that CCI induces a microglial inflammatory response in rats.

**Figure 1:** MiR-339 was reduced in dorsal horn of spinal cord in CCI rats. (A) Mechanical allodynia analysis of rats post-CCI establishment. (B) Thermal hyperalgesia analysis of rats post-CCI establishment. (C) Expression of miR-339 in dorsal horn of spinal cord in CCI rats. (D) Expressions of IL-6 and TNF-α in dorsal horn of spinal cord in CCI rats. (E) Expression of Iba1 in dorsal horn of spinal cord in CCI rats; **p < 0.01 vs. sham

**Forced miR-339 expression attenuated neuropathic pain development**

To investigate the functional role of miR-339 in neuropathic pain, rats in which CCI had been induced were injected intrathecally with miR-339 agomir. Mechanical allodynia (Figure 2 A) and thermal hyperalgesia (Figure 2 B) were reduced by injection of miR-339 agomir, compared to NC agomir, suggesting that miR-339 alleviates neuropathic pain. Rats injected with miR-339 agomir exhibited increased miR-339 expression (Figure 2 C), and decreased IL-6 and TNF-α secretion (Figure 2 D). Moreover, expression of Iba1 protein was reduced in CCI rats injected with miR-339 agomir (Figure 2 E), suggesting that forced miR-339 expression may attenuate neuropathic pain development through inhibition of the microglial inflammatory response.

**Figure 2:** Forced miR-339 expression attenuated neuropathic pain development. (A) Mechanical allodynia of rats injected intrathecally with miR-339 agomir or NC agomir. (B) Thermal hyperalgesia analysis of rats injected intrathecally with miR-339 agomir or NC agomir. (C) Expression of miR-339 in dorsal horn of spinal cord in CCI-induced rats injected intrathecally with miR-339 agomir or NC agomir. (D) Expression of IL-6 and TNF-α in dorsal horn of spinal cord in CCI-induced rats injected intrathecally with miR-339 agomir or NC agomir. (E) Expression of Iba1 in dorsal horn of spinal cord in CCI-induced rats injected intrathecally with miR-339 agomir or NC agomir. # and ## denote p < 0.05 and p < 0.01 vs. CCI + NC agomir, respectively; **p < 0.01 vs. sham

**PFKFB3 was targeted by miR-339**

PFKFB3, containing a potential binding site for miR-339, was then identified as a target gene of miR-339 (Figure 3 A). Luciferase activity of PFKFB3-WT was decreased by transfection with miR-339 mimic, compared to NC mimic (Figure 3 B). Moreover, mutation of the miR-339 binding site in PFKFB3 abolished the suppressive effect of miR-339 on luciferase activity (Figure 3 B), suggesting that miR-339 binds to PFKFB3. To further investigate regulation of PFKFB3 by miR-339, HEK293T cells were transfected with miR-339 mimic or inhibitor, and the expression of PFKFB3 was then evaluated via qRT-PCR and western blot. Results showed that miR-339 decreased mRNA (Figure 3 D) and protein (Figure 3 E) expression of PFKFB3, while transfection with miR-339 inhibitor enhanced the expression of PFKFB3 (Figure 3 D and E). These results showed that miR-339 targeted PFKFB3.
PFKFB3 was targeted by miR-339. Potential binding site between miR-339 and PFKFB3. (B) Effect of miR-339 on luciferase activities of PFKFB3-WT and PFKFB3-MUT. (C) Transfection efficiency of miR-339 mimic and inhibitor in HEK293T cells. (D) Effect of miR-339 on mRNA expression of PFKFB3. (E) Effect of miR-339 on protein expression of PFKFB3.

** Forced miR-339 expression suppressed LPS-induced inflammation in microglia cells

Primary microglial cells were isolated and induced with LPS, to evaluate the role of miR-339 on microglia inflammation in vitro. LPS treatment stimulated increased levels of IL-6 and TNF-α (Figure 4 A), while transfection with miR-339 mimic reduced LPS-induced increases in IL-6 and TNF-α (Figure 4 A), suggesting that forced miR-339 expression suppressed LPS-induced inflammation in microglia cells. Moreover, expression of PFKFB3 protein was enhanced in microglia cells after LPS treatment (Figure 4 B), but the response was reduced by miR-339 mimic transfection (Figure 4 B), indicating that miR-339 may regulate the microglial inflammatory response through targeting of PFKFB3.

** DISCUSSION

Previous research has shown that an inflammatory response in the central nervous system, modulated by activated microglia cells, results in neuronal cell death and aggravates neurodegenerative diseases, including neuropathic pain [10]. Therefore, miRNAs with the ability to regulate the microglia-mediated immune response [5] might provide a promising approach to ameliorate neuropathic pain [11].

** Figure 3: PFKFB3 was targeted by miR-339. Potential binding site between miR-339 and PFKFB3. (B) Effect of miR-339 on luciferase activities of PFKFB3-WT and PFKFB3-MUT. (C) Transfection efficiency of miR-339 mimic and inhibitor in HEK293T cells. (D) Effect of miR-339 on mRNA expression of PFKFB3. (E) Effect of miR-339 on protein expression of PFKFB3. ** ## \( p < 0.01 \) vs. NC mimic or vs. NC inhibitor, respectively

Because miR-339 suppressed inflammation in alcohol-induced microglia cells [8], favorable effects of miR-339 on the microglia inflammatory response in neuropathic pain were investigated in this study.

Ligation of the left sciatic nerve in the CCI model can lead to inflammation and swelling, thus resulting in nociceptive outcomes with decreased MWT and PWTL [12]. Therefore, CCI induction has been widely used as a neuropathic pain model. In addition to nociceptive outcomes, CCI also induces neuroinflammation with increased inflammatory cytokines, including TNF-α and IL-6 [13]. This study successfully established the CCI-induced neuropathic model, with reduced thresholds for mechanical allodynia and thermal hyperalgesia as well as increased TNF-α and IL-6. Moreover, activation of microglia cells is related to inflammation in neuropathic pain [14]. Microglia activation could induce neuronal damage during neuropathic pain through release of inflammatory cytokines [10]. The biomarker of microglia cell activation, Iba1, was also up-regulated post-CCI establishment, suggesting that CCI induction promotes microglia activation in neuropathic pain.

** Figure 4: Forced miR-339 expression suppressed LPS-induced inflammation in microglia cells. (A) Expression of IL-6 and TNF-α in LPS-induced microglia cells, with or without transfection of miR-339 mimic. (B) Protein expression of PFKFB3 in LPS-induced microglia cells, with or without transfection of miR-339 mimic. ** ## \( p < 0.01 \) vs. control or vs. LPS + NC mimic, respectively

A previous study has shown that miR-339 was reduced in dorsal root ganglion of rats following spinal nerve ligation [7]. The present study also indicated a significant decrease of miR-339 in
dorsal horn of the spinal cord in CCI rats. Functional assays further revealed that intrathecal injection of miR-339 agomir exerted anti-nociceptive and anti-inflammatory roles in CCI rats, resulting in increased MWT and PWTL, and decreased TNF-α and IL-6. Moreover, suppression of microglia activation has been regarded as a potential therapeutic strategy for neurodegenerative disease [10]. Activation of microglia cells was suppressed by intrathecal injection of miR-339 agomir, suggesting that miR-339 attenuates the microglia inflammatory response in CCI rats. An *in vitro* neuropathic pain model, established by isolated microglia cells treated with LPS [15], also showed increased inflammatory cytokines, TNF-α and IL-6. Forced miR-339 expression also decreases TNF-α and IL-6 and suppresses LPS-induced inflammation in microglia cells, confirming the anti-inflammatory role of miR-339 in microglia cells.

I-kappa B kinase was validated as a miR-339 binding target in alcohol-induced brain inflammation [8]. In this study, PFKFB3 was determined as a target gene of miR-339. Previous study has shown that LPS promotes an inflammatory state, with increased PFKFB3, in microglia cells [16]. Therefore, PFKFB3 was implicated in microglial inflammation. Data obtained in the present study showed that LPS treatment increased the PFKFB3 level in microglia cells, and promoted microglial inflammation, with enhanced TNF-α and IL-6. Over-expression of PFKFB3 has been shown to increase IL-1β and IL-6 to stimulate microglial polarization in the pro-inflammatory M1 phenotype [17]. Switching of microglial polarization from M1 to an anti-inflammatory M2 phenotype could suppress CCI-induced neuropathic pain [18]. This study showed that forced miR-339 expression decreased the LPS-induced up-regulation of PFKFB3, attenuating microglial inflammation. However, the role of miR-339 in microglial polarization should be investigated further. Furthermore, intrathecal injection of lentiviruses expressing siRNA to target PFKFB3 expression in CCI-induced rats should also be performed to elucidate the functional role of miR-339/PFKFB3 in neuropathic pain.

CONCLUSION

MiR-339 suppressed the microglial inflammatory response to ameliorate neuropathic pain through down-regulation of PFKFB3. The miR-339/PFKFB3 axis might be a promising therapeutic strategy for the treatment of neuropathic pain.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Xiaolian Fan and Renqing Zeng designed the study, supervised the data collection, and analyzed the data. Longxue Cai interpreted the data, prepared the manuscript for publication, and reviewed the draft of the manuscript. All authors have read and approved the manuscript.

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