Original Article

Downregulation of miR-206 contributes to neuropathic pain in rats by enhancing RASA1 expression

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Abstract: Neuropathic pain (NP), chronic pain resulted from dysfunction or diseases of the somatosensory nervous system, is a major challenge to currently available clinical therapy of peripheral nerve injury. Although there are significant improvements in drug and surgical therapy, the efficiency of the therapeutic approach for NP is low. And the molecular mechanisms of NP maintenance are still not clear. Herein, we investigated the novel role of miR-206 in regulating NP development in spinal nerve ligation (SNL) and chronic constriction injury (CCI) pain rats. RT-PCR analysis showed that the expression of miR-206 is aberrantly down-regulated in the dorsal root ganglion (DRG) of rats at days 7 and 14 after SNL or CCI compared to the control side. After transfection by miR-206 agomir, the paw withdrawal threshold to mechanical stimulus of SNL and CCI rats were alleviated by miR-206 overexpression, implying a role of miR-206 in NP. By bioinformatics and luciferase reporter analysis, RASA1 was proven to be a direct target of miR-206 with the binding site in 3’-UTR of mRNA. Further studies indicated that the protein level of RASA1 was increased in SNL and CCI rats, while miR-206 overexpression significantly down-regulated the expression of RASA1 protein. In contrast, the mRNA expression of RASA1 in DRG of SNL and CCI rats were unaffected by miR-206 agomir. In conclusion, we demonstrated that miR-206 represents a potential suppressor in NP development through targeting RASA1. This may provide a novel understanding of miR-206 in negatively regulating peripheral sensitization to pain threshold and offer a potential therapeutic target in treating NP.

Keywords: Neuropathic pain, miRNA-206, RASA1, SNL, CCI, PC12 cells

Introduction

Neuropathic pain (NP), chronic pain resulted from dysfunction or diseases of the somatosensory nervous system, is a major challenge to currently available clinical therapy of neurogenic pain syndromes. NP occurs in patients with peripheral nerve injury, which might be caused by diverse factors, including metabolic disorders, inflammation, accident or surgical traumas [1, 2]. Expression and functional changes of key molecules induced by primary sensory neurons injury is the main reason underlying the progression of NP [3]. Although there are significant improvements in drug and surgical therapy, the efficiency of these two therapeutic approaches for NP is low. So the knowledge about mechanisms whereby NP is developed is necessary to improve the clinical therapeutic strategies of chronic NP.

MicroRNAs (miRNAs) are 21 to 23 nucleotides long, non-coding, single-stranded RNAs that present a large class of endogenous regulators for gene expression. Most miRNAs bind to target genes with distinct degrees of complementarity, and play negatively regulatory roles in the expression of multiple target genes at post-transcriptional levels via the repression of translation or degradation of target mRNA [4, 5]. It is known that a single miRNA can bind diverse target genes and regulate multiple genes and cellular functions, including cell differentiation, growth and apoptosis. Dysregulation of miRNAs has been proven to contribute to various human diseases pathogenesis [6]. For NP, changes in microRNAs expression profiling have been reported, including miR-1, miR-16, miR-206, miR-221, miR-203, miR-21, miR-182, miR-183, miR-429 and others [7-11]. It has been reported that altered expression of microRNAs profiling is associated with the multiple regulatory mechanisms of NP, such as the pathogenesis of diabetic NP, neuronal adaptive responses in the nucleus accumbens under NP.
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and mechanical hypersensitivity induced by spinal nerve ligation [12-14]. Among the miR-NAs dysregulated in the serum and dorsal root ganglion (DRG) of rats pain models, miR-206 expression has been found to be decreased after peripheral nerve injury [15]. MiR-206 has a very wide range of physiological functions. In muscle cells, miR-206 has been well elucidated to promote skeletal muscle differentiation [16]. Furthermore, miR-206 was characterized as an potential tumor suppressor miRNA downregulated in tumorigenesis [17]. However, its accurate role in neuronal cells and the pathogenesis of NP is poorly understood.

RAS p21 protein activator (GTPase activating protein) 1 (RASA1) is an important member of Ras family, which is an crucial molecular regulator for downstream mitogen-activated protein kinases (MAPK) pathway [18]. In neurons, synapses and microglia cells, MAPKs signaling cascades, composed of RAS, RAF and ERK, regulates various physiological and pathologic processes including neural plasticity and pain [19].

In present study, we investigated the novel role of miR-206 in regulating the development of NP in rats after nerve injures. MiR-206 was found to be down-regulated in rat pain models, and negatively regulated the NP by directly targeting RASA1. Our data provided a potential role of miR-206 deregulation in the pathogenesis of NP, might represent a novel therapeutic target for analgesics.

Materials and methods

Animal models

Male Sprague-Dawley rats (6 weeks of age) were offered by the laboratory animal center of Hebei province (China). Animals were housed under a photoperiod of 14 h of light and provided food and water ad libitum. Rat NP models were conducted as described previously [20, 21]. In brief, the lumbar fifth (L5) spinal nerve of rats was exposed and tightly ligated with silk thread in two regions to obtain spinal nerve ligation (SNL) model. For chronic constriction injury (CCI) model, the sciatic nerve was loosely ligated in four regions. All experimental procedure was performed on the left side of rats, and the right side was left as control. The protocols were approved by the Experimental Animal Care and Use Committee of Cangzhou Central Hospital (China) and performed according to the guidelines of the International Association for the Study of Pain.

Cell culture

Neuronal PC12 cells were purchased from ATCC (Manassas, VA, USA) and cultured in RPMI-1640 medium with 10% fetal bovine serum (FBS, Invitrogen) at 37°C in 5% CO₂ atmosphere in incubator for 72 h. 50 ng/mL NGF-2.5 s (Millipore, MA, USA) was added into the medium, then cells were cultured for another 7 days.

RT-PCR

RNA was isolated using Trizol (Sigma). Expression of miR-206 was detected by MicroRNA First-Strand Synthesis and miRNA Quantitation kits (Takara, Dalian, China) according to the instructions. The preparation of qRT-PCR of RASA1 was performed using CellAmp Direct RNA Prep kit for qPCR and the Protein Analysis kit (Takara). Reaction was as follows: 10 min 95°C; 40 cycles of 1 min 95°C, 2 min 63°C, and 1 min 72°C; 72°C for 10 min. Ct values of U6 was used to normalize the relative expression of miR-206, and GAPDH was used to normalize the relative expression of RASA1. All PCRs were performed in triplicate.

Western blotting

Equal amounts of proteins were isolated and separated using SDS-PAGE gels (Invitrogen), then transferred electrophoretically to PVDF membrane. Antibodies (rabbit anti-RASA1 1:1000 dilution Abcam; mouse anti-β-actin 1:3000 dilution ABclonal) were reacted with blots overnight at 4°C. Secondary antibodies were then incubated with blots for 1 h at room temperature, followed by enhanced chemiluminescence (ECL, Amersham Pharmacia, NJ).

Behavioral tests

Behavioral tests were performed as described previously [22]. Briefly, rat was placed on a mesh bottom cages for 15 minutes. A von Frey filament was applied through the mesh floor to the plantar surface of the hind paw in five trials. The force inducing withdrawal of the stimulated paw was recorded. The average value was used as the latency of response.
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Transfection

For cells transfaction, 100 nM miR-206 mimic and negative control miRNA (RiboBio, Guangzhou China) were transfected into cultured PC12 cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Further, miR-206 agomir (RiboBio) was used to induce the overexpression of miR-206 in NP rat models. Before SNL and CCI, 10 nM miR-206 agomir or negative control miRNA was locally injected into dorsal root ganglion of rats once every 3 days for 2 weeks. The transfection efficacy was assessed by RT-PCR.

Luciferase reporter assay

The 3’UTR of RASA1 mRNA was cloned into the pGL3 luciferase promoter vector (Promega, Madison, WI) by PCR. Site-directed mutagenesis in the miR-206 binding site of RASA1 mRNA was performed using QuikChange Lightning Site-Directed Mutagenesis Kits (Stratagene). PC12 cells were seeded (1×10^6 cells/well) into 96-well plate and co-transfected with 200 ng pGL3 plasmids and 100 nM miR-206 mimic using Lipofectamine 2000. After 36 h, reporter assays were measured via the Dual Luciferase Assay (Promega, Madison, WI). PRL-TK vector (Promega) was used as the control.

Statistical analysis

All experiments were performed at least three times. The data were expressed as mean ± SEM. Differences between groups were measured using GraphPad Prism 5 (La Jolla, CA) by one way ANOVA. P<0.05 was considered statistically significant.

Results

MiR-206 expression is downregulated in neuropathic pain rats

To determine whether miR-206 is responsible for development of neuropathic pain, we firstly
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investigated the expression levels of miR-206 in dorsal root ganglion (DRG) of SNL and CCI model rats. At 7 days after SNL, SNL model was well established (Figure 1A). Quantitative RT-PCR analysis showed that miR-206 was aberrantly down-regulated in DRG of SNL models at days 7 and 14 after SNL compared to the control side (Figure 1B). Furthermore, we examined the expression of miR-206 in DRG of CCI models. As seen in Figure 1C, the paw withdrawal threshold to mechanical stimulus of CCI rats was significantly decreased at 7 days after CCI. Consistently, the miR-206 expression in DRG of CCI rats was restrained at low levels at days 7 and 14 compared to the control (Figure 1D). These data implied that miR-206 might represent a negative regulator in development of neuropathic pain.

**MiR-206 alleviates the neuropathic pain in rat pain models**

To further identify the potential role of miR-206 low expression in maintenance of neuropathic pain, we induced miR-206 expression specifically in DRG neurons of rats before SNL and CCI using the miR-206 agomir. The efficiency of overexpression of miR-206 was confirmed by RT-PCR. Following direct injection of agomir, the expression of miR-206 was markedly increased in DRG after 7 days, and remained increased after 21 days. No significant difference of miR-206 expression was observed in the negative control group (Figure 2A).

At days 7 after injection, rats were performed with SNL or CCI. The mechanical sensitivity threshold of SNL and CCI rats were then determined after another 7 days. As shown in Figure 2B and 2C, the paw withdrawal threshold to mechanical stimulus of SNL and CCI rats was significantly restored by miR-206 overexpression at days 10 and 14, implying a role of miR-206 in neuropathic pain.

**RASA1 is a predicted target gene of miR-206, and involved in the regulation of miR-206 to NP**

To explore the potential role of miR-206 in NP, we analyzed the possible targets of miR-206 through miRNA databases (miRDB and TargetScan). And RASA1 was found to be a target gene of miR-206 with a possible binding site in the 3'-UTR. We then performed luciferase reporter analysis in neuronal PC12 cells to identify the interaction between RASA1 and miR-206. The 3'-UTR of RASA1 containing predicted binding site of miR-206 or mutant site were cloned into the pGL3 luciferase vector (Figure 3A). The luciferase activity of RASA1-3'UTR-wt reporter in PC12 cells was suppressed by miR-206 mimic transfection, however, no inhibitory effects of miR-206 agomir on the activities of the mutant reporter was observed (Figure 3B), indicating that RASA1 is a direct target of miR-206.

Further, we investigate the expression of RASA1 in DRG of SNL and CCI rats using RT-PCR and western blotting. The protein level of RASA1 was increased in SNL and CCI rats, while miR-206 overexpression significantly down-regulated the expression of RASA1 protein (Figure 3C). In contrast, the mRNA expression of RASA1 in DRG of SNL and CCI rats were unaffected by...
MiRNA-206 targets RASA1. A. Predicted and mutant miR-206 target sequences in the 3'-UTR of RASA1. B. Luciferase assay on PC12 cells transfected with RASA1-3'UTR-wt (UTR-wt) and RASA1-3'UTR-mut (UTR-mut). C. The mRNA and protein expression of RASA1 were detected by qRT-PCR and Western blot in rats without treatment and transfection (NT) or those treated with SNL or CCI in absence (Control) or presence of miR-206 agomir and or non-targeting control miRNA (s-Mock) injection (*P<0.05 vs. NT; # P<0.05 vs. control).

Discussion

MiRNAs play regulatory roles in multiple physiological processes, and dysregulation of miRNAs contribute to diverse pathological processes. As important regulator, miR-206 has a very wide range of physiological functions. In muscle cells, miR-206 has been well characterized to promote skeletal muscle differentiation in physiological growth and muscle regeneration in response to injury [23]. Additionally, aberrant expression of miR-206 has been also observed in tumorigenesis. In gastric cancer, miR-206 was identified as an potential tumor suppressor to suppress gastric cancer cells proliferation partially by inhibiting the Cyclin D2 expression [24]. In sensory neurons, previous miRNA array analysis indicated that miR-206 is downregulated in the serum and DRG of rats with SNL surgery or CFA-induced inflammation, suggesting the potential regulatory function of miR-206 in development of NP [25, 26]. However, the distinct role of miR-206 in NP is still not clear. In present study, we reported a substantial decrease in miR-206 expression in the dorsal root ganglion (DRG) of rats with SNL injuries. CCI injury, another type of model causes representative NP symptoms, also decreased the miR-206 expression, implying that miR-206 might represent a suppressor and a potential therapeutic target in NP.

Aberrant miRNA expression has been associated to many diseases, and improvement of miRNA activity has demonstrated potential therapeutic value for diseases [27]. In nervous system diseases, miRNAs expression is temporarily and stimulus dependent. Previous studies showed that miRNA play critical roles in the neurobehavioral dysfunction caused by schizophrenia or autism, and in altering paw withdrawal thresholds induced by inflammatory pain [28, 29]. However, little was known about the accurate role of inappropriate expression of miRNAs and the therapeutic potential of modulating miRNA expression in NP. In this paper, our results show that the representative NP symbol, mechanical sensitivity threshold, was alleviated by miR-206 overexpression in SNL and CCI rats, indicating the negative role of miR-206 in development of NP caused by nerve injury.
In our study, miRNA databases predicted RASA1 to be a target of miR-206. RASA1 is an important member of Ras family of small G-protein. The Ras family is an crucial molecular regulator and mediates a large number of downstream signaling pathways, including MAPK pathway [30, 31]. In sensory neurons, MAPks signaling cascades, composed of RAS, RAF and ERK, regulates various cellular functions associated with physiological and pathologic processes, including alterations in expression and modification of target proteins. Alterations of key protein expression can cause nerve cell death and the changes of synaptic plasticity, resulting in increased sensitivity to mechanical stimuli [32, 33]. In other report, Rap1a protein, another member of the Ras family, has been proven to be also upregulated significantly and involved in the modulation of miR-203 to NP development in spinal dorsal horns of CCI rat [19]. These studies provide evidence that Ras family and the subsequent signaling pathways play critical roles in sensitization of NP. Here, we found that RASA1 is a direct target of miR-206 with the binding site on its 3'-UTR of mRNA. Further study show that the protein level of RASA1 is up-regulated in SNL and CCI rats and significantly reduced by miR-206 overexpression, while the mRNA expression of RASA1 is not affected by miR-206, suggesting that the interaction of miR-206 and RASA1 may be functionally relevant in the development of NP.

In summary, we demonstrated that miR-206 represents a potential suppressor in NP development through targeting RASA1. Our study provides a novel understanding of miR-206 in negatively regulating peripheral sensitization to pain threshold, and affords the evidence that miR-206 may be clinically useful as potential predictor or novel therapeutic target in treating NP.

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Disclosure of conflict of interest

None.

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