MiR-96-5p relieves neuropathic pain by targeting ZEB1

Fang Wang  
Huazhong University of Science and Technology

Li Wang  (kq000682@whu.edu.cn)  
Wuhan University

Research

Keywords: miR-96-5p, neuropathic pain, ZEB1, CCI rats

DOI: https://doi.org/10.21203/rs.3.rs-124601/v1

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Abstract

Background

MicroRNAs (miRNAs) gradually attract researchers’ attention in regulating the development of neuropathic pain, and many miRNAs have been reported that were able to alleviate neuropathic pain. Meantime, neuroinflammations promote the process of neuropathic pain. MiR-96-5p was associated with many pathological diseases, including many kinds of cancers. However, we knew little about the biological function of miR-96-5p in the regulation of neuropathic pain development. Hence, we focused our study on the biological function of miR-96-5p in neuropathic pain.

Results

As the result, a decrease of miR-96-5p expression was observed in CCI rats model. Meanwhile, the overexpression of miR-96-5p resulted in inhibition of inflammation-correlated biomarkers of IL-6, IL-1β and Cox-2. In addition, we predicted the zinc finger E-box binding homeobox 1 (ZEB1) was one target of miR-96-5p, with a conserved interaction site at 3’-untranslated region of ZEB1. Furthermore, we observed the increased expression of ZEB1 in CCI rats at both of transcriptional and translational level and miR-96-5p could regulate that negatively. And overexpressing ZEB1 would disrupt the miR-96-5p inducing alleviation of neuropathic pain, along with IL-6, IL-1β and Cox-2 up-regulated. Conclusions

Our study demonstrated that miR-96-5p could relieve neuropathic pain by targeting ZEB1 in vivo.

Introduction

As a result of the somatosensory nervous system affected by lesions or disease, neuropathic pain could caused by trauma, compression, autoimmune disease and diabetes, which had disrupted the quality of many patients’ live, with a population prevalence estimate of neuropathic pain ranging from 6.9%-10% (Baron, Binder, & Wasner, 2010; Campbell & Meyer, 2006; van Hecke, Austin, Khan, Smith, & Torrance, 2014). The neuropathic pain development was associated with neuronal pathways, peripheral immune system, satellite cells, Schwann cells, spinal microglia and astrocytes (Scholz & Woolf, 2007; Woolf & Mannion, 1999). However, the mechanism of neuropathic pain is still to be investigated deeply (de Moraes Vieira, Garcia, da Silva, Mualem Araujo, & Jansen, 2012). According to resent reports, there is a strong recommendation for drug proposal in neuropathic pain for pregabalin and gabapentin (Finnerup et al., 2015). But neuropathic pain remains difficult to be cure, because of which the development of effective therapeutic treatments is necessary.

MicroRNAs are approximated 20–30 nucleotide molecules, which could negatively regulate the genes expression at posttranscriptional level through binding to mRNAs (Bai, Ambalavanar, Wei, & Dessem, 2007; Carthew & Sontheimer, 2009). Along with the deepening of investigation to miRNA, it has draw our attention to the linkage between miRNAs and pain. MiRNAs modulation in pains in animal pain models has been reported widely, which indicate that miRNAs could be modulators and biomarkers of neuropathic pain (Andersen, Durox, & Gazerani, 2014; Bali & Kuner, 2014). For example, miR-103 could regulate neuron pain by targeting Cav1.2 (Favereaux et al., 2011). Intrathecal injection of miR-124 could inhibit persistent inflammatory and neuropathic pain (Willemen et al., 2012). MiR-7a alleviate neuropathic pain by regulating neuronal excitability (Sakai et al., 2013). MiR-146a-5p could target TRAF6 to suppresses neuropathic pain (Lu, Cao, Jiang, Yang, & Gao, 2015). MiR-183 contribute to neuropathic pain by mTOR/VEGF target (Xie, Ma, Xi, Zhang, & Fan, 2017). By targeting SLA17A6, miR-190a-5p attenuate diabetic neuropathic pain (Yang et al., 2017).

It has been reported that oncogenic miR-96-5p could promote the proliferations and migrations of breast cancer cells, ovarian cancer cells and cervical cancer cells and was associated with colorectal cancer, pancreatic carcinoma and hepatocellular carcinoma (Gao et al., 2018; Iwai et al., 2018; Li et al., 2014; Liu, Zhang, & Yang, 2019; Ress et al., 2015; Shao, Wang, Wang, Zhang, & Zhang, 2019). Meanwhile, miR-96-5p prevents hepatic cells activation and rescues impairment of human trophoblastic cells induced by TiO2 NP by inhibiting autophagy (Mao et al., 2018; Yu et al., 2018). Also, miR-96-5p is able to regulate wound healing through BNIP3/FAK pathway and act as a neuroprotective role via regulating glutathione level (Kinoshita et al., 2014; Wu, Cao, Zhao, & Wang, 2019). So miR-96-5p raises our interest about its role in neuropathic pains.
Here, our recent study was focused on the functions of miR-96-5p in regulating neuropathic pain. First of all, we observed a down-regulation of miR-96-5p in chronic constriction injury (CCI) rats. Meanwhile, the overexpression of miR-96-5p repressed inflammatory cytokines in our rats model. Further, we predicted that ZEB1 was one of targets of miR-96-5p, because it could be negatively regulation by miR-96-5p. As the result of overexpressing ZEB1, the alleviation of neuropathic pain by miR-96-5p was reversed. So we hypothesized miR-96-5p could relieve neuropathic pain by inhibiting ZEB1 level.

**Experimental Section**

**Animal studies**

In our study, S-D rats (adult, female, weighed 190-210 g) were purchased from Shanghai Animal Laboratory Center. Every cage kept five rats in a constant environment at 25 °C with 55% ± 5% humidity. All rats were observed to detect any abnormal behaviors every day and randomly assigned after seven days. The intrathecal administration was performed after CCI surgery for 7 days. The spinal cord of rats were collected after rats sacrificed for next experiments. The procedures of our all experiments were under the requirements of Guide for the Care and Use of Laboratory Animals by National Institutes of Health.

**CCI rats model**

The neuropathic pain rats model was established through CCI method(Bennett & Xie, 1988). Sham control groups were sciatic nerve exposed and isolated and not ligated. At days 0, 3, 7, 14, and 21, the transected and midline-cut dorsal spinal cords were harvested and stored at −80 °C for further experiments.

**Intrathecal injection**

Lidocaine (Sigma, USA) was injected for paralysis of hind limbs. LV-NC, LV-miR-96-5p and LV-ZEB1 (Genepharma, China) were injected through a intrathecal catheter linking microsyringe, 3 days before CCI surgery.

**qRT-PCR**

RNAiso Plus (Takara Bio, China) was used to extract total RNA of rats tissues. Then reverse transcription and quantitative PCR were performed through the PrimeScript™ RT Master Mix and SYBR Premix Ex Taq™ II (Takara Bio, China), respectively. The primers were shown in Table 1. All reactions were performed in the QuantStudio6 Flex (Fujifilm, Japan).

| Genes    | Forward(5'-3')                     | Reverse(5'-3')                      |
|----------|------------------------------------|-------------------------------------|
| miR-96-5p| ACACTCCAGCTGGTTTGGCACTAGCACATT    | CTCAACTGCTGTCGGAGTCGGGAATTCCAGTGGAGACAAAA |
| ZEB1     | GCCAATAAGCAACAGATTTCTTG           | TTTGGCTGGATCACHTTTCAAG              |
| GAPDH    | GATGCTGGTGCTGATGATGRC             | GTGGTGCGAGATGCATTGCTGGA             |
| U6       | CTCGCTTCCGACGAC                 | AACGCTTCCAGAATTTCG                 |

**Behavioral tests**

Paw withdrawal threshold (PWT) was performed to assesses mechanical allodynia. Each hind paw of rats was exposed to the stimuli created by Electronic von Frey filament (IITC, USA). The time span between the onset of stimulation and the withdrawal of paws was recorded. Paw withdrawal latency (PWL) was employed to evaluate thermal hyperalgesia. The time of paws withdrawal responding to heat was recorded.

**ELISA assay**

ELISA kits (Abcam, UK) were used to examine the level of IL-6, IL-1β and Cox-2.
Cell culture
HEK-293T cell line was purchased from the American Type Culture Collection. DMEM medium (Gibco, USA), adding 10% heat inactivated FBS (Gibco, USA), was used to culture cells.

Luciferase activity assay
The wildtype and mutant ZEB1 3′-UTR were subcloned into the pGL3 Luciferase Reporter Vector (Promega, USA), respectively. After co-transfecting the miR-96-5p mimics (RiboBio, China) with WT-ZEB1 or MUT-ZEB1, Promega Dual-Luciferase™ Reporter (DLR™) Assay Systems (Promega, USA) was used.

Western blot analysis
Proteins were separated by 10% SDS-PAGE and transferred onto PVDF membranes. Then 5% defatted milk was used to block membranes. Primary antibodies [anti-ZEB1 and anti-GAPDH (Abcam, UK)] and the secondary antibodies (Abcam, UK) were incubated in succession. Pierce ECL Plus (Thermo Scientific, USA) was used.

Statistical analysis
With at least three independent experiments, all data were exhibited as the form of mean ± standard deviation. Student’s t-test and One-Way ANOVA analysis were employed to compare quantitative variables. When the P values were less than 0.05, the differences were significant. The SPSS 19.0 (SPSS Inc, USA) was used.

Results

MiR-96-5p was decreased in CCI rats
To investigate whether miR-96-5p possesses biological functions which could regulate neuropathic pain, we firstly examined the levels of miR-96-5p in the spinal cord of CCI rats through qRT-PCR. The levels of miR-96-5p in CCI rats was significantly decreased compared to the expression in sham-operated rats, after CCI surgery for 0, 3, 7, 14 and 21 days (Fig. 1A). Then, we made miR-96-5p stably overexpress in CCI rats through intrathecal injection of LV-miR-96-5p and the up-regulating expression was observed in LV-miR-96-5p infecting CCI rats (Fig. 1B). Next, the relationship between miR-96-5p and neuropathic pain was measured by evaluating mechanical allodynia and thermal hyperalgesia. Both mechanical allodynia (Fig. 1C) and thermal hyperalgesia (Fig. 1D) were restrained in CCI rats after overexpressing miR-96-5p, which implied that miR-96-5p acted as a repressive role in regulation neuropathic pain.

Overexpressed miR-96-5p inhibited neuroinflammations in vivo
Neuroinflammations promote neuropathic pain, so we examined the expression levels of neuroinflammation-associated cytokines in CCI rats and miR-96-5p overexpressed CCI rats to figure out the effect of miR-96-5p to neuropathic pain. ELISA was employed to detect the expression level of common inflammation-associated cytokines IL-6, IL-1β and Cox-2. As exhibited, the inflammatory cytokines increased significantly after CCI surgery and the expression of IL-6 (Fig. 2A), IL-1β (Fig. 2B) and Cox-2 (Fig. 2C) were down-regulated after miR-96-5p overexpressed, which implied that miR-96-5p could inhibit inflammations in CCI rats.

ZEB1 was a direct binding target of miR-96-5p
Further, we employed Starbase, TargetScan, miRDB, and miRanda to predict potential binding targets of miR-96-5p. According to the results of bioinformatic prediction, it was found that a binding region of miR-96-5p was at ZEB1 3′UTR (Fig. 3A). Then we employed dual-luciferase report system to detect the direct binding of miR-96-5p and ZEB1. The wild type (WT) or mutant (MUT) ZEB1 3′UTR was constructed into a dual-luciferase vector and then co-transfected into HEK-293T cells with miR-96-5p mimics. The reduction of luciferase activity indicated the binding of miR-96-5p and WT-ZEB1 mRNA (Fig. 3B). Meanwhile, we
examined the expression of ZEB1 in vivo. A significant decrease of ZEB1 was observed after overexpressing miR-96-5p in CCI rats through qRT-PCR (Fig. 3C). In addition, the protein level of ZEB1 was also detected and the down-regulation of ZEB1 protein was observed after miR-96-5p overexpressed (Fig. 3D,E). All of these indicated that miR-96-5p could bind to ZEB1 mRNA and repress the expression of ZEB1 directly.

### ZEB1 reversed the alleviating effect of miR-96-5p

To figure out if miR-96-5p alleviated neuropathic pain through inhibiting ZEB1, a rescue experiment of co-infecting CCI rats of LV-miR-96-5p and LV-ZEB1 was performed. After co-infecting of LV-ZEB1, both of transcriptional (Fig. 4A) and translational level (Fig. 4B,C) of ZEB1 were significantly up-regulated in LV-miR-96-5p infecting CCI rats. Then, as indicated in Fig. 4D-F, the miR-96-5p inducing down-regulation of IL-6, IL-1β and Cox-2 was reversed by ZEB1 overexpression, suggesting that miR-96-5p relieves neuropathic pain by inhibiting ZEB1 in vivo.

### Discussions

In two decades years, miRNAs had raised people's attention, including their biological functions in the development of neuropathic pains (Andersen et al., 2014; Bai et al., 2007; Lu et al., 2015; Willemen et al., 2012; Xie et al., 2017; Yang et al., 2017). Many miRNAs have been reported could regulate neuropathic pains through different pathways. We investigated that miR-96-5p could alleviate neuropathic pains by targeting ZEB1, along with the down-regulation of inflammation-associated cytokines. Through establishing CCI rats model, we observed the decrease of miR-96-5p expression and down-regulation of neuroinflammation-associated cytokines in CCI rats. Then, through employing bioinformatic predictions, ZEB1 was found possessed a binding site of miR-96-5p. Overexpressing miR-96-5p in CCI rats could inhibit ZEB1 by targeting its 3’-UTR conserved binding region. The direct interaction of miR-96-5p and ZEB1 3’-UTR was verified, and miR-96-5p mimics could significant repress ZEB1. Moreover, we observed an up-regulation of ZEB1 in CCI rats and it would be neutralized by miR-96-5p overexpressing. Also, anaplerotic expression of ZEB1 in miR-96-5p overexpressing CCI rats would reverse the alleviating effect miR-96-5p. Taken together, miR-96-5p regulated neuropathic pain through suppressing ZEB1 in vivo. In addition, with more and more reports, lncRNA may play a role in regulating miRNAs such as molecular sponges (Gao et al., 2018), which points a direction for our further study of miR-96-5p.

It has been reported that ZEB1 was associated with many kinds of cancers including breast cancer, renal clear cell carcinoma, and prostate carcinoma (Graham et al., 2008; Guaita et al., 2002; Krishnamachary et al., 2006; Ma et al., 2016). Meantime, there were some reports that miRNAs was able to be regulators in some diseases by targeting ZEB1, such as miR-409-3p, miR-204, miR-708, miR-873, miR-28-5p and miR-429 (Bao et al., 2018; Jiao, Guo, & Fu, 2019; Luo et al., 2019; Ma et al., 2016; Sun et al., 2019; G. C. Wu et al., 2019). In this study, it was observed that the ZEB1 was up-regulated at both transcriptional and translational level in CCI rats. In addition, ZEB1 was one of binding targets of miR-96-5p in regulating neuropathic pain. MiR-96-5p could inhibit ZEB1, and overexpression of ZEB1 could reverse the alleviating effect of miR-96-5p. In conclusion, miR-96-5p alleviate neuropathic pain through down-regulating ZEB1 in vivo, and depress the neuroinflammations at the same time, which indicated miR-96-5p possesses the potential for clinical therapeutic of neuropathic pain.

### Abbreviations

CCI : chronic constriction injury;

### Declarations

**Authors’ contribution**

LW designed the study. FW performed the experiments. FW and LW performed the analysis. FW prepared the manuscript draft. LW revised the manuscript. All authors approved the final manuscript.
Acknowledgements
None.

Funding
None.

Conflicts of Interest
The authors declare that they have no competing interests.

Availability of data and materials
All data generated or analyzed during this study are included in this published article.

Consent for publication
Not applicable.

Ethics approval
This study was approved by the ethics committee at the State Key Laboratory Breeding Base of Basic Science of Stomatology (Hubei-MOST)& Key Laboratory of Oral Biomedicine Ministry of Education, School & Hospital of Stomatology, Wuhan University.

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