miR-590-3p Alleviates diabetic peripheral neuropathic pain by targeting RAP1A and suppressing infiltration by the T cells

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Background: MicroRNAs play a crucial role in diabetic peripheral neuropathic pain (DPNP). miR-590-3p is a novel miRNA and involved in multiple diseases. However, the pathological mechanism of miR-590-3p in DPNP needs to be elucidated. Materials and methods: The db/db mice and db/m mice were selected to mimic diabetes and control, respectively, for in vivo studies. The miR-590-3p agomir was injected into db/db mice and pain-related behavioral tests were performed. The interaction of miR-590-3p with target gene was confirmed by dual-luciferase reporter assay. The expression of target gene was determined by qRT-PCR and western blot assay. The levels of inflammatory cytokines were measured by enzyme-linked immunosorbent assay (ELISA). Results: miR-590-3p was down-regulated in diabetic peripheral neuropathy mice. More importantly, miR-590-3p agomir alleviated pain-related behavior, reduced TNF-α, IL-1β, and IL-6 concentrations, and inhibited neural infiltration by immune cells in db/db mice. Interestingly, RAP1A was predicted to be the target of miR-590-3p by Targetscan, and was actually regulated by miR-590-3p. Finally, the rescue experiments proved that overexpression of RAP1A partially abrogated the suppressive impact of miR-590-3p on T cells proliferation and migration. Conclusion: miR-590-3p ameliorates DPNP via targeting RAP1A and inhibiting T cells infiltration, indicating that exogenous miR-590-3p may be a potential candidate for clinical treatment of DPNP.

Key words: miR-590-3p, diabetic peripheral neuropathic pain, pro-inflammatory cytokines, T cell

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Abbreviations: DPN, diabetic peripheral neuropathic pain; DRG, dorsal root ganglion; PN, diabetic peripheral neuralgia; ELISA, enzyme-linked immunosorbent assay; IHC, immunohistochemistry; IL-1β, interleukin-1 beta; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TBST, tris buffered saline with Tween; TNF-α, tumor necrosis factor alpha

INTRODUCTION

Diabetes has a high prevalence rate and has become a common chronic disease all over the world. It also induces a complication — diabetic neuropathy, which causes diabetic peripheral neuropathic pain (DPNP) (Davies et al., 2006). Diabetic patients with DPNP experience limb numbness, spontaneous pain, hyperalgesia, or allodynia (Calcutt 2004). Given that diabetic peripheral neuralgia (DPN) is a typical chronic neuropathic pain, it is difficult to treat, affects the physiological functions and quality of life of the patients, and brings a heavy burden to individuals and society (Boulton et al., 2004). The pathogenesis of DPNP is complex, including inflammation, repair process, and gene expression (Vanotti et al., 2007). Although the diverse treatment strategies have advanced, DPN is still difficult to cure. Therefore, the pathogenesis of DPNP should be further clarified and new treatment strategies should be developed.

MicroRNA (miR), is a non-coding RNA, which generally has a length of 18–22-nucleotides. MiRs can bind to the 3’-UTR of the target gene to modulate the gene expression (Wu et al., 2013). Currently, the expression of miRs has been proved to be closely related to DPN. miR-146a influences the severity of DPN through the regulation of inflammation (Feng et al., 2018). Moreover, knockdown of miR-25 can significantly aggravate diabetic peripheral neuralgia via the production of the reactive oxygen species (Zhang et al., 2018). Overexpression of miR-146a can suppress hyperglycemia-induced proinflammatory genes and alleviate diabetic peripheral neuralgia (Liu et al., 2017). Moreover, miR-590-3p is a novel miRNA involved in multiple diseases. The expression of miR-590-3p is reduced in Alzheimer’s disease, suggesting that it may be related to neural degradation (Villa et al., 2011). More importantly, miR-590-3p is down-regulated in DPN model (Gong et al., 2015). However, the specific role and mechanism of miR-590-3p in DPN remain unknown.

RAP1A (Ras-associated protein 1A) is a member of the Ras-like GTPases family and plays a vital role in the cell-matrix and cadherin-mediated cell-cell contacts (Duchnie-wicz et al., 2006). In addition, RAP1A regulates T cells via augmenting lymphocyte responses and activating integrins (Sebzda et al., 2002). Moreover, inhibition of RAP1A alleviates neuropathic pain (Li et al., 2015; Fang et al., 2019). Also, accumulating evidence has proved that pro-inflammatory T cells migrate into the spinal cord in several pain models, and the immune cell infiltration is involved in diabetic neuropathy (Costigan et al., 2009; Grace et al., 2011; Agarwal et al., 2018). Given all these results, RAP1A might be related to the pathogenesis of DPNP.

The aim of our study was to elucidate the role of miR-590-3p in DPNP. We found that miR-590-3p was downregulated in db/db mice (type 2 diabetes model). Overexpression of miR-590-3p alleviated DPNP in db/db mice by targeting RAP1A, providing strong evidence for the role of altered miR-590-3p/RAP1A axis in the development of DPNP.

MATERIALS AND METHODS

Animals. All animal experiments were performed in accordance with the Guide for the Care and Use of
The collected DRG pleons from 3RAP1A. The pain-related behavior tests were performed. Before the end of the animal experiments, the blood glucose in one drop of tail blood and body weight were determined with a blood glucose monitoring system (Bayer, Germany) and electronic balance (Meitele, Switzerland), respectively. Finally, the dorsal root ganglion (DRG) tissues were extracted, frozen and stored for further experiments.

**Isolation of T cells and transfection.** Pleons from db/m mice were collected and disaggregated, and then the erythrocytes were lysed with Lysing Buffer (BD Biosciences, USA). The T cells were isolated using CD4+ T cell Isolation Kit (Miltenyi Biotec, Germany). Next, the T cells were cultured and stimulated with 0.5 μg/ml anti-CD3e (BD Biosciences, USA) at 37°C. After transfection, T cells were transfected with pcDNA3 or pcDNA3-RAP1A (GenePharma, China) and miR-590-3p mimic or NC mimic (GenePharma, China) using Lipoctamine 2000 (Invitrogen, USA). The sequences for NC mimic were: 5'-UUCUCCGAACGUGUCACGU-3' and for miR-590-3p mimic: 5'-UAAUUUAUGUAUAAAGCUAGU-3'.

**Pain-related behavioral tests.** The pain-related behavioral tests were performed as follows: the mice were placed on a hot plate (approx. 55°C), and then the reaction time (latency of the first recoil or struggle of the rear paw) was recorded. To avoid skin damage, we applied a cutoff time of 10 seconds. The shortened withdrawal latency indicated hyperalgesia (Chopra et al., 2010). In addition, each mouse’s tail-swelling delay was determined by dipping the distal end of the tail 2–3 cm into a vessel filled with cold water (10±1°C). The data on the duration of the struggle, tail-swinging reaction, or tail-swinging were recorded. The threshold value was set at 15 seconds. A shorter soaking time indicated hyperalgesia which is also attributed to a central mechanism (Kamboj et al., 2010).

**qRT-PCR assay.** The total RNA from DRG tissues or T cells was extracted using TRIzol reagent (Takara, Japan), and cDNA was synthesized using PrimeScript™ RT Master Mix (Takara, Japan). The expression of the target gene was evaluated using SYBR Green PCR Master Mix (Takara, Japan) based on 2ΔΔCT method. U6 or β-actin was used as an endogenous control for the normalization. The primer sequences were as follows: miR-590-3p forward: 5'-AAAGATTCGAGAAGCTTAAGGGTG-3' and reverse: 5'-CTAATCCTTTTGGTTCCGTGCTCTGGCTA-3'; U6 gene forward: 5'-GCTGCGCCAGCAGATATAAA-3' and U6 gene reverse: 5'-CCGGTTCAATTGTTGCGGTCA-3'; RAP1A forward: 5'-TTGCTGCATCTGCACTTCA-3' and RAP1A reverse: 5'-GACTTTCAAGCTGTTCAAT-3'; β-actin gene forward: 5'-TACCCACACTTGCCCATCTACA-3' and β-actin gene reverse: 5'-CAGCCGAACCGCTCATTGCCAATGG-3'.

**Western blot.** The cells or DRG tissues were lysed with RIPA lysis buffer (Beyotime, China) to isolate the proteins, and the protein concentration was determined using a BCA kit (Beyotime, China). The equal amount of protein was separated by SDS-PAGE and transferred onto a PVDF membrane. Then the membranes were washed with TBST (Tris Buffered Saline with Tween) buffer three times and incubated with anti-RAP1A antibody (1:800; Abcam, UK) or anti-β-actin antibody (1:800; Abcam, UK) overnight at 4°C. After washed with TBST buffer for three times, the membranes were then incubated with Goat Anti-Rabbit IgG H&L (HRP) (1:800; Abcam, UK) or Goat Anti-Mouse IgG H&L (HRP) (1:800; Abcam, UK) for 2 h. Finally, an electrochemiluminescence kit (Beyotime, China) was used to measure the corresponding protein expression levels.

**Luciferase reporter assay.** The 3'-UTR of RAP1A and the potential target sequences of miR-590-3p were predicted by Targetscan (http://www.targetscan.org) and inserted into pGL3 plasmids (Promega, USA). When isolated T cells grew to about 80% confluence, they were co-transfected with pGL3-RAP1A-WT vectors (RAP1A WT) or pGL3-RAP1A-MUT vectors (RAP1A MUT) along with miR-590-3p mimic or NC mimic (GenePharma, China) using Lipofectamine 2000 (Invitrogen, USA). After 24 hours, the luciferase activity was evaluated.

**ELISA assay.** The levels of IL-1β, TNF-α and IL-6 in DRG tissues were evaluated by ELISA. After transfection, T cells were stimulated with 0.5 μg/ml anti-CD3e (BD Biosciences, USA) in RPMI-1640 medium (Gibco, USA) containing 10% fetal bovine serum (FBS; Gibco, USA) and 1% penicillin-streptomycin (Gibco, USA) at 37°C. The T cell supernatant was collected, and IL-1β and TNF-α in the sample supernatant was determined with IL-1β, IL-6, IL-12 and TNF-α ELISA kit (Elabscience, China), respectively. Each measurement was repeated three times.

**Immunohistochemistry (IHC).** The collected DRG tissues were subjected to IHC staining. After fixation with 10% parafomaldehyde, the samples were sliced horizontally, blocked with goat serum (Jackson ImmunoResearch, USA), and probed with anti-Cd4 antibody (1:200; Abcam, UK). DAB and hematoxylin were used to stain the tissue slices. RAP1A staining was visualized using a light microscope (Olympus, Japan).

**Cell Counting Kit-8 (CCK-8) assay.** For CCK-8 assay, after transfection, T cells were seeded into 96-well plates at the density of 3×10³ cells/well. After cultured for 48 h, the cell proliferation was detected using Cell Counting Kit-8 (CCK-8) assay (Beyotime, China). Finally, the absorbance at the 450 nm was determined.

**Transwell assay.** After transfection, T cells were seeded onto the upper chamber of a Transwell plate (filters diameter: 6.5 mm, pore size: 5 μm; Corning, USA). The lower chamber was filled with medium containing sphingosine-1-phosphate (SIP; Sigma, USA). The lower chamber was harvested and counted under a light microscope (Olympus, Japan).

**Data statistics.** The data in this study were analyzed with SPSS 20.0 (SPSS, USA) and presented as mean and Standard Deviation (S.D.). The Student’s t-test or one-way analysis of variance was performed for comparison between two groups or more than two groups, respectively. P<0.05 was considered to indicate a significant difference.

**RESULTS**

miR-590-3p was down-regulated in diabetic peripheral neuropathy mice

Compared to db/m mice, the blood glucose in db/db mice was markedly elevated, suggesting the suc-
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Successful establishment of diabetes models (Fig. 1A). Besides, compared to db/m mice, the body weight was higher in db/db mice. For pain-related behavior test, the hind paw reaction time and tail withdrawal latency were lower in db/db mice compared to those in db/m mice, suggesting that db/db mice were in hyperalgesia and suffered from diabetic neuropathy pain (Fig. 1B). Interestingly, compared to db/m mice, the db/db mice exhibited a reduction of miR-590-3p levels in DRG tissue (Fig. 1C). These results confirmed that diabetic peripheral neuropathy mice models were successfully established and miR-590-3p was down-regulated in diabetic peripheral neuropathy mice.

To further clarify how miR-590-3p alleviates peripheral neuropathic pain in diabetic mice, the 20-week-old db/db mice were injected with miR-590-3p agomir or NC agomir (10 mg/kg) weekly for 4 weeks. Compared to db/m mice, miR-590-3p level in db/db mice was significantly declined, while miR-590-3p agomir markedly promoted miR-590-3p expression compared to NC agomir (Fig. 2A). Moreover, blood glucose and body weight were significantly elevated in db/db mice, whereas miR-590-3p agomir did not influence blood glucose and body weight (Fig. 2B). As for pain-related behavior of mice, the hind paw reaction time and tail withdrawal latency were increased by miR-590-3p agomir, indicating that miR-590-3p agomir alleviated diabetic neuropathy pain (Fig. 2C). Thus, these findings proved that miR-590-3p decreased peripheral neuropathic pain in vivo.

miR-590-3p alleviated peripheral neuropathic pain in diabetic mice

An excessive amount of blood glucose stimulates the production of pro-inflammatory cytokines (including IL-6, IL-1β and TNF-α) and directs the cells toward inflammation (Rains & Jain 2011). When compared to the control, the levels of IL-6, IL-1β, and TNF-α in db/db mice were increased, however, they were significantly reduced by miR-590-3p agomir (Fig. 3A). Accumulating evidence has shown a significant neural infiltration by the immune cells in DPNP patients (Younger et al., 1996; Alexandraki et al., 2006). The infiltration by the immune cells was determined by IHC staining of CD4. The expression of CD4 in DRG tissue of db/db mice was elevated (Fig. 3B). After the injection of miR-590-3p agomir into db/db mice, the expression of CD4 was reduced (Fig. 3B). These findings proved that exogenous miR-590-3p inhibited the production of pro-inflammatory mediators and neural infiltration by immune cells in db/db mice.
Next, the potential target of miR-590-3p was identified. RAP1A, a vital protein, was predicted to be a validated target of miR-590-3p by Targetscan. As illustrated in Fig. 4A, miR-590-3p binds to a conserved site of RAP1A 3'-UTR. The luciferase activity assay confirmed that miR-590-3p mimic markedly decreased the luciferase activity of RAP1A 3'-UTR WT, but not RAP1A 3'-UTR MUT (Fig. 4B). Moreover, miR-590-3p mimic or miR-590-3p inhibitor significantly increased or decreased miR-590-3p expression in T cells (Fig. 4C), respectively. Interestingly,
RAP1A
β-actin

Figure 5. miR-590-3p inhibited T cells proliferation and migration via targeting RAP1A

DISCUSSION

In the current study, the diabetic peripheral neuropathy mice models were successfully established and we found that miR-590-3p was down-regulated in diabetic peripheral neuropathy mice. Moreover, miR-590-3p agonist reduced the production of pro-inflammatory mediators and neural infiltration by the immune cells in db/db mice. Interestingly, RAP1A was predicted to be the direct target of miR-590-3p by Targetscan, and miR-590-3p regulated the expression of RAP1A. Finally, the rescue experiments proved that overexpression of RAP1A partially rescued the suppressive effects of miR-590-3p on T cells proliferation and migration. Together, these findings suggested that exogenous miR-590-3p may be beneficial to the clinical treatment of DPNP.

Neuropoietic cytokines (such as TNF-α, IL-6, and IL-1β) are imperative to keep homeostasis of peripheral neurons (Skundric & Lisak 2003). Previous reports demonstrated that IL-1β, TNF-α and IL-6 levels are elevated in diabetic patients and animals compared to the controls (Abdel Aziz et al., 2001; Lee et al., 2013; Cox et al., 2017). In this study, we demonstrated that miR-590-3p agomir reduced the IL-1β, TNF-α and IL-6 concentrations, and alleviated DPN pain-related behavior in db/db mice, indicating that miR-590-3p suppressed DPNP via an anti-inflammatory function. More importantly, diabetes-induced inflammation occurs with inflammatory infiltrations (Thaietthawatkul et al., 2018). Regarding this,
we demonstrated that miR-590-3p inhibited neural infiltration by T cells. Besides, under hyperglycemia, TNF-α production aggravates in neural tissues, thereby inducing nerve damage, and finally resulting in the development of diabetic polyneuropathy (Satoh et al., 2003). Many central nervous system paradigms proved that IL-6 induces axonal regeneration, which may be related to immune cells proliferation and migration (Leibinger et al., 2013; Carmel et al., 2015). Thus, we suspected that miR-590-3p affects the T cells migration and proliferation, which needed more experiment in the next step.

_RAP1A_ is confirmed to be a target of a series of miRNAs and is involved in cell migration and proliferation in non-small cell lung cancer, breast cancer, cervical cancer and prostate cancer (Xiang et al., 2015; Zhang et al., 2018; Cao 2019; Lu et al., 2020). In this study, _RAP1A_ was proved to be a direct target of miR-590-3p. As we suspected, miR-590-3p inhibited T cells proliferation and migration in DPNP, which was reversed by over-expression of RAP1A. These findings suggested that miR-590-3p inhibited T cells proliferation and migration via targeting RAP1A in DPNP, which was consistent with the previous research. A recent study pointed out that lncMIR205HG acts as a natural decoy for miR-590-3p and leads to the process of head and neck squamous cell carcinoma (Di Agostino et al., 2018). Besides, RAP1A contributes to cell migration via regulating the MAPK/ERK pathway (Fujita et al., 2005; Zhang et al., 2018). Given all these reports, we hypothesized that miR-590-3p may target miR-590-3p and activate the MAPK/ERK pathway in DPNP. However, this hypothesis needs more experiments to confirm it in the future.

In summary, our data demonstrated for the first time that miR-590-3p inhibited the production of pro-inflammatory mediators and neural infiltration by T cells in DPNP. Moreover, additional _in vitro_ studies indicated that miR-590-3p inhibited T cells proliferation and migration by targeting RAP1A. Our findings provide a novel insight into the molecular mechanism and molecular basis of treating DPN with exogenous miR-590-3p. Although the treatment strategy of exogenous miR-590-3p can be implemented, the interaction network of the miR-590-3p in diabetes remains to be elucidated.

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Competing interests

The authors state that there are no conflicts of interest to disclose.

Ethics approval

All animal experiments were in accordance with the Guide for the Care and Use of Laboratory Animals and were approved by the Ethics Committee of the First Affiliated Hospital of Soochow University.

Statement of Informed Consent

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors’ contributions

Yihua Wu and Ye Gu designed the study, supervised the data collection, analyzed the data, Bimin Shi interpreted the data and prepared the manuscript for publication, supervised the data collection, analyzed the data and reviewed the draft of the manuscript. All authors have read and approved the manuscript.

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