

The role of miR-190a-5p contributes to diabetic neuropathic pain via targeting SLC17A6

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Introduction: MicroRNAs play a key role in neuropathic pain. In a previous study, miR-190a-5p was significantly downregulated in diabetic neuropathic pain (DNP). However, the role and pathological mechanism of miR-190a-5p in DNP still remain unclear.

Materials and methods: DNP model was established. The paw withdrawal thresholds were measured to assess the mechanical nociceptive response. Dual-luciferase reporter assay was used to confirm the target gene of microRNA. The expressions of microRNA, gene, and protein were detected by the quantitative real-time polymerase chain reaction or Western blot. The levels of IL-1 β and IL-6 were detected with the enzyme-linked immuno sorbent assay.

Results: Compared with the control sample, the expression of miR-190a-5p was decreased and SLC17A6 was increased in the spinal tissue from those developing DNP. The bioinformatics and luciferase reporter assay demonstrated that SLC17A6 is a direct target of miR-190a-5p. Up-regulation of miR-190a-5p and inhibition of SLC17A6 could significantly weaken the painful behavior and reduce IL-1 β and IL-6 level in DNP.

Conclusion: miR-190a-5p is involved in DNP via targeting SLC17A6, and miR-190a-5p and SLC17A6 may be the therapeutic targets of this disease.

Keywords: miR-190a-5p, DNP, spinal tissue, painful behavior, IL-1 β and IL-6, SLC17A6

Background

Diabetes neuropathy is the floorboard of the multiple lesions in the nervous system and is one of the most common, complex, and serious complications for diabetic patients, which is caused by the status of chronic high blood glucose.¹⁻³ It is involved in any part of whole-body peripheral nervous system, including sensory nerve, motor nerve, and autonomic nerve, characterized by physical pain, hypoesthesia, hot and cold numbness, and spontaneous pain, and seriously affects the life quality of these patients.^{2,4,5} Nowadays, diabetic neuropathy is considered to be a very painful condition and urgent treatment is required.^{6,7} Therefore, it is important to understand the mechanisms underlying diabetic neuropathic pain (DNP) and provide a new strategy to treat this disorder.

MicroRNAs are a kind of small non-coding RNA, which are involved in the post-transcriptional regulation of gene expression by binding the translation section leading to either mRNA degradation or translational inhibition and have been found to regulate crucial biological processes, including proliferation, differentiation, and apoptosis.^{8,9} Emerging evidence has shown that some microRNAs are implicated in the gene regulation of neural plasticity, pathological nerve pain, and pain sensitization.¹⁰⁻¹² Recently,

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some studies have shown that abnormal microRNAs are present in those patients with painful disorders, such as complex regional pain syndrome, osteoarthritis, and fibromyalgia.^{13–15} In addition, abnormal microRNAs have been reported to be involved in the progress of pain generation and maintenance.¹⁶

In a previous experiment, Gong et al showed that 21 microRNAs were significantly upregulated and other 21 microRNAs were downregulated in diabetes neuropathy through microRNA microarrays, and miR-190a-5p was validated to be the most significantly downregulated in diabetes neuropathy.¹⁷ However, the pathological mechanism of this microRNA under diabetes neuropathy still remains unclear. Therefore, in this study, the roles and mechanisms of this microRNA in diabetes neuropathy were investigated, and the results may provide a new strategy of treating this disease.

Materials and methods

Animals

Male Balb/c mice (8 weeks) were purchased from the Medical Research Center of Sichuan Academy of Medical Sciences & Sichuan Provincial People's Hospital. They were housed in separate cages at constant room temperature, under a 12:12 light–dark cycle, with optional food and water. The animals were kept to acclimate for 1 week before the experiment. Test evaluations were performed between 8:00 AM and 6:00 PM. All experiments were approved by the animal care committee of Sichuan Academy of Medical Sciences & Sichuan Provincial People's Hospital and were carried out according to the guidelines of the National Institutes of Health on animal care, and the protocol was approved by the ethics committee of Sichuan Academy of Medical Sciences & Sichuan Provincial People's Hospital.

DNP model

DNP model was established as described in a previous study.¹⁷ Briefly, diabetes model was induced under a single intraperitoneal injection of streptozocin (STZ) (Sigma, St Louis, MO, USA) at a dose of 200 mg/kg body weight after an overnight fast.¹⁸ STZ was dissolved with fresh citrate buffer (0.1 N; pH 4.5) before injection. Control mice were only injected with citrate buffer without STZ. After 6 weeks, if the blood glucose levels of the mice were >300 mg/dL, they were considered to be diabetic and were allowed for the development of neuropathic changes.

Assessment of mechanical nociceptive responses

The up-down method was used to assess the mechanical sensitivity, which was described previously.^{17,19,20} Briefly, the von

Frey filament with logarithmically incremental stiffness was used to measure the paw withdrawal thresholds, 0.04–2.04 g (0.04, 0.07, 0.16, 0.4, 0.60, 1.0, 1.4, 2.04 g); the mid-value of the series was 0.4 g, which was applied first. The animals were firstly adapted for 1 week in the test room, then, the corresponding stimulation method was evaluated in advance for 30 minutes before the formal testing. If paw lifting, licking, and shaking appeared within 5 seconds of the application of the filament for the mouse, it was regarded as a positive response. Then, a thin filament was applied. A significant reduction in the paw withdrawal threshold indicated a tactile allodynia. This method was first performed 3 times before STZ injection to establish the basal pain level. Then, for the next few weeks, mechanical nociceptive response was assessed once per week.

RNA isolation and quantitative real-time polymerase chain reaction (qRT-PCR)

The lumbar spinal dorsal horn was separated from mice, and total RNA from tissues was isolated with the RNeasy plus mini kit (Qiagen, Hilden, Germany), according to the manufacturer's protocols. Total RNA concentrations were detected by the NanoVue plus (GE Healthcare, Pittsburgh, PA, USA) for RNA concentrations. About 5 μ L RNA was added into 15 μ L reverse transcribed (RT) reaction to generate complementary DNA (cDNA) by the PrimeScript RT reagent Kit (TaKaRa, Dalian, China) according to the manufacturer's protocols. MicroRNAs were measured with the TaqMan miRNA assays, and U6 was used as an internal control (at 95°C for 10 minutes, followed by 95°C for 15 seconds [40 cycles] and 60°C for 1 minute [40 cycles]). The primers of the relative gene were as follows: SLC17A6: 5'-gcgaggcacaagttatcaag-3' (forward), 5'-cctggaatctgggtgatgat-3' (reverse). The expression of microRNAs or gene was evaluated based on the threshold cycle (Cq) as $n=2^{-\Delta\Delta Cq}$, where $\Delta Cq=Cq$ related microRNA–Cq U6 and $\Delta\Delta Cq=\Delta Cq$ experimental– ΔCq control.

Prediction of the target gene

miRanda, TargetScan, and PicTar databases were used to predict the potential target genes of miR-190a-5p. Among those target genes, solute carrier family 17 (sodium-dependent inorganic phosphate cotransporter), member 6 (SLC17A6) had a binding site in the 3'-untranslated regions (UTR) of miR-190a-5p.

Dual-luciferase reporter assay

Luciferase reporter vector was purchased from Saierbio (Tianjin, China), HEK293T cells were purchased from the Institute of Biochemistry and Cell Biology (Chinese Academy of Sciences, Shanghai, China), and the QuickChange

Lightning kit (Stratagene, Shanghai, China) was used for site-directed mutagenesis. Expressions of miR-190a-5p plasmid, SLC17A6 wild-type (WT), and mutated (Mut) 3'-UTR luciferase reporter were co-transfected into HEK293T cells. Renilla luciferase (Promega, Shanghai, China) served as control. The luciferase signal was measured with the Dual-Luciferase Reporter Assay System (Promega).

Western blot

The lumbar spinal dorsal horn was homogenized in a urea protein lysis buffer. Lysis buffer was used to extract whole cell lysates for Western blotting, and protein assay kit (Beyotime, Shanghai, China) was used to determine the total protein concentration according to the manufacturer's instructions. Total protein (20 µg) was boiled, then chilled and separated, and finally transferred to the polyvinylidene fluoride membrane (Millipore Corporation, Billerica, MA, USA). The membranes were incubated overnight with primary antibody, and then incubated with secondary anti-rabbit or anti-mouse horseradish peroxidase-conjugated antibodies according to the manufacturer's protocols. The primary antibodies included a rabbit anti-SLC17A6 antibody (Synaptic Systems, Goettingen, Germany) and a mouse anti-β-actin antibody (Sigma). The protein expression of SLC17A6 was normalized to the β-actin.

ELISA

The lumbar spinal dorsal horn samples were suspended in a lysis buffer and phenylmethanesulfonyl fluoride, incubated on ice for 5 minutes, and centrifuged (10,000×g, 4°C) for 10 minutes. Supernatants were collected, and the protein content was detected by the MicroBCA assay (Thermo Scientific, Bonn, Germany). IL-1β and IL-6 were detected with the ELISA kit (R&D Systems, Wiesbaden, Germany), according to the manufacturer's instructions. The iMark microplate reader (Bio-Rad, Munich, Germany) was used to detect the absorbance at 450 nm (with 540 nm as reference).

Transfection of miR-190a-5p

Lentiviral vectors were generated by Saierbio, which had the ability to upregulate miR-190a-5p and were enriched with overspeed centrifugal method. Lentivirus carrying up-regulation of miR-190a-5p was injected into subarachnoid space of DNP mice by microinjection and induce up-regulation of miR-190a-5p in the lumbar spinal dorsal horn of DNP mice. Lentivirus carrying empty vectors was also injected into subarachnoid space of DNP mice by microinjection and these DNP mice were served as the control group. Briefly, the

enriched lentiviral vectors with upregulation of miR-190a-5p were injected into the subarachnoid space twice, 2 µL every time. While injecting along the spinal cord, if the mice had tail swing behavior, the enriched lentiviral vectors were injected within 60 seconds. After injection, the DNP mice were housed in separate cages for 3 days, and mechanical nociceptive responses were assessed at fourth week after injection.

SLC17A6 inhibitor injection

The SLC17A6 inhibitor, Chicago Sky Blue 6B (CSB6B; Sigma-Aldrich, St. Louis, MO, USA), was dissolved in artificial cerebrospinal fluid (NaCl 147 mmol/L, KCl 2.7 mmol/L, CaCl₂ 1.2 mmol/L, MgCl₂ 0.85 mmol/L, Na₂HPO₄ 1.0 mmol/L, pH 7.4), and the concentration was 5 µg/µL. Then, artificial cerebrospinal fluid with SLC17A6 inhibitor and without SLC17A6 inhibitor was injected into subarachnoid space twice, 5 µL every time. After injection, the DNP mice were housed in separate cages for 3 days, and mechanical nociceptive responses were assessed at the first week after injection.

Statistical analysis

All data were analyzed by using SPSS 20.0 and GraphPad Prism 5.0. The data were presented as the mean ± standard deviation. Unpaired and paired Student's *t*-tests were used for statistical analysis of the continuous data. Spearman's correlation analysis was used to evaluate the correlation. A *p*-value <0.05 was considered a statistically significant difference.

Results

Characteristics of diabetic mice induced by STZ and mechanical allodynia

STZ was used to induce diabetes in mice. The results showed that compared with the basal blood glucose level, blood glucose level was significantly increased from the second week in mice induced by STZ. Compared with the basal blood glucose level, blood glucose level showed no statistical difference in the control mice. Compared with the control mice, the blood glucose level was significantly increased from the second week in mice induced by STZ (Figure 1A).

The results also showed that the basal body weight and paw withdrawal thresholds significantly decreased from the third week in diabetic mice. Compared with the basal body weight and paw withdrawal thresholds, there was no statistical difference in the control mice. Compared with the control mice, these were also significantly decreased from the third week in diabetic mice (Figure 1B and C).

Spearman's correlation analysis was used to evaluate the correlation between blood glucose level and paw withdrawal

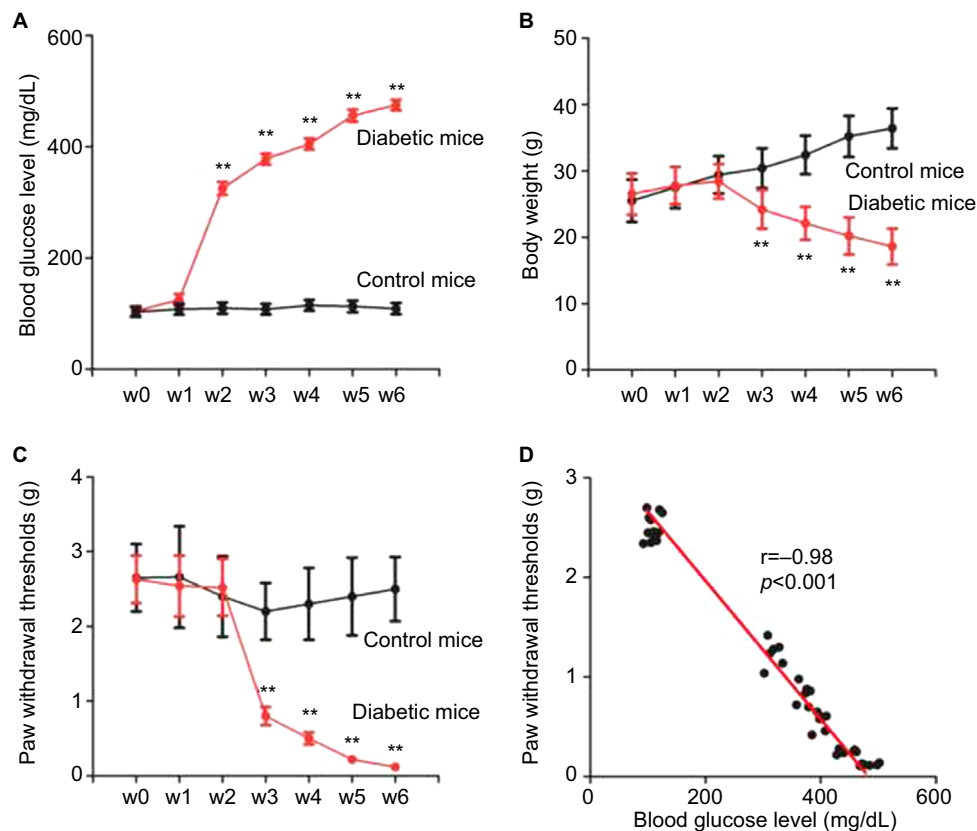


Figure 1 The characteristic of diabetic mice induced by streptozocin (STZ) and mechanical allodynia. **(A)** Change in blood glucose level; **(B)** change in body weight; **(C)** change in paw withdrawal thresholds; **(D)** correlation between blood glucose level and paw withdrawal thresholds.

Note: ** p -value < 0.01 was considered to represent a statistically significant difference.

Abbreviation: w, week.

thresholds. The results showed that there was a negative correlation between the blood glucose level and mechanical threshold, $r = -0.98$ (95% confidence interval = -0.994 to -0.981) (Figure 1D).

The expression of miR-190a-5p in lumbar spinal dorsal horn from DNP

The lumbar spinal dorsal horn was separated from mice, and the expression of miR-190a-5p was detected by qRT-PCR. The results showed that compared with the basal expression and control mice, miR-190a-5p was significantly downregulated in diabetic mice from the fourth week to the sixth week (Figure 2A).

SLC17A6 was the target gene of miR-190a-5p

According to target gene prediction analysis, SLC17A6 had a putative binding site in the 3'-UTR of miR-190a-5p and might be the target gene of miR-217 (Figure 3A). The dual-luciferase reporter assay method was used to further

investigate whether miR-190a-5p directly targeted SLC17A6. The results showed that miR-190a-5p inhibited luciferase activity under 3'-UTR of WT SLC17A6 (Figure 3B). In addition, miR-190a-5p did not inhibit luciferase activity under 3'-UTR of Mut SLC17A6 (Figure 3C).

The expression of SLC17A6 in lumbar spinal dorsal horn from DNP

The lumbar spinal dorsal horn was separated from mice, and the expression of SLC17A6 was detected by qRT-PCR and Western blot. The results showed that compared with the basal expression and control mice, the gene and protein expression of SLC17A6 was significantly upregulated in diabetic mice from the fourth week to the sixth week (Figure 2B and C).

The therapeutic effect of miR-190a-5p in DNP

The lentivirus carrying up-regulation of miR-190a-5p or empty vectors was injected into subarachnoid space of DNP mice by microinjection, and the levels of miR-190a-5p, SLC17A6,

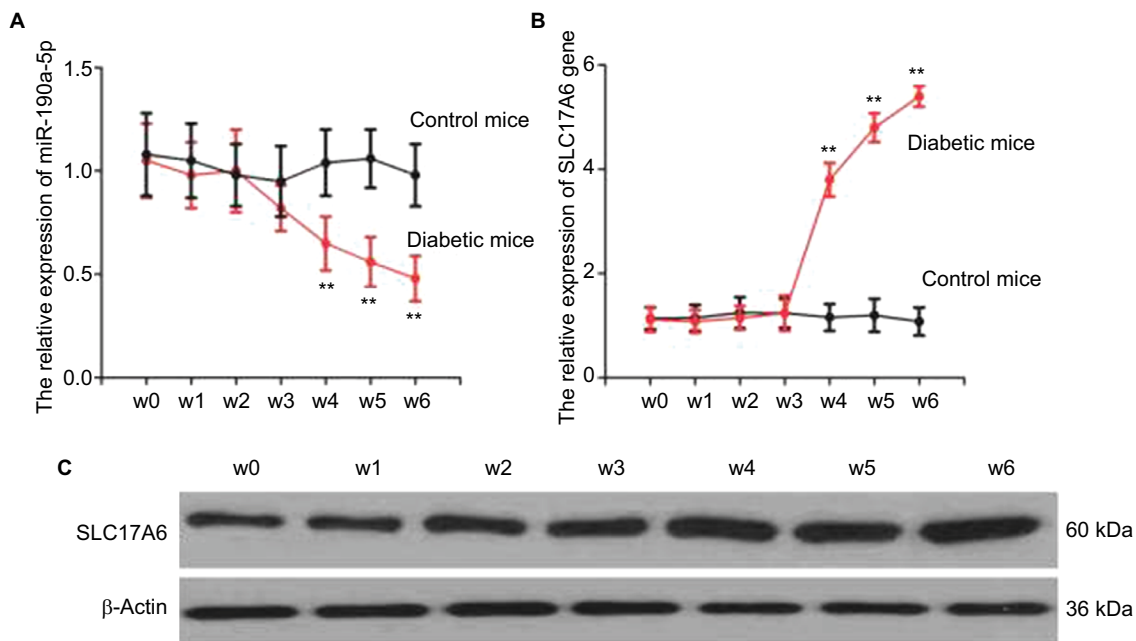


Figure 2 The expression of miR-190a-5p and SLC17A6 in lumbar spinal dorsal horn from diabetic mice. **(A)** The expression of miR-190a-5p; **(B)** the gene expression of SLC17A6; **(C)** the protein expression of SLC17A6. **Note:** **p-value<0.01 was considered to represent a statistically significant difference. **Abbreviation:** w, week.

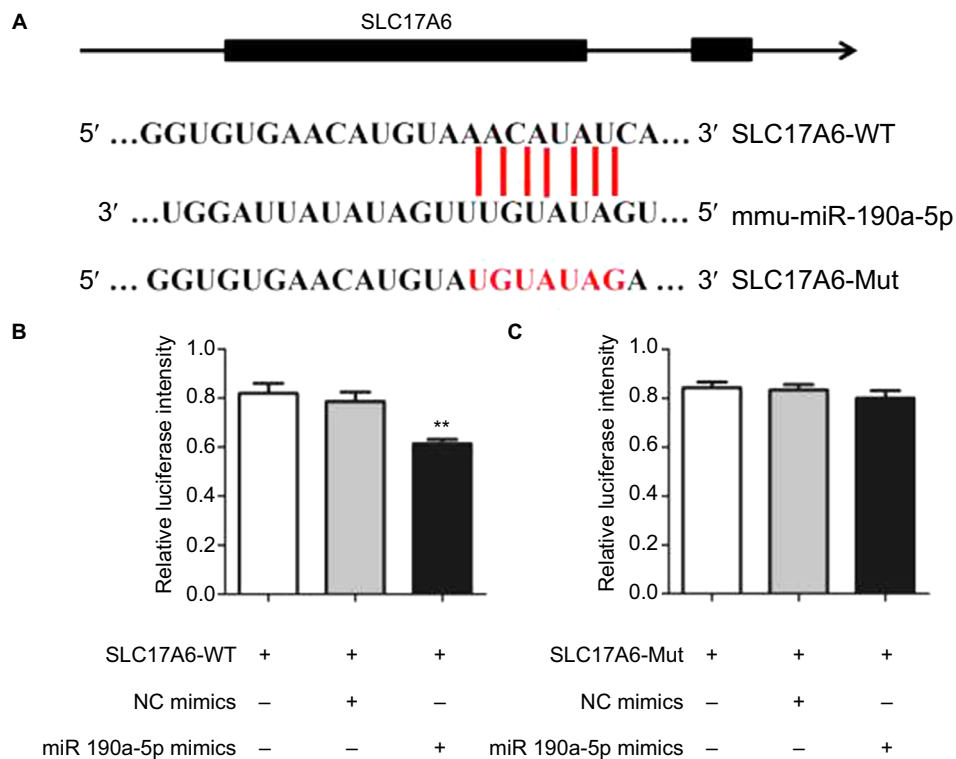


Figure 3 The dual-luciferase reporter assay of miR-190a-5p. **(A)** Putative mmu-miR-190a-5p-binding sequence in the SLC17A6 3'-UTR and the site-directed mutant SLC17A6 3'-UTR. **(B, C)** The WT or Mut reporter plasmids or NC or miR-190a-5p mimics were co-transfected into HEK293T cells. **Note:** **p-value<0.01 was considered to represent a statistically significant difference. **Abbreviations:** Mut, mutated; NC, normal control; WT, wild-type.

IL-1 β , and IL-6 were detected by qRT-PCR, Western blot, and ELISA method. The results showed that after injection, compared with the blank control lentivirus, miR-190a-5p was upregulated, SLC17A6 was downregulated, IL-1 β and IL-6 were decreased in lumbar spinal dorsal horn under lentiviral vectors with the upregulation of miR-190a-5p (Figure 4A–E). Moreover, compared with paw withdrawal thresholds before injection and control lentiviral vectors, paw withdrawal thresholds were significantly increased under lentiviral vectors with upregulated expression of miR-190a-5p (Figure 4F).

The therapeutic effect of SLC17A6 inhibitor in DNP

SLC17A6 inhibitor was injected into subarachnoid space in vivo through intrathecal injection, and the expression of SLC17A6, IL-1 β , and IL-6 was detected by Western blot and ELISA. The results showed that after injection, compared with the control, SLC17A6 was downregulated and IL-1 β and IL-6 were also decreased in the lumbar spinal dorsal horn under SLC17A6 inhibitor (Figure 5A–C). Moreover, compared with paw withdrawal thresholds before injection and control, paw withdrawal thresholds were also significantly increased under SLC17A6 inhibitor (Figure 5D).

Discussion

The animal model induced by STZ is a well-recognized animal model of diabetes.²¹ When the blood glucose levels of diabetic mice are significantly increased and body weights are significantly decreased after STZ injection, a supporting diabetes model is successfully established. After the diabetes model, when the paw withdrawal thresholds from diabetes mice are significantly decreased, the DNP model is successfully established. Furthermore, some studies have shown that this method could be used to duplicate the DNP model.^{17,22} These confirm that the DNP model developed by this method is successful. Although there are many studies in the field of neuropathic pain, the research on DNP is still less studied. Nowadays, diabetic patients are increasing, and DNP is one of the most common, complex, and serious complications for diabetic patients. Therefore, it is necessary to understand the pathophysiological mechanisms of DNP, and better treatments for this disease need to be provided.

MicroRNAs are small, non-coding RNAs that have the ability of regulating protein-coding genes by binding the translation section, leading to either mRNA degradation or translational inhibition. More evidences show that microRNAs are dysregulated in the progress of DNP and play

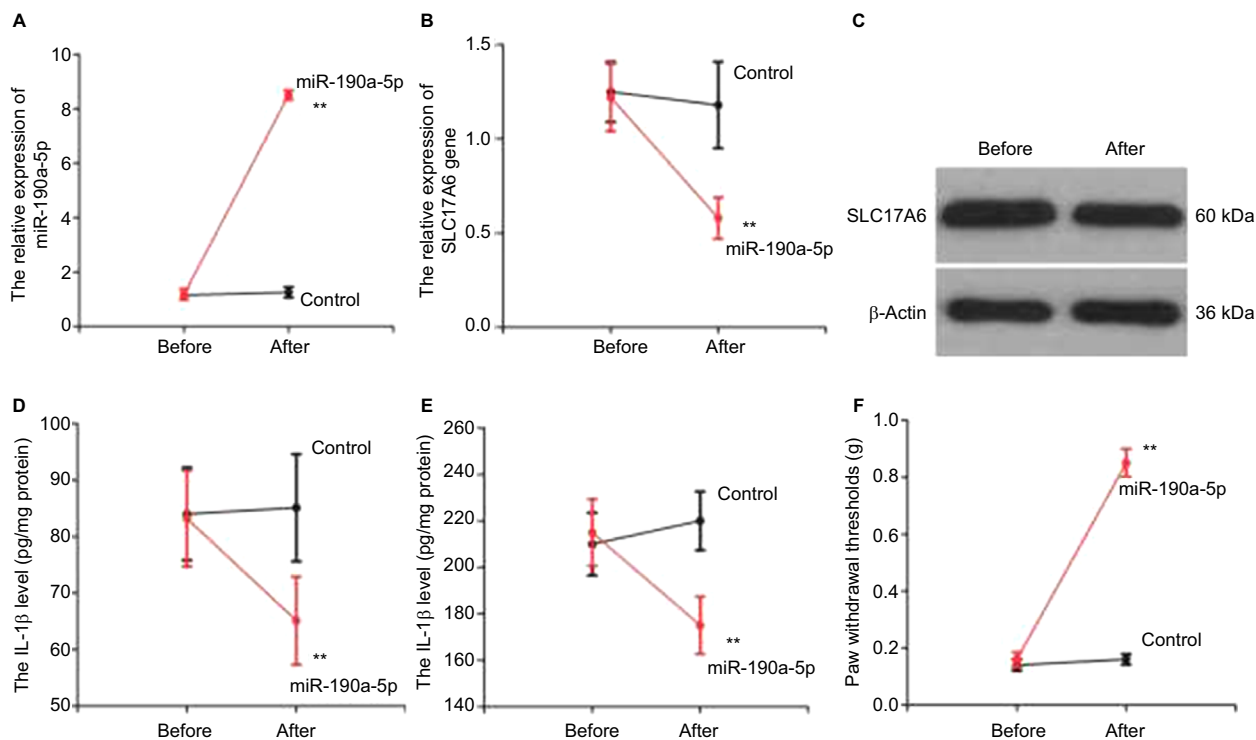


Figure 4 The therapeutic effect of miR-190a-5p in diabetic neuropathic pain. (A) The expression of miR-190a-5p; (B) the gene expression of SLC17A6; (C) the protein expression of SLC17A6; (D, E) the level of IL-1 β and IL-6; (F) change in paw withdrawal thresholds.

Note: ** p -value<0.01 was considered to represent a statistically significant difference.

Abbreviations: Before, before injection with lentiviral vectors; After, at the fourth week after injection with lentiviral vectors.

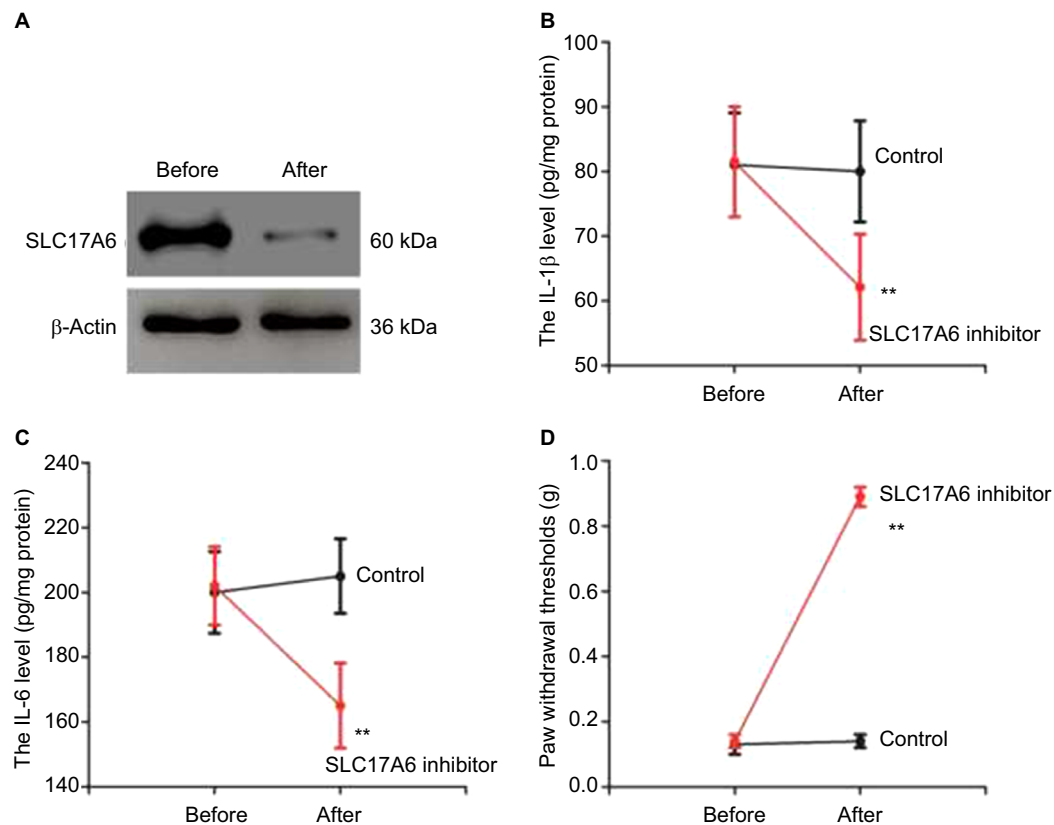


Figure 5 The therapeutic effect of SLC17A6 inhibitor in diabetic neuropathic pain. (A) The protein expression of SLC17A6; (B, C) the level of IL-1 β and IL-6; (D) change in paw withdrawal thresholds.

Note: ** p -value<0.01 was considered to represent a statistically significant difference.

Abbreviations: Before, before injection with SLC17A6 inhibitor; After, at first week after injection with SLC17A6 inhibitor.

important roles in these diseases.²³ In previous experiments, miR-155 was significantly upregulated, and the L-arginine and ibuprofen could delay the development of tactile allodynia and inhibit the expression of miR-155 in DNP.²² Another study also showed that a large number of microRNAs were dysregulated in DNP, and miR-190a-5p was validated to be the most significantly downregulated in DNP.¹⁷ However, the pathophysiological mechanism of miR-190a-5p in DNP remains unclear. In this study, the DNP model was duplicated and the miR-190a-5p was detected in DNP; the results showed that miR-190a-5p was significantly downregulated in the lumbar spinal dorsal horn from DNP induced by STZ. In addition, lentivirus carrying up-regulation of miR-190a-5p was injected into subarachnoid space of DNP mice by micro-injection and could improve pain behavior of these mice. Therefore, miR-190a-5p played an important role in DNP and could be considered as the therapeutic target for DNP.

Then, the dual-luciferase reporter assay was used to confirm miR-190a-5p directly targeted SLC17A6, in addition, the gene and protein expression of SLC17A6 was significantly upregulated in DNP. The solute carrier family

17 (sodium-dependent inorganic phosphate cotransporter), member 6 (SLC17A6) is also known as VGLUT2, the family of transporters that package glutamate into synaptic vesicles, and plays a key role in the progress of most fast excitatory synaptic transmission in the vertebrate nervous system.²⁴ It had been reported that this was involved in neuropathic pain, for example, SLC17A6 was significantly upregulated in related nociceptors, deletion of SLC17A6 from these reduced acute heat and mechanical and chemical pain responsiveness.²⁵ SLC17A6 was significantly upregulated in rat dorsal root ganglia and spinal cord following spared nerve injury.²⁶ Finally, SLC17A6 was transiently upregulated, and the level was returned to the basal level by 36 hours in neuropathic pain in spared nerve injury model.²⁷ However, the expression of SLC17A6 in DNP remains unclear. In this study, the results found that SLC17A6 was also significantly upregulated in DNP. Therefore, SLC17A6 was also involved in the progress of DNP and played an important role in DNP. In addition, the therapeutic effect of SLC17A6 inhibitor in DNP was explored, and after treatment with SLC17A6 inhibitor, painful behavior was also improved. Therefore,

SLC17A6 played an important role in DNP and could be the therapeutic target for DNP.

For the mechanisms of SLC17A6 in neuropathic pain, some studies have shown that SLC17A6 was involved in neuropathic pain after nerve injury through the aggravation of glutamate imbalance and upregulation of glutamatergic signaling and also played a key role in the crucial mechanism of neuropathic pain.²⁷ In addition, the upregulation of SLC17A6 lasted for a very short time, and this expression was returned to the basal level, which might be due to the inactivation of the TrkB signaling pathway.^{27,28} Moreover, upregulation of SLC17A6 might be related to brain-derived neurotrophic factor (BDNF), which was reported to increase the VGLUT expression in cultured neurons.^{27,28} Finally, Chicago Sky Blue 6B (CSB6B) selectively inhibited the function of VGLUTs and was reported to weaken inflammatory pain in related mouse model.^{29,30} In this study, the results showed that when miR-190a-5p was upregulated, SLC17A6 was downregulated, IL-1 β and IL-6 were decreased in lumbar spinal dorsal horn, and painful behavior was improved. In addition, when SLC17A6 was inhibited, IL-1 β and IL-6 were also decreased in lumbar spinal dorsal horn, and pain behavior was also improved. Some studies have shown that the activation of glial and immune cells was involved in the pathogenesis of neuropathic pain in the peripheral and central nervous systems.^{31,32} Glial cells regulated the release of many inflammatory mediators, including chemokines and cytokines, which were very important to establish and maintain neuropathic pain.^{31,33} In diabetes model, microglia had been reported to be activated in the dorsal horn of the lumbar spinal cord and released many inflammatory mediators leading to neuropathic pain.^{3,34} Therefore, glial cells may play an important role in the development of diabetes neuropathy pain; in these cells, downregulation of miR-190a-5p regulates the expression of SLC17A6 and raises inflammatory response and leads to diabetes neuropathy pain. In addition, in these cells, miR-190a-5p is increased and SLC17A6 is decreased, inflammatory response is weakened, and diabetes neuropathy pain can be improved.

Conclusion

In conclusion, first, the results of this study confirm the expression of miR-190a-5p in DNP. Then, the results verify that *SLC17A6* is the target gene of miR-190a-5p. Moreover, they show that miR-190a-5p contributes to DNP via targeting SLC17A6. Finally, they provided a new strategy of treating this disease.

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Disclosure

The authors report no conflicts of interest in this work.

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