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Low-Level miR-199 Contribute to Neuropathic Low Back Pain via TRPV1 by Regulating the Production of Pro-Inflammatory Cytokines on Macrophage

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ABSTRACT

AIM: To explore the post-translational regulation of TRPV1, which plays an important role in neuropathic low back pain (NLBP).

MATERIAL and METHODS: qPCR was used to examine the gene mRNA levels. Western blot was used to examine the protein level. NLBP rat model was established for confirming what we observed in clinical samples. Dual-luciferase assay was used to verify the miR-199 targets on the 3'UTR of TRPV1. Cell coculture was used to explore the interaction between macrophages and nerve cells.

RESULTS: We found the mRNA level of TRVP1 decreased in the sinuvertebral nerve biopsy of NLBP. With bioinformatics prediction, miR199 would involve the post-transcription regulation of TRPV1. As the prediction, the miR199 level decreased in the clinical samples. Correlation regression analysis showed a negative correlation between miR-199 and TRPV1. The same phenomenon was confirmed in the rat NLBP model. With dual-luciferase assay, we confirmed that miR199 directly binds to the 3'UTR of TRPV1. Through co-culture of macrophage (THP1) and sNF96.2, we found that up or down-regulates miR-199 in macrophage and sNF96.2 could relieve or aggravate the injury of nerve cells strain.

CONCLUSION: These results suggest that the occurrence of NLBP may be caused by the lower expression of miR-199 in macrophages and nerve via TRPV1.

KEYWORDS: NLBP, miR-199, TRPV1, co-culture

INTRODUCTION

Neuropathic low back pain (NLBP) is caused by damaged lumbar intervertebral discs directly affecting the spine and lower limbs and pathologically invasive innervation of (2), often accompanied by depression, panic disorder, anxiety disorder and sleep disorders and other complications (9,10,12). Neuropathic low back pain, a disabling, costly and complex heterogeneous disease, is prevalent in global clinical practice (2), which seriously affects patients' working ability, quality of life and psychological state, and requires a large amount of personal and social healthcare costs (4,19,23). However, neuropathic low back pain is currently underrecognized and undertreated, and better understanding, diagnosis, and treatment are needed to improve patient outcomes and reduce the burden on the healthcare system.

Transient Receptor Potential Cation Channel Subfamily V Member 1 (TRPV1) receptor is a ligand-gated non-selective cation channel, mainly expressed in the sensory nerves that innervate the heart and blood vessels (21,26). There is

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increasing evidence that TRPV1 expressed in primary sensory neurons is involved in mediating various inflammatory and neuropathic pain in the peripheral nervous system (8). TRPV1 can be used as a molecular synthesizer of various chemical and physical stimuli, including protons, toxic heat, endothelin, etc. (7). At the same time, studies have shown that TRPV1 activation can lead to ischemic heart pain (21). However, the exact molecular mechanism by which TRPV1 causes pain remains controversial.

NLBP is one type of neurological pain which is caused by neuro-immune cell interactions mediated by inflammatory cytokines. Macrophage with its produced cytokines is one of the key players in the process (22).

MicroRNAs (miRNAs) are non-coding RNAs with regulatory functions in eukaryotes, which can regulate gene expression and protein synthesis by binding to mRNA and controlling mRNA degradation (6). miRNAs play a key role in the development of most diseases. Therefore, miRNA is also considered as a new drug therapy target. Several microRNAs showed an anti-inflammatory effect on the nerve. Such as mIR-146a is closely related to the occurrence of neuropathic low back pain (17) and miR182 alleviates NP via Nav1.7 (5). Therefore, microRNAs are likely to be a new target for the diagnosis and treatment of NLBP.

In this study, we found a negative relationship in expression level between miR-199 and TRPV1 in NLBP patients. We confirmed the relations in a rat model. With dual-luciferase assay, miR199 was verified that can bind to 3' UTR of TRPV1. At the same time, we found that the occurrence of NLBP may also be caused by the abnormal expression of miR199 in immune cells, which leads to the increase of inflammatory factors and never cells' hypersensitivity to stimulation. This study provides a new perspective on the occurrence and treatment of NLBP.

MATERIAL and METHODS

Human Subjects

A total of 16 NLBP patients were collected from the Department of Orthopedics, Wuhan Hanyang Hospital between June 2015 and Feb 2021 where they underwent herniated disc surgery, including sinuvertebral nerve biopsy. The study was approved by the Wuhan Hanyang Hospital Ethics Committee (No: 01, Date: 2013 June 04th) and all the patients signed the informed consent before the study. The diagnosis of neuropathy was based on characteristic symptoms and signs in the neurological examination. NLBP patient incorporation Criteria: 1, Low back pain lasting more than 3 months (Patients reported pain with an intensity of 3 or more on a visual analogue scale (VAS) ranging from 0 to 10. Patients with a score of less than 3 meaning "no pain") (Table I). 2, Combined with lumbar disc herniation (or lumbar spinal stenosis and other degenerative diseases of the lumbar spine, with CT or MRI examination indicating nerve compression), conservative treatment has failed, and surgery (nerve root decompression) is needed. 3, Apart from radiating pain in the lower extremities (sciatica) and low back pain, there is no pain in other parts of the body. 4,

Table I: Demographic and Clinical Characteristics of Patients with Neuropathic Low Back Pain

Patient ID	Age	Gender	VAS score
1	25	Female	7
2	53	Female	6
3	44	Female	6
4	54	Female	4
5	37	Female	7
6	38	Female	6
7	35	Female	7
8	35	Female	7
9	27	Female	8
10	48	Male	7
11	27	Male	8
12	23	Male	6
13	42	Male	7
14	35	Male	7
15	41	Male	8
16	41	Male	7

VAS: *Visual analogue scale.*

Low back pain is generally characterized by burning, shooting, and tingling sensations in the lower back (20).

NLBP patients exclusion Criteria: 1, Low back has previously been injured; 2, There are lesions in the lower back such as wounds or tumours; 3, The patient has a history of cerebral and brain diseases such as stroke, intracranial tumours, or traumatic brain injury. 3, Patients with autoimmune, chronic, inflammatory, neoplastic, or psychiatric diseases were excluded.

The control group consisted of 10 patients who underwent herniated disc surgery but without neuropathic pain. Inclusion Criteria: 1, Combined with lumbar disc herniation (or lumbar spinal stenosis and other degenerative diseases of the lumbar spine, with CT or MRI examination indicating nerve compression), conservative treatment has failed, and surgery (nerve root decompression) is needed. 2, No lower back pain.

The patient was placed in a prone position, and the C-arm X-ray machine was used to locate the intervertebral space. After the surgical area was routinely disinfected and spread with towels, a 5 cm posterior median longitudinal incision was made. Subcutaneous tissue and deep fascia were incised, and paraspinal muscle was stripped under the periosteum. Clear the laminar space on the affected side, Part of the upper and lower lamina were bitten off to expand the laminar space and ligamentum flavum was resected, exposing the dura and nerve root. The branch that comes out along the nerve root

and returns to the dura and disc, which is the sinus nerve, could be exposed and then excised. Protruding nucleus pulposus were removed on or under the shoulder of the nerve root, releasing the nerve roots to a relaxed state. The incision was sutured after flush.

All the research procedures are conformed to the principle of the Declaration of Helsinki (1).

Rats

In this study, 10 weeks of Sprague Dawley rats weighing 250-330 g were selected for modelling and experiment. Rats were randomly divided into control and NLBP groups with 12 rats in each group. NLBP group rats were induced by chronic compression of the L5 DRG (dorsal root ganglion) on the right side according to Hu and Xing's reports on the NLBP rats model construction method (14). All the rats were anaesthetized by thiopental (2.5%, 30 mg/kg) before surgery. All the surgeries took place from 2:00 PM to 5 PM. Two rats were dead 3 days after the surgery. Therefore, we chose 10 rats from each group for the following assay.

When the surgery finished, the foot withdrawal latencies to noxious heat stimuli were measured to evaluate whether the NLBP Rat model was successfully established. A simple apparatus for measuring the withdrawal latencies were made according to Hargreaves's reports (13). Before testing, the apparatus was loaded with 100°C water and was covered by a metal plate for a rat to step on it (Figure 2A). And the withdrawal latency standard difference (SD) is +-0.66 seconds according to Bennett's work (3). When the NLBP rat model was established, the rats were anaesthetized and sacrificed. The DRG of the rats were collected for the following research.

Cell Culture and Transfection

HEK293T human embryonic kidney cells and human malignant peripheral nerve tumour cell lines, sNF96.2 were obtained from American Type Culture Collection (ATCC). Cells were cultured in Dulbecco's Modified Eagle Medium (Hyclone, Logan, UT, USA) containing 10% fetal bovine serum (Hyclone, Logan, UT, USA), 100 IU/ml penicillin and 10 mg/mL streptomycin. All cells were maintained at 37°C under an atmosphere of 5% CO2.

After inoculation, wait for the cell convergence rate to reach 60%. Lipofectamine 2000 was used to transfect 20 nM miR-199 mimic, 100 nM mirRNA-199 inhibitors or TRPV1 regulatory plasmids at doses specified by the manufacturer. RNA and protein were extracted 48 h later for subsequent detection.

Co-culture

THP-1 cell lines and sNF96.2 cell lines were cultured in Dulbecco's Modified Eagle Medium (Hyclone, Logan, UT, USA) containing 10% fetal bovine serum (Hyclone, Logan, UT, USA), 100 IU/ml penicillin and 10 mg/mL streptomycin. THP-1 cells were implanted into the upper layer of Transwell, and sNF96.2 cells were planted in the lower layer. After 24 hours of co-culture, The sNF96.2 cells were planted in a 96 wells plate and their proliferation rate was detected by Cell Counting Kit-8 (CCK8) kit according to the manufacturer's instruction (Beyotime, C0037, PR. China.).

RT-qPCR

Total RNA was extracted from the Sinuvertebral nerve biopsy and was performed in 16 patients and 10 control participants or cells using TRIzol (Invitrogen, USA) according to the manufacturer's instructions. RNA concentration and purity were determined using a model ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). Only samples with absorbance ratios 260 nm/280 nm of ~2.0 and 260 nm/230 nm of 1.9–2.2 were considered for inclusion in the study. MiRNA-199 and U6 were retrotranscribed using a reverse transcription kit (Takara, Japan). Dosage and reaction conditions refer to the instructions. 0.5μg total RNA was synthesized using the first strand cDNA to detect the content of the target gene. qPCRs were detected with SYBR Green PCR Master Mix (Takara, Japan) reagent.

Endogenous U6 and B-actin were used as internal references to calibrate sample loading. The relative value of gene expression was calculated by the $2^{-\Delta\Delta CT}$ method. All qPCRs were three replicates. All primers were ordered from Sangon Biotech (Sangon, China).

Western Blot Analysis

Cells were collected by centrifugation at 4°C and washed with PBS. The cells were lysed with RIPA buffer (ABCAM, England) and then boiled in sodium dodecyl sulfate/β-mercaptoethanol sample buffer for 5 min at 95°C. 20μg protein was loaded on 10% SDS-PAGE and transferred to a polyvinylidene fluoride membrane (Amersham Pharmacia Biotech, St. Albans, Herts, UK) by electrophoretic transfer. The membrane was firstly blocked by 5% BSA and then incubated with one of the primary antibodies for 2 h at 37°C. After incubating with horseradish peroxidase-conjugated secondary antibody, the specific protein-antibody complex was detected by using an ECL kit (Pierce, Appleton, WI, USA). The β-actin signal was used as a total cell lysate loading control.

Antibody information: Rabbit anti-TRPV1 polyclonal antibody (1:1000 dilution, ab3487, Abcam, Cambridge, MA, USA), rat anti-TRPV1 polyclonal antibody (1:1000 dilution, ab10295, Abcam, Cambridge, MA, USA), mouse anti-β-actin monoclonal antibody (1:5000 dilution, Cat.no. sc-8432, m, r, h, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA).

Dual-luciferase assays

The full length of 1368bp TRPV1 3'UTR was cloned into pmirGLO vector (Promega, Madison, WI USA), to generate a reporter vector, using TRPV1 3'UTR as the 3'UTR of firefly luciferase. miRNA mimics and inhibitors were the product of GenePharma Co., Ltd (Shanghai, China). HEK293T cells were seeded in 48-well plates with a density of 5×10^4 per well. Luciferase reporter vectors were co-transfected with miRNA mimic or inhibitor into HEK293T cells using lipofectamine 2000 (Invitrogen). Forty-eight hours post-transfection, cells were harvested and subjected to dual luciferase assay. Each treatment was performed in triplicate in three independent experiments. The results are expressed as relative luciferase activity (Firefly Luciferase/Renilla Luciferase).

Statistical Analysis

Data analysis used in this study was obtained from three bioinformatics replicates. GraphPad Prism 9.5 was used for statistical analysis. The two were statistically compared using the student T-test. One-way ANOVA was used to compare three or more groups. The Group Normality test was used by D'Agostino & Pearson test, the Anderson-Darling test, the Shapiro-Wilk test, and the Kolmogorov-Smirnov test. One of the four methods passed was considered that this data passed the Normality test (Table II). A simple linear regression method was used for Pearson correlation regression analysis. $P<0.05$ is represented by an asterisk (*).

RESULTS

The Expression of miR-199 was Abnormal in NLBP Patients

We measured TRPV1 expression level in clinical samples from the sinuvertebral nerve of NLBP patients who were diagnosed with VAS (Table I) and CT image (Figure 1 A-D) and the lumbar disc protrusions were marked by red circle. We found that the mRNA and protein levels of TRPV1 were significantly increased in the sinuvertebral nerve of NLBP and the expression. In the NLBP sinuvertebral nerve, the relative TRPV1 level is 2.49 which means 2.49 times higher than the control group (Figure 2 A, B). For exploring the upper stream regulators, we predicted the potential upstream microRNAs of TRPV1 by bioinformatics database (https://www.targetscan.org/vert_80/). The data indicated that miR-199 might be one of the upstream microRNAs of TRPV1 (Figure 2C). Therefore, the expression of miR-199 in the tissues of NLBP patients was examined by Q-PCR to evaluate the levels. As shown in Figure 2D, the expression of miR-199 decreased significantly in the sinuvertebral nerve of NLBP patients. The mean of relative expression is 0.59. Correlation regression analysis on the mRNA level of miR-199 and TRPV1 in the sinuvertebral nerve of NLBP patients showed that TRPV1 and miR-199 are negatively related with the Pearson r = -0.8917 (Figure 2E).

These results suggested that miR-199 plays a negative regulatory role on TRPV1 by potentially targeting TRPV1 and may be one of the main post-transcriptional regulators of TRPV1 in NLBP patients.

Table II: Normality Distribution Test

Figures	Test for normal distribution
Fig. 2A	Passed
Fig. 2D	Passed
Fig. 3B-p	Passed
Fig. 3B-4	Passed
Fig. 3B-8	Passed
Fig. 3B-12	Passed
Fig. 3B-16	Passed
Fig. 3B-20	Passed
Fig. 3B-24	Passed
Fig. 3B-28	Passed
Fig. 3C	Passed
Fig. 3D	Passed
Fig. 4A	Passed
Fig. 4B	Passed
Fig. 4C	Passed
Fig. 5A	Passed
Fig. 5B	Passed
Fig. 5C	Passed

Expression of miR-199 and TRPV1 in an NLBP Model

To confirm what we found in NLBP patients, we constructed a rat model of NLBP induced by the surgery method developed by Hu and Xing (14). After the surgery, 2 rats were dead in the surgery group and there were left 10 rats in this group. The NLBP rat model was validated by the paws withdraw test with our simply developed equipment (Figure 3 A). After surgery, we put the rats in our equipment and test the paws' withdrawal time when the paws were put on the hot metal plate (Figure 3A). The withdrawal latency standard difference (SD) is +-0.66 seconds according to Bennett and Xie (3). Results showed that, in the surgery group, the paws' withdrawal time SD varied from -2 SD to -4SD in all time points and SD values, which indicated that the paws' withdrawal delay, is significant in the surgery group. These paw withdrawal tests showed that the NLBP rats model was well established (Figure 3B).

With this animal model, we detected miR-199 and TRPV1 levels in the DRG of NLBP rats. As in patients with NLBP, the TRPV1 mRNA expression in the DRG of NLBP rats averagely increased 2.65 times compared with control DRG tissues ($p<0.001$, Figure 3C). And miR-199 levels in rat DRG were significantly decreased in NLBP rats with means is 0.417 ($p<0.001$, Figure 3D). In addition, western blot results also showed that TRPV1 protein was elevated in NLBP rats compared with the control group (Figure 3E). These results confirmed that miR-199 and TRPV1 expression levels were negatively correlated in NLBP rats which is consistent with NLBP patient tissues.

The Expression Level of TRPV1 is Directly Regulated by miR-199

To explore whether there is physical interaction between miR-199 and TRPV1, we employed a dual luciferase reporter assay for exploring it in HEK293 cells. Luciferase assay showed that transfection of miR-199 cells inhibited the activity of the luciferase reporter gene fused to the WT 3'UTR of TRPV1, while the activity of the mutated 3'UTR reporter was not inhibited (Figure 4A).

To more rigorously verify the direct regulatory effect of miR-199 on TRPV1 in vitro. We further examined the expression

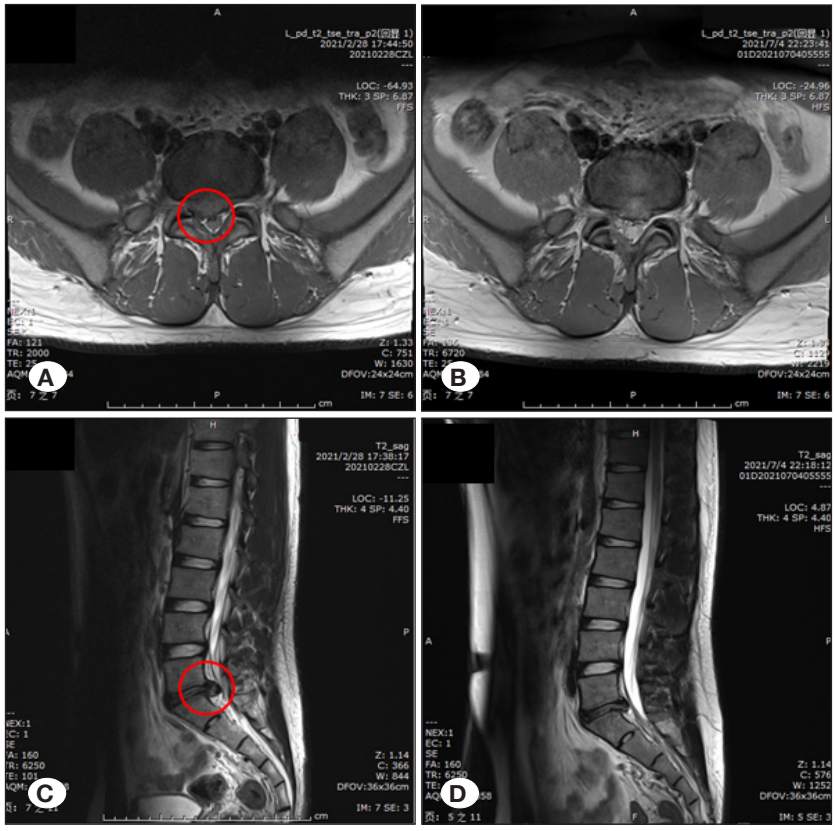

Figure 1: Magnetic resonance imaging (MRI) studies for one of the NLBP patients enrolled in this study. **A)** T2W axial MRI scan of the lumbar vertebra. The herniated disk was circled. **B)** The same patient's T2W axial MRI scan of the lumbar vertebra after surgery. **C)** T2W sagittal MRI scan of the lumbar vertebra. The herniated disk was circled. **D)** The same patient's T2W sagitaal MRI scan of the lumbar vertebra after surgery.

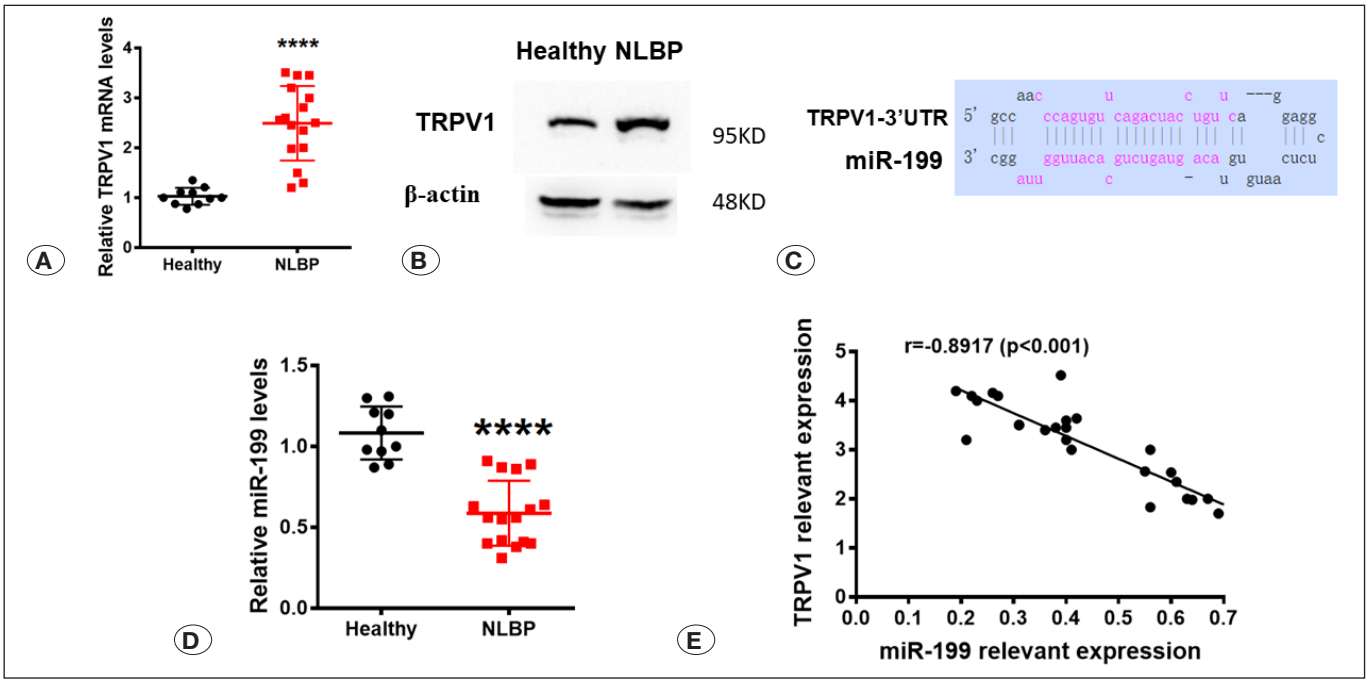

Figure 2: The expression of miR-199 was abnormal in NLBP patients. **A)** QRT-PCR results for evaluating the TRPV1 mRNA Level in the sinuvertebral nerve of NLBP patients and normal LBP patients. TRPV1 significantly increased in the sinuvertebral nerve of NLBP patients ($p < 0.001$). **B)** Western detection of TRPV1 expression level in one of the NLBP patients. **C)** Prediction results of the upstream microRNA of TRPV1 on Targetscan (https://www.targetscan.org/vert_80/). miR-199 targets on the 3'UTR of TRPV1. **D)** QRT-PCR result of the expression level of miR-199 in sinuvertebral nerve of NLBP patients and normal LBP patients. miRNA levels significantly increased in the sinuvertebral nerve of NLBP. **E)** Correlation analysis of miR-199 and TRPV1 mRNA expression in patients with NLBP. mRNA level of miR-199 negative related with TRPV1. *** $p < 0.001$.

of TRPV1 being regulated by the level of miR-199 in sNF96.2 cells. The results showed that the expression of TRPV1 mRNA and protein decreased after being transfected with a miR-199 mimic. In contrast, TRPV1 mRNA and protein levels increased in miR-199 inhibitor-treated sNF96.2 cells (Figure 3B-D). All these data demonstrated that miR-199 targets 3'UTR of TRPV1 physically.

The miR-199 Regulates Cytokine Expression in Macrophages

miR-199 was reported to play roles in anti-inflammatory and regulates cytokines production (11,27). Thus, we investigated the regulatory effects of miR-199 on cytokines in THP1 cells, a cell model for macrophage. As shown in Figure 5A, the levels of IL-6, TNF-A and CCL3 decreased in THP1 cells after miR-199 overexpression. In contrast, when miR-199 levels in THP1 were inhibited, the IL-6, TNF-A, and CCL3 mRNA levels were not changed compared with the control (Figure 5B). This result confirmed the literature's reports that miR-199 has an anti-inflammatory effect (27).

The miR-199 Regulates the THP1 Interacting with sNF96.2 and Influences the Survival Rate of sNF96.2

Once we confirmed the anti-inflammatory role of miR199 in macrophage, we want to know the miR199 roles on NLBP in macrophage. Thus, we co-cultured THP1 cells with sNF96.2 and employed a CCK-8 assay to test the survival rate of sNF96.2. As shown in Figure 5C, in the co-culture environment, the survival rate of sNF96.2 was decreased when TRPV1 was upregulated. When Down-regulation of miR-199 by miR-199 inhibitor in THP1, sNF96.2 and both, the survival rate of sNF96.2 significantly decreased in the three groups (THP1, sNF96.2, and both). When we up-regulated miR-199 by miR-199 mimic, The survival rate in the three groups increased. Especially, in both groups, the survival rate is higher than in THP1 and sNF96.2 groups which suggested there is a synergistic effect for keeping sNF96.2 cell survival when increasing miR-199 level in both THP1 and sNF96.2 cells (Figure 5B).

DISCUSSION

Neuropathic pain (NP) is a long-term recurrent disease and NLBP ranks highest in global disease burden for years lived with disability. TRPV1 sensitization contributes to the development of neuropathic pain in a rat chronic constriction injury model (CCI) (15,18).

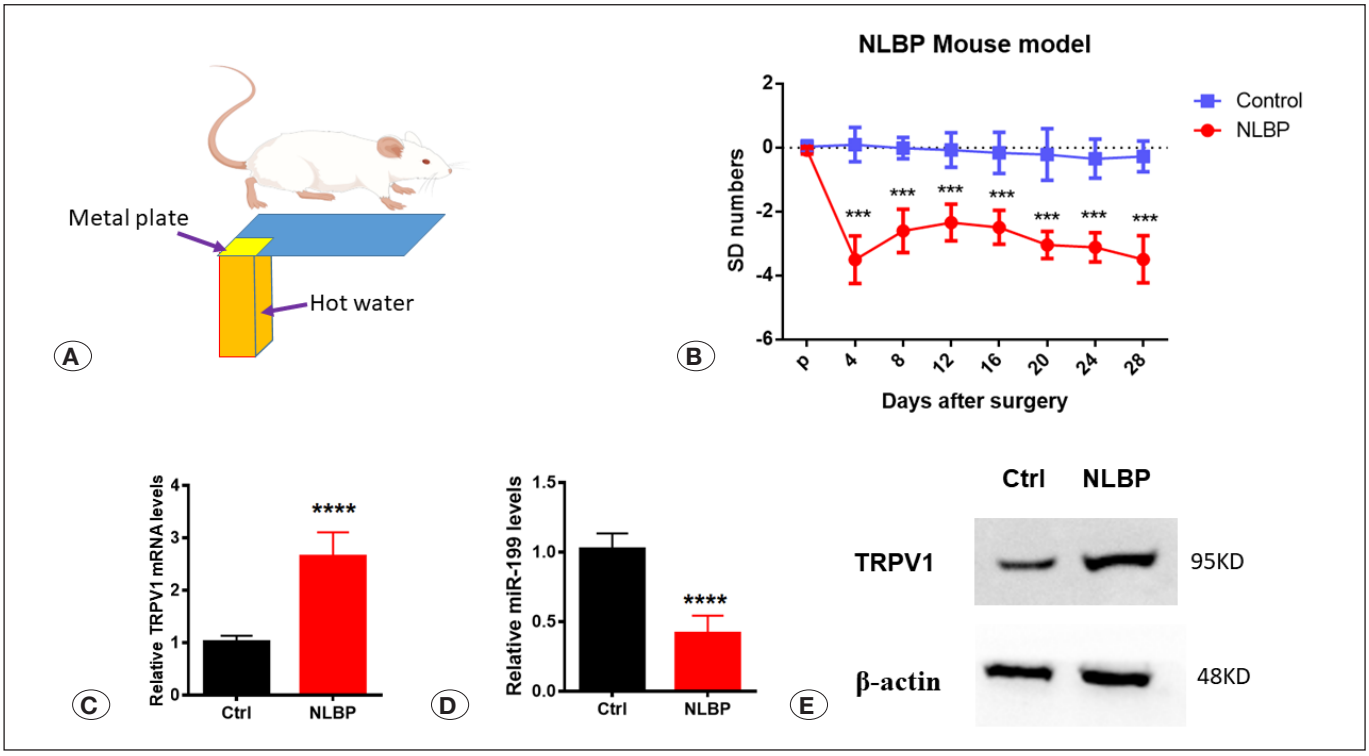

Figure 3: Expression of miR-199 and TRPV1 in an NLBP Rat model. **A)** Diagram of our simple equipment for examining the NLBP Rat Model. The hot water is 100°C and covered with a metal plate on it. When a rat's right leg steps on it, it will feel hot and move away. **B)** The time differences in latency to noxious heat for the two paws in the control and surgery group. the x-axis is days after surgery. The y-axis is the number of standard differences (SD) at each time point. The SD is 0.66 sec. Lower numbers of the SD stand for more serious NLBP on the Rat. **C)** TRPV1 mRNA level in DRG of NLBP rat model were detected by QRT-PCR. The level of TRPV1 in NLBP rats was significantly higher than in normal rats. **D)** miR-199 level in DRG of NLBP rat model which were detected by QRP-PCR. The levels of miR-199 in NLBP rats significantly decreased. **E)** Western blot detected TRPV1 protein levels in the DRG of rats. The band intensity is stronger in the NLBP group. ***p<0.001, ****p<0.0001.

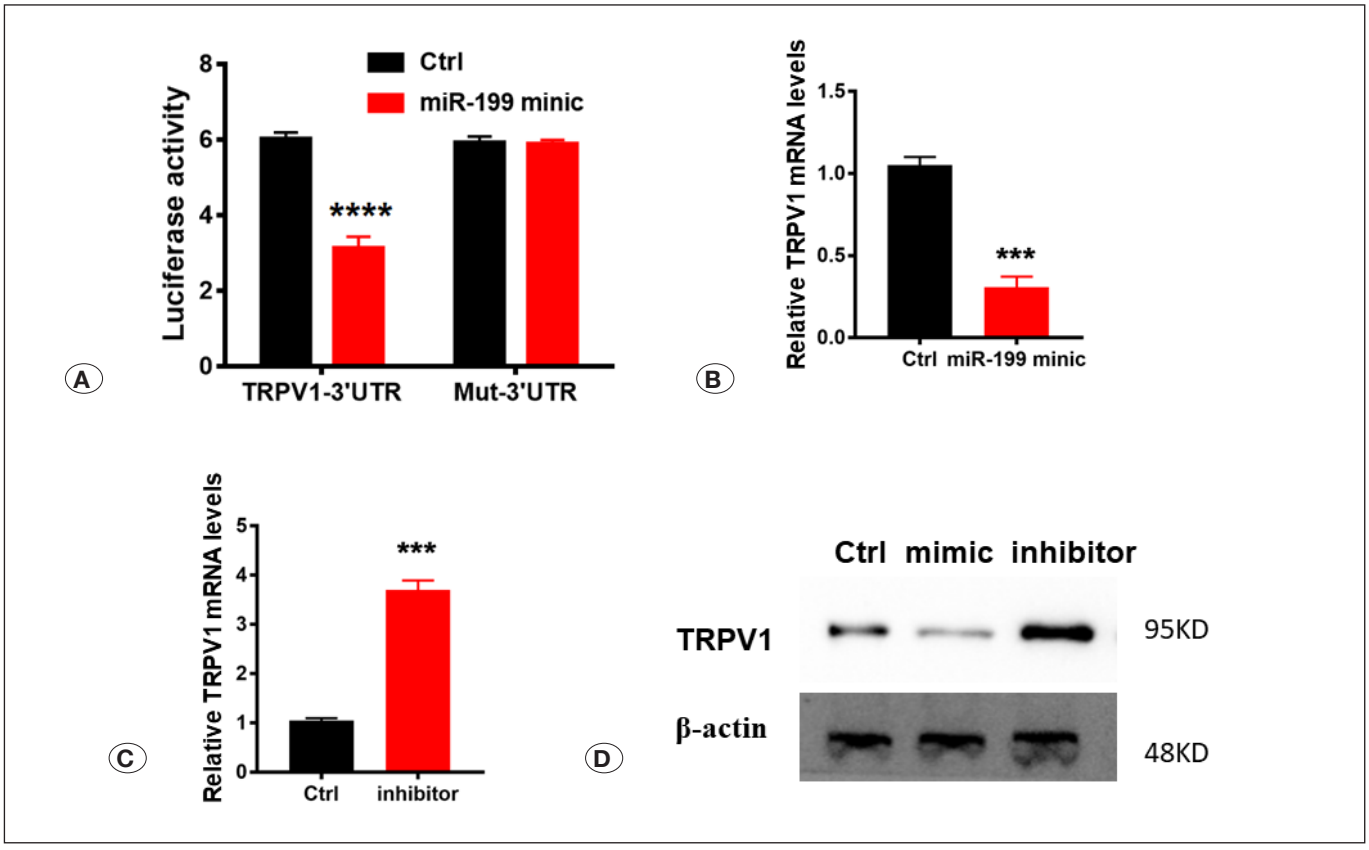

Figure 4: 3'UTR of TRPV1 physically binding to miR-199. **A)** Luciferase assay was used to detect the targeted regulation of miR-199 on TRPV1 in HEK293T cells. A Mut-3'UTR on TRPV1 was used as a negative validation on the binding of miR-199 to the 3'UTR of TRPV1. **B)** QRT-PCR was used to detect the mRNA levels of TRPV1 after transfection with Mir-199 mimic in sNF96.2 cells. **C)** QRT-PCR was used to detect the mRNA levels of TRPV1 after transfection with miR-199 inhibitor in sNF96.2; **D)** TRPV1 protein level in sNF96.2 after miR-199 was regulated by western detection. ***p<0.001, **** p<0.0001.

Due to the importance of TRPV1 in NP, we confirmed again that the TRPV1 level increased in 16 NLBP patients. Considering the modulation of TRPV1 in neuropathic pain was under attention, and several chemicals were verified to relieve neuropathic pain by inhibiting TRPV1 (16,25), we explored the upper post-translational regulator of TRPV1 by bioinformatics prediction, in which miR-199 potentially targets 3'UTR of TRPV1. We validated that miR-199 was significantly decreased in the sinuvertebral nerve of NLBP patients. Correlation analysis between the mRNA level of TRPV1 and miR-199 showed that they shared a negative relation. These results strongly suggested that miR-199 would play a negative regulatory role on the mRNA level of TRPV1. To confirm what we observed in NLBP patients, we established a rat model. In the rat model, the TRPV1 increased and the miR-199 decreased. The negative relation between miR199 and TRPV1 appeared again.

After confirming the miR-199 levels negatively relates to TRPV1 in NLBP, the next question is how miR-199 negatively post-translational regulates TRPV1. Does miR-199 physically interact with the mRNA of TRPV1? Thus, we employed a dual luciferase assay to answer this question. The wt 3'UTR or a mutated 3'UTR of TRPV1 was cloned to a luciferase reporter vector. miR-199 can activate the wt TRPV1 3'UTR contained reporter while could not activate the mutated TRPV1 3'UTR contained reporter. This proved that miR-199 does physically bind to TRPV1 3'UTR. Meanwhile, we up or down-regulated the miR-199 level in sNF96.2, and the TRPV1 mRNA level was also changed. This proved that the miR-199 negatively regulates the mRNA of TRPV1 in nerve cells by binding on it.

Peripheral nerve injuries often lead to pain persisting after the resolution of damage (24). Neurological pain (NP) involves neuro-immune cell interactions mediated by inflammatory cytokines and macrophage with its produced cytokines is one of the key players in the process (22). miR-199 is reported that it has anti-inflammatory effects (27). Thus, we explored the roles of miR-199 in macrophage on NLBP. To research the macrophage-nerve cell interaction, we employed a co-culture system to mimic the interaction. THP1 cells were used as the macrophage cell model. In our result, upregulated miR-199 in THP1 cells decreased the production of IL-6, TNF-a and CCL-3 which are pro-inflammatory cytokines. While inhibiting the miR-199 level in THP1 with an inhibitor, the mRNA level of IL-6, TNF-a and CCL3 in THP1 did not change. These results showed that miR-199 also regulates the macrophage-secreting inflammatory cytokines which may influence the pathological process of NP.

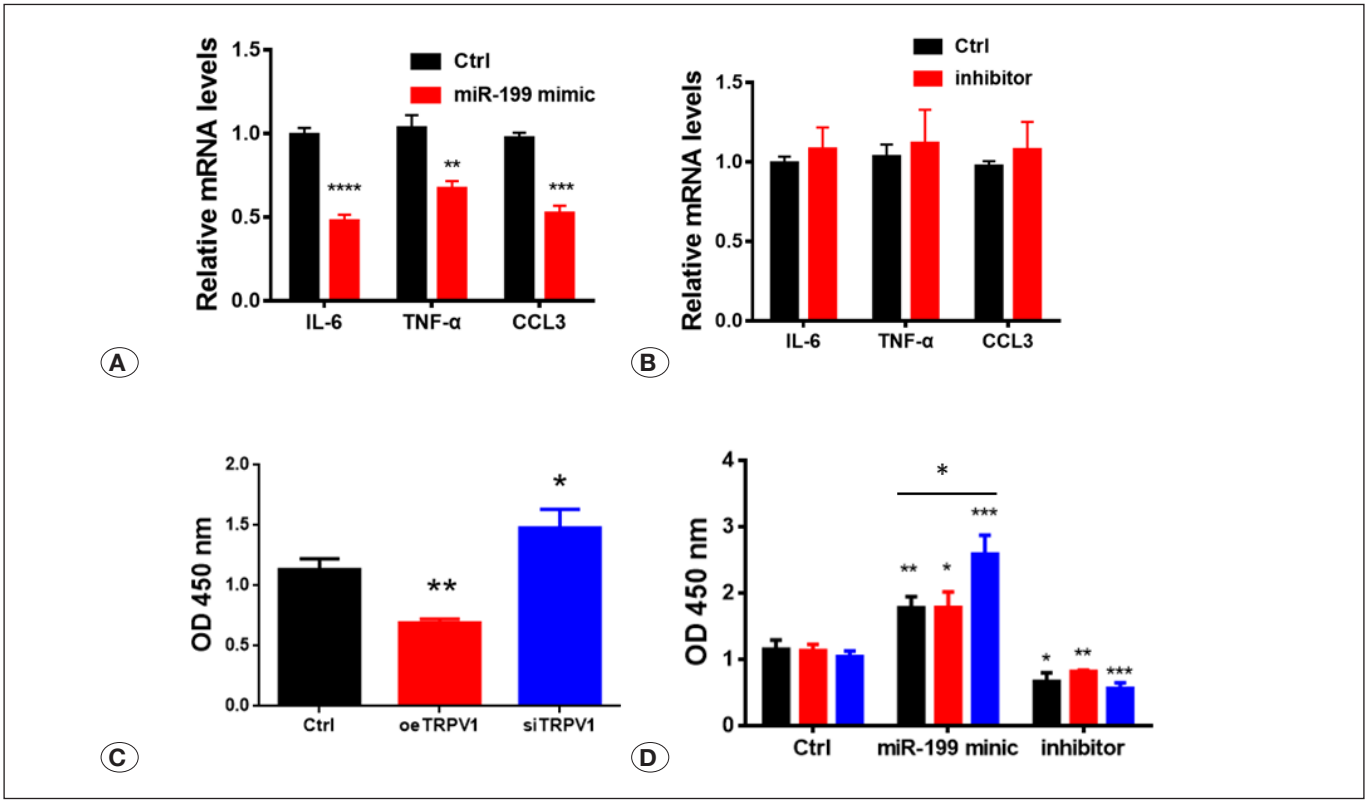

Figure 5: The miR-199 regulates the THP1 interaction with sNF96.2. **A)** mRNA level of inflammatory cytokines (IL-6, TNF-a and CCL3) levels in THP-1 cells after transfection with miR-199 mimic. All three cytokines in miR-199 transfected THP1 cells were significantly downregulated. **B)** mRNA level of inflammatory cytokines (IL-6, TNF-a and CCL3) levels in THP-1 cells after transfection with miR-199 inhibitor. All three inflammatory cytokines levels have not changed. **C)** sNF96.2 was over-expressed or knockdown TRPV1 and co-cultured with THP1 cells for 24h. Then, sNF96.2 were collected and the survival rate of cells was examined by CCK-8 assay. **D)** THP1 cell and sNF96.2 were transfected with miR-199 mimic or mir-199 inhibitor. And these THP1 cells and sNF96.2 were cocultured in one plate for 24h. Then sNF96.2 were collected and the survival rate of cells was examined by CCK-8 assay. The proliferation rate of sNF96.2 in both regulating groups is the lowest. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

Based on this observation, we tested the interactions on THP1 and sNF96.2 in a co-culture system. In the co-culture system, the over-expression of TRPV1 in sNF96.2 inhibited the proliferation and the silence of TRPV1 in sNF96.2 will promote the proliferation of sNF96.2. This demonstrated TRPV1 negatively regulates the survival of nerve cells when macrophage exists. When we up or down miR-199 in macrophage and sNF96.2, the proliferation of sNF96.2 increased or decreased respectively. This showed that no matter whether miR-199 is in macrophage or sNF96.2, it can regulate the survival rate of sNF96.2. Interestingly, when we both up or down-regulated miR-199 in THP1 and sNF96.2, the proliferation change rates are more than regulates miR-199 in one type of cells. This suggested miR-199 in macrophage and nerve cells has a synergistic effect on anti-inflammation.

In this study, we preliminary explored the mechanism of miR-199 on NLBP. And there are also some important questions are need to be clear, such as the mechanism of synergistic effect on anti-inflammation of miR-199 in macrophage and nerve cells; whether the synergistic effect was achieved by the exosome secreted by the macrophage or not? We will explore this interesting question in our future research.

CONCLUSION

miR-199 regulates TRPV1 in macrophages and nerve cells. The low level of miR-199 expression in peripheral nerve injuries of NLBP patients is likely to be one of the pathogenesis of NLBP. Also, miR-199 level in macrophage surrounding injuries nerve of NLBP patients would contribute the NLBP development. This study provides a new target for NLBP treatment.

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AUTHORSHIP CONTRIBUTION

Study conception and design: ZL
Data collection: ZiL
Analysis and interpretation of results: YL
Draft manuscript preparation: ZiL, YL
Critical revision of the article: ZiL
Other (study supervision, fundings, materials, etc...): ZL
All authors (ZiL, YL, ZL) reviewed the results and approved the final version of the manuscript.

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