Evaluation of circulating microRNA expression in patients with trigeminal neuralgia
An observational study

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Abstract
Trigeminal neuralgia (TN) is a chronic neuropathic pain that seriously affects the daily life of patients. There is increasing evidence that microRNAs (miRNAs) play an important role in the development of neuropathic pain.

In this study, the TaqMan Low Density Array (TLDA) was used to analyze the serum miRNA levels of 28 TN patients, and 31 healthy people without any neuropathic pain were used as controls. The results showed that the expression profile of serum miRNA in TN patients was different from that in healthy controls. Compared with the control group, 13 miRNAs in the serum of TN patients were up-regulated and 115 miRNAs were down-regulated by \textgreater 2 times. Quantitative reverse transcription PCR (RT-qPCR) analysis and receiver operating characteristic (ROC) curve were performed. The analysis further confirmed that the expression levels of 4 miRNAs, including miR-132-3p, miR-146b-5p, miR-155-5p, and miR-384, were significantly higher than those of healthy controls, and the difference was statistically significant. This study preliminarily confirmed the changes of serum miRNA expression profile in TN patients. Among them, 4 kinds of serum miRNA are likely to be related to the occurrence and development of TN.

Abbreviations: ARE = antioxidant response element, GO = gene ontology, HO = heme oxygenase, miRNAs = microRNAs, Nrf2 = nuclear factor-E2 related factor 2, ORF = open reading frame, ROC = receiver operating characteristic, RT-qPCR = quantitative reverse transcription PCR, TLDA = TaqMan Low Density Array, TN = trigeminal neuralgia.

Keywords: microRNA, serum, trigeminal neuralgia

1. Introduction
Trigeminal neuralgia (TN) is a common neuropathic pain, clinically manifested as repeated short-term electric shock-like pain in the area of trigeminal nerve distribution, sudden onset and termination, speaking, washing face, brushing teeth, or touching the trigger point can induce severe pain, seriously affect the daily life of patients.\textsuperscript{1,2} Epidemiological data show that female patients are slightly more than men, more on the right than on the left, and the incidence can increase with age, the prevalence is about 0.03\%.\textsuperscript{3,4} The etiology and pathogenesis of TN are still unclear, which may be related to demyelination caused by microvascular compression of trigeminal nerve or epileptic

MicroRNA (miRNA) is an endogenous non-coding RNA with a length of about 22 nt, which is widely found in organisms.\textsuperscript{5} MiRNA plays a key regulatory role in organisms and is one of the most important regulatory factors of gene expression.\textsuperscript{6,7} MiRNAs typically target one or more mRNAs and regulate gene expression through translation level inhibition. Studies have shown that circulating miRNA levels are very stable compared with mRNAs and are highly resistant to degradation, serving as biomarkers or diagnostic tools for diseases.\textsuperscript{8,9} Although miRNAs have been reported to be associated with neuropathic pain, the mechanism is not very clear.

In this study, we compared the changes in serum miRNA expression profiles between patients with TN and healthy controls, and investigated the possible physiological functions of circulating miRNA in patients with TN.

2. Methods
2.1. Subjects
From December 2018 to January 2020, 59 participants from Nanjing Integrated Traditional Chinese and Western Medicine Hospital were selected, including 28 TN patients and 31 healthy
subjects (randomly selected from healthy individuals participating in physical examination). All patients included in this study met the diagnostic criteria of classical TN as described in the International Classification of Headache Disorders (3rd edition).\textsuperscript{[1]} Inclusion criteria: older than 18 years; recurrent paroxysms of unilateral facial pain fulfilling criteria for TN; MRI showed neurovascular compression. Exclusion criteria: secondary TN caused by tumors, intracranial lesions, multiple sclerosis, or herpes zoster; with mental disorders or intellectual disability; with severe hepatic and renal insufficiency; with coagulation disorders; with malignancy; and with chronic inflammatory diseases. The serum samples were sent to the laboratory as soon as possible and stored at −80°C within 4 hours. The study was approved by the ethics committee of Nanjing Integrated Traditional Chinese and Western Medicine Hospital, and all participants provided written informed consent before participating in the study. Demographic information is gathered through electronic medical records or charts.

2.2. RNA extraction

Serum samples from TN patients and healthy controls were placed in test tubes respectively and total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Add 200 μL/mL chloroform, place it at room temperature for 15 minutes, and centrifuge it at 12,000 × g at 4°C for 15 minutes. Then absorb the upper water phase, add isopropanol to precipitate RNA, and centrifuge the supernatant. At last add ethanol, centrifuge the supernatant, and dry it at room temperature. The dry RNA precipitation was suspended in RNase-free H₂O and stored at −80°C until use.

2.3. TaqMan low density array

TaqMan low density array (TLDA) analysis was used to determine the expression profile of serum miRNAs. Seven hundred sixty eight miRNAs in the serum of TN patients and 31 healthy controls were repeatedly measured in the experiment. TLDA reaction system contains 4.5 μL reverse transcription (RT) mixture and 3 μL extracted RNA. According to the manufacturer’s instructions, through the pre-amplification step, the sensitivity of TLDA was improved. 7900 HT Fast Real-Time PCR System was used for quantitative RT-PCR (qRT-PCR) analysis. SDS software analyzed qRT-PCR data and used RQ Manager to calculate the relative level of serum miRNAs. The threshold cycle (CT) values >35 were considered undetectable. The miRNAs expression level uses U6 snRNA as an internal reference.

2.4. Real-time qRT-PCR analysis

Serum miRNA levels were quantified by TaqMan qRT-PCR. The TaqMan miRNA Reverse Transcription Kit and stem ring primers targeting miRNA were used to complete reverse transcription reactions. 5 μL reaction system contained 1.67 μL extracted RNA. PCR reaction was carried out in 7900 HT Fast real-time PCR System: the reaction was conducted at 95°C for 10 minutes, then maintained at 95°C for 15 seconds, finally cooled to 60°C for 60 seconds through 40 cycles, the final volume was 10 μL. The reaction system contained 5 μL TaqMan Universal PCR Master Mix, 0.5 μL TaqMan miRNA Assay primer, and 4.5 μL cDNA template solution. All experiments were repeated 3 times.

2.5. Prediction of target genes

The Target Scan was used to predict the potential target genes of candidate miRNA, and the Database for Annotation, Visualization and Integrated Discovery (DAVID) platform was used to analyze the possible functions or signaling pathways involved in miRNA target genes.\textsuperscript{[10]}

2.6. Luciferase assay

The Human Nrf2 3’UTR fragments were amplified by PCR using human genome DNA as a template. PCR products were cloned to the SpeI and HindIII sites in the pMIR-reporter plasmid polyclonal region and subsequently sequenced to confirm successful insertion. According to the manufacturer’s guidelines, control RNA, mimic miR-155-5p and mutant mimic miR-155-5p, luciferase reporter plasmid, and galactosidase expression vector were transfected into HEK293T cells for luciferase reporter gene assay. Twenty four hours after transfection, luciferase assay kit was used for analysis cells. All experiments were repeated 3 times.

2.7. Statistical analysis

All cases were analyzed using SPSS 20.0 statistical software (IBM Corp, Armonk, NY, USA). The continuous variables are expressed as mean ± standard deviation, and the categorical variables are expressed as numbers and percentages. The receiver operating characteristic (ROC) curves were used to determine the individual specificity and sensitivity of each miRNA and to predict TN in combination with other miRNA. \( P < .05 \) was considered statistically significant.

3. Results

3.1. Participant characteristics

There were 59 participants in this study, including 28 TN patients and 31 healthy controls. There was no significant difference in age and sex distribution between the 2 groups of participants \( (P > .05) \). Among the 28 patients with TN, 5 patients had pain in the V1 and V2 division of the trigeminal nerve, 8 patients had pain in the V2 division, 12 patients had pain in the V3 division, and 3 patients had pain in the V2 and V3 division. Details of the participants are shown in Table 1.

| Characteristics of the study subjects. |
|----------------------------------------|
| Characteristics | TN Group | Healthy Control Group |
|-----------------|----------|-----------------------|
| Case, n         | 28       | 31                    |
| Age, y          | 55.1 ± 13.7 | 49.5 ± 18.3 *         |
| Sex             | n        | n                     |
| Male            | 9 (32.1%) | 11 (35.5%)*           |
| Female          | 19 (67.9%)| 20 (64.5%)*           |
| TN localization |          |                       |
| V1 and V2 division | 5 (17.8%) | 0                     |
| V2 division     | 8 (28.6%) | 0                     |
| V3 division     | 12 (42.9%)| 0                     |
| V2 and V3 division | 3 (10.7%) | 0                     |

\( TN = \) trigeminal neuralgia.

* \( P > .05 \).
3.2. Differential expression of serum miRNAs by TLDA

The expression profiles of miRNAs in serum samples of TN group and healthy control group were analyzed by TLDA. MiRNAs in serum samples of TN group are considered to be significant differences if the Ct value is >35 and the miRNAs concentration differs by >2 times compared with the healthy control group. The results showed that among 768 miRNAs in serum samples of TN patients, 13 miRNAs were up-regulated and 115 miRNAs were down-regulated (Table 2).

### Table 2

| miRNA       | ΔCtTN  | ΔCtControl | ΔΔCt |
|-------------|--------|------------|------|
| miR-523     | 0.244123 | 21.14534   | -20.901219 |
| miR-132-3p  | 2.897932 | 21.14534   | -18.24741  |
| miR-661     | 7.926822 | 21.14534   | -13.21652  |
| miR-888     | 8.388779 | 21.14534   | -12.756552 |
| miR-202     | 5.483215 | 14.72213   | -9.238917  |
| miR-146b-5p | 12.29036  | 21.14534   | -8.850966  |
| miR-155-5p  | 2.319208 | 11.04153   | -8.72324   |
| miR-603     | 12.53269  | 21.14534   | -8.61245   |
| miR-520b    | 7.276987  | 15.49726   | -8.220926  |
| miR-212     | 5.737188  | 13.45477   | -7.717579  |
| miR-34a     | 11.20689  | 14.65523   | -3.448338  |
| miR-197     | 2.876415  | 5.175547   | -2.299132  |
| miR-384     | 3.36635   | 5.55322    | -2.18687   |

The different Ct value between 2 groups was calculated by ΔΔCt method: \( \Delta \Delta Ct = \Delta CtTN - \Delta CtControl \), where \( \Delta Ct = \Delta Ct_{\text{target miRNA}} - \Delta Ct_{U6snRNA} \). TN = trigeminal neuralgia.

3.3. Validation of the serum miRNAs expression by qRT-PCR

The expression profiles of 13 candidate miRNAs in TN patients screened by TLDA were different from those in healthy controls. Further qRT-PCR was used for verification. It was confirmed that 4 miRNAs (miR-132-3p, miR-146b-5p, miR-155-5p, and miR-384) were highly upregulated in the individual patient samples (Fig. 1, \( P < .05 \)). Meanwhile, miR-523, miR-888, miR-202, miR-661, miR-603, miR-320b, miR-212, miR-34a, and miR-197 showed no significant differences between the TN group and the healthy control group (\( P > .05 \)).

3.4. Evaluating the diagnostic value of miRNAs in TN with ROC curves

We performed ROC curve analysis on 4 circulating miRNAs with significantly higher expression levels in TN group than in the healthy control group to evaluate their diagnostic value in TN. As shown in Fig. 2 and Table 3, the area under the ROC curve (AUC) of miR-132-3p, miR-146b-5p, miR-155-5p, and miR-384 were 0.871 (95% CI 0.757–0.986), 0.785 (95% CI 0.662–0.908), 0.838 (95% CI 0.702–0.974), 0.812 (95% CI 0.697–0.927), respectively, indicating that these miRNAs have a good
Further regression analysis of the 4 candidate miRNAs revealed an AUC value of 0.836 (95% CI 0.713–0.958), indicating that these miRNAs can be used as biomarkers for TN and have certain diagnostic value.

3.5. Target gene prediction and functional analysis

In recent years, the influence of miRNAs on neuropathic pain has attracted some attention. We used the Target Scan to predict the target genes of miR-132–3p, miR-146b-5p, miR-155-5p, and miR-384 to determine their potential physiological functions. Gene ontology (GO) analysis suggested that some target genes may be involved in the development of neuropathic pain (Table 4). To verify our target gene predictions, we used a luciferase assay to verify whether Nrf2 is a potential target for miR-155-5p. Human Nrf2 3’UTRs were cloned into the luciferase open reading frame (ORF), and we generated 3 nucleotide mutants at the 3’UTR sequence of Nrf2 (Fig. 3A). The

| miRNA       | AUC   | Std. error | Asymptotic sig. | Lower bound | Upper bound |
|-------------|-------|------------|-----------------|-------------|-------------|
| miR-132-3p  | 0.871 | 0.058      | <0.001          | 0.757       | 0.986       |
| miR-146b-5p | 0.785 | 0.059      | <0.001          | 0.662       | 0.908       |
| miR-155-5p  | 0.838 | 0.069      | <0.001          | 0.702       | 0.974       |
| miR-384     | 0.812 | 0.062      | <0.001          | 0.697       | 0.927       |
| Combined miRNAs | 0.836 | 0.063 | <0.001 | 0.713 | 0.958 |

AUC = area under the ROC curve; ROC = receiver operating characteristic.
patients develop facial numbness after surgery, which seriously percutaneous neurosurgery may pose surgical risks, and some term use can cause side effects. Microvascular decompression and Although anticonvulsants are effective in some patients, long- mechanism of demyelination leading to TN is still unclear. heterotopic impulses that cause touch transmission, but the 5p (MUT). (miR-384 Nervous system NTS, NKAIN2, NDNF, NEUROD4

miR-155-5p Nervous system NGEF, NRG3, NRF2, NFASC, NAV3

miR-146b-5p Nervous system NOVA1, NRAS, NF2, NRP2

miRNAs Go Term Genes

The list of genes predicted to be targeted by the candidate miRNAs. results showed that miR-155-5p mimics significantly downregulated the expression of luciferase fused to Nrf2, while miR-155-5p mutants did not (Fig. 3B). We transfected HEK293T cells with a luciferase vector fused with mutant Nrf2, neither miR-155-5p mimics nor mutant miR-155-5p affected luciferase activity (Fig. 3B). These results indicate that miR-155-5p can directly target the Nrf2 sequence.

4. Discussion

TN is a chronic neuropathic pain characterized by repetitive, transient electrical shock-like pain. Researches have suggested that TN is related to nerve demyelination that occurs around nerve compression sites.[1,2] Demyelination injury can produce heterotopic impulses that cause touch transmission, but the mechanism of demyelination leading to TN is still unclear. Although anticonvulsants are effective in some patients, long-term use can cause side effects. Microvascular decompression and percutaneous neurosurgery may pose surgical risks, and some patients develop facial numbness after surgery, which seriously affects the quality of life. Some patients even develop depression due to inadequate treatment.[6]

The relatively stable properties of circulating miRNAs in serum or plasma make them biomarkers for diagnosis and prognosis of various diseases.[11-14] This study evaluated the expression profile of serum miRNAs in TN patients for the first time. The results based on the ROC curve showed that miR-132-3p, miR-146b-5p, miR-155-5p, and miR-384 in serum of TN patients were significantly increased compared with healthy controls. Leinders et al.[15] believed that miR-132-3p played a harmful-promoting role in chronic neuropathic pain. Upregulation of miR-146b can down-regulate KLF7, reduce the proliferation and migration of Schwann cells, and inhibit nerve regeneration.[16] MiR-155-5p can target and inhibit Nrf2 in diabetic peripheral neuropathy, resulting in sciatic nerve injury.[17,18] Down-regulation of miR-384 can activate the NF-kB signaling pathway to promote SOX9, inhibit apoptosis, promote chondrocyte proliferation, and thus prevent the development of osteoarthritis.[19,20] These miRNAs play a role in the occurrence and development of neuropathic pain.

In addition, target genes were predicted based on significantly up-regulated candidate miRNAs, and the results showed that multiple target genes were closely related to the development of the nervous system. For example, nuclear factor-E2 related factor 2 (Nrf2) can combine with the antioxidant response element (ARE) in the nucleus to initiate the expression of a series of downstream molecules, such as phase II detoxification enzymes, heme oxygenase (HO), and glutathione anabolic enzymes, subsequently play a broad neuroprotective role.[17] In this study, we demonstrated that miR-155-5p could directly target the 3’UTR sequence of Nrf2 mRNA and down-regulate Nrf2 expression. Therefore, it can be speculated that miR-155-5p upregulation may be one of the possible mechanisms in the development of TN, but the exact mechanism remains to be clarified.

In conclusion, our study preliminarily believes that miRNAs may play a substantial role in the occurrence and development of TN. There are still some deficiencies in this study, such as the small number of participants in this study, and only the changes of serum miRNAs in TN patients were compared, more clinical samples are needed to further demonstrate in the future. In addition, the specific biological mechanism of differential expression of miRNAs in TN patients still needs to be further revealed.

Author contributions

Xihan Li and Dongxu Wang designed the study. Xihan Li, Dongxu Wang and Jianbin Zhou carried the study. Xihan Li, Yanfeng Yan and Leiyao Chen drafted the paper and revised it.

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