



Deep Sequencing Identification of Differentially Expressed miRNAs in the Spinal Cord of Resiniferatoxin-Treated Rats in Response to Electroacupuncture

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Abstract

Electroacupuncture (EA) is an effective treatment to relieve pain in patients with postherpetic neuralgia. However, the mechanisms of EA involved therein are still unknown. We first injected resiniferatoxin (RTX) into Sprague Dawley rats to construct the neuralgia model. One week after injection, the rats were treated with EA at the “Huantiao” (GB30) and “Yanglingquan” (GB34) acupoints for 5 weeks. Nociceptive behavioral tests were performed to analyze the changes in thermal sensitivity and mechanical allodynia after RTX induction and EA treatment. Deep sequencing was performed to identify differentially expressed miRNAs in the spinal cord of RTX-induced rats in response to EA treatment. The nociceptive behavioral tests showed that EA at the left GB30 and GB34 acupoints significantly reduced RTX-induced tactile sensitivity and increased RTX-inhibited thermal sensitivity. The sequencing data indicated that RTX resulted in one upregulated and five downregulated miRNAs, and EA treatment resulted in two upregulated miRNAs. Furthermore, seven upregulated and two downregulated miRNAs were found between rats subjected to EA and sham operation. Functional analysis suggested that the targets of differentially expressed miRNAs were enriched in many nervous system-related pathways. The pathway-gene-miRNA net analysis showed that miR-7a-5p had the most target genes. Moreover, miR-233-3p was downregulated after RTX injection and upregulated by EA treatment. We speculated that the upregulation of miR-7a-5p and miR-233-3p is involved in the analgesic effects of EA. Our analysis on the EA-induced differential expression of miRNAs provides novel insights into the mechanisms of EA analgesia in postherpetic neuralgia.

Keywords Postherpetic neuralgia · Electroacupuncture · Analgesia · miRNAs · Deep sequencing

Jing Zou and Xueyang Dong contributed equally to this work.

Significance

The present study identified changes in miRNA expression induced by electroacupuncture in the spinal cord in a rat model of postherpetic neuralgia. The results elucidate the potential role of miRNAs in electroacupuncture-mediated analgesia.

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Introduction

Postherpetic neuralgia (PHN) caused by varicella-zoster virus (VZV) is a type of neuropathic pain characterized by severe burning pain, hypersensitivity, and numbness in the affected area (Baron and Saguer 1993; Virgin et al. 2009). More than 95% of the worldwide population has been exposed to VZV. When the immune system is weakened, the virus is reactivated and causes herpes zoster (HZ), also known as shingles (Levin et al. 2003). The risk of developing HZ is markedly increased in the elderly (Yawn et al. 2007), and nearly 20% of HZ patients develop PHN (Klompas et al. 2011). Therefore, it is necessary to find an efficient analgesic therapy against PHN.

Acupuncture and electroacupuncture (EA) originated from China and have been demonstrated to be effective in relieving pain. The possible mechanisms of acupuncture and EA have been studied in animal models (Zhang et al. 2014), and clinical and experimental results have verified the effect of EA on pain

relief in patients suffering from PHN (Grachev Iu et al. 1998; Wang and Fang 2012). Previous studies have found that systemic treatment of adult rats with resiniferatoxin (RTX), an ultrapotent agonist of transient receptor potential vanilloid 1, resulted in persistent mechanical and thermal sensitivities, which seemed to be unique clinical features of PHN patients (Pan et al. 2003). Therefore, RTX has been used in models of PHN for the search of new therapies to alleviate persistent pain (Chen and Pan 2005; Tanaka et al. 2010). Though it has been shown that EA reduced RTX-induced tactile allodynia and improved thermal perception (Wu et al. 2013), the mechanisms underlying the effect of EA on PHN are unclear.

MicroRNAs (miRNAs) regulate gene expression by translation inhibition or mRNA degradation and play important roles in many biological progresses (Shukla et al. 2011). Studies have suggested the involvement of miRNAs reducing pain (Lopez-Gonzalez et al. 2017) and thus, they are potential targets for investigations in analgesics, in particular the analgesic effect of EA on PHN-related pain.

In the present study, we selected two acupoints, “Huantiao” (GB30) and “Yanglingquan” (GB34), according to their effects in reducing thermal hyperalgesia and mechanical allodynia in an RTX rat model (Wu et al. 2013; Zhao 2008). We performed deep sequencing to determine the differential expression of miRNAs in the spinal cord of RTX-treated rats subjected to EA at the left GB30 and GB34 acupoints.

Materials and Methods

Animal Models

A total of 30 male adult Sprague Dawley rats (250–280 g) were obtained from the First Affiliated Hospital of Tianjin Medical University (Tianjin, China). All rats were individually kept in cages under a 12/12-h light/dark cycle (20–25 °C, 45–55% relative humidity) and given free access to food and water during the experiment. All experimental procedures were approved by the Animal Care Committee at Huazhong University of Science and Technology and conformed to the ethical guidelines of the International Association for the Study of Pain (Zimmermann 1983).

RTX (250 µg/kg, LC Laboratories, USA) dissolved in a mixture of 10% Tween 80 and 10% ethanol in normal saline (Khan et al. 2002) was injected in the peritoneum of experimental rats under halothane (2% in O₂) anesthesia. A mixture of 10% Tween 80 and 10% ethanol in normal saline was injected as a control.

EA Treatment

EA was performed 1 week after RTX injection. A pair of needles (diameter, 0.35 mm; length, 25 mm) were inserted

into the GB30 and GB34 acupoints (Chen et al. 2009; Wu et al. 2013) and connected to an electrical acupuncture apparatus (HANS LH 202 H, Huawei, China). Corresponding to the location of GB30 and GB34 in humans, GB30 is found at the junction of the lateral 1/3 and medial 2/3 of the distance between the greater trochanter and the hiatus of the sacrum, and GB34 lies on the lateral aspect of the leg, in the depression anterior and inferior to the head of the fibula in rats (Wang et al. 2006). EA was carried out at a frequency of 2 Hz and an intensity of 1 mA for 30 min at 10:00 am. The sham EA group was treated in the same way as the EA group but without electrical current.

Nociceptive Behavioral Tests

Behavioral tests were performed three times before and after RTX injection. Prior to testing, the animals were habituated to the testing environment for 30 min. Thermal sensitivity was assessed by exposing the mid-plantar surface of the hindpaw to a beam of radiant heat through a transparent glass surface using a plantar analgesia meter (UgoBasile, Italy) (Hargreaves et al. 1988). The mean value of the withdrawal latency on three consecutive trials was calculated. Mechanical allodynia was assessed by placing the rats on an elevated mesh floor, and the tactile threshold was measured by using the “up-down” method (Chaplan et al. 1994). After an acclimation period of 30 min, a series of calibrated von Frey filaments (Stoelting, USA) were applied perpendicularly to the plantar surface of both hindpaws with sufficient force to bend the filament for 6 s. Brisk withdrawal or paw flinching was considered as a positive response. The test was repeated three times in each rat.

Small RNA Library Construction and Deep Sequencing

After 5 weeks of EA treatment (6 weeks after RTX injection), three rats in each group were deeply anesthetized with 10% chloral hydrate (3.5 ml/kg, i.p.) and transcardially perfused with 500 ml of normal saline at 37 °C, followed by 500 ml of paraformaldehyde at 4 °C in 0.1 M phosphate buffer (pH = 7.4). The L4–L6 lumbar segment of the spinal cord was quickly removed. Total RNA from 12 individual samples (three biological repeats per group) was extracted by Trizol reagent (Invitrogen, USA), and purified by DNase I. The RNAs were reverse-transcribed with the NEBNext Small RNA Kit (Illumina, USA), and cDNAs were amplified by polymerase chain reaction (PCR). The “TruSeq Small RNA Sample Preparation kit” (Illumina Inc., USA) was used to construct libraries according to the manufacturer’s instructions. Small RNA isolation, library construction, and deep sequencing were carried out using an Illumina HiSeq 2500 Genome Analyzer (Illumina Inc., USA) by Genoseq Biotechnology Co., Ltd. (Wuhan, China).

Analysis of Differentially Expressed miRNAs

Poor-quality reads (quality < 20), 5' adapter reads, reads without 3' adapters, reads containing poly (A) tails, rRNA, or tRNA, and reads < 18 nt in length were removed from initial reads acquired by deep sequencing using the Fastx-Toolkit software (http://hannonlab.cshl.edu/fastx_toolkit) to obtain clean reads. The clean reads were mapped to the rat genome to annotate the location on the chromosomes by Burrows-Wheeler Aligner. The known miRNAs were blasted using miRBase 21 (<http://www.mirbase.org/>), and the expression level of miRNAs was determined by the number of reads per million clean tags. Differentially expressed miRNAs were analyzed using the DEGseq software (<http://www.bioconductor.org/packages/release/bioc/html/DESeq.html>). The criteria of differentially expressed miRNAs were *p* value ≤ 0.05 and fold change ≥ 2 .

Prediction of Target Genes and Functional Analysis

Potential target genes of differentially expressed miRNAs were predicted using miRanda (<http://www.microrna.org/microrna/home.do>) software. Gene

Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways of target genes were analyzed by the Database for Annotation, Visualization and Integrated Discovery 6.8 Functional Annotation Tool (<http://david.abcc.ncifcrf.gov/>). The Reactome plug-in in the Cytoscape software (version 3.2.1) was used to construct the functional interaction (FI) network of potential targets, and the key nodes were identified as having more edge counts by network analysis. The path-gene-miRNA net was constructed using Cytoscape software (version 3.2.1).

Quantitative PCR

Total RNA from each sample was reverse-transcribed into cDNA with a stem-loop primer using the Prime Script™ RT reagent kit with gDNA eraser (TaKaRa, Japan). Quantitative PCR (qPCR) was performed in a 10- μ l volume containing 5 μ l of 2 \times SYBR Green Supermix (KAPA Biosystems, USA) and 0.2 μ l of each of forward and reverse primers (Table 1) on a T100-Thermal Cycler (Bio-Rad, USA). U6 was used as an internal control, and the relative mRNA levels were calculated using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen 2001).

Table 1 Primer sequence for qPCR

miRNAs	Sequence (5'-3')
miR-133c-RT	CTCAACTGGTGTCTGGAGTCGGCAATTCAGTTGAGTTGGTCCC
miR-133c-R	GGGCAGCTGTTGAAG
miR-223-3p-RT	CTCAACTGGTGTCTGGAGTCGGCAATTCAGTTGAGGGGGTATT
miR-223-3p-R	GGGTGTCAGTTTGTCA
miR-451-5p-RT	CTCAACTGGTGTCTGGAGTCGGCAATTCAGTTGAGAACTCAGT
miR-451-5p-R	GGGAAACCGTTACCATT
miR-486-RT	CTCAACTGGTGTCTGGAGTCGGCAATTCAGTTGAGCTCGGGGC
miR-486-R	GGGTCTGTACTGAGCT
miR-135a-5p-RT	CTCAACTGGTGTCTGGAGTCGGCAATTCAGTTGAGTCACATAG
miR-135a-5p-R	GGGTATGGCTTTTTATTTC
miR-144-3p-RT	CTCAACTGGTGTCTGGAGTCGGCAATTCAGTTGAGAGTACATC
miR-144-3p-R	GGGTACAGTATAGAT
miR-196b-5p-RT	CTCAACTGGTGTCTGGAGTCGGCAATTCAGTTGAGCCCAACAA
miR-196b-5p-R	GGGTAGGTAGTTTCCTG
miR-196a-5p-RT	CTCAACTGGTGTCTGGAGTCGGCAATTCAGTTGAGCCCAACAA
miR-196a-5p-R	GGGTAGGTAGTTTCATG
miR-19a-3p-RT	CTCAACTGGTGTCTGGAGTCGGCAATTCAGTTGAGTCAGTTTT
miR-19a-3p-R	GGGTGTGCAAATCTATGC
miR-7a-5p-RT	CTCAACTGGTGTCTGGAGTCGGCAATTCAGTTGAGAC AACAAA
miR-7a-5p-R	GGGTGGAAGACTAGTGAT
U6-F	CTCGCTTCGGCAGCAC
U6-R	AACGCTTCACGAATTTGCGT

Statistical Analysis

Each experiment was repeated three times. Differences between the two treatment groups were evaluated by the least significant difference test ($p < 0.05$).

Results

Effects of EA on RTX-Induced Thermal Hypoalgesia and Mechanical Allodynia

To evaluate the effect of EA on thermal hypoalgesia and mechanical allodynia, we evaluated the tactile and thermal sensitivities in rats after the administration of RTX. The baseline of withdrawal threshold was similar in all groups before RTX administration. EA was applied every day starting from 7 days after RTX injection (Fig. 1). After RTX administration, the tactile sensitivity increased significantly, whereas the thermal sensitivity was decreased.

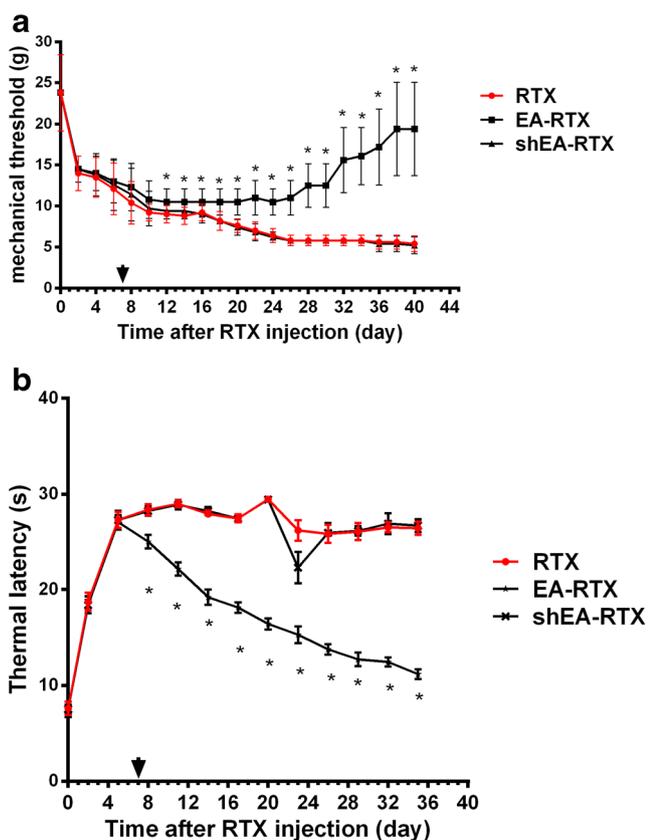


Fig. 1 The effects of EA on RTX-induced thermal hypoalgesia and mechanical allodynia. **a** Time course of mechanical withdrawal threshold in response to von Frey filaments. **b** Time course of paw withdrawal latency to a noxious heat stimulus. EA treatment started 1 week after RTX injection as indicated by arrows. Data are expressed as means \pm standard error of the mean ($n = 10$ rats per group). * $p < 0.01$, compared with the RTX group

EA significantly increased the mechanical threshold starting at 4 days after EA treatment, and this effect was enhanced gradually until 12 days post-EA (Fig. 1a). The thermal sensitivity was significantly increased the day after EA treatment, and this effect was enhanced gradually (Fig. 1b).

Identification of Differentially Expressed miRNAs

To determine the miRNA expression pattern in the spinal cord of treated rats, miRNA sequencing was performed in each library. The clean reads were mapped to the rat genome and matched to several non-coding RNA databases. More than 400 mature miRNAs and 30 to 90 novel miRNAs were found in each sample (Table 2).

We designed four pairwise comparisons to determine the differentially expressed miRNAs: RTX group (RTX vs. control), EA group (EA-RTX vs. RTX), shEA group (shEA-RTX vs. RTX), and treatment group (EA-RTX vs. shEA-RTX). Using the criteria of fold change ≥ 2 ($|\log_2| \geq 1$) and p value ≤ 0.05 , we identified 6, 2, and 9 differentially expressed miRNAs in the RTX, EA, and treatment groups, respectively. In the RTX group, compared with the control, the administration of RTX resulted in one upregulated and five downregulated miRNAs. In the EA group, compared with RTX-induced rats, EA resulted in two upregulated miRNAs. However, shEA treatment induced no differentially expressed miRNAs. Furthermore, seven upregulated and two downregulated miRNAs were found in the treatment group (Supplementary data 1). To verify the results of deep sequencing, we randomly chose 16 miRNAs for qPCR and showed that the results between sequencing and qPCR were similar (Table 3).

Table 2 The number of clean reads, detected miRNA, and novel miRNA in each sample

Sample	Clean reads	Detected miRNA	Novel miRNA
Control-1	29,250,313	499	77
Control-2	28,271,567	486	71
Control-3	29,270,724	484	36
RTX-1	27,560,552	477	70
RTX-2	32,809,581	483	79
RTX-3	29,719,691	468	61
EA-RTX-1	32,676,434	496	72
EA-RTX-2	31,208,905	502	58
EA-RTX-3	37,380,287	511	93
shEA-RTX-1	34,095,444	492	90
shEA-RTX-2	28,847,055	461	62
shEA-RTX-3	31,290,238	484	60

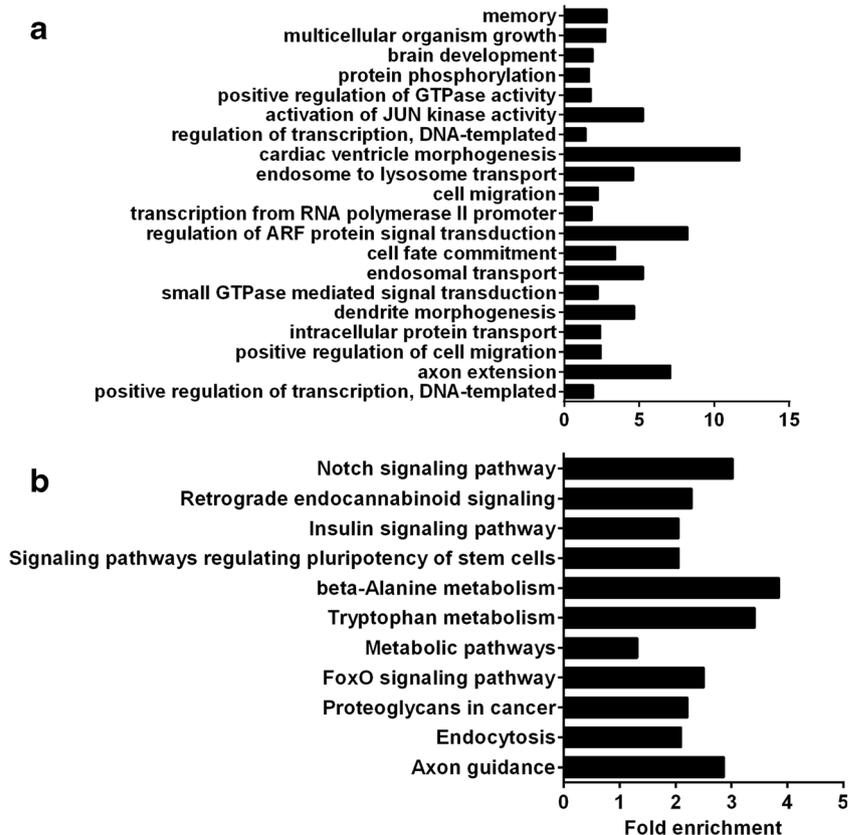
Table 3 The relative expression of potential miRNAs response to EA treatment (log₂(fold change))

miRNAs	EA group		Treatment group	
	seq	qPCR	seq	qPCR
miR-133c	1.11	1.27	0.98	1.41
miR-223-3p	1.90	0.50	1.22	0.52
miR-451-5p	0.63	0.15	1.29	0.09
miR-486	0.42	0.16	1.09	0.33
miR-135a-5p	0.67	0.04	1.09	0.22
miR-144-3p	0.56	0.52	1.56	0.68
miR-196b-5p	-0.52	-0.01	-1.15	-0.13
miR-196a-5p	-0.43	-0.15	-1.13	-0.15
miR-19a-3p	0.73	0.57	1.01	1.27
miR-7a-5p	0.79	3.85	1.11	4.19

Functions of Altered miRNAs in the RTX Group

After RTX administration, there were one upregulated and five downregulated miRNAs. To identify the functions of these miRNAs, we predicted their potential target genes and analyzed the functions of these target genes by GO annotation analysis, pathway analysis, and network analysis. We found 771 potential target genes for six miRNAs. GO enrichment

Fig. 2 **a** Top twenty enriched GO terms of biological processes and **b** KEGG pathways for the predicted target genes of potential miRNAs in the RTX group



analysis showed that the targets of potential miRNAs were enriched in 65 “biological process” categories, including “axon extension” ($p = 3.50 \times 10^{-4}$) (Fig. 2a). KEGG pathway analysis showed that the targets were significantly mapped in 12 pathways, including “axon guidance” ($p = 0.001$) and “FoxO signaling pathway” ($p = 0.005$) (Fig. 2b).

To further confirm the function of targets regulated by miRNAs, we conducted FI network analysis using the Reactom plug-in in the Cytoscape Software. The results showed that some genes had more connection to others (edge counts), such as MAPK1 (target of miR-451-5p), DVL1 (target of miR-214-3p), ITGB1 (target of miR-214-3p), SMAD3 (target of miR-743b-3p), and NOTCH1 (target of miR-743b-3p and miR-881-3p) (Fig. 3, Supplementary data 2).

Functions of Altered miRNAs in Response to EA Treatment

In the comparison analysis, we did not find any altered miRNAs in the shEA group, which suggested that shEA treatment had no significant effect on rats subjected to RTX. Compared with RTX-treated rats, two miRNAs were induced by EA treatment. Furthermore, nine miRNAs were altered between EA and shEA, and miR-233-3p was the overlapping altered miRNA between the EA and treatment groups

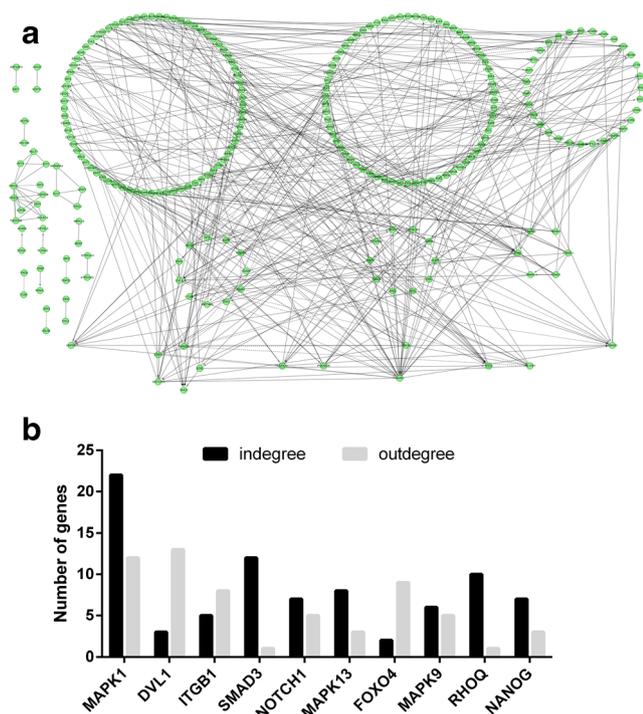


Fig. 3 FI network of predicted target genes of potential miRNAs in the RTX group. **a** The effects of the interaction are represented by arrows, bar-headed lines, straight lines, and imaginary lines. \rightarrow : activating/catalyzing; $-|$: inhibition; $-$: FIs were extracted from complexes or inputs; $-$: predicted FIs. **b** The top ten edge counts of genes

(Fig. 4a). Therefore, we chose these ten miRNAs as the potential miRNAs to perform functional analysis (Table 2) and found 752 target genes. GO analysis showed that the target genes were enriched in 73 “biological process” categories, including “positive regulation of neuron projection development” ($p = 0.01$), and other neuron-related terms (Fig. 4b). The results of KEGG pathway analysis suggested that the targets were significantly enriched in 30 pathways, including “cAMP signaling pathway” ($p = 2.19 \times 10^{-4}$) and “AMPK signaling pathway” ($p = 6.20 \times 10^{-4}$) ($p = 0.04$) (Fig. 4c).

To further confirm the function of the targets regulated by miRNAs, we conducted FI network analysis using the Reactom plug-in in the Cytoscape Software. The results implied that MAPK1 (target of miR-7a-5p and miR-451-5p), GNG2 (target of miR-486), HRAS (target of miR-486), SP1 (target of miR-135a-5p and miR-7a-5p), TBL1XR1 (target of miR-135a-5p), PPARA (target of miR-19a-3p), and AP2B1 (target of miR-133c) might be key genes (Fig. 5, Supplementary data 2).

The Potential Function of Altered miRNA Expression in the Nervous System

To explore the potential mechanisms of EA on RTX-induced pain, we selected enriched pathways that were

related to the nervous system to construct the miRNA-gene-pathway net. Six pathways in the EA and treatment groups were selected, namely “cholinergic synapse” ($p = 0.002$), “dopaminergic synapse” ($p = 0.002$), “GABAergic synapse” ($p = 0.012$), “glutamatergic synapse” ($p = 0.02$), “axon guidance” ($p = 0.04$), and “MAPK signaling pathway” ($p = 0.04$). The results showed that in these pathways, miR-7a-5p had the most target genes, and MAPK1 was involved in the most pathways. Furthermore, we found that UNC5C, MAPK1, PPP2R3B, GABRA2, SHANK2, KCNJ6, PDGFB, and MRAS were the targets of more than one miRNA (Fig. 6).

Discussion and Conclusions

PHN is a persistent neuropathic pain that causes health problems with serious social and economic consequences. Acupuncture is a common therapy in traditional Chinese medicine that has been used to relief pain throughout history, of which EA is a modified mode through electrical stimulation on specific acupoints. In the present study, we investigated the potential mechanism underlying the analgesic effects of EA through deep sequencing of altered miRNA profiles in the spinal cord of RTX-treated rats.

RTX was a potent TRPV1 agonist, which expressed on primary sensory neurons that mediate thermal pain perception. Administration of RTX to neuronal perikaryal selectively ablated nociceptive neurons. The results suggested that RTX could be effective and broadly applicable as strategies for pain management (Laszlo et al. 2004). However, Pan et al. (2003) found intraperitoneal injection of smaller amounts of RTX reduced thermal sensitivity and resulted in profound and persistent tactile allodynia in adult rats, which are similar to those observed in patients with PHN (Pan et al. 2003). Subsequently, many studies used this method to construct the pain model for studying the mechanism of neuralgia (Wu et al. 2013; Yu-Lin et al. 2012). In our study, we found that RTX injection immediately increased the tactile sensitivity and decreased the thermal sensitivity. In the deep sequencing data, we found one up-regulated and five downregulated miRNAs after RTX injection, and many targets of these miRNAs were involved in functions of the nervous system. The gene net analysis suggested that MAPK1 had the greatest number of connections to other genes.

The analgesic effect of EA has been confirmed by many clinical observations and experimental studies (Berman et al. 2004; Vickers et al. 2012). Our results showed that the stimulation of the left GB30 and GB34 acupoints with EA at 2 Hz significantly reduced RTX-induced tactile sensitivity and increased RTX-inhibited thermal sensitivity, which were similar to those from previous studies (Wu

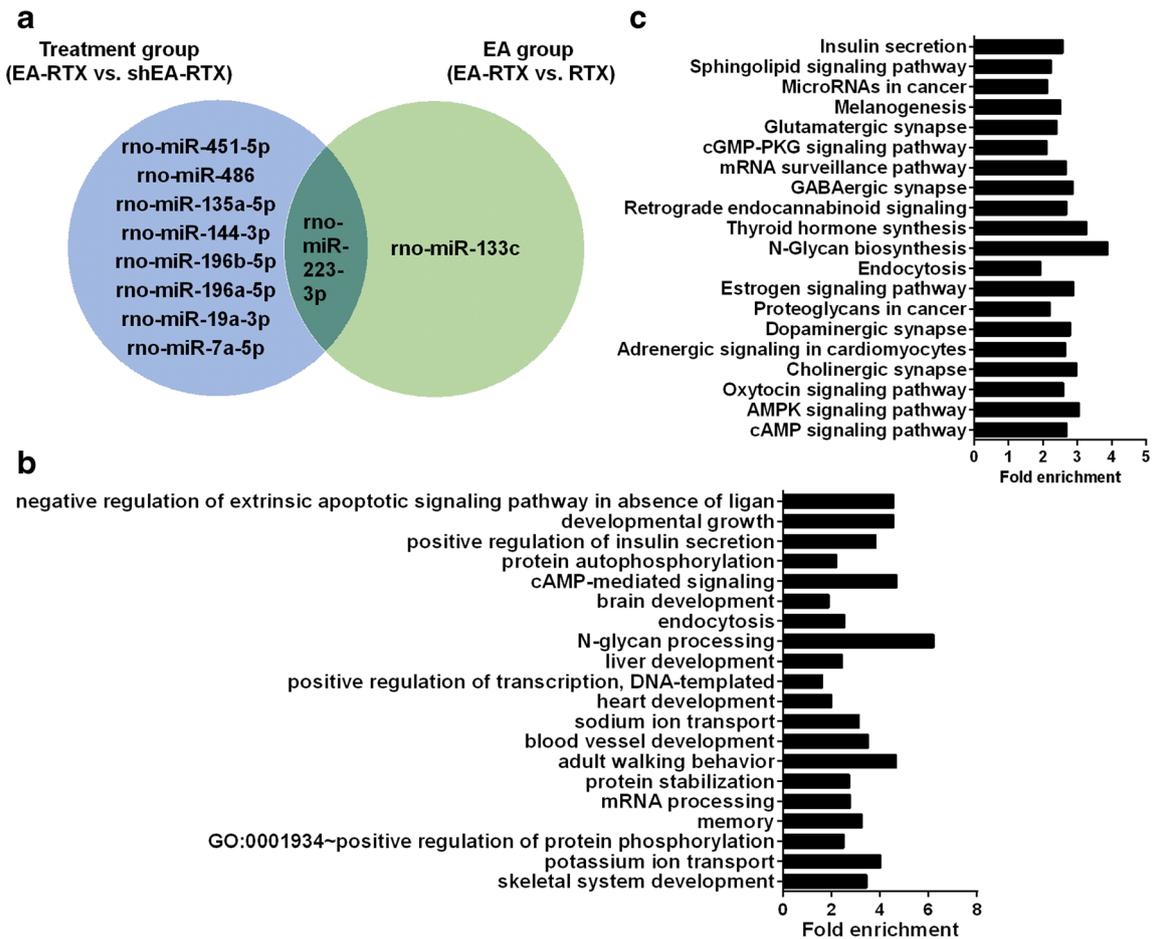


Fig. 4 GO and KEGG pathway analyses of the predicted target genes of potential miRNAs in the EA and treatment groups. **a** Differentially expressed miRNAs in the EA and treatment groups. **b** The top twenty enriched GO terms of biological processes and **c** KEGG pathways

et al. 2013). It has been suggested that the analgesic effect of acupuncture on neuropathic pain involved many factors, including opioids, serotonin, norepinephrine, amino acids, and glial cell/cytokines (Zhang et al. 2014). Deep sequencing data showed that the targets of differentially expressed miRNAs were enriched in many nervous system-related pathways, including “c synapse,” “dopaminergic synapse,” “GABAergic synapse,” “glutamatergic synapse,” and “axon guidance.” Glutamate is a major excitatory neurotransmitter, and gamma-aminobutyric acid (GABA) is the most abundant inhibitory neurotransmitter in the mammalian central nervous system. EA significantly reduced the concentrations of spinal glutamate, aspartate, and glutamine and increased the contents of glycine, GABA, and taurine in the spinal cord (Ma et al. 2008; Yan et al. 2011). Moreover, gabazine and saclofen, antagonists of GABA (A) and GABA (B) receptors, blocked EA inhibition of cold allodynia (Park et al. 2010). These results suggested that EA relieved pain by inhibiting the release of excitatory neurotransmitters and promoting the release of inhibitory neurotransmitters. In our study, nine

genes (GABRA2, KCNJ6, SLC6A1, ADCY6, GABBR1, GNG2, CACNA1S, GAD1, and CACNA1A) were involved in the GABAergic synapse pathway, and ten genes (MAPK1, PLD1, GRM2, GRIA2, ADCY6, GNG2, GRIA3, SHANK2, CACNA1A, and ITPR2) were involved in the glutamatergic synapse pathway. However, the functions of these genes in the analgesic effect of EA require further exploration.

In the pathway-gene-miRNA net analysis, we found that miR-7a-5p had the most target genes. MiR-7a is known as a regulator of the dopaminergic phenotype during development (de Chevigny et al. 2012). It was robustly downregulated by neuronal injury, consequently causing the persistence of neuropathic pain, and exogenous miR-7a induction specifically alleviated neuropathic pain (Sakai et al. 2013). Moreover, miR-223-3p was downregulated after RTX injection and upregulated by EA treatment. A recent study reported that miR-223 suppressed the activities of inflammasomes (nucleotide-binding and oligomerization domain-like receptor 3, apoptosis-associated speck-like protein, and caspase-1) to relieve morphine analgesic

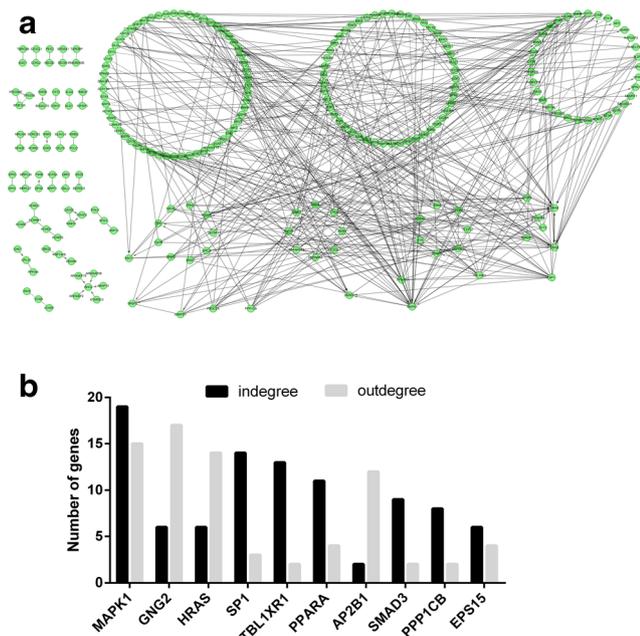


Fig. 5 FI network of predicted target genes of potential miRNAs in the EA and treatment groups. **a** The effects of the interaction are represented by arrows, bar-headed lines, straight lines, and imaginary lines. →: activating/catalyzing; -: inhibition; -: FIs were extracted from complexes or inputs; —: predicted FIs. **b** The top ten edge count of genes

tolerance in rats by downregulating nucleotide-binding and oligomerization domain-like receptor 3 (Xie et al. 2017). Therefore, we speculated that the upregulation of miR-7a-5p and miR-233-3p is involved in the analgesic function of EA. However, the specific mechanism of these miRNAs requires further investigation.

In conclusion, we explored the expression profiles of miRNAs involved in the analgesic effect of EA on RTX-induced pain. Our findings revealed that EA treatment reduced RTX-induced tactile sensitivity and increased RTX-inhibited thermal sensitivity. Functional analysis of target genes of differentially expressed miRNAs suggested that the potential mechanism of EA in relieving pain was associated with the release of neurotransmitters. Furthermore, we found that miR-7a-5p and miR-233-3p might be the key miRNAs in this process, but the mechanism of these two miRNAs requires further study. Our study provides new insights into the analgesic effect of EA.

Author Contributions Jing Zou and Guofu Huang designed the experiments. Xueyang Dong and Yanling Li drafted and revised the manuscript. Siqi Tong and Jingwen Wang assisted with data analysis and interpretation. Mingxuan Liao was a major contributor to the writing of the manuscript. All authors have read and approved the final manuscript.

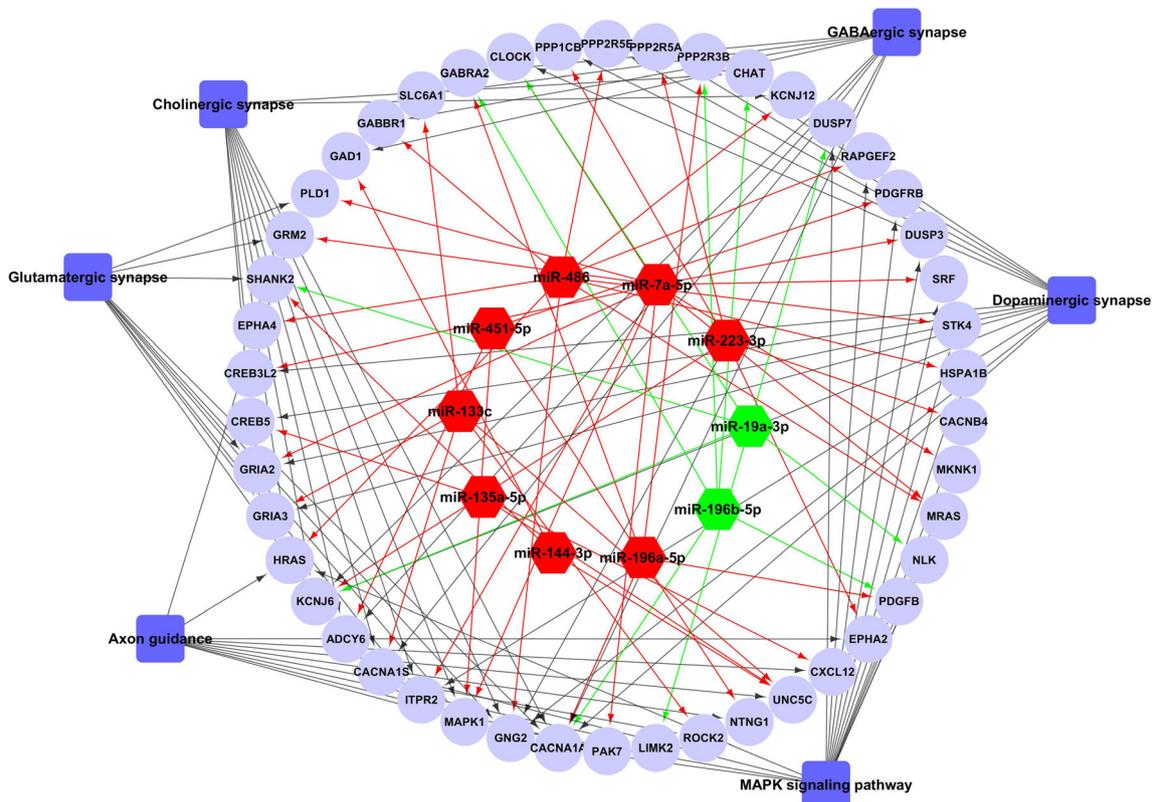


Fig. 6 Pathway-gene-miRNA network in nervous system-related pathways. Circle: gene; square: pathway; diamond: miRNA. Green: downregulated miRNA; red: upregulated miRNA. Green lines:

relationship between downregulated miRNAs and their target genes; red lines: relationship between upregulated miRNAs and their target genes

Compliance with Ethical Standards

All experimental procedures were approved by the Animal Care Committee at Huazhong University of Science and Technology and conformed to the ethical guidelines of the International Association for the Study of Pain.

Conflict of Interest The authors declare that they have no conflict of interest.

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