Circulating microRNA expression profile: a novel potential predictor for chronic nervous lesions

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The mechanisms of chronic neuropathic pain are not clear. Serum microRNAs (miRNAs) might show a special feature for chronic nervous lesions. However, little is known about the changes in circulating miRNAs for the neuropathic pain. Therefore, changes in the circulating miRNAs expression profile for the neuropathic pain were investigated. Serum was collected from rats before and after spinal nerve ligation (SNL) surgery, and a microarray analysis was performed to determine the changes in miRNA expression profile. The expression of inflammatory cytokines in serum from the same individuals, including interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), and monocyte chemotactic protein-1 (MCP-1), was also measured. The results showed that the expression levels of IL-6, TNF-α, and MCP-1 were significantly elevated in SNL rats which were significantly correlated with pain levels. Nine miRNAs with significantly different expression levels before and after SNL surgery were identified by microarray analysis, which were further validated by quantitative real-time polymerase chain reaction analyses. Compared with naive rats without SNL surgery, the expression of five miRNAs (hsa-miR-221, hsa-miR-34c, hsa-miR-21, hsa-miR-30a-5p, and hsa-miR-206) in the serum of rats after SNL surgery was decreased and four miRNAs (hsa-miR-31-5p, hsa-miR-133b, hsa-miR-22, and hsa-miRPlus-A1087) were increased, suggesting that miRNA changes may involve in the regulation of neuropathic pain. TargetScan was used to predict mRNA targets for these miRNAs, and the results showed that the transcripts with multiple predicted target sites belonged to neurologically important pathways. Bioinformatics analysis revealed that several target genes are related to the activation of cell signaling associated with nervous lesions. In this study, the changes to miRNA profiles in serum under neuropathic pain conditions were shown for the first time, suggesting that circulating miRNAs profile in serum is a potential predictor for neuropathic pain.

Keywords circulating microRNA; neuropathic pain; predictor; spinal nerve ligation

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Introduction

Neuropathic pain is a major challenge in clinical medicine with diverse (metabolic, inflammatory, traumatic) neuropathic/neurogenic pain syndromes, which might occur in up to 50% of patients with traumatic peripheral nerve injury resulting from accidents or surgical procedures [1,2], or occur as a complication of bone fracture, tissue injury, or surgical interventions [3]. However, the mechanisms involved are still unclear.

MicroRNAs (miRNAs) were reported to be associated with various pathological processes [4,5] that may play critically important roles in regulating chronic pain. Different miRNA expression patterns may serve as biomarkers for pain and help to predict individual risks for chronic neuropathic pain and responsiveness to analgesic drugs, and some specific miRNAs could become new molecular targets for pain prevention and relief. Therefore, miRNA-based diagnostics are expected to improve the mechanism-based treatment and targeted prevention strategies [6]. Previous studies have shown that circulating miRNAs that are associated with cancer progression are detectable in body fluids, including blood and cerebrospinal fluid. Hence, they may be used as novel biomarkers, which are suitable for clinical diagnostic applications with many kinds of diseases [7–11]. Therefore, it is promising to carefully assess which circulating miRNAs are associated with chronic nervous lesions and could be used as reliable diagnostic biomarkers for painful neuropathy or nerve injury pain. However, direct evidence of changes of circulating miRNA expression in chronic nervous lesions is still lacking. Therefore, understanding the changes of serum miRNAs in neuropathic pain is crucially needed.
Since aberrant miRNA expression is a common feature in a variety of human diseases, an understanding of circulating miRNA expression profiles in neuropathic pain could provide an avenue for the identification of biomarkers or the discovery of novel therapeutic targets [8]. Recent studies have shown that miRNAs in body fluids were stable enough to be used as novel biomarkers for the clinical diagnosis in translational medicine [12–15], with the observed specificity to be used as novel biomarkers for the clinical diagnosis in neuropathic pain. Our results showed that miRNAs could be used as novel predictors of chronic nervous lesions.

**Materials and Methods**

**Animals**

Male Sprague-Dawley rats (220–260 g) were provided by the Experimental Animals Center of Shanghai Jiaotong University (Certificate No. 201000082, Grade II). All experimental protocols were reviewed and approved by the Animal Care and Use Committee of the Medical School of the Shanghai Jiaotong University, and all experiments were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals. The protocol was approved by the Animal Research Committee of Shanghai Jiaotong University in China.

**Behavioral testing**

A 50% paw withdrawal threshold was used to evaluate the behavior of mechanical allodynia, as previously reported [17]. Rats were placed into a transparent plastic cage with a wire mesh bottom. After acclimatization for 30 min, a series of Frey filaments (0.4, 0.6, 1.0, 1.4, 2.0, 4.0, 6.0, 8.0, 10.0, and 15.0 g; Stoelting, Wood Dale, USA) were sequentially applied to the plantar surface of the left paw. Brisk withdrawal and hindpaw licking were recorded as positive responses. If continuous positive or negative responses occurred until the exhaustion of the stimulus set, values of 15.0 and 0.4 g were assigned, respectively. All behavior tests were performed between 10:00 and 13:00.

**Neuropathic pain model**

Left lumbar 5 (L5) SNL was performed, as described previously [18–20]. Briefly, rats were anesthetized with pentobarbital (40–60 mg/kg, intraperitoneally) and placed in a prone position. A midline incision was made at the L4–S2 levels. The left paraspinal muscles were separated from the spinous processes. The L6 transverse process was then removed with particular attention to avoid damaging the nerves underneath. The left L5 spinal nerve was identified and tightly ligated with 4-0 silk thread. Sham surgery was carried out by the same procedure but without ligation of the L5 spinal nerve.

**Serum collection and RNA isolation**

Before and 7 days after SNL surgery, the rats were anesthetized with ketamine HCl and their blood samples were collected by ocular puncture via heparinized capillary tubes into well-labeled heparinized bottles. Whole blood samples were left at room temperature for 30 min to allow complete coagulation and then spun at 1500 g for 15 min at 4°C. Serum samples were immediately frozen at −80°C until the RNA was extracted.

The miRNeasy kit (Qiagen, Chatsworth, USA) was used for total RNA extraction using 200 µl of serum. All serum samples were completely thawed on ice before RNA extraction, followed by centrifugation once at 20,000 g for 15 min at 44°C to remove cell debris. Serum was mixed with a denaturing buffer as described in the manufacturer’s protocols. The mixture was placed on a vortex mixer, and then left at room temperature for 5 min to allow complete inactivation of serum RNases. Then, 20 fmol of synthetic miRNA was spiked into the mixture. From here, the manufacturer’s protocols for RNA extraction were followed. Total RNA was diluted with 50 µl of nuclease-free water for microRNA profiling experiments. The RNA concentration was measured using a NanoDrop 2000C spectrophotometer (Thermo Scientific, New York, USA).

**Determination of cytokines/chemokines by enzyme-linked immunosorbent assay**

The concentrations of cytokines/chemokines in the serum were measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits specific for interleukin-6 (IL-6), tumor necrosis factor alpha (TNF-α), and the chemokine monocyte chemotactic protein-1 (MCP1). The protein concentration was calculated from a standard curve. Blood samples from different groups were centrifuged at 2500 g for 20 min and the serum was collected. The expression of cytokines was analyzed according to the manufacturer’s instructions. Specific antibody was coated onto the wells of the microtiter plates. Samples, including standards of known rat cytokines content, control specimens and unknowns, were added into these wells. Then, a biotinylated antibody specific for rat IL-6, TNF-α, or MCP1 was added. After removal of excess second antibody, streptavidin–peroxidase (enzyme) was added. After a third incubation and wash to remove the unbound enzyme, a substrate solution was
added. The intensity of the colored product was directly proportional to the concentration of rat cytokines present in the blood specimen, which was read at 450 nm.

**Expression profiling determined by miRNA microarray and data analysis**

Microarrays were produced using an LNA-based oligo-Q4 nucleotide probe library (miRCURY LNA array ready to spot v.7.1; Exiqon, Woburn, USA). Oligonucleotides were spotted in duplicate in a phosphate buffer on CodeLink microarray glass slides (GE Health Care, Bethesda, USA) using a VersArray Chipwriter Pro system (Bio-Rad, Hercules, USA), as previously described [21]. Briefly, the RNA was directly labeled with Hy3 using the miRCURY LNA array labeling kit (Exiqon). For each experiment, RNA was labeled with Hy5 using the LNA array labeling kit (Exiqon). Hybridization and washing of the microarray slides were performed as recommended. Scanning was performed using a ScanArray 4000 scanner (GSI Lumonics; PerkinElmer, Waltham, USA) and TIGR Spotfinder 2.23 software was used to generate raw intensity data, which was then normalized using TIGR MIDAS 2.19 software [22]. Average log 2 ratios were calculated from the normalized data based on two measurements of each miRNA. The microarray analysis was performed using three arrays per miRNA sample, and the results were averaged.

**Quantitative real-time polymerase chain reaction of mature miRNAs**

The miRNAs were quantified using real-time polymerase chain reaction (PCR) based on SYBR, as previously described [23,24] with minor modifications. In the absence of a unified internal reference for serum miRNA detection, the exogenous synthetic cel-miR-39 was used as a control [13,25]. Synthetic *Caenorhabditis elegans* miRNA (cel-miR-39; 50 pM in 5 µl) was added as a control before chloroform extraction during total RNA extraction using 200 µl of serum. Real-time quantification was performed in an Applied Biosystems 7500 Sequence Detection system. After the reaction, the Ct data were determined using default threshold settings and the mean Ct was determined from the duplicate PCR. The relative amount of each miRNA was calculated using the comparative threshold (Ct) method with ΔCt = Ct(miRNA) - Ct(cel-miR-39). The ratio of SNL serum miRNA to naive serum miRNA was calculated using the 2^-ΔΔCt method. Quantitative RT-PCR was performed in triplicate, and the entire experiment was repeated several times. All reactions were run in triplicate.

**Statistical analysis**

The results are presented as the mean ± SEM from three or more independent experiments, and the differences were considered statistically significant at *P* < 0.05 using Student’s *t*-test.

**Results**

**Differential expression of serum cytokines in neuropathic pain model**

The SNL model, which has been widely used to investigate neuropathic pain mechanisms and as an assay for the development of new analgesic drugs, produces long-lasting behavioral signs of mechanical hyperalgesia and tactile allodynia. Here, the ligation of L5 was used and tested for tactile allodynia to confirm the success of the surgery in eliciting a pain response. Compared with animals that underwent a sham operation, tight ligation of the L5 spinal nerve produced a robust decrease in mechanical withdrawal thresholds of the ipsilateral hindpaw. The change was started from day 1 after surgery (35%, *P* < 0.01), with a peak on day 3 (10%, *P* < 0.01), and this increased mechanical sensitivity was maintained throughout the duration of the study (Fig. 1).

Whole blood samples were obtained from rats before and after the ligation of the spinal nerve. The expression of cytokines was examined by ELISA to identify the different characters of neuropathic pain. Analysis of inflammatory cytokines in the blood from the same individuals showed changes of several markers after the SNL model was established. Compared with the naive rats before SNL, the results showed that IL-6, TNF-α, and MCP1 levels were significantly elevated in the serum of the SNL rats (Fig. 2). These results are consistent with previous studies [26,27], suggesting that cytokines might function as a downstream regulator involved in the etiology of neuropathic pain.

![Figure 1. Time course of mechanical allodynia for the ipsilateral hind paws in the rats that underwent tight ligation of the L5 spinal nerves (n = 5 per group)](image-url) SNL, but not sham operation, produces a significant decrease in 50% paw withdrawal threshold, starting immediately at day 1 after surgery and persisting for the following observing days. *P* < 0.01, compared with baseline (day 0).
Expression profile of miRNAs in serum after SNL

To identify miRNAs that were specifically regulated in the serum of rats with neuropathic pain, we performed a comprehensive analysis of miRNA expression in the serum of rats before and 7 days after SNL surgery using miRNA expression microarrays. A total of 1200 miRNAs were included in the miRNA microarray, which represents all of the human miRNAs known to date. Each of the three samples of the naive serum and SNL serum was examined for the changes of miRNA expression (Fig. 3). A total of nine miRNAs were identified to be differentially expressed in SNL serum (P < 0.05). Therefore, the nine miRNAs were analyzed. Compared with naive serum, the expression of five miRNAs was decreased and four was increased in the SNL serum (Table 1). Among these miRNAs, miR-21, miR-30a-5p, miR-31-5p, and miR-133b showed the largest fold change in SNL serum compared with naive serum: miR-21 (0.13 folds), miR-30a-5p (0.10 folds), miR-133b (3.16 folds), and miR-31-5p (2.85 folds).

Validation of miRNA expression in serum after SNL by quantitative RT-PCR

To validate the microarray results, quantitative RT-PCR was performed to compare miRNA expression between the serum of rats before and 7 days after SNL surgery. Few studies have been done on hsa-miRPlus-A1087 because it is a relatively new member of the miRNA family. So, we did not do the validation of hsa-miRPlus-A1087. Eight of the nine altered miRNAs that exhibited more than 2-fold change were analyzed (Table 2). In agreement with the microarray results, miR-221, miR-34c, miR-21, miR-30a-5p, and miR-206 were down-regulated, whereas miR-31-5p, miR-133b, and miR-16 were up-regulated in the SNL serum compared with naive sera, and these differences were statistically significant (Table 2). Overall, the microarray data were considered sufficient to warrant further analyses in a clinical setting.

Effects of SNL on miRNA expression in serum

To identify the effect of SNL on miRNA expression in serum, we then analyzed the detectable levels of the eight miRNAs in the serum of rats after SNL. The blood samples were collected by ocular puncture at days 0, 1, 3, 7, and 13. This neuropathic pain model allowed us to monitor the development of tactile stimulus-induced neuropathic pain hypersensitivity. Peripheral nerve lesion induced a marked mechanical allodynia from days 1 to 13 post-surgery, whereas sham-operated animals showed no change in mechanical sensitivity, as described above (Fig. 1).

The expression of hsa-miR-221, hsa-miR-34c, hsa-miR-21, and hsa-miR-30a-5p in serum showed no change at...
day 1 after nerve injury, but a significant down-regulation was observed at days 3, 7, and 13 post-surgery (Fig. 4A). However, the expression of hsa-miR-206 showed a remarkable down-regulation as early as day 1, and persisted at days 3, 7, and 13 (Fig. 4A). On the contrary, hsa-miR-31-5p, hsa-miR-133b, and hsa-miR-22 showed no difference at the expression level at day 1, but had a significant up-regulation at days 3, 7, and 13 post-surgery (Fig. 4B). It is well established that pain from different origins may induce specific phenotypic changes in serum.

Prediction of miRNA targets and pathway analysis
To explore the possible biological consequences of SNL-derived miRNA expression changes, we identified mRNA transcripts containing predicted target sites for these eight miRNAs. The TargetScan algorithm was used as previously reported. Each miRNA of the eight miRNAs potentially regulates many targets. A total of 909 potential targets were predicted, and the number of potential targets of each miRNA varied from 3 to 297. Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses were carried out to elucidate target pathways of the miRNAs. The DAVID platform was used to identify the putative targets of the miRNAs. Our results showed that many targets played significant roles in several important signaling pathways that are closely associated with the signaling of pain, such as the transforming growth factor-β (TGF-β) family and mitogen-activated protein kinase (MAPK) pathways, ion channels, Bdnf, Calm2, and Igf1 (Table 3).

Discussion
Recently, specific miRNAs have been found to be associated with pathological pain [28,29]. Unique signatures of miRNAs are associated with altered neuron function [30–33], and alterations in miRNA expression may account for the variation in susceptibility to certain types of pain, or even for the responsiveness to analgesics and opioid tolerance [34]. Understanding the role of miRNAs in pain mechanisms may provide great benefit for clinical diagnostic and therapeutic applications. However, the functional roles of miRNAs in neuropathic pain are unraveled, and no investigation has been reported about serum miRNAs in neuropathic pain. Therefore, blood-based miRNA biomarkers that predict clinical behavior and/or therapeutic response should be identified.

Serum cytokine expression profiles in chronic pain conditions were reported to be significantly different from those of people without chronic pain [26]. Chronic pain is associated with lower gene and protein expression levels of anti-inflammatory cytokines and higher gene and protein expression levels of pro-inflammatory cytokines [35]. Therefore, we determined the expression of cytokines in serum by ELISA. Our results showed an increase of IL-6, TNF-α, and MCP-1 expression in serum in the SNL model, which is in line with

Table 2. Validation of miRNA microarray results using quantitative RT-PCR: expression levels of miRNAs in rat serum after spinal nerve ligation

<table>
<thead>
<tr>
<th></th>
<th>Before</th>
<th>After</th>
<th>P value</th>
</tr>
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<tbody>
<tr>
<td>hsa-miR-221</td>
<td>12.68 ± 0.34</td>
<td>15.33 ± 0.35</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>hsa-miR-34c</td>
<td>14.28 ± 0.37</td>
<td>18.76 ± 0.26</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>hsa-miR-21</td>
<td>13.08 ± 0.31</td>
<td>16.75 ± 0.32</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>hsa-miR-30a-5p</td>
<td>18.53 ± 0.29</td>
<td>21.37 ± 0.53</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>hsa-miR-206</td>
<td>19.58 ± 0.56</td>
<td>17.23 ± 0.52</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>hsa-miR-31-5p</td>
<td>12.76 ± 0.45</td>
<td>9.69 ± 0.73</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>hsa-miR-133b</td>
<td>19.58 ± 0.24</td>
<td>10.66 ± 0.33</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>hsa-miR-22</td>
<td>17.4 ± 0.64</td>
<td>15.09 ± 0.47</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

Low values indicate high levels of miRNA. Data derived from quantitative reverse transcriptase PCR validation of miRNAs.
There is evidence showing that chronic pain and analgesic response is highly variable between individuals. Although much of the variability showed a sufficient heritability, the functional genetic polymorphisms were found to be related to this variability [36–38]. Polymorphisms of specific molecular targets may be associated with certain pain phenotypes [39]. Changes of miRNA expression profile or their target sites were not only bioinformatically predicted to be associated with the pathogenesis of diseases, but also were experimentally validated [40,41]. However, the polymorphisms in miRNA genes and/or their binding sites, the downstream targets of miRNAs, and the mechanisms involved are entirely unclear.

In the present study, differential expression of nine miRNAs in serum from whole blood of SNL rats was observed compared with control samples. It was found that five microRNAs (hsa-miR-221, hsa-miR-34c, hsa-miR-21, hsa-miR-30a-5p, and hsa-miR-206) were down-regulated and four miRNAs (hsa-miR-31-5p, hsa-miR-133b, hsa-miR-22, and hsa-miRPlus-A1087) were up-regulated. The results indicated that multiple miRNAs were significantly different between neuropathic pain subjects and control subjects. miRNAs recognize their target mRNAs using the 2–8 nucleotide sequence at the 5' region of the miRNA, called the seed sequence. Target prediction algorithms use different parameters to provide candidate target genes for miRNAs. Some of the miRNAs are of special interest concerning their putative targets, so TargetScan was used to identify putative targets and to provide a foundation for functional analyses of neuropathic pain as done in previous reports [42–44].

Many of miRNAs identified in our study are known to be associated with the cell cycle differentiation. Some of the miRNAs identified in this study have been shown to play
important roles in biological processes of cellular mechanisms [29]. Bioinformatics prediction of the significantly altered eight miRNAs showed that these miRNAs can potentially modulate the mRNAs of a number of genes relevant to neuropathic pain, including inflammatory mediators, ion channels, and G protein-coupled receptors, as shown in Table 3. For example, a bioinformatics-based prediction indicates that hsa-miR-22 and hsa-miR-206 can target Bdnf, MAPK3, Calm2, Ngfr, Pla2g4a, and Igf1 [27]. One frequently identified target of hsa-miR-221 is ADP-ribosylation factor 4 (ARF4). ARF4 interacts with EGFR, thereby enhancing oncogenic processes [45,46]. Some studies have reported that hsa-miR-221 expression is negatively correlated with p27 expression [46,47], and can target the KIT gene [48]. However, only a few miRNA targets have been validated in neuropathic pain. Further studies are needed to determine the function of the miRNAs identified in the serum of SNL rats.

Additional studies using real-time quantitative polymerase chain reaction assays to validate these predictions and functional consequences of miRNA alterations can provide mechanistic insight into the mode of action of miRNAs in neuropathic pain. The miRNAs we found, shown in Fig. 3 from the neuropathic pain study, were compared with previous reports in rodent models for pain investigation. This study is the first report about the association between miRNAs and neuropathic pain in SNL rats, although there was some overlap with previous studies, which focused on the expression changes of miRNAs [27,29,42,44,49].

In summary, the expression profiles of miRNAs in the serum of SNL and naive rats were distinct. As each individual miRNA could have hundreds of mRNA targets, miRNAs that are differentially regulated in serum may have a variety of functions in neuropathic pain condition. The mechanisms of the reduced expression of miRNAs remain to be elucidated. However, miRNAs could be attractive therapeutic targets, and will be an exceptionally valuable tool for assisting physicians in choosing treatment options for patients in clinical trials. Our studies suggested that clinically monitoring of the patient population is possible on the basis of alterations in miRNA expression and that miRNA expression profiling can serve as a novel approach. However, the target mRNA(s) of the differentially expressed miRNAs, which were predicted by computational algorithms, need to be validated. Another limitation of the study is its relatively small sample size. In the future, researchers could gain further insight into mechanistic aspects of neuropathic pain by performing similar miRNA profiling in more animal models to cross validate the human data.

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