Association Between LncRNA NONRATT021972 and TNF-αin Diabetes

Kai Li1, Shan LU1 and Shan-shan YU1,*

1Anesthesia Department, China-Japan Union Hospital of Jilin University, Changchun, Jilin
*Corresponding author

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Abstract. Objective to explore potential association between LncRNA NONRATT021972 and TNF-αin diabetic rat models. Methods Streptozocin (STZ) was treated for SD adult male rats to establish diabetes model. 40 STZ-treated rats were randomly into two groups, and siRNA group received NONRATT021972 siRNA treatment, while the rest received saline treatment (D2M). 20 Normal rats were received saline treatment in Control group. Vein blood sampling was performed in 3 groups. Quantitative Real-time PCR was performed to examine expression of LncRNA NONRATT021972 and TNF-α in blood. Mechanical withdrawal threshold and the thermal withdrawal latency test were performed in1w, 2w, 3w, 4w later for 3 groups’ rats. Results Compared with D2M group, blood samples in Control group and siRNA group contained significantly lower concentration of glucose and LncRNA NONRATT021972 (P<0.05) . Compared with D2M group, blood samples of 3 weeks later in Control group and siRNA group contained significantly lower concentration of TNF-α (P<0.05). Moreover, the concentration of TNF-αamong D2M rats was positively associated with LncRNA NONRATT021972. Compared with control group, D2M group showed decreased mechanical withdrawal threshold and the thermal withdrawal latency. Further experiment showed that inhibition of LncRNA NONRATT021972 by siRNA indeed alleviated neuropathic pain, verified by mechanical withdrawal threshold and the thermal withdrawal latency in siRNA group compared with D2M group. Conclusion LncRNA NONRATT021972 was increased in type 2 diabetes rat and was positively associated with TNF-α. LncRNA NONRATT021972 exacerbated neuropathic pain via glucose and TNF-α related pathways. Inhibition the gene was a therapeutic strategy.

Introduction

Long non-protein-coding RNA (LncRNAs) belongs to family of small RNAs, and is characterized with transcripts that are >200 nucleotides in molecular length. LncRNAs can be transcribed, ranging from 195 bp up to a couple of kilobases in length according to the sequence of LncRNA nearby protein-coding genes [1, 2]. Previous studies have indicated that LncRNA was involved in occurrence and progressing of diabetes, and produced a complex regulatory network through interactions with transcription factors in type 2 diabetes. On the other hand, LncRNAs were also involved in the pathological progression of nervous system, and had multiple effects on neuropathic pain [3].

Diabetes mellitus has become a global health issue in recent years, with a global incidence of 14.6 %. In clinical scenario, neuropathic pain is one of the most common chronic complications in patients with type 2diabetes, and character with typical symptoms of pathological pain, including hyperalgesia, spontaneous pain and specific alldynia [4, 5]. Thus, it is urgent to develop novel therapy for neuropathic pain of type 2diabetes due to the enormous number of diabetes patients and its adverse effects.

NONRATT021972 (NONRA) is an LncRNA which was proved with diabetes promoting effect, and its sequence was well determined (http://www.noncode. org/show, rna.php? id= NONRATT021972). Animal experiments have showed that expression of NONRATT021972 increased in diabetic mice, and participated in the transmission of nociceptive signaling, especially in neuropathic pain. What’s more, there are data suggesting NONRA silence was related to decreased inflammation, however, although researchers found NONRA could regulate P2X7 and
P2X3 receptors in dorsal root ganglia, no detailed mechanisms were explored in the field of inflammation. In the clinical application, it is unclear whether NONRA could be a predictor for type 2 diabetes, and there is also no report about how NONRA influenced neuropathic pain of type 2 diabetes in clinical scenario. Accordingly, we hypothesized that NONRA could be a potential biomarker for neuropathic pain of type 2 diabetes in clinical scenario.

Materials and Methods

Serum Isolation of NONRATT021972 and RT-PCR

Total RNA was isolated from the serum using the RNA pure Circulating Reagent (CWBIO, Beijing, cat. no. CW2281) with routine protocols. Briefly, serum sample (300μL) was added to three times volumes of RNA pure Circulating Reagent and mixed thoroughly via vortex. Storage samples to maintain reaction at room temperature for five minutes. Further, 1/5 volume of chloroform was added, mixed vigorously for 30s, and incubated for 5 min at room temperature. Centrifuge mixture for 20 min (12,000×g, 4 °C). The supernatant was transferred to a new sterile EP tube, and the same volume of isopropanol was added and mixed thoroughly for 30 min at room temperature. Centrifuge mixture for 20 min (12,000×g, 4 °C). The precipitate was transferred and rinsed twice with 1 mL of 75 % ethanol (dilute with DD H2O). Isolated RNA was eluted by RNase-free water (20 μL) with routine protocols. Total RNA (1000 ng) was prepared as standard template for reverse transcription with the Revert Aid First Strand cDNA Synthesis Kit (Thermos, USA). PCR amplification of NONRATT021972 and GAPDH (control) was performed according to reported method. The primers were as follows: NONRATT021972, 5-TGTTCTGCAACTGTCAGCT-3, antisense 5-GGATGGTTCAAAGCCTCA-3; the estimated length of the PCR product was 250 bp and 199 bp for NONRATT021972 and GAPDH, respectively. The thermal cycling protocols were as follows: 95°C for 30 s; 40 cycles of amplification at 95°C for 5 s; 60°C for 30 s. The results of PCR were analyzed by the software with PCR instrument (ABI 7500).

Measurement of TNF-α

The TNF-α levels of blood samples were quantified with enzyme-linked immunosorbent assay (ELISA) and antibodies used in our experiment were commercially available. The protocol was provided by the ELISA kit supplier (Senxioming Company, Shanghai, China). The reactions were performed and assayed using standard ELISA reader (Rayto, RT-6000, USA) at 450 nm. The concentrations of TNF-α were determined with routine protocol.

NONRATT021972 Small Interference RNA Treatment

The small interference RNA (siRNA) of NONRATT021972 was purchased from Invitrogen (Carlsbad, CA). siRNA targeted to NONRATT021972 was used in our experiment. The siRNA was diluted with 80 μL of RNA free water and added 80 μL of 10% glucose as mixture A. Mixture B was produced by mixing 80 μL of Transfection Reagent and 80 μL 10% glucose. Mix mixture B and mixture A for 15 min reaction. The siRNA target sequence was 5-GAATGTGGTCATACAAA-3 (Invitrogen).40 SD adult male rats were prepared for diabetes model, and intraperitoneal injection of STZ (30 mg/kg) was performed to establish animal models of diabetes with reported protocols. Fasting blood glucose >7.8 mmol/L and non-fasting blood glucose >11.1 mmol/L were considered to successful diabetes model. 20 diabetic rats were randomly injected with NONRATT021972 siRNA (320 μL) per week

Behavioral Studies of Diabetic Rats

The thermal withdrawal latency was determined with the Thermal Paw Stimulation System (BME410C, Tianjin). Rats were placed in a transparent box (50 cm×35 cm× 50 cm) with glass plate under which a thermal light source was located. After a 30-min adaptive phase, the paw of rats was exposed to a beam of radiant heat applied through the glass floor. Activation of the thermal light source simultaneously activated a timer, and both were immediately turned off by paw withdrawal or at the 25-s cut-off time. Mechanical withdrawal threshold was assessed by observing withdrawal
responses to mechanical stimulation using von Frey filaments (Stoelting, Wood Dale, IL, USA) with reported protocols. Both examinations were performed at 4-time points after establishment of diabetic model (1-week, 2-week, 3-week and 4-week)

Statistical Analysis
SPSS 16.0 software was used for data processing. Measurement data are normal distribution to mean ± SD. T test was performed for data with normal distribution and equal variance. Logistic Linear regression was performed to determine association among variate.

Result
NONRA Increased in Type 2 Diabetes
Compared with control group, serum concentration of NONRA indeed increased in patients with type 2 diabetes (P<0.05), suggesting increase of NONRA was associated with increased blood glucose or occurrence of type 2 diabetes and NONRA might be a novel biomarker of type 2 diabetes (Table 1).

Table 1. Analysis of blood glucose and NONRATT021972 relative expression.

<table>
<thead>
<tr>
<th>Group</th>
<th>blood glucose</th>
<th>NONRA relative expression</th>
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<tbody>
<tr>
<td>Control</td>
<td>5.12±1.023</td>
<td>1.00±0.107</td>
</tr>
<tr>
<td>D2M</td>
<td>13.27±2.972*</td>
<td>2.98±0.693*</td>
</tr>
<tr>
<td>SiRNA</td>
<td>8.63±1.564▲</td>
<td>1.08±0.423▲</td>
</tr>
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Compared with Control group, *P<0.05. Compared with D2M group, ▲P<0.05.

Serum Level of TNF-α Increased in Type 2 Diabetes
ELISA showed that, compared with control groups, patients with type 2 diabetes had a remarkably higher level of TNF-α (P<0.05, Figure 1), and such increase was associated with NONRA (P<0.05, Figure 2).

Figure 1. TNF-α in different group after 3W intraperitoneal injection Compared with Control group, *P<0.05. Compared with D2M group, ▲P<0.05.

Figure 2. Association between TNF-α and NONRA in D2M group.
LncRNA NONRATT021972 siRNA Decreased TNF-α in Diabetic Rats

STZ-induced diabetes models were successfully established in SD adult male rats. Further NONRA siRNA decreased blood glucose level and decreased TNF-α, verified by ELISA (P<0.05, Figure 1), suggesting inhibition of NONR attenuated inflammation of STZ-induced diabetes.

**NONRA siRNA Alleviated Neuropathic Pain in Diabetic Rats**

Compared with normal rats, STZ-induced diabetic rats had more severe symptoms of neuropathic pain, verified by increased duration of mechanical withdrawal threshold and the thermal withdrawal latency (Figure 3). NONRA siRNA significantly decreased duration of mechanical withdrawal threshold and the thermal withdrawal latency after 4 weeks treatment (Figure 3), suggesting NONRA siRNA alleviated neuropathic pain in diabetic rats.

![](image1)

**Figure 3A 3B. Temporal trend of mechanical withdrawal and thermal withdrawal latency in rats after intraperitoneal injection. Compared with Control group, *P<0.05. Compared with D2M group, ▲P<0.05.**

**Discussion**

Although LncRNAs accounts for a quite high proportion of RNA profile in mammalian, current studies only revealed a small number of LncRNAs which have been functionally proved, and functions of most LncRNAs are unclear. Recent evidence from animal experiments indicated that the abnormal expression of LncRNAs participates in the occurrence and progression of type 2 diabetes, in which LncRNA ONRATT021972 was a biomarker with promising clinical potential [6].

Our studies firstly shown that increased serum level of NONRA was positively associated with grade neuropathic pain in patients with type 2 diabetes, suggesting NONRA was biomarker or predictive factor for neuropathic pain of type 2 diabetes. Further clinical trials explored that there was no association between neuropathy and NONRA in patients without diabetes, suggesting NONRA was a biomarker for type 2 diabetes and not exclusively a marker for neuropathy.

Our studies firstly shown that increased serum level of NONRA was positively associated with type 2 diabetes, suggesting NONRA was biomarker or predictive factor for type 2 diabetes. Further experiment explored that ELISA of the blood samples indicated that LncRNA NONRATT021972 could enhance inflammation via TNF-α related pathways, which was consistent with reported animal studies. What’s more, NONRA siRNA decreased mechanical withdrawal threshold and the thermal withdrawal latency of STZ-induced diabetic rats, suggesting inhibition of LncRNA NONRATT021972 could alleviate neuropathic pain. Our study give a clue that inhibition of
LncRNA NONRATT021972 might be a feasible way to relieve symptoms of neuropathic pain for patients with type 2 diabetes.

Previous reports indicated that NONRA was involved in type 2 diabetes and mostly induced hypersensitivity of nervous system, which finally resulted in neuropathic pain [7]. Moreover, animal studies have showed that NONRA aggravated diabetic neuropathic pain via P2X3 or other P2 receptors in dorsal root ganglia [8]. However, as described above, such studies were mainly focused on animal studies or underlying mechanisms, while no concrete data were offered in clinical scenario. This report made the first excellent answer in clinical research.

In summary, LncRNA NONRATT021972 was positively associated with type 2 diabetes via TNF-α related pathways. Inhibition of LncRNA NONRATT021972 could alleviate neuropathic pain via TNF-α related pathways.

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References


