Effects of miR-129-5p on inflammation and nucleus pulposus cell apoptosis in rats with intervertebral disc degeneration through JNK signaling pathway

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Abstract

This study aimed to explore the effects of miR-129-5p on inflammation and nucleus pulposus (NP) cell apoptosis in rats with intervertebral disc degeneration (IVDD) through the c-Jun N-terminal kinase (JNK) signaling pathway. A total of 20 rats were randomly divided into control group (n=10) or IVDD group (n=10). The mRNA expressions of miR-129-5p and apoptosis index Fas in IVDD tissues were determined using RT-PCR. NP cell apoptosis rate was detected via TUNEL assay. NP cells were extracted from IVDD tissues for primary culture. Subsequently, the cells were transfected with miR-129-5p inhibitor or mimic to inhibit or overexpress miR-129-5p, respectively. Furthermore, the changes in the JNK pathway indexes and apoptosis indexes were detected using Western blotting. In IVDD group, the expression of miR-129-5p was significantly down-regulated, while the transcriptional level of Fas was up-regulated compared with those in control group. Pearson correlation analysis revealed a negative correlation between the expressions of miR-129-5p and Fas mRNA (r=-0.75, P<0.05). IVDD group exhibited significantly higher levels of serum TNF-α, IL-6 and IL-1 than control group. Subsequent TUNEL assay indicated that the apoptosis rate was evidently higher in IVDD group (60.6%) than control group (2.5%). The results of Western blotting showed that the protein expressions of JNK1, JNK2 and Fas remarkably rose in IVDD group compared with those in control group. However, they declined remarkably in miR-129-5p mimic group compared with those in control group. Furthermore, such trends were significantly reversed in miR-129-5p inhibitor group. MiR-129-5p was significantly down-regulated in IVDD, whose overexpression has anti-inflammatory and anti-apoptotic effects.

Keywords: MiR-129-5p, Intervertebral disc degeneration (IVDD), Inflammation, Apoptosis.

1. Introduction

Intervertebral disc degeneration (IVDD) induced lumbar has become a serious global problem, affecting people’s life quality [1]. Histologically, IVD consists of three interdependent and structurally different parts: (1) nucleus pulposus (NP) containing a large number of extra-cellular matrixes (ECM), such as proteoglycan and collagenII, (2) annulus fibrosus (AF) composed of a large amount of collagen I, and (3) upper and lower cartilage endplate (CEP) responsible for regulating the diffusion of nutrients in the vertebral body and IVD [2]. Due to these complex structures, IVD possesses unique biomechanical properties to keep the flexibility and mechanical stability of the spine. The changes in IVD will alter the arrangement and mechanical environment of the vertebral body, facet joints, spinal ligaments and muscles, eventually leading to spinal pain [3]. In degenerated IVD, a series of inflammatory factors and chemical mediators can be produced. Meanwhile, they can trigger pain in peripheral nerve endings and induce the growth of blood vessels and nerves into IVD circular defects [4].

Previous researches have found that IVDD is a chronic process of excessive destruction of ECM. It is also considered as the main cause of lumbago as well [5]. However, the potential mechanism of IVDD has not been fully clarified. Recent studies have shown that apoptosis is an important form of IVD cell death [6]. The reason is that apoptosis-induced cell loss contributes to the degradation of ECM and plays an important role during IVDD [7]. However, the specific mechanism of apoptosis in degenerated IVD remains unknown.

Microribonucleic acids (miRNAs) are a class of endogenous non-coding RNA molecules with about 22 nucleotides in length. They can regulate gene expression through base pairing with the 3’ untranslated region of target mRNAs [8,9]. MiRNAs have been confirmed involved
in several life processes, such as cell proliferation, apoptosis and invasion. Meanwhile, their dysregulation is correlated with many human diseases, including IVDD [10,11]. For example, miR-129-5p inhibits Fas-mediated apoptosis of various human cells via regulating Fas [12]. However, the specific role of miR-129-5p during the progression of IVDD has not been fully elucidated.

In this study, inflammatory factors and NP cell apoptosis were used as indexes of IVDD. The indexes of the c-Jun N-terminal kinase (JNK) signaling pathway were analyzed as well. All our findings might help to explore the potential mechanism of miR-129-5p in IVDD.

2. Materials and Methods

2.1. Animal Modeling

A total of 20 male Sprague-Dawley rats (4 weeks old, 200 g) were randomly selected and divided into two groups, including control group (n=10) and IVDD group (n=10). The IVDD model was successfully established via endplate injection. In brief, a 21 G puncture needle was locally punctured at the upper and lower endplates of the L5/6 IVD into the bone marrow cavity. From there, 30 μL of absolute ethanol was injected under pressure to block the nutrient penetration from the cartilage endplate to AF and NP, resulting in metabolic disorders. In control group, the same amount of normal saline was injected. After 1 month, subsequent experiments were performed. This study was approved by the Animal Ethics Committee of Heilongjiang University of Chinese Medicine Animal Center.

2.2. Cell Culture

NP cells were sterilely isolated from IVDD tissues. Briefly, IVDD tissues were washed with phosphate-buffered saline 3 times and cut into fragments the size of mung bean. After treatment with 0.25% trypsin for 30 min, the tissues were digested with collagenase II (Thermo Fisher Scientific, Waltham, MA, USA) containing 15% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) at 37°C. After culturing for 2 d, the cells were collected for subsequent experiments.

2.3. MiR-129-5p Interference and Cell Transfection

The miR-129-5p sequence (5'-CGGCAGGCATTA-GAGATGAACAGCA-3') was synthesized into the first-strand complementary deoxyribonucleic acid (cDNA) using the reverse transcription kit (Invitrogen, Carlsbad, CA, USA). RT-PCR was then performed using the standard SYBR Green PCR kit in the StepOne Plus System (Applied Biosystems, Foster City, CA, USA). Primers used in this study are as follows: miR-129-5p: sense strand: 5'-ACCCAGTGCGATT-TGTCA-3', antisense strand: 5'-ACTGTAGTGAAGA-TGGACC-3'; U6: sense strand: 5'-CTCCTTGGCCAGCACA-3'; antisense strand: 5'-AACGTTCAAGTATTGTGC-3'; Fas: sense strand: 5'-CTGCATCATGATGGGCAATTCGCTG-3'; antisense strand: 5'-ATGACATTAAATGTCAGTTAAGGC-3'; GAPDH: sense strand: 5'-GGGAGCCAAAAGGTCAT-3'; antisense strand: 5'-AGTCCTTCAGGATACCAA-3'. The expression of miR-26 was detected using TaqMan microRNA reverse transcription kit and TaqMan Master Mx II (Applied Biosystems, Foster City, CA, USA). The fold change of gene expression was calculated by the 2^−ΔΔCt method.

2.4. Detection of MiR-129-5p and Fas mRNA Levels Via Fluorescence Quantitative Real Time-Polymerase Chain Reaction (RT-PCR)

Total RNA was first isolated using TRIzol reagent (TaKaRa, Dalian, China). Subsequently, extracted RNA was synthesized into the first-strand complementary deoxyribonucleic acid (cDNA) using the reverse transcription kit (Invitrogen, Carlsbad, CA, USA). RT-PCR was then performed using the standard SYBR Green PCR kit in the StepOne Plus System (Applied Biosystems, Foster City, CA, USA). Primers used in this study are as follows: miR-129-5p: sense strand: 5'-ACCCAGTGCGATT-TGTCA-3', antisense strand: 5'-ACTGTAGTGAAGA-TGGACC-3'; U6: sense strand: 5'-CTCCTTGGCCAGCACA-3'; antisense strand: 5'-AACGTTCAAGTATTGTGC-3'; Fas: sense strand: 5'-CTGCATCATGATGGGCAATTCGCTG-3'; antisense strand: 5'-AGTCCTTCAGGATACCAA-3'. The expression of Fas was analyzed as well. All our findings might help to explore the relative expression of target protein.

2.5. Determination of Protein Expression Using Western Blotting

40 μg of protein samples were separated and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After sealing with 5% skimmed milk at room temperature for 1 h, the membranes were incubated with primary antibodies of JNK1 (1:1,000, Proteintech, Rosemont, IL, USA), JNK2 and Fas (1:1,000, Abcam, Cambridge, MA, USA) overnight. After washing with Tris-Buffered Saline and Tween (TBST), the membranes were incubated again with corresponding horseradish peroxidase-labeled secondary antibodies (1:5,000, Shanghai Beyotime Biotechnology Co., Ltd., Shanghai, China). Immuno-reactive bands were visualized with the enhanced chemiluminescence (ECL) system (Bio-Rad, Hercules, CA, USA), and gray value was analyzed using a gel analyzer. The ratio of the gray value of target protein to that of corresponding internal reference indicated the relative expression of target protein.

2.6. Statistical Analysis

GraphPad Prism 5.01 software (La Jolla, CA, USA) was used for all statistical analysis. Measurement data were expressed as x±s. The differences in indexes between the two groups were compared using the independent-samples t-test. The correlation between expressions of miR-129-5p and Fas in IVDD rats was analyzed via Pearson correlation analysis. P<0.05 was considered statistically significant.

3. Results

3.1. MiR-129-5p and Fas mRNA Levels in IVDD Tissues Detected Via RT-PCR

To investigate the biological role of miR-129-5p in IVDD, the expressions of miR-129-5p and Fas were determined in normal and IVDD rats via qRT-PCR. It was found that the expression of miR-129-5p was significantly down-regulated, while the transcriptional level of Fas was
Meanwhile, they may play important roles in the occurrence and development of IVDD.

3.5. Effects of MiR-129-5p on JNK Pathway and Apoptosis Protein in NP Cells Detected In Vitro

To verify whether the anti-inflammatory and anti-apoptotic effects of miR-129-5p on IVDD are realized via the JNK signaling pathway, NP cells were extracted from IVDD tissues. Subsequently, the cells were transfected with the inhibitor or mimic to interfere with the expression of miR-129-5p. The changes in JNK pathway and apoptosis protein were then explored. As shown in Figure 4, the protein expressions of JNK1, JNK2 and Fas rose remarkably in miR-129-5p inhibitor group, and such trends were significantly reversed in miR-129-5p mimic group ($P<0.05$).

4. Discussion

Currently, studies have demonstrated that the occurrence and development of IVDD are closely related to the up-regulated in IVDD tissues ($P<0.05$; Figure 1). According to the Pearson correlation analysis, there was a negative correlation between expressions of miR-129-5p and Fas mRNA ($r=-0.75$, $P<0.05$). It can be seen that miR-129-5p and Fas are involved in the progression of IVDD.

3.2. Levels of Serum Inflammatory Factors in Both Groups

IVDD group exhibited significantly higher levels of serum tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and IL-1 than control group ($P<0.05$) (Table 1).

3.3. Apoptosis Rate of NP Cells in Both Groups Determined Using TUNEL Assay

The results of RT-PCR demonstrated that miR-129-5p and Fas were involved in the progression of IVDD. To verify such a conclusion at the functional level, the apoptosis of NP cells was detected using TUNEL assay in both groups. The results indicated that the apoptosis rate was higher in IVDD group (60.6%) than in control group (2.5%) ($P<0.05$) (Figure 2).

3.4. Levels of JNK Pathway and Apoptosis Protein Detected Via Western Blotting

Total protein was first extracted from IVD tissues, and protein expressions of JNK1, JNK2 and Fas were determined using Western blotting. As shown in Figure 3, the protein expressions of JNK1, JNK2 and Fas remarkably rose in IVDD group compared with those in control group ($P<0.05$). It can be seen that the JNK pathway and apoptotic pathway are significantly activated in the IVDD model.

Table 1. Levels of serum inflammatory factors in both groups ($\bar{x}\pm s$).

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>TNF-α (pg/mL)</th>
<th>IL-6 (pg/mL)</th>
<th>IL-1 (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group</td>
<td>10</td>
<td>12.34±1.88</td>
<td>10.58±0.51</td>
<td>8.22±0.35</td>
</tr>
<tr>
<td>IVDD group</td>
<td>10</td>
<td>29.06±3.65*</td>
<td>23.62±2.01*</td>
<td>19.54±1.62*</td>
</tr>
</tbody>
</table>

Note: *$P<0.05$ vs. control group.
production of various inflammatory cytokines and metabolites. During the process of IVDD, a large number of inflammatory, degradation and decomposition molecules will be produced, including proteolytic and degrading enzymes, oxygen free radicals, nitric oxide, ILs and prostaglandins [13]. Risbud and Shapiro [14] have found that spinal instability and structural changes caused by the increase in inflammatory cytokines and decrease in hydrophilic matrix molecules are the major causes of protrusion, sciatica and stenosis. These inflammatory factors mainly include IL-1, IL-6, TNF-α, lipopolysaccharides and reactive oxygen species. IL-1 plays a major role in degenerative musculoskeletal diseases. It increases the matrix penetration rate in IVD, reduces the proteoglycan synthesis rate of IVD cells, and induces the expression of prostaglandin E2. As a pro-inflammatory cytokine, TNF-α plays a key role in discogenic pain and sciatica [15]. In this study, the expressions of serum inflammatory factors were explored in IVDD rats. The results found that the levels of serum TNF-α, IL-6 and IL-1 significantly rose in IVDD group.

As a common form of programmed cell death, apoptosis is initiated upon the stimulation of inflammation, damage, DNA injury and oxidative stress. It is widely involved in the pathophysiological process of a variety of degenerative diseases, including IVDD [16]. Wei et al. [17] have found that bone morphogenetic protein-7 protects human IVD cells against apoptosis in vitro. Fas is a membrane-bound receptor, which binds to cells with Fas ligand to initiate apoptosis [18]. It has been demonstrated that Fas and its receptors are not carried in normal IVD cells. However, they begin to be expressed shortly after IVDD [19]. In the present study, the levels of miR-129-5p and Fas mRNA were detected via RT-PCR. The results revealed that miR-129-5p was remarkably down-regulated, while Fas mRNA was significantly up-regulated in IVDD rats. Meanwhile, there was a negative correlation between the two molecules. Histologically, the results of TUNEL assay manifested that the apoptosis of NP cells in IVDD group was evidently enhanced. The above findings suggest that miR-129-5p plays an important role in the apoptosis of NP cells in IVDD. Previous studies have shown that IVDD occurs due to excessive degradation and impaired synthesis of ECM secreted by NP cells [20-23]. Therefore, primary NP cells were extracted from IVDD tissues, and transfected with the inhibitor or mimic to interfere with the expression of miR-129-5p. Meanwhile, the changes in JNK pathway and apoptosis protein were explored. The results demonstrated that miR-129-5p regulated the apoptosis of NP cells through the JNK pathway.

Furthermore, Benakis et al. [24] have shown that the mitogen-activated protein kinase (MAPK) pathway is an important downstream inflammatory pathway in cartilage tissues. In the pathway, p38, extracellular signal-regulated kinase (ERK) and JNK are main members. According to the research by Li et al. [25] on cartilage degeneration, the MAPK pathway plays a key role in the inflammatory process and apoptosis. Ren et al. [26] have also found that miR-138 protects against H2O2-induced BV-2 cell apoptosis via regulating the MLK3/JNK/MAPK pathway. In this study, it was confirmed that miR-129-5p exerted an anti-apoptotic effect through the JNK pathway.

5. Conclusions

Altogether, these data demonstrate that miR-129-5p exhibits good anti-inflammatory and anti-apoptotic effects in the IVDD model. In addition, such a finding may provide a basis for the treatment of IVDD with miR-129-5p.

Conflict of Interests
The author has no conflicts with any step of the article preparation.

Consent for publications
The author read and approved the final manuscript for publication.

Ethics approval and consent to participate
This study was approved by the Animal Ethics Committee of Heilongjiang University of Chinese Medicine Animal Center.

Informed Consent
The authors declare not used any patients in this research.

Availability of data and material
The data that support the findings of this study are available from the corresponding author upon reasonable request.

Authors’ contributions
Chen Li and Xi Gao designed the study and performed the experiments, Zhiwen Sun and Hongjun Lou collected the data, Yenong Sun and Chengyuan Li analyzed the data, and Xi Gao prepared the manuscript. All authors read and approved the final manuscript.

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References


