Mechanism of microRNA-21 regulating IL-6 inflammatory response and cell autophagy in intervertebral disc degeneration

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Abstract. This study investigated the mechanism of microRNA-21 in regulating IL-6 inflammatory response and cell autophagy in intervertebral disc degeneration. A total of 10 patients with lumbar disc herniation accompanied by nerve root pain (observation group) and 10 patients with lumbar burst fractures (control group) were selected. The nucleus pulposus tissues of the lesion were obtained during operation for cell culture. Real-time quantitative polymerase chain reaction (PCR) was used to detect the expression of microRNA-21. The ELISA method was used to detect the levels of IL-6, and type II collagen (Col II). Aggrecan and western blotting was used to detect autophagy-related gene 7 (ATG7) and microtubule-associated protein 1 light chain 3 (LC3)-II/-I. As a result, the levels of microRNA-21 and IL-6 in the observation group were significantly higher than those in the control group, but the levels of Col II and aggrecan were significantly lower than those in the control group. The differences were statistically significant ($P<0.05$). The levels of ATG7 and LC3-II/-I in the observation group were significantly decreased ($P<0.05$). In conclusion, the expression of microRNA-21 is abnormally high in the nerve root pain of the lumbar intervertebral disc, which can increase the IL-6 inflammatory response and reduce the capacity of cell autophagy.

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Introduction

Intervertebral disc degeneration (IDD) is the main cause of low back pain and ~20-40% of middle and old-aged patients can suffer from nerve root pain to different degrees (1). The microRNA chip technique was used to screen differentially expressed microRNAs in degenerative nucleus pulposus tissues (2) and it was found that the abnormal expression of microRNA-21 is positively correlated with the degenerative Pfirrmann grade. The occurrence of IDD is related to aging, cell dehydration, inflammatory response, cell apoptosis and autophagy (3). Herniation of the nucleus pulposus activates the body's immune system, leading to leukocyte activation, release of a variety of inflammatory factors and nerve cell degeneration, resulting in neuropathic pain (4). Rat models have confirmed (5) the prolonged inflammatory response with significant increase of IL-6 and TNF-α. Decreased contents of type II collagen (Col II) and aggrecan are typical pathological changes of IDD, and autophagy is an important way to regulate the extracellular matrix (ECM) metabolism of the intervertebral disc (6). Studies (7) have confirmed that the autophagy-related gene 7 (ATG7) is closely related to the formation of autophagic vacuoles, and it is also the target gene of microRNA-21. Based on this, our study further analyzed the degenerative nucleus pulposus tissue obtained *in vivo* to investigate the mechanism of microRNA-21 expression of regulating IL-6 inflammatory response and cell autophagy so as to provide new treatment ideas.

Materials and methods

Patient data. A total of 10 patients diagnosed with lumbar disc herniation accompanied by nerve root pain from May to October 2016 in our hospital (observation group) and 10 patients with lumbar burst fractures (control group) were continuously selected. In the observation group, there were 6 males and 4 females aged 52-67 with an average age of 56.9±7.2 years. In terms of sampling site, there were 2 cases of L2-3, 1 case of L3-4, 4 cases of L4-5 and 3 cases of L5-S1. In the control group, there were 5 males and 5 females aged 48-68 with an average age of 55.7±6.9 years. In terms of sampling site, there were 3 cases of L2-3, 2 cases of L3-4, 3 cases of L4-5 and 2 cases of L5-S1. Baseline data of the two groups was

comparable. This study was approved by the Ethics Committee of Clinical College of Maanshan, and signed written informed consents were obtained from the patients.

Research methods. The nucleus pulposus tissues of the lesions were obtained for cell culture during the operation. Real-time quantitative polymerase chain reaction (PCR) was used to detect the expression of microRNA-21. ELISA method was used to detect the levels of IL-6, Col II and aggrecan and western blotting was used to detect ATG7 and LC3-II/-I.

Cell culture and identification. The tissue samples were washed with PBS and placed in a DMEM/F12 (1:1) (both from Biosharp, Hefei, China) sterile culture flask containing 10% fetal bovine serum and stored at low temperature. The residual components in nucleus pulposus tissues were removed using ophthalmic forceps on a sterile operation desk, and nucleus pulposus tissues were washed using 1% double resistant flushing fluid, and about 1 mm^3 tissue was cut into pieces. A total of 1.5-time-volume of 0.25% trypsin (Biosharp) was added into the centrifuge tube for digestion for 30 min, followed by centrifugation at 2,000 x g for 5 min; isopyknic 0.2% Col II was added into the sediment and mixed for about 4 h and then isopyknic DMEM/F12 complete culture solution was added to terminate the digestion, followed by screening via cell strainer and centrifugation at 1,000 x g for 5 min; 4 ml DMEM/F12 (1:1) complete culture solution containing 15% fetal calf serum was added to blow away cells; re-suspension density was $1x10^5$/ml; and cells were incubated in an incubator containing 5% CO_2 at 37˚C. The solution was changed the next day for subculture. The cell adherence, growth and morphological changes were observed under inverted microscope (BX-42; Olympus, Tokyo, Japan). The second generation of cells was taken for immunocytochemical staining to identify Col II expression, confirmed to be nucleus pulposus cells.

Real-time quantitative PCR. The total RNA in cells was extracted using the conventional TRIzol reagent, and its concentration and purity were determined by ultraviolet spectrophotometer. cDNA was synthesized using a reverse transcription kit, and primer sequences were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China) according to GenBank sequence. MicroRNA-21: forward, 5'-GGTTTCATCCAGGATCGAGCAGG-3' and reverse, 5'-ACAAAGATGGTCACGGTCTGCC-3', 445 bp; GAPDH forward, 5'-CGCGAGAAGATGACCCAGAT-3' and reverse, 5'-GCACTGTGTTGGCGTACAGG-3', 225 bp. Reaction system: 2 μl cDNA + 3 μl upper primer and 3 μl lower primer + 0.5 μl Taq polymerase + 1 μl dNTPs + 3 μl $MgCl_2$ + 5 μl 10X buffer, and water was added until the total volume was 20 μl. Reaction conditions: 95˚C for 5 min, 95˚C for 30 sec, 58˚C for 30 sec, 72˚C for 60 sec, a total of 30 cycles, ending at 72˚C for 10 min. PCR products were identified via 2% agarose gel electrophoresis, followed by imaging via gel imaging analysis system and gray value analysis via digital photograph. The results are presented using $2^{-\Delta\Delta Cq}$ method.

ELISA method. IL-6, Col II and aggrecan reagents were purchased from Jiangsu Beyotime Technology Co. Ltd., (Jiangsu, China); cell sap was detected three times via a microplate reader after being centrifuged at 3,000 x g for 20 min, and the average was taken.

Western blotting. RIPA lysate was added to extract the total cell protein, followed by rough quantification via Coomassie brilliant blue method and dose standardization via β-actin antibody. A total of 30 μg total protein was taken and separated via 8% SDS-PAGE, and the separated zone was transferred to a PVDF membrane and mouse anti-human ATG7, LC3-II and LC3-I monoclonal antibodies (1:2000; Sigma, St. Louis, MO, USA) was added overnight; then rabbit anti-mouse polyclonal secondary antibody (1:500; Sigma) was added to incubate at room temperature for 4 h, followed by washing via PBS and development via ECL. Results were scanned and saved, and semi-quantitative analysis was performed using Lab Works 4.5 gel imaging software (Invitrogen, Carlsbad, CA, USA).

Statistical analysis. SPSS 20.0 software (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis; measurement data was presented as mean ± standard deviation, and independent sample t-test was used for intergroup comparison; enumeration data were presented as case or percentage (%), and Chi-square test was used for intergroup comparison. $P<0.05$ indicates that the difference was statistically significant.

Results

Identification of nucleus pulposus cells. The second generation of cells was observed under the inverted phase contrast microscope; in both groups, cells were mainly short fusiform with good refractivity, and short and thick protrusions spread all around in arborescent type, and there were secondary protrusions. The number of cells in the observation group was decreased and that of protrusions was increased (Fig. 1).

Immunostaining showed that the cytoplasm of the control group was stained brown yellow and that the control group was stained pale yellow (Fig. 2).

Results of real-time quantitative PCR. The level of microRNA-21 in the observation group was significantly higher than that in the control group and the difference was statistically significant ($P<0.05$, Fig. 3).

Results of ELISA method. The level of IL-6 in the observation group was significantly higher than that of the control group, but the levels of Col II and aggrecan were significantly lower than those in the control group; the differences were statistically significant ($P<0.05$, Table I).

Results of western blotting. The levels of ATG7 and LC3-II/-I in the observation group were significantly lower than those of the control group, and the differences were statistically significant ($P<0.05$, Fig. 4).

Discussion

It is agreed that IDD is a physiological and pathological process related to ECM synthesis and catabolic imbalance,

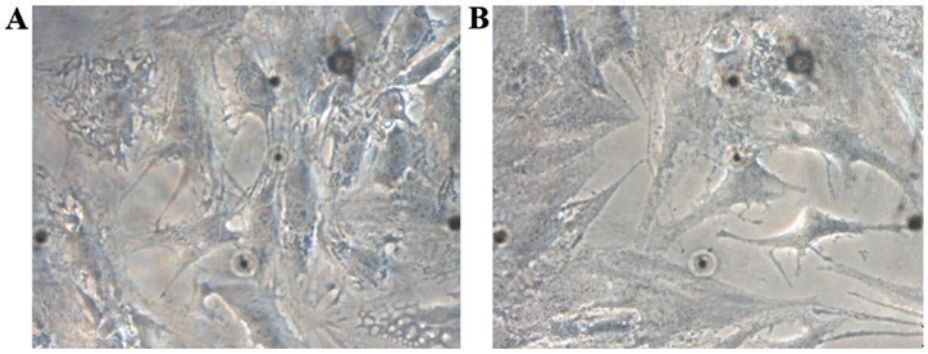

Figure 1. Observation of the second generation of cells under the inverted phase contrast microscope (magnification, x200). (A) Control group. (B) Observation group. In both groups, cells were mainly short fusiform with good refractivity and short and thick protrusions spread all around in arborescent type and there were secondary protrusions. The number of cells in the observation group was decreased and that of protrusions was increased.

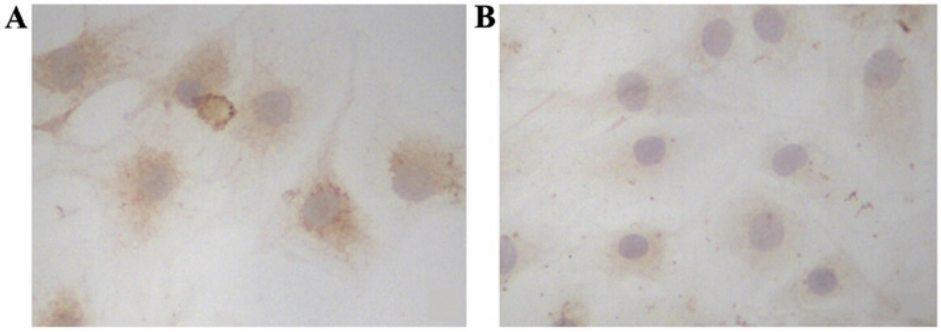

Figure 2. Immunostaining of the second generation of cells Col II (magnification, x200). (A) Control group. (B) Observation group. The cytoplasm of the control group was stained brown yellow and that of the control group was stained pale yellow. Col II, type II collagen.

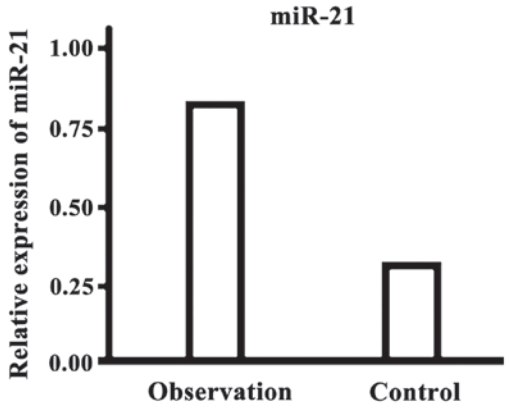

Figure 3. Detection of microRNA-21 levels via real-time quantitative PCR (the level of microRNA-21 in the observation group was significantly higher than that of the control group). PCR, real-time quantitative polymerase chain reaction.

Table I. Results of ELISA method (μmol/l).

Group	IL-6	Col II	Aggrecan
Observation group	125.6±34.9	223.1±65.9	125.8±54.2
Control group	64.7±22.5	352.8±82.7	264.7±72.9
t-test	12.635	10.234	15.285
P-value	<0.001	<0.001	<0.001

Col II, type II collagen.

cell apoptosis, inflammatory response and vascular proliferation. Some biological treatments, such as active substances of intervertebral disc regeneration, stem cell transplantation, autologous chondrocyte transplantation and gene therapy can promote intervertebral disc regeneration or repair in different degrees and reverse the degeneration process, but most of these results are in animal experiments or *in vitro* animal model stages (8,9).

This study showed that the levels of microRNA-21 and IL-6 in the observation group were significantly higher than those of the control group, but the levels of Col II and aggrecan were significantly lower than those in the control group, and the levels of ATG7 and LC3-II/-I in cells were significantly decreased, suggesting that the expression of microRNA-21 is abnormally high in lumbar intervertebral disc nerve root pain with increased IL-6 inflammatory response and reduced cell autophagy. MicroRNAs are endogenous non-coding RNAs widely distributed in the human body and mainly bind to the 3' untranslated region (3'UTR) in target mRNA, inhibiting the translation process or increasing the target mRNA degradation, which affects the expressions of target genes and 30% genome coding protein, which plays an important role in a variety of pathological and physiological processes. Liu *et al* (10) confirmed that miR-21 expression is

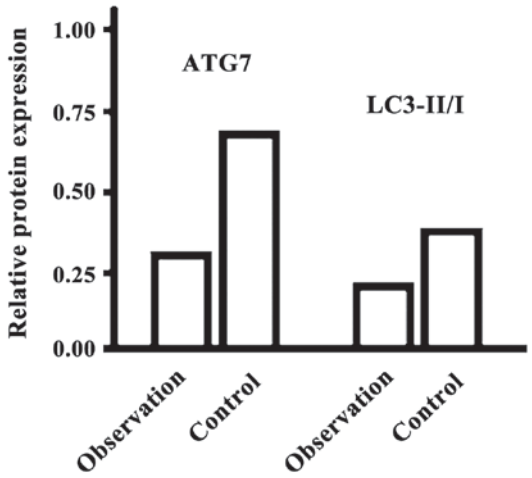

Figure 4. Detection of ATG7 and LC3-II/-I levels via western blotting (the levels of ATG7 and LC3-II/-I in the observation group were significantly lower than those of the control group). ATG7, autophagy-related gene 7; LC3-II/-I, microtubule-associated protein 1 light chain 3.

significantly increased in the degenerative nucleus pulposus tissues and miR-21-transfected human nucleus pulposus cells can stimulate cell proliferation. Studies on overexpression of miR-21 or silent miR-21 expression showed that the overexpression of miR-21 can aggravate cell degeneration, increase the expression of MMP and ECM degradation, upregulate the inflammatory responses, such as IL-6, and promote cell apoptosis and autophagy (11). Therefore, it is thought that miR-21 expression is closely related to the occurrence of IDD.

This study found that (12) IL-6 and TNF-α inflammatory factors in intervertebral disc tissue play important roles in the occurrence of IDD damage and neuropathic pain. They can be significantly increased at an early stage. The injection of recombinant IL-6 in dorsal root ganglion can cause hyperalgesia, and IL-6 inhibitors can significantly reduce the pain of rats in a chronic compression model, and the application of IL-6 gene silencing treatment can significantly reduce the mechanical hyperalgesia in rats in a spinal nerve abruption model (13). At the same time, anti-inflammatory factors, such as IL-10, have significant analgesic effects (14). Jiang *et al* (15) pointed out that the number of autophagosomes in human degenerative nucleus pulposus cells was significantly reduced, and LC3-II/-I and beclin-1 expression levels were decreased. Autophagy inhibitor 3-methyladenine can significantly reduce the number of autophagic vacuole (16). Wang *et al* (17) pointed out that resveratrol can increase the capacity of cell autophagy, and inhibit TNF-α-induced MMP-3 expression. ATG7 is an important target of autophagy. ATG7 mRNA 3'UTR has complementary sequences with miR-21. A dual-luciferase reporter gene test showed that miR-210 mimic significantly reduces the activity of wild-type ATG, but has no effect on mutant type, confirming that ATG7 is the target gene of miR-21 (10).

The innovation of this study is that it obtained the degenerative nucleus pulposus tissue from the human body and established a cell culture method, providing an important basis for follow-up studies. The deficiency of this study is that it failed to further analyze the mechanism of miR-21 expression for IL-6-mediated inflammatory response and cell autophagy, and whether miR-21 is a potential target for intervention treatments remains to be verified.

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