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Interleukin-21 Is Associated with the Pathogenesis of Lumbar Disc Herniation

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ABSTRACT

Inflammation is an important reaction underlying lumbar disc herniation (LDH). Th17 cells play a critical role in immune activation. Interleukin (IL)-21 controls the functional activity of effector T-helper cells and the differentiation of Th17 cells, and promotes B-cell differentiation. It plays important roles in chronic inflammation and autoimmune diseases. However, little is known about relationship between IL-21 and LDH. This study was aimed to determine the association between IL-21 levels and pain scores in LDH patients compared to healthy controls.

We enrolled 34 LDH patients and 20 healthy controls in this study. The LDH patients underwent surgery. Pain intensity was recorded using visual analogue scale (VAS) scores preoperatively. Serum IL-21 and IL-17 levels in the peripheral blood were determined using enzyme-linked immunosorbent assay. Disc tissue was examined using western blot and quantitative reverse-transcription polymerase chain reaction to determine IL-21, IL-17, and cyclooxygenase (COX)-2 expression, and using immunohistochemistry to assess IL-21 expression.

LDH patients exhibited significantly higher levels of serum IL-21 and IL-17 than healthy controls. Moreover, higher expression of IL-21, IL-17, and COX-2 was found in the protein and mRNA levels in disc tissues from LDH patients than in normal disc tissues. Different parameters like VAS pain scores, IL-17, and COX-2 were positively correlated with the IL-21 levels. Enhanced production of IL-21 in disc tissues of LDH patients was also confirmed using immunohistochemical analyses.

We concluded that inflammation was responsible for the pain associated with LDH, and that increased IL-21 expression may be associated with the pathogenesis of LDH.

Keywords: COX-2; IL-17; IL-21; Intervertebral disc; Lumbar disc herniation

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INTRODUCTION

Lumbar disc herniation (LDH) is a common affliction among adults. LDH is not only associated with low back pain/sciatica and reduced quality of life but also poses a heavy economic burden on society. Most back pain and sciatica are related to degeneration, especially herniation and disruption of the lumbar intervertebral disc (IVD).^{1,2} Spinal nerve compression is responsible for numbness, not pain; inflammation is the main cause of low back pain.³

In recent years, an increasing number of studies have investigated inflammation and immune activation in the pathogenesis of lumbar disc disease. The cytokines interleukin (IL)-1 and tumor necrosis factor (TNF)- α are overexpressed in degenerated IVDs,^{4,5} and IL-1 expression is significantly elevated in painful IVDs.⁶ Kraychete et al showed that patients with chronic low back pain due to disc herniation had high levels of TNF- α and IL-6.⁷ Phospholipase A2 is responsible for the pain associated with LDH, and is involved in the rate-limiting step in the production of prostaglandin E (PGE) and leukotrienes.⁸ PGE-2 is elevated in herniated lumbar discs⁹ and has been linked to pain induction.^{10, 11} Karppinen et al reported that IL-6 is related to sciatica.¹² Gabr et al proved that IL-17 acts synergistically with TNF- α to promote inflammatory mediator release in human IVD cells.¹³ Increased levels of Th17 lymphocytes and IL-17 contribute to the pain associated with LDH.¹⁴

IL-21 was discovered by Parrish in 2000.¹⁵ It is a pleiotropic cytokine that belongs to the IL-2 cytokine family, which is produced by activated CD4+ T-cells and natural killer T (NKT) cells^{16,17}. IL-21 regulates the reciprocal differentiation of Th17 cells and regulatory T-cells by promoting Th17 cell expansion and inhibiting the generation and function of induced regulatory T-cells.¹⁸ IL-21 also promotes B-cell differentiation.¹⁹ The overexpression of IL-21 in mice results in hypergammaglobulinemia and autoantibody production.²⁰ Recently, studies have shown that IL-21 is associated with the development of a variety of immune-mediated diseases, for example, systemic lupus erythematosus (SLE), rheumatoid arthritis,

Sjogren syndrome, multiple sclerosis, and type 1 diabetes.²¹⁻²⁵

However, little is known about the relationship between IL-21 and LDH. The purpose of this study was to determine the levels of IL-21 in LDH patients and to explore their association with pain in these patients.

MATERIALS AND METHODS

Patients

This study involved 34 LDH patients with single lumbar IVD herniation who were treated in a hospital affiliated to Nantong University and 20 healthy adult volunteers. Patients with typical back pain or sciatica symptoms, a positive Lasegue's sign, typical MRI and CT findings, and no signs of calcification were included. Patients with a history of autoimmune disease, acute or chronic inflammatory disease, metabolic disease, and tumors were excluded. All medications, including non-steroidal anti-inflammatory drugs and hormones, were discontinued 2 weeks before blood-sample collection and surgery.

Normal IVD tissue was obtained from 8 patients with acute burst fractures of the lumbar vertebra, but without a history of low back pain or signs of disc degeneration on MRI. Written informed consent was obtained from all patients and controls. The study was approved by the ethics committee of the hospital affiliated to Nantong University. The characteristics of the study subjects are presented in Table 1.

Pain intensity was recorded using a visual analogue scale (VAS) before the operation (early in the morning of the surgery). At the same time, 5 ml blood was collected into a tube containing the anticoagulant heparin sodium; 5 ml blood was also drawn from the healthy volunteers (controls). Both the patients and volunteers were in a fasting state at the time of blood collection. The patients underwent posterior fenestration discectomy, posterior lumbar interbody fusion, or transforaminal lumbar interbody fusion. During the surgery, the nucleus pulposus was removed, rinsed with phosphate-buffered saline (PBS), and placed in a cryopreservation tube under sterile conditions. The sample was preserved at -80°C.

Table 1. Patient Characteristics

	LDH patients	Fracture patients	Healthy controls
number	34	8	20
male/female	20/14	5/3	12/8
Age(years)	51.71(21-70)	42.75(26-51)	38.65(25-50)

Serum Levels of IL-21 and IL-17

The serum concentrations of IL-21 and IL-17 in the 34 LDH patients and 20 healthy controls were measured using sandwich enzyme-linked immunosorbent assay (ELISA). In brief, monoclonal capture antibodies (eBioscience, San Diego, CA, USA) were added to a 96-well plate (Nunc, Thermo Fisher Scientific, Waltham, MA, USA) and incubated overnight at 4°C. Then, the plates were washed five times with PBS containing 0.05% Tween-20 (Sangong Biotech, Shanghai, China). Following incubation with a blocking solution for 1 h at room temperature, the test samples and recombinant IL-21 or IL-17 (eBioscience) standards were added to the plates. The plates were incubated for 2 h at room temperature, after which they were washed five times. Biotin-conjugated anti-human IL-21 or IL-17 antibodies (eBioscience) were added, and the plates were incubated for 1 h at room temperature and then washed. Avidin-horseradish peroxidase (eBioscience) was added, and the reaction was allowed to proceed for 30 min at room temperature. The plates were then washed five times, and tetramethylbenzidine solution was added to induce the color reaction, which was stopped by adding 2 N H₂SO₄. The optical density at 450 nm was measured using an automated microplate reader (VERSAmax, Molecular Devices, Palo Alto, CA, USA). A standard curve was drawn by plotting the optical density against the log of the concentration of IL-21 or IL-17.

Tissue Levels of IL-21, IL-17, and Cyclooxygenase-2 Proteins

Tissue and cell proteins were immediately homogenized in a homogenization buffer containing 50 mM Tris-HCl (pH 7.5) (Sangong Biotech), 150 mM NaCl (Sangong Biotech), 0.1% NP-40 (Sangong Biotech), 5 mM EDTA (Sangong Biotech), 60 mM β-glycerophosphate (Sangong Biotech), 0.1 mM sodium orthovanadate (Sangong Biotech), 0.1 mM NaF (Sangong Biotech), and complete protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland), and then centrifuged at 12,000 g for 20 min to collect the supernatant. Protein concentrations were measured with a Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA). The supernatant was diluted in 2× sodium dodecyl sulfate (Sangong Biotech) loading buffer and boiled for 15 min. Proteins were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred to polyvinylidene

difluoride membranes (Millipore, Bedford, MA, USA). The membranes were blocked with 5% fat-free milk in Tris-buffered saline containing Tween-20 (TBST; 150 mM NaCl, 20 mM Tris, and 0.05% Tween-20) for 2 h at room temperature. Thereafter, the membranes were washed with TBST three times and incubated overnight with the primary antibodies and later with horseradish peroxidase-linked IgG (eBioscience) as the secondary antibody for 2 h at room temperature. Band density was measured with a computer-assisted image-analysis system (Adobe Systems, San Jose, CA, USA) and normalized against GAPDH (Sangong Biotech) levels. Values were calculated for at least three independent reactions.

IL-21, IL-17, and Cyclooxygenase-2 mRNA Levels in the Nucleus Pulposus

TRIzol reagent (Invitrogen, Carlsbad, CA) was used to extract total RNA. An aliquot of total RNA (1 μg) was reverse-transcribed into single-stranded complementary DNA by using the M-MLV First Strand Kit (Invitrogen). Relative RNA levels were determined using the ABI Prism 7000 sequence detection system (Life Technologies, NY) with SYBR-Green Mix (Takara Bio, Otsu, Japan) and GAPDH as the internal control. The primer sequences used for the quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis are shown in Table 2.

Immunohistochemical Staining

Immunostaining was performed using the avidin-biotin-peroxidase complex. The sections were deparaffinized with a graded ethanol series, and endogenous peroxidase activity was blocked by soaking in 3% hydrogen peroxide (Sangong Biotech) for 30 min. Then, the sections were processed in 10 mmol/L citrate buffer (Sangong Biotech) (pH, 6.0) and heated to 121°C in an autoclave for 20 min to retrieve the antigens. After being rinsed with PBS (pH, 7.2), the sections were incubated with anti-SCYL1BP1 (eBioscience) antibody (dilution, 1:100) and anti-Mdm2 (eBioscience) antibody (dilution, 1:400) for 2 h at room temperature. Negative control slides were processed in parallel using non-specific IgG (Sigma Chemical Co., St. Louis, MO, USA) at the same concentration as the primary antibody. All slides were processed using the peroxidase-anti-peroxidase method (DAKO, Hamburg, Germany). The sections were rinsed with PBS, and the peroxidase reaction was

Table 2. Sequences of primers for the qRT-PCR assays

Gene	Sense	Sequence 5'→3'
IL-21	F	5'-GTCATCTGTCTGATGGTCATCTTCTT-3'
	R	5'-CAGGGACCAAGTCATTCACATAATT-3'
IL-17	F	5'-ACTACAACCGATCCACCTCAC-3'
	R	5'-ACTTTGCCTCCCAGATCACAG-3'
COX-2	F	5'-TGCATTCTTTGCCAGCACT-3'
	R	5'-AAAGGCGCAGTTTACGCTGT-3'
GAPDH	F	5'-GAAGGTGAAGGTCGGAGTC-3'
	R	5'-GAAGATGGTGATGGGATTC-3'

visualized by incubating the sections with the following liquid mixture: 0.02% diaminobenzidine tetrahydrochloride, 0.1% PBS, and 3% hydrogen peroxide. After being rinsed with water, the sections were counterstained with hematoxylin, dehydrated, and coverslipped.

Statistical Analysis

All values were expressed as the mean \pm standard deviation. Statistical evaluation of the western blot, ELISA, and qRT-PCR data was performed by one-way ANOVA of variance with post hoc analysis. Between-group differences in tissue immunoreactivity scores were assessed using the chi-square test. Bivariate correlation analysis was used to determine the relationship among IL-17 mRNA levels, cyclooxygenase (COX)-2 mRNA levels, and VAS scores and the IL-21 levels. All tests were performed using SPSS 22.0 (SPSS Inc., Chicago, IL). Values of $P < 0.05$ were considered significant.

RESULTS

Serum IL-21 Levels Are Higher in LDH Patients Than in Healthy Controls

The serum IL-21 levels in LDH patients ($n = 34$) and healthy controls ($n = 20$) were measured using ELISA (Figure 1). The levels in the patients were notably higher than those in the control subjects. Significant increase in IL-17 levels was also observed in LDH patients.

IL-21 Expression Is Higher in IVD Tissue from LDH Patients Than in Normal IVD Tissue

Western blot analysis (Figure 2) revealed that IL-21 expression was significantly higher in IVD tissue obtained from LDH patients than in normal IVD tissue.

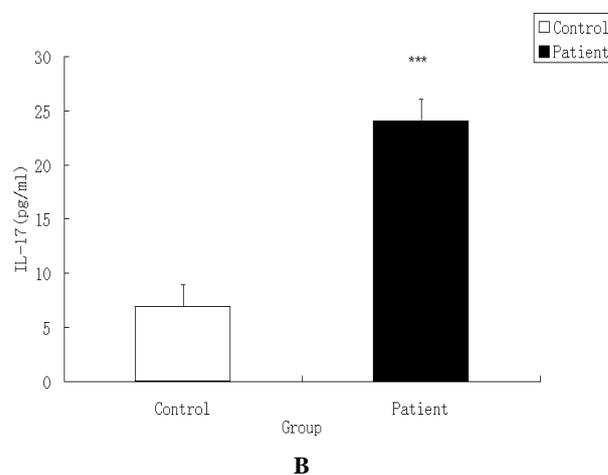
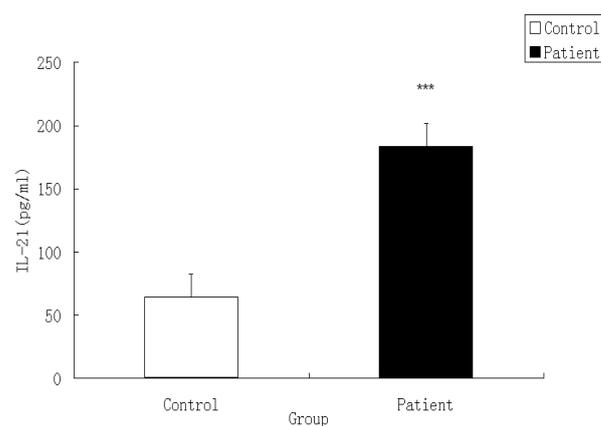


Figure 1. ELISA. (A) Serum IL-21 levels were significantly higher in LDH patients (183.5 \pm 65.1773 pg/ml) than in healthy controls (64.20 \pm 9.4345 pg/ml), $p=2.16*10^{-12}$. (B) Serum IL-17 levels were significantly higher in LDH patients (24.0765 \pm 7.169 pg/ml) than in healthy controls (6.940 \pm 1.2479 pg/ml), $p =8.15*10^{-16}$.

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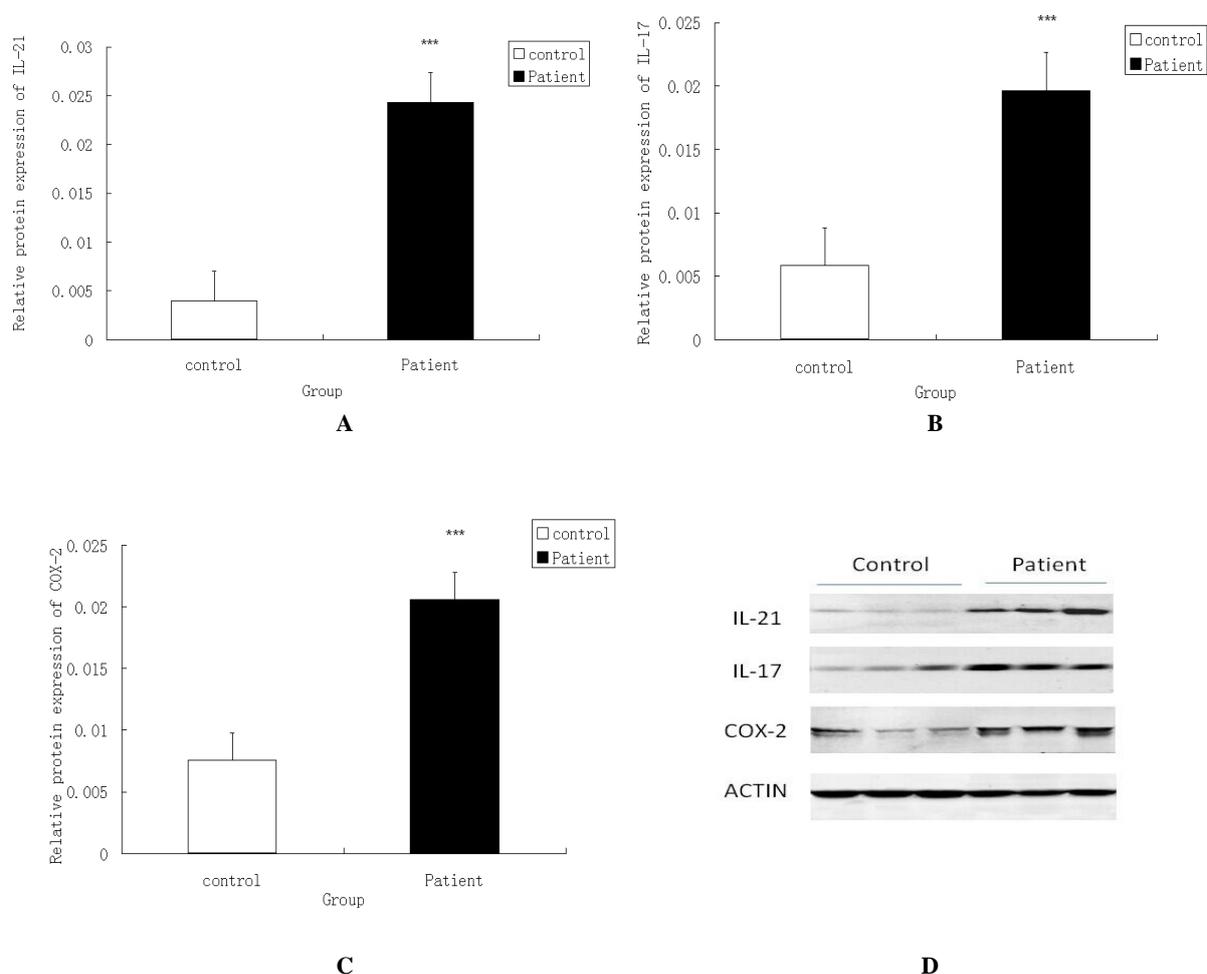


Figure 2. Western blot analysis. (A) IL-21 protein levels were significantly higher in herniated disc tissue (0.024322 ± 0.004188) than in normal disc tissue (0.003994 ± 0.001331). $p = 1.43 \times 10^{-23}$. (B) IL-17 protein levels were obviously higher in herniated disc tissue (0.019638 ± 0.006288) than in normal disc tissue (0.005836 ± 0.001576). $p = 4.75 \times 10^{-14}$. (C) COX-2 protein levels were obviously higher in herniated disc tissue (0.020568 ± 0.004106) than in normal disc tissue (0.007567 ± 0.002047). $p = 8.36 \times 10^{-12}$. (D) Photographs of the results of western blot analysis of the IL-21, IL-17, and COX-2 levels in herniated and normal disc tissues.

Elevated expressions of tissue IL-17 and COX-2 were also found in the disc tissues of LDH patients.

IL-21, IL-17, and COX-2 mRNA levels in disc tissue were also measured using qRT-PCR. The results showed a significant difference between the patients and the controls. The IL-21, IL-17, and COX-2 mRNA levels were obviously elevated in LDH patients (Figure 3). Pearson correlation coefficients for the correlation of pain (VAS) scores, COX-2 mRNA levels, and IL-17 mRNA levels with IL-21 mRNA levels are shown in

Figure 4. The VAS scores were very strongly correlated with IL-21 expression. IL-17 and COX-2 mRNA levels were strongly related to IL-21 expression.

Immunohistochemical Staining

The results of immunoreactivity grading of IL-21 in the cells in the IVDs are shown in Figure 5. IL-21 expression was higher in the tissue obtained from LDH patients than in normal IVD tissue.

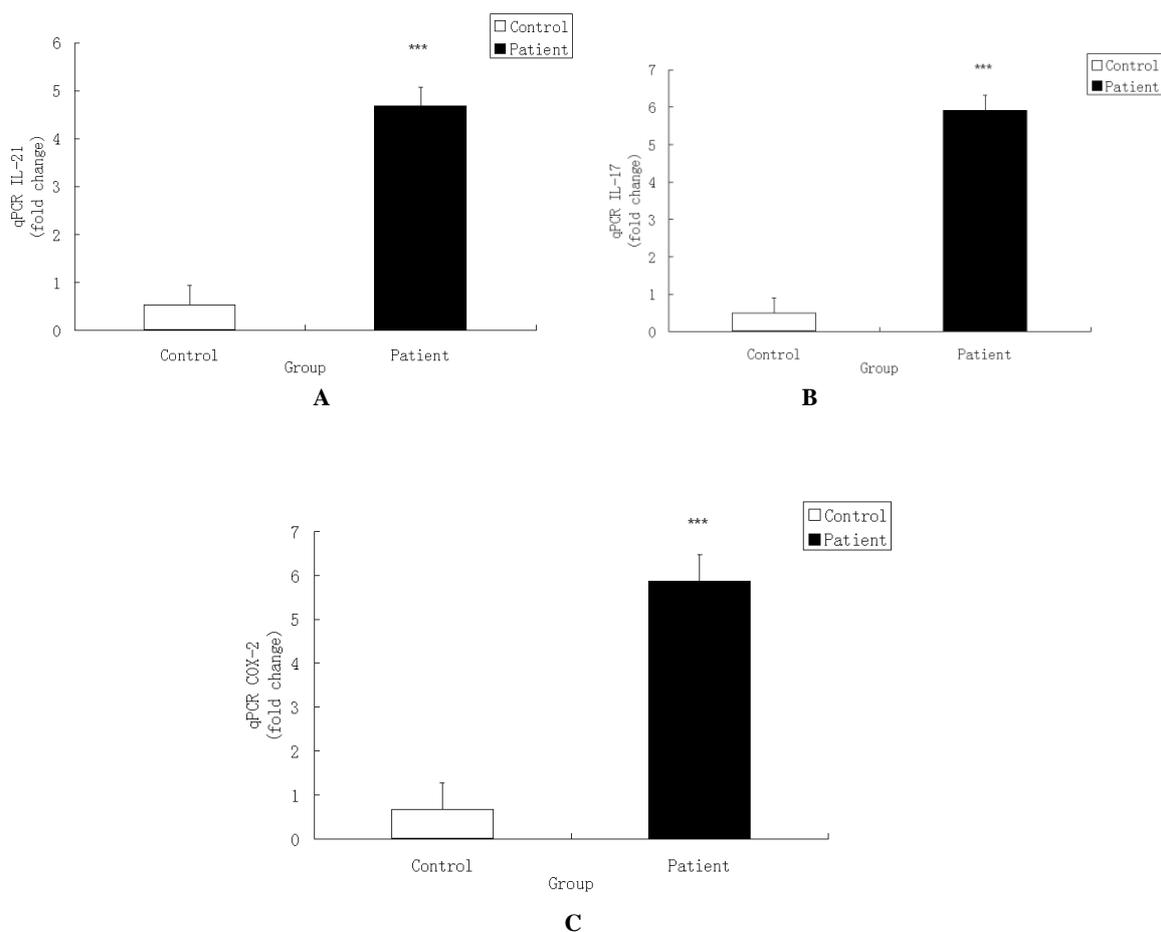


Figure 3. qRT-PCR. (A) IL-21 mRNA expression levels were significantly higher in herniated disc tissue (4.674165 ± 3.132175) than in normal disc tissue (0.533289 ± 0.247399), $p = 6.79 \times 10^{-9}$. (B) IL-17 mRNA levels were obviously higher in herniated disc tissue (5.914564 ± 3.599703) than in normal disc tissue (0.490636 ± 0.272799), $p = 3.36 \times 10^{-10}$. (C) COX-2 mRNA levels were obviously higher in herniated disc tissue (5.865896 ± 3.701188) than in normal disc tissue (0.665831 ± 0.247947), $p = 1.75 \times 10^{-9}$.

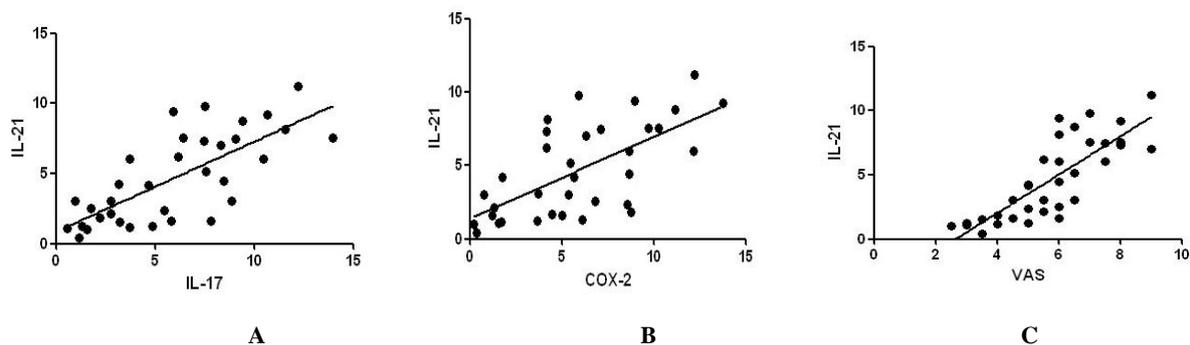


Figure 4. Scatter plots of the correlation of VAS pain scores, COX-2, and IL-17 with IL-21. (A) $r = 0.739$, $p = 6.07 \times 10^{-7}$; (B) $r = 0.659$, $p = 2.29 \times 10^{-5}$; (C) $r = 0.809$, $p = 6.97 \times 10^{-9}$.

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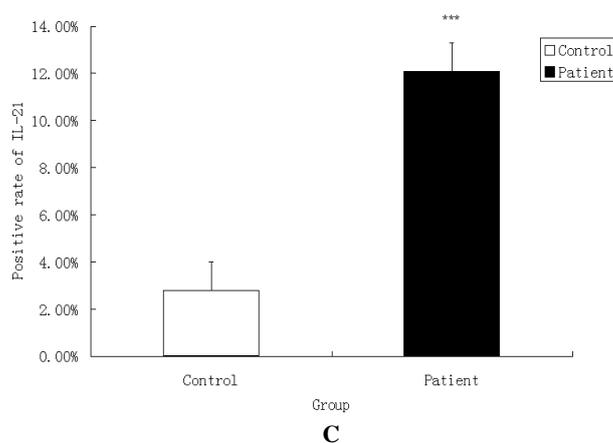
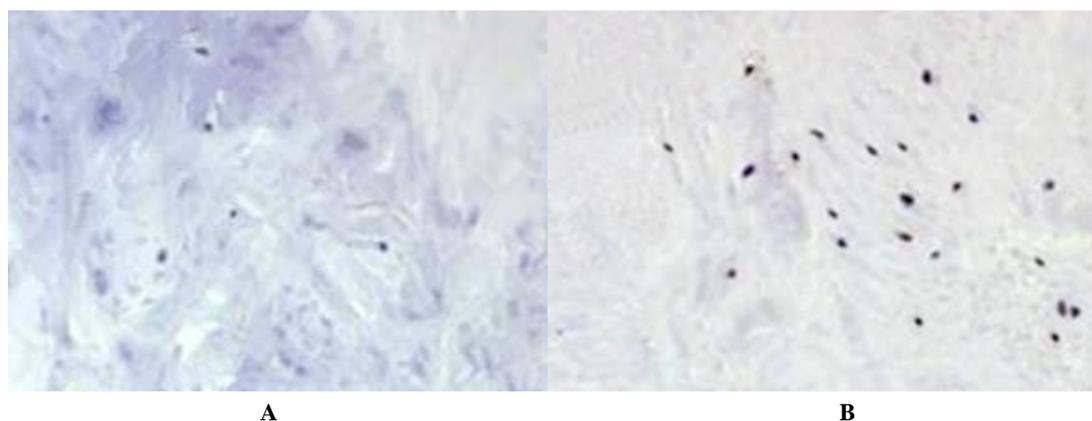


Figure 5. Immunohistochemical staining showing IL-21 expression in intervertebral discs from LDH patients and normal controls. (A) Intervertebral disc from a normal control subject; no positive expression is noted. (B) Intervertebral disc from an LDH patient; the number of positive cells is significantly increased. (C) The rates of IL-21 immune-positive cells in the nucleus pulposus of the patients ((12.1±4.4)%) and the healthy controls ((2.8±2.0)%). $p = 9.14 * 10^{-7}$.

DISCUSSION

In the present study, we investigated the involvement of IL-21, IL-17, and COX-2 in LDH by evaluating peripheral blood and disc tissue samples from LDH patients and healthy controls. Our results demonstrated that LDH patients exhibited significantly increased IL-21 expression compared with the healthy controls. IL-21 might be involved in the pathogenesis of LDH.

Mechanical compression, spinal instability, and immune activation are the three conventionally accepted factors underlying the pathogenesis of LDH¹⁴. However, the first two cannot fully explain LDH pathogenesis. Macnab pointed out that inflammation was the main factor leading to pain in LDH²⁶. Disc herniation contributes to the injury-induced

inflammation mediated by biochemical and immunological factors, which determine the pathophysiology of radiculopathy.²⁷ In the past decade, an increasing number of studies have investigated inflammation and immune activation in the pathogenesis of lumbar disc disease. It has been proved that autoimmunity plays an important role in the inflammation and pain associated with IVD disease.^{14,28-32}

IL-21 and IL-21 receptor (IL-21R) were first cloned and identified in 2000.^{15,33} IL-21 is mainly produced by activated CD4+ T-cells.³⁴ Th17 cells, which have been shown to be elevated in LDH patients and to correlate with pain,¹⁴ appear to be the most prolific producers of IL-21.^{35,36} Th17 cells are characterized by IL-17 production, but they also produce TNF- α , IL-21, and IL-22. IL-17 is thought to be a major effector cytokine

of Th17 cells. It can induce the production of inflammatory cytokines such as IL-6 granulocyte colony-stimulating factor and PGE-2, thus enhancing the inflammatory reaction.³⁷ Wei³⁸ reported that IL-21 is produced by Th17 cells, and its production is Signal transducer and activator of transcription 3 (STAT3) dependent. IL-21 activates STAT3 to promote Th17 cell differentiation, thus induces IL-17 production.³⁸ Geri et al showed that IL-21 promotes Th17 cell response, suppresses regulatory T-cells, and correlates with disease activity in Behcet disease.³⁹ IL-21 can stimulate Th17 cell proliferation in an autocrine manner.^{35,36,40} IL-21 has also been reported to be important for the growth of CD8+ T-cells.^{17,41}

Recent studies have shown that IL-21 plays an important role in the development of SLE, rheumatoid arthritis, Sjogren syndrome, multiple sclerosis, and type 1 diabetes.²¹⁻²⁵ This study shows that serum IL-21 and IL-17 levels are much higher in LDH patients than in healthy controls. This means that IL-21 may be associated with LDH. We next demonstrated the presence of IL-21, IL-17, and COX-2 within the nucleus pulposus. COX-2 mRNA levels and VAS scores were positively correlated with IL-21 mRNA levels in LDH patients. IL-17 mRNA levels, which contribute to the pain related to LDH¹⁴, were also positively correlated with IL-21 mRNA levels. This further proved our hypothesis.

Mice in which IL-21 signaling is blocked and mice that are genetically deficient in IL-21 or IL-21R are protected from autoimmune diseases. Herber et al showed that in the MRL-Fas^{lpr} mouse model of SLE, the blocking of IL-21 with IL-21R-Fc fusion protein (IL-21R-Fc) ameliorated renal disease, lymphadenopathy, and skin lesions and reduced circulating autoantibody and IgG levels.⁴² DBA/1 mice treated with IL-21R-Fc showed reduced clinical and histological signs of collagen-induced arthritis⁴³. IL-21R deficiency rendered non-obese diabetic mice resistant to insulinitis, production of insulin autoantibodies, and onset of type 1 diabetes.⁴⁴ This raises the question of the effects of the blockage of IL-21 signaling in LDH patients.

However, it should be noted that this study has several limitations. First, the number of subjects included is relatively small. Second, we could not acquire fresh cadavers, and so did not have normal nucleus pulposus specimens. The nucleus pulposus specimens obtained from patients who underwent

emergency surgery for fractures of the lumbar vertebra were used as the normal IVD control tissue. Third, this study only examined changes in IL-21 levels in patients with LDH; further studies are needed to elucidate the mechanism via which IL-21 induced IL-17 and COX-2 production in LDH patients.

Collectively, our results imply that inflammation is responsible for the pain associated with LDH, and that the increased expression of IL-21 may play a role in the pathogenesis of LDH. This finding could potentially be used to develop novel therapeutic strategies for the relief of related-to-LDH pain.

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