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Expression and significance of telomerase in the nucleus pulposus tissues of degenerative lumbar discs

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Abstract. The pathogenesis of lumbar disc degeneration is extremely complex, and the expression and role of telomerase in degenerative lumbar disc tissues remains unclear. The aim of the present study was to detect telomerase expression in nucleus pulposus tissues of degenerative lumbar discs and to explore the correlation between telomerase expression and other factors typical of disc degeneration. A total of 8 patients with degenerative nucleus pulposus were included as the experimental group and compared with 8 control patients without evident lumbar disc degeneration. The expression of telomerase in nucleus pulposus tissues was detected by immunohistochemical staining. ELISA was performed to determine the differential expression of telomerase, type II collagen and chondroitin sulfate between the two groups. In addition, a correlation analysis was performed to form associations between these factors. Finally, 5 cases in the experimental group and 5 in the control group were involved in the analysis. Immunohistochemistry results showed that telomerase expression in the experimental group was significantly lower compared to the control group and the percentage in the unit field of view showed significant differences between the two groups ($P<0.05$). Similarly, the ELISA test results showed lower expression levels of telomerase, type II collagen and chondroitin sulfate in the experimental group when compared with the control group ($P<0.05$). The correlation analysis revealed that telomerase was positively correlated with type II collagen and chondroitin sulfate (correlation coefficients, 0.673 and 0.528, respectively; $P<0.01$). In conclusion, telomerase is involved in the degeneration process of nucleus pulposus tissue in lumbar discs and has a positive correlation with other factors typically associated with degeneration.

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Introduction

Lumbar disc degeneration is a common disease and a leading cause of lower back pain in humans (1). It is estimated that ~5.7 million people suffer from lumbar disc disease each year in China, and the incidence is the highest in adults aged 45-64 years (2). At present, the prevalence of intervertebral disc degeneration is evidently increased, and more young people suffer from this disease. Intervertebral disc degeneration is an age-related disease that is caused by a number of factors, and among them the common feature is the reduced number and dysfunction of nucleus pulposus cells, and decline and disproportion in the extracellular matrix content. These are indicators of intervertebral disc cell senescence or apoptosis. Pathogenesis of lumbar intervertebral disc degeneration is relatively complex, the senescence and apoptosis of intervertebral disc cells are the extremely critical factors of degeneration (3). The mechanistic studies have identified that intervertebral disc degeneration commences from the senescence of nucleus pulposus cells, followed by a programmed degeneration process (4). The nucleus pulposus cells in adults have an extremely short viability and a limited self-renewal capacity. As the nucleus pulposus cells divide continuously, the telomere length declines; when the terminal restriction fragment is 5-7 kb long, the cells enter the crisis M1 and accordingly trigger a signal. The signal is blocked at the checkpoint in the cell cycle, thus nucleus pulposus cells exit the cell cycle, ultimately leading to cell cycle arrest or cell senescence. A previous study demonstrated that telomerase can regulate the cycle and activity of intervertebral disc cells (5). Therefore, the correlation between telomerase and intervertebral disc degeneration is of important significance. In the present study, the expression of telomerase was detected in the nucleus pulposus tissue of degenerative lumbar discs and compared with non-degenerative lumbar discs in a broader attempt to explore the etiology of lumbar disc degeneration.

Materials and methods

Harvesting nucleus pulposus tissue from lumbar discs. The study was performed in the Central Hospital of Bazhong City (Sichuan, China) between March and May 2014. Experimental procedures were approved by the Ethics Committee of the Central Hospital of Bazhong City. In the experimental group, degenerative lumbar nucleus pulposus tissues were harvested

from 8 patients (5 males, 3 females; mean age, 58; range, 52-68 years) who underwent posterior discectomy, grafting fusion and fixation due to lumbar degenerative disease. Intervertebral disc degeneration was graded as Pfirrmann grade IV by magnetic resonance imaging (MRI). In the control group, non-degenerative nucleus pulposus tissues were harvested from 8 patients (5 males, 3 females; mean age, 55; range, 47-65 years) who received interbody fusion surgery due to lumbar fractures. Intervertebral disc degeneration was graded as Pfirrmann grade I-II by MRI. None of the control patients had a previous history of lower back pain. The informed consents for participation in the study were obtained from all the patients.

During surgery, the intervertebral discs were separated and the cartilage endplate, nucleus pulposus and annulus fibrosis were immediately isolated. The specimens were rinsed with sterile saline to remove blood and the nucleus pulposus was stored in liquid nitrogen and transferred to -80˚C for further tests.

Outcome measures. The expression levels of telomerase, type II collagen and chondroitin sulfate in the nucleus pulposus tissues were determined to analyze the correlation between telomerase expression and degeneration of the nucleus pulposus.

Immunohistochemistry. The cell suspension was collected and 10 mmol/l citrate buffer (pH 6.0) was added. The sample was subjected to microwave antigen retrieval for 5 min and naturally cooled to room temperature. Subsequently, 3% H_2O_2 deionized water was added to the cell suspension and incubated at 37˚C for 10 min, following blocking with goat serum blocking solution at 37˚C for 30 min. Subsequent to discarding the serum, the cells were incubated with the diluted antibody (rabbit anti-TERT, 1:200; cat. no. ABIN265627) in a wet box overnight at 4˚C and with universal biotinylated secondary antibody at 37˚C for another 30 min. Horseradish peroxidase-conjugated streptavidin was added at 37˚C for 30 min. Except for the serum blocking step, specimens were rinsed with phosphate-buffered saline (PBS) 3 times for 5 min each, between any two steps. Cells were developed using diaminobenzidine for 5-10 min and the developing time under a microscope was controlled. Cells were counterstained with hematoxylin, rinsed with tap water and mounted with neutral gum. PBS was applied for the blank control, instead of a primary antibody. Specimens were observed under a microscope, and the positive cells exhibited brown granules in the nucleus or cytoplasm. All the above reagents were purchased from Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd. (Beijing, China).

ELISA detection. All the reagents were purchased from USCN Life Sciences Inc. (Wuhan, China). Telomerase, type II collagen and chondroitin sulfate were detected with ELISA using the kits for type II collagen (SEA572Ra), telomerase (E01541) and chondroitin sulfate (SBJ-H1520), respectively. In brief, nucleus pulposus tissue samples were triturated into pieces using an appropriate quantity of saline and centrifuged at 300 x g for 10 min. Following suspension, the supernatant was discarded.

The sample (10 μl) was combined with 40 μl of sample dilution buffer in the wells of a 96-well plate. To this, 100 μl of horseradish peroxidase-conjugated antibody was added to the wells and the reaction was sealed and incubated in a 37˚C water bath or incubator for 60 min. This allowed the reaction to take place and the protein to adhere to the surface of the plate. Following incubation, the solution in each well was discarded and the wells containing the sample-antibody complex were dried. Each well was washed with washing solution for 1 min. The washing solution was subsequently discarded and the specimens were dried again. This washing procedure was repeated 5 times. Substrates A and B (50 μl each), provided in the kit, were added to each well and the specimens were incubated at 37˚C in the dark for 15 min. The incubation was terminated using 50 μl of termination solution for 15 min. The absorbance was measured at 450 nm and compared against blank (no antibody) and standard controls, as per the manufacturer's instructions.

Statistical analysis. Data are expressed as the mean ± standard deviation (SD) and statistically analyzed using SPSS v19.0 software (SPSS, Inc., Chicago, IL, USA). The differences between the groups were compared using multiple samples χ^2 test. The indexes between the groups were compared using the least significant difference t-test and a correlation analysis was performed using the Spearman rank correlation analysis. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Subjects. Due to specimen preservation and experimental surgery, 5 cases (including 3 males and 2 females, aged 52-68 years with a mean of 58 years) in the experimental group and 5 cases (including 3 males and 2 females, aged 47-65 years with a mean of 55 years) in the control group were involved in the final analysis. In the experimental group, 5 cases with degenerative nucleus pulposus underwent posterior discectomy, bone grafting and internal fixation due to lumbar degenerative disease and were graded as IV level using the Pfirrmann rating scale by MRI detection. The 5 cases without degenerative nucleus pulposus in the control group underwent intervertebral fusion due to lumbar fractures and were graded as I-II level using the Pfirrmann rating scale by MRI detection.

Immunohistochemistry for telomerase expression. Telomerase positive signals were the appearance of brown particles, which were mainly located in the nucleus. Using the Image-Pro Plus software (Media Cybernetics Co., Silver Spring, MD, USA), telomerase expression in the nucleus pulposus tissue of the experimental group was significantly lower compared with the control group (experimental group, 2.1%; control group, 4.3%). There were significant differences in the percentage in each field of view between the experimental and control groups (χ^2=132.432 and 87.658, $P<0.05$) (Fig. 1).

Comparison of telomerase expression in the experimental and control groups. A total of 5 specimens in each group were selected for. The ELISA test results showed that the expression

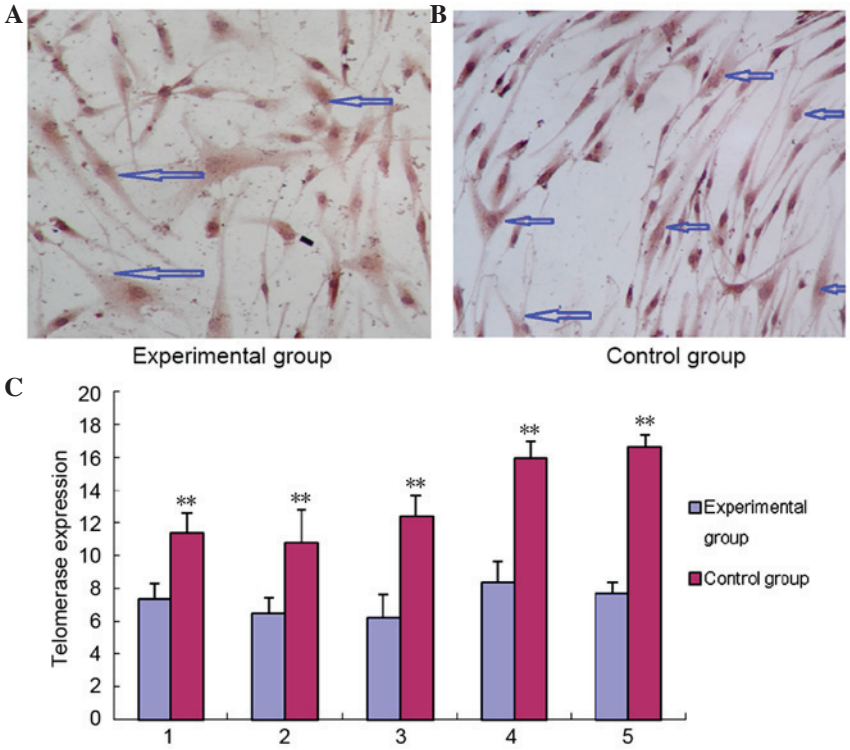

Figure 1. Telomerase expression in the (A) experimental (arrow) and (B) control groups (arrow). (C) Comparison of telomerase expression in the experimental and control groups. **P<0.05.

of telomerase in the experimental group was lower compared with the control group, which was statistically significant (P<0.05; Fig. 1).

Comparison of chondroitin sulfate expression in the experimental and control groups. A total of 5 specimens in each group were selected for. The ELISA test results showed that the expression of chondroitin sulfate in the experimental group was lower compared with the control group, which was determined to be statistically significant (P<0.05; Fig. 2).

Comparison of type II collagen fiber expression in the experimental and control groups. A total of 5 specimens in each group were selected for. The ELISA test results showed that the expression of type II collagen fiber in the experimental group was lower compared with the control group, which was statistically significant (P<0.05; Fig. 3).

Telomerase, chondroitin sulfate and type II collagen ELISA analyses. In the experimental group, significant correlations were identified between telomerase and chondroitin sulfate (0.8087; P<0.01) and telomerase and type II collagen fibers (0.5837; P<0.01). This indicates that telomerase has a significantly positive correlation with chondroitin sulfate and type II collagen fibers. The higher expression of telomerase occurs in parallel with the high expression of chondroitin sulfate and type II collagen fibers (Figs. 4 and 5).

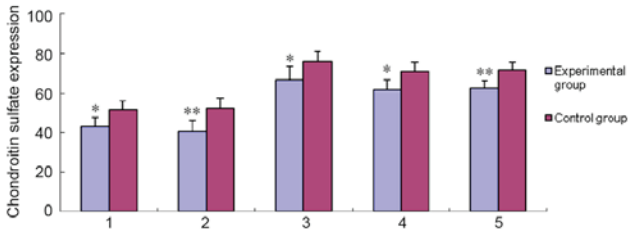

Figure 2. Comparison of chondroitin sulfate expression in the experimental and control groups. *P<0.01, **P<0.05.

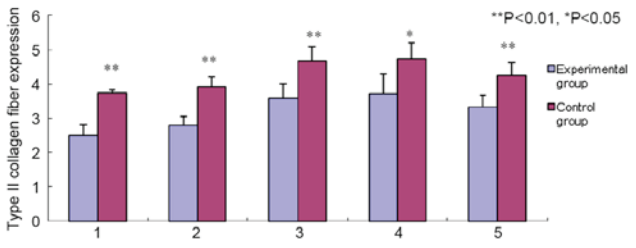

Figure 3. Comparison of type II collagen fiber expression in the experimental and control groups.

Discussion

Degenerative disc disease is caused by intervertebral disc degeneration, characterized by clinical symptoms, such as pain in the neck, hip and leg. Degenerative disc disease includes neck and lumbar disc herniation, cervical spondylosis, degenerative lumbar spinal stenosis, degenerative lumbar spondylolisthesis and degenerative scoliosis. The incidence of degenerative disc disease is extremely high in adults and 70-80% of people experience lower back pain in their lifetime (1). Approximately 15% of patients have a clearly determined cause of lower back pain, in which >50% is induced by degenerative disc disease (1). In

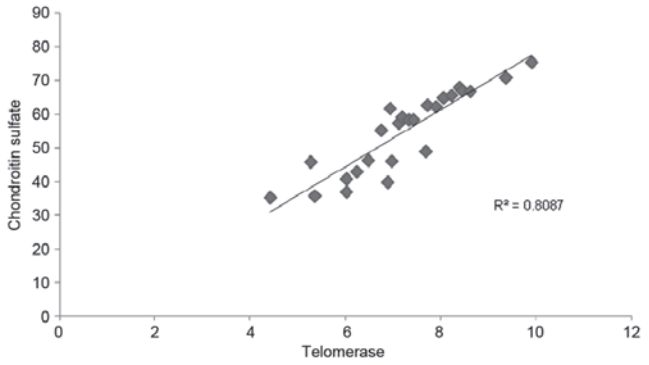

Figure 4. Linear regression of telomerase and chondroitin sulfate expression.

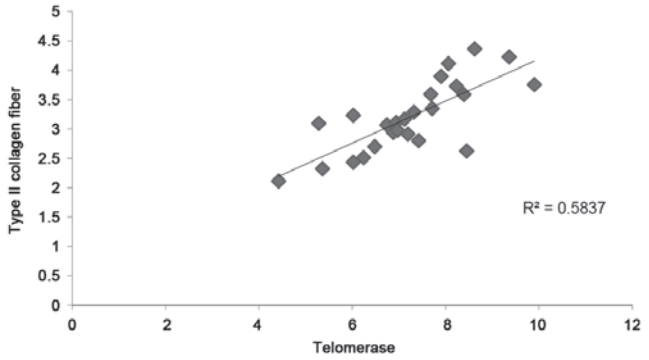

Figure 5. Linear regression of telomerase and type II collagen fiber expression.

the United States, degenerative disc disease is the most common disease in the orthopedic outpatient department and also one of the top 5 causes of hospitalization, with an incidence rate as high as 70-90% and annual medical expenses of >50 billion USD.

In China, the prevalence of degenerative disc disease in adults is ~6% (2); however, this rate is estimated to increase to 50 million people by 2020. Degenerative disc disease poses a serious threat to patient health and quality of life due to the partial or complete loss of mobility and independence in afflicted patients; this, in turn, is associated with a significant economic and social burden of the disease. Therefore, it is urgent to investigate ways to prevent, diagnose and treat chronic degenerative diseases of the spine.

The reductions in type II collagen and chondroitin sulfate are the main characteristics of the nucleus pulposus during the lumbar disc degeneration process. Preliminary studies have shown that degenerative disc disease is associated with age, gender, occupation, smoking and mechanical factors, among which long-term, heavy lifting is the dominant cause (6). With the development of basic medicine and molecular biology, our previous study identified that the decline in type II collagen and chondroitin sulfate is mediated by a series of cytokines (7).

Telomerase is a ribonucleoprotein complex enzyme with reverse transcriptase functions that include the telomerase RNA (TR), catalytic protein subunit or telomerase reverse transcriptase (TERT) and telomerase accessory protein (6). The quantity, arrangement and pattern of TR nucleic acid sequences are associated with the species. The human TR component was first successfully cloned in 1995 and there are 451 nucleotides in human TR. The TR sequence and length have a conserved secondary structure and relatively stable tertiary structure. Cohen *et al* (8) detected that the molecular mass of the human telomerase complex was 650-670 kDa through chromatography analysis, and found that it is a dimer consisting of TERT (127 kDa), TR (153 kDa) and dyskerin (57 kDa).

Intervertebral disc cell proliferation and growth are also attributed to telomerase activity. Previous studies have shown that telomerase activity is inhibited or destroyed in the degenerating intervertebral disc cells, thus it cannot effectively maintain chromosome balance, leading to continuous shortening of the telomere in the replication process and promoting cell senescence and intervertebral disc degeneration (9,10). Kim *et al* (11) explored the mechanisms of cellular aging and the signal transduction pathway of intervertebral disc cells, and identified that telomerase activity and telomere length gradually decreased or shortened along with the deterioration of disc degeneration, and a decline in the telomerase activity could promote the accumulation of senescent cells in the intervertebral disc. The present study found that telomerase expression in the nucleus pulposus tissues of degenerative lumbar vertebral discs was significantly lower compared with the non-degenerative tissue (control group), indicating the involvement of telomerase in the degeneration process of the nucleus pulposus.

Telomerase can delay intervertebral disc degeneration through cell maintenance. Liang *et al* (12) transfected nucleus pulposus cells with the telomerase gene and found that the survival of normal nucleus pulposus cells was significantly prolonged. Chung *et al* (13) transfected nucleus pulposus cells with a non-viral liposome-mediated telomerase gene, and the results showed that telomerase enhanced the division ability of nucleus pulposus cells and prolonged the lifespan to 496 days; the nucleus pulposus cell matrix sustained expression within 282 days and the synthesis of type II collagen protein was 46 and 32 times as high as the control group. Following transfection of the nucleus pulposus with the telomerase gene, the percentage of senescent cells to the total nucleus pulposus cells was 3-30% (following telomerase gene transfection) and 4-64% (control group). In addition, the generation of extracellular matrix products, such as collagen, were significantly increased within 120 days and the notable difference was identified at day 21 with a yield that was 7.2 times as high as the control group (14,15). In the present study, the expression levels of chondroitin sulfate and type II collagen in the nucleus pulposus were also significantly lower in the experimental group compared with those in the control group. This finding is consistent with other pathological studies addressing intervertebral disc degeneration.

At present, the specific mechanism of intervertebral disc degeneration remains unclear. Tabach *et al* (16) explored the function of nucleus pulposus cells and telomerase, as well as the expression of 168 downstream genes induced by p53 activity, and the results showed that the mechanism responsible for telomerase delaying intervertebral disc degeneration is mediated by the regulation of the p53-p21-pRb and p16-pRb pathways (17). Previous studies have found that when the telomere length in the disc cells reached a certain Hayflick limit, DNA damage and telomere dysfunctions occurred, which triggered a DNA damage response (DDR). DDR is associated with phosphorylated form of H2AX histone variant and DDR proteins p53-binding proteins 1, Nijmegen breakage syndrome 1 and mediator of DNA damage checkpoint 1, while

DDR-associated kinases, such as ataxia telangiectasia-mutated (ATM) and Rad3-related, are also activated, thus causing cell senescence and apoptosis (18). Kim *et al* (11) investigated the mechanisms of human disc degeneration and identified that telomerase activity was significantly inhibited or destroyed in all the senescent nucleus pulposus cells, and the expression of p53, p21 and pRb that is regulated by telomerase activity was strongly positive. Liang *et al* (12) transfected the telomerase gene into the nucleus pulposus cells and found that telomerase activity was significantly enhanced, while p53 expression was reduced (13). Certain investigators have also found that telomerase, cofactor of the Wnt/β-catenin pathway, can regulate the synthesis and secretion of Wnt proteins and the associated protective proteins to maintain the viability of intervertebral disc cells (19). The correlation analysis results showed that telomerase expression has a significant positive correlation with chondroitin sulfate and type II collagen expression: The lower expression of telomerase paralleled the lower expression of chondroitin sulfate and type II collagen. The possible mechanisms are that telomerase may be involved in the proliferation and apoptosis processes of nucleus pulposus cells. When telomerase is low, the corresponding signal transduction is also reduced, thus slowing the transcription of RNA polymerase and TERT promoter, accelerating the degeneration and apoptosis of nucleus pulposus cells and decreasing the expression of chondroitin sulfate and type II collagen fibers and ultimately leading to the intervertebral disc degeneration.

In conclusion, degenerative nucleus pulposus tissues have a significantly lowered expression of telomerase compared with the non-degenerative nucleus pulposus tissues, indicating that telomerase may be involved in the pathological process of intervertebral disc degeneration. Furthermore, this reduced level of telomerase is positively correlated with lowered expression levels of chondroitin sulfate and type II collagen, two factors typically affected during the degeneration process. Further studies are required to reveal the molecular biological mechanisms of disc degeneration.

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