Effect of miR-202-5p-mediated ATG7 on autophagy and apoptosis of degenerative nucleus pulposus cells

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Abstract. – OBJECTIVE: This study aimed to research the effect of miR-202-5p-mediated ATG7 on autophagy and apoptosis of degenerative nucleus pulposus cells.

PATIENTS AND METHODS: The intervertebral disc nucleus pulposus (NP) tissue of patients with intervertebral disc degenerative disease and normal intervertebral disc nucleus pulposus (NP) tissue of patients with spinal fractures was collected as the research object. Normal NP cells and degenerative NP cells were isolated. Low expression of miR-202-5p and overexpression of ATG7 were carried out in degenerative NP cells. The expression of miR-202-5p and ATG7 mRNA was detected by RT-PCR. The expression of ATG7, LC3-II, Bax, and Bcl-2 proteins was detected by Western blot. The autophagy of cells was detected by MDC staining. The apoptosis of NP cells was detected by flow cytometry. The targeting relationship between miR-202-5p and ATG7 was detected by Dual-Luciferase reporter.

RESULTS: In the degenerative NP tissues, miR-202-5p was highly expressed and ATG7 was low expressed. The inhibition of miR-202-5p expression can effectively promote autophagy of NP cells, increase the expression of ATG7 and LC3-II, inhibit the apoptosis of NP cells, inhibit the expression of pro-apoptotic proteins Bax, and promote the expression of pro-apoptotic proteins Bcl-2 proteins. The upregulation of ATG7 expression in degenerative NP cells alone had the same effect as the downregulation of miR-202-5p. The assay of the Dual-Luciferase reporter confirmed the targeting relationship between miR-202-5p and ATG7.

CONCLUSIONS: MiR-202-5p can affect the autophagy and apoptosis of degenerative nucleus pulposus cells through targeted adjustment of ATG7, which may be a new therapeutic target for intervertebral disc degenerative diseases.

Key Words:
MiR-202-5p, ATG7, Degenerative nucleus pulposus cells, Autophagy, Apoptosis, Effect.

Introduction

Intervertebral disc degenerative disease (IDDD) is a common chronic disease of the locomotor system in clinic. At present, about 630 million people around the world are currently suffering from IDDD. Moreover, intervertebral disc degeneration (IDD) is also one of the main causes of disability of human body functions, which has a serious impact on patients' quality of life and causes heavy burden on patients' families and society[1,2]. In the past, most studies[3,4] have considered that the onset of IDD was caused by the interaction between genetic and environmental factors, but the molecular mechanism of its pathogenesis is not clear.

Nucleus pulposus (NP) tissue is a key region of the intervertebral disc organ, which contains some NP cells. NP cells play a key role in the synthesis and secretion of extracellular matrix and in supporting the mechanical function of intervertebral disc[5]. Autophagy of cells is a conservative cellular protective process. In recent years, more and more attention has been paid to the role of autophagy of cells in degenerative disease[6]. Ao

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et al[7] have reported that autophagy and apoptosis of cells play an important role in the degeneration of intervertebral disc, and it is considered that autophagy of cells has a protective effect on the apoptosis of cells. MiRNA is a short non-coding RNA that can affect the biological function of cells by binding to the 3'-UTR of target genes[8]. In the past, there have been reports in the research on IDD that there is a close relationship between the abnormal expression of miRNA and the occurrence and development of IDD. Kang et al[9] indicated that miR-494 can induce apoptosis of degenerative NP cells by combining with SOX9, but few studies have explored the content related to autophagy. MiR-202-5p is a miRNA with abnormal expression in many diseases. However, it has been reported[10] that it can regulate the autophagy of myocardial cells through the PETN/AKT/mTOR pathway. We found that one of its target genes is ATG7 through the prediction of the online target genes. As an autophagy-related protein, ATG7 plays an important role in the formation of autophagosomes. However, a large number of previous studies[11,12] have shown that autophagy exists in a variety of degenerative disease, including IDD.

Therefore, we speculated whether miR-202-5p could regulate ATG7 to affect the autophagy of NP cells. However, there are no studies on the mechanism of miR-202-5p in IDD, so, in order to seek more possibilities for the treatment of IDD patients, we conducted the following research.

Patients and Methods

Experimental Materials and Reagents

The intervertebral disc nucleus pulposus tissue of 65 cases with intervertebral disc degenerative disease that came to our Hospital to treat from March 2016 to June 2018 was collected as a study group. The normal intervertebral disc nucleus pulposus tissue of 65 cases with spinal fractures that conduct surgery in our hospital was selected as the control group. All tissues were obtained with the consent of patients and their families and signed an informed consent. All tissues stored in liquid nitrogen jar at -80°C. This study was approved by the Hospital Ethics Committees. All patients were inspected with a routine nuclear magnetic resonance imaging before surgery and we graded the degree of progressive transmutation of the intervertebral disc. I was normal, II was mild IDD, III was moderate, IV was severe. Rabbit anti-human ATG7, LC3-II, Bax, Bcl-2 monoclonal antibody (USA Abcam, 52472, 48394, 32503, 185002) and β-actin monoclonal antibody of rabbit anti-human were purchased from ProteinTech Wuhan, LTD (Wuhan, China). Bicinchoninic acid (BCA) protein concentration assay kits were purchased from Shanghai Weiao Biotechnology Co., Ltd (Shanghai, China). Real-time quantitative PCR instrument was purchased from BioRad company (Hercules, CA, USA). Flow cytometry CytoFLEX LX was purchased from Beckman (Brea, CA, USA). Dulbecco's Modified Eagle's Medium (DMEM) was purchased from Gibco (Rockville, MD, USA). Fetal bovine serum (FBS) and trypsin were purchased from HyClone (South Logan, UT, USA). TRIzol reagents were purchased from Applied Invitrogen (Carlsbad, CA, USA). qPCR and reverse transcription kits were purchased from TransGen Biotech (Beijing, China, AQ201-01, AQ202-01). Dual-Luciferase reporter gene assay kits were purchased from Solarbio (Beijing, China). MiR-129-5p, miRNA NC, internal reference U6 and β-actin primers were synthesized and designed by Shanghai GenePharma Co., Ltd (Shanghai, China). CAnnexin V-FITC/PI apoptosis of cells kit was purchased from Jiangsu KeyGEN BioTECH Corp., Ltd (Jiangsu, China).

The Isolation, Cultivation, and Transfection of Human NP Cells

First, the NP tissues were washed by PBS and was cut into small pieces of 1x1 mm and placed in a centrifuge tube. Then, 0.2% of type II collagenase was added to the centrifuge tube and placed in a 37°C water bath for digestion with 10 min. After digestion, the tissues were centrifuged at a rate of 3000 r/min for 5 min. After removing the supernatant, the hyaluronidase was separated in a water bath by 10 U/mL for 2 hours. After separation, the tissues were centrifuged again at a speed of 3000 r/min for 5 min, then, we washed them three times by PBS. After washing, the cells were inoculated into DMEM containing 10% FBS. 100 u/ml penicillin and 100 microns/ml streptomycin were added. Then, they were placed in an incubator at 37°C and 5% CO_2 for incubating 48 h. Then, the cells were inoculated into a 6-pore plate at the density of $1x10^6$ cells/pore. The cells were grouped and transfected when cell fusion was observed to reach 80%. The cells were divided into blank control group (NP cells from normal intervertebral disc nucleus pulposus tissue were

not transfected), miR-NC group (transfection miR-NC of NP cells from degenerative intervertebral disc nucleus pulposus tissue), miR-202-5p inhibition group (transfection miR-202-5p inhibition of NP cells from degenerative intervertebral disc nucleus pulposus tissue), Sh-ATG7 group (transfection Sh-ATG7 of NP cells from degenerative intervertebral disc nucleus pulposus tissue), and Sh-NC group (transfection Sh-NC of NP cells from degenerative intervertebral disc nucleus pulposus tissue).

Detection of MiR-202-5p and ATG7 Expression in Tissues and Cells by RT-PCR

The total of RNA from tissues and cells was extracted by using TRIzol reagent. The purity and concentration of RNA were detected by ultraviolet spectrophotometer. Then, the total of RNA of 5 μg was taken to reverse transcription cDNA according to the kit instructions. The reaction parameters were: 37°C 10 min, 42°C 30 min, 70°C 5 min. MiR-202-5p amplification system: cDNA 1 μL, upstream and downstream primers each 0.4 μL, 2×TransTaq® Tip Green qPCR SuperMix 10 μL, passive Reference Dye (50X) 0.4 μL. In the end, ddH_2O was added to complete to 20 μL. Amplification conditions: PCR reaction conditions: pre-degeneration at 94°C for 45 s, degeneration at 94°C for 15 s, anneal and extension at 60°C for 40 s, then followed a total of 40 cycles. ATG7 amplification system: cDNA 1 μL, upstream and downstream primers each 0.4 μL, 2X TransScript® Tip Green qPCR SuperMix 10 μL, Passive Reference Dye (50X). In the end, the Nuclease-free Water was added to complete to 20 μL. Amplification conditions: pre-degeneration at 95°C for 30 s, degeneration at 95°C for 10 s, anneal and extension at 60°C for 35 s, then, followed by a total of 40 cycles and 3 replicate pores per sample. The experiment was carried out 3 times. MiR-202-5p used U6 as the internal parameters; ATG7 used β-actin as the internal parameters. More details of the primer sequence are shown in Table I. $2^{\Delta\Delta ct}$ was used to analyze the data.

Detection of Western Blot

We collected NP cells from each group after culture. The total protein was extracted by radioimmunoprecipitation assay (RIPA) lysis. The protein concentration was detected by bicinchoninic acid (BCA). The protein concentration was adjusted to 4 μg/μL. By 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis we separated and transferred film to polyvinylidene difluoride (PVDF) after ionization; next, we dyed with ponceau working fluid; finally, we soaked and washed in Phosphate-Buffered Saline and Tween (PBST) for 5 min. We blocked with 5% skim milk powder for 2 h. ATG7 (1:1000), LC3-II (1:1000), Bax (1:1000), Bcl-2 (1:1000) and β-Actin first antibody (1:1000) were added and sealed overnight at 4°C. The first antibody was removed by washing the film, and HRP-conjugated sheep anti-mouse second antibody (1:1000) was added, incubated at 37°C for 1 h, and rinsed 3 times with PBS for 5 min each time. The excess liquid on the film was dried with a filter paper, and the ECL was illuminated and developed. The protein bands were scanned and the gray values were analyzed in the Quantity One. The relative expression level of its protein = the gray value of the target protein band / the gray value of the β-Actin protein band.

Detection of Dual-Luciferase Reporter

The Targetscan7.2 were used to predict the downstream target genes of miR-202-5p. ATG7-3'UTR wild type (Wt), ATG7-3'UTR mutant (Mut), miR-202-5p-mimics, and miR-NC were transferred into NP cells using Lipofectamine™ 2000 kit. Luciferase activity was determined by Dual-Luciferase reporter gene assay kit (Promega, Madison, WI, USA) at 48 h after transfection.

Detection of Autophagy by MDC Staining

The transfected NP cells in each group were continued to be cultured in an incubator at 37°C and 5% CO_2 for 48 h, and then, washed by PBS

Table I. Primer sequences.

Factors	Upstream primers	Downstream primers
miR-202-5p	5′-TTCCTATGCATATACTTCT-3′	5′-CGTATCCAGTGCGAATA-3′
U6	5′-ATTGGAACGATACAGAGAAGATT-3′	5′-GGAACGCTTCACGAATTTG-3′
ATG7	5-ATTGCTGCATCAAGAAACCC-3	5-GATGGAGAGCTCCTCAGCA-3
β-Actin	5′-CCACCATGTACCCAGGCATT-3′	5′-GAGCCACCAATCCACACAGA-3′

in culture medium for 3 times. 200 μL MDC dyes (0.05 mmol/L) was added and incubated at 37°C in the dark for 60 min. Then, the MDC dyes was absorbed and discarded, washed 3 times by PBS, and fixed with 4 ¥ paraformaldehyde for 10 min. After air-drying, it was observed immediately with a fluorescence microscope (excitation wavelength was 425 nm, emission wavelength was 525 nm). Under the 200×, 100 cells were randomly selected to count the cells containing punctiform autophagic vacuoles and calculate the autophagy of cells ratio = cells containing autophagic vacuoles / 100 cells. The average value was calculated.

Detection of Apoptosis of Cells by Low Cytometry

The transfected cells were digested by 0.25% trypsin, then, washed by PBS after digestion for 2 times, added 100 μL binding buffer, then, configured it as 1x10^6 ind/mL suspension, added AnnexinV-FITC and PI in turn, and incubated at room temperature for 5 min in the dark. FC500MCL flow cytometry system was used for detection. The experiment was repeated for 3 times to take the average value.

Statistical Analysis

In this study, SPSS 20.0 (IBM Corp., Armonk, USA) was used for statistical analysis of the collected data. The desired images were drawn by GraphPad 7. Independent *t*-test was used for comparison between groups. The single factor analysis of variance was used to compare the multiple groups. LSD-*t* test was used for pairwise comparison after the event. The difference was statistically significant with $p<0.05$.

Results

Expression of MiR-202-5p and ATG7 in NP Tissues

Compared with the normal NP tissues, expression of miR-202-5p in degenerative NP tissues was significantly upregulated, but the expression of ATG7 was significantly downregulated. The difference was statistically significant (all $p<0.05$). Moreover, the expression of miR-202-5p and ATG7 was negative correlation (all $p<0.05$). More details are shown in Figure 1.

Effect of MiR-202-5p Over-Expression on Autophagy and Apoptosis of Degenerative NP Cells

Compared with the blank control group, the autophagy rate of NP cells in the miR-NC group was significantly decreased. ATG7 expression and lc3-ii/lc3-i ratio were significantly downregulated. However, NP cell apoptosis rate significantly increased. Pro-apoptotic proteins Bax were significantly upregulated. Anti-apoptotic protein Bcl-2 was significantly downregulated (all $p<0.05$). Compared with the miR-NC group, the autophagy rate of NP cells in the miR-202-5p inhibition group significantly increased. ATG7 expression and LC3-II/LC3-I ratio were significantly upregulated. However, the apoptosis rate of NP cells significantly decreased. Pro-apoptotic proteins Bax were significantly downregulated. Anti-apoptotic protein Bcl-2 was significantly upregulated (all $p<0.05$). More details are shown in Figure 2.

ATG7 Was a Direct Target Gene of MiR-202-5p

Bioinformatics predicted that ATG7 is the direct target gene of miR-202-5p. Therefore, we speculated that miR-202-5p may participate in the autophagy of NP cells by ATG7 of targeting regulation. Therefore, we performed a Dual-Luciferase assay. The results showed that the ATG7-3'UT Wt Luciferase activity was significantly decreased after miR-202-5p overexpression (all $p<0.05$), but had no effect on ATG7-3'UTR Mut Luciferase activity (all $p>0.05$). WB assay showed that the expression of ATG7 protein in NP cells after transfection with miR-202-5p mimic was significantly decreased. The expression of ATG7 protein in NP cells was significantly increased after transfection with miR-202-5p (all $p<0.05$). More details are shown in Figure 3.

Effect of ATG7 on Autophagy in NP Cells

In order to verify that the effect of miR-202-5p on autophagy in NP cells occurred by targeting ATG7, we transfected Sh-ATG7 to upregulate the expression of ATG7 in NP cells. The subsequent results showed that compared with the blank control group, the autophagy rate of NP cells in Sh-NC group was significantly decreased. The expression of ATG7 and LC3-II/LC3-I ratio were significantly downregulated, but the apoptosis rate of NP cells was significantly increased. Pro-apoptotic proteins Bax were significantly upregulated and anti-apoptotic protein Bcl-2 was

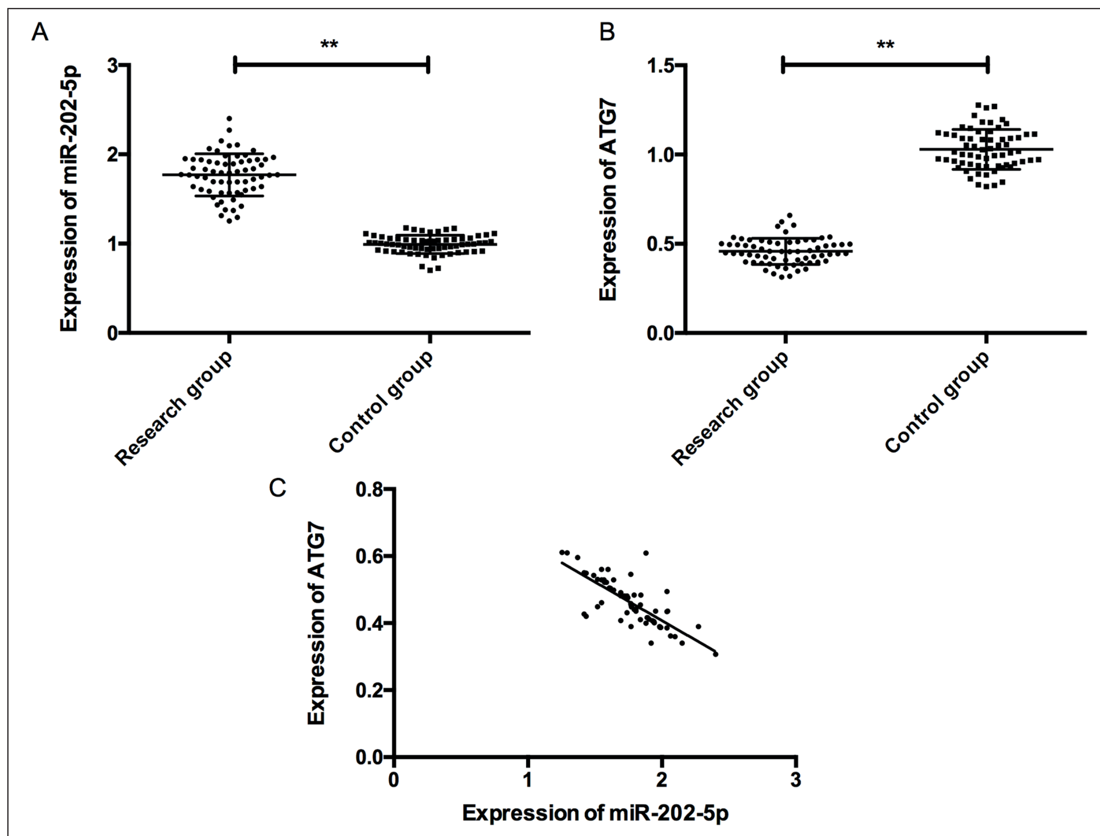

Figure 1. Expression of miR-202-5p and ATG7 in NP tissues. **A,** Compared with the normal NP tissues, the expression of miR-202-5p in the degenerative NP tissues was significantly upregulated. **B,** Compared with the normal NP tissues, the expression of ATG7 in the degenerative NP tissues was significantly downregulated. **C,** The expression of miR-202-5p and ATG7 was negative correlation. **Indicates $p<0.05$.

significantly downregulated (all $p<0.05$). Compared with the Sh-NC group, the autophagy rate of NP cells in Sh-ATG7 group significantly increased. The expression of ATG7 protein and LC3-II/LC3-I ratio were significantly upregulated. The apoptosis rate of NP cells significantly decreased. Pro-apoptotic proteins Bax were significantly downregulated and anti-apoptotic protein Bcl-2 was significantly upregulated (all $p<0.05$). More details are shown in Figure 4.

Discussion

Autophagy, as a conservative lysosome degradation pathway, mainly maintains homeostasis of cytoplasm by eliminating protein aggregates and damaged organelles[13]. In the past, many studies have reported that autophagy plays an important role in the pathological process of intervertebral disc degenerative[14]. Jiang et al[15] have found that glucosamine can induce autophagy through mammalian rapamycin signaling as a protective mechanism of normal NP cells. Yang et al[16] indicated that the death of NP cells involves many changes, such as cell senescence, apoptosis, and autophagy. Autophagy is considered to play a very important role in the pathogenesis of IDD due to its cell stabilization and anti-apoptotic effect.

In recent years, miRNAs have been considered to play an important role in regulating autophagy of cells. In our study, we also found that the expression of miR-202-5p was upregulated in degenerative NP tissues. In addition, we also found that the changes of expression of miR-202-5p and ATG7 were related to autophagy and apoptosis of degenerative NP cells. The ATG family is a class of genes that play the most important role in autophagy. ATG7 is one of the essential molecules to be verified in the formation of autophagy in mammals, and

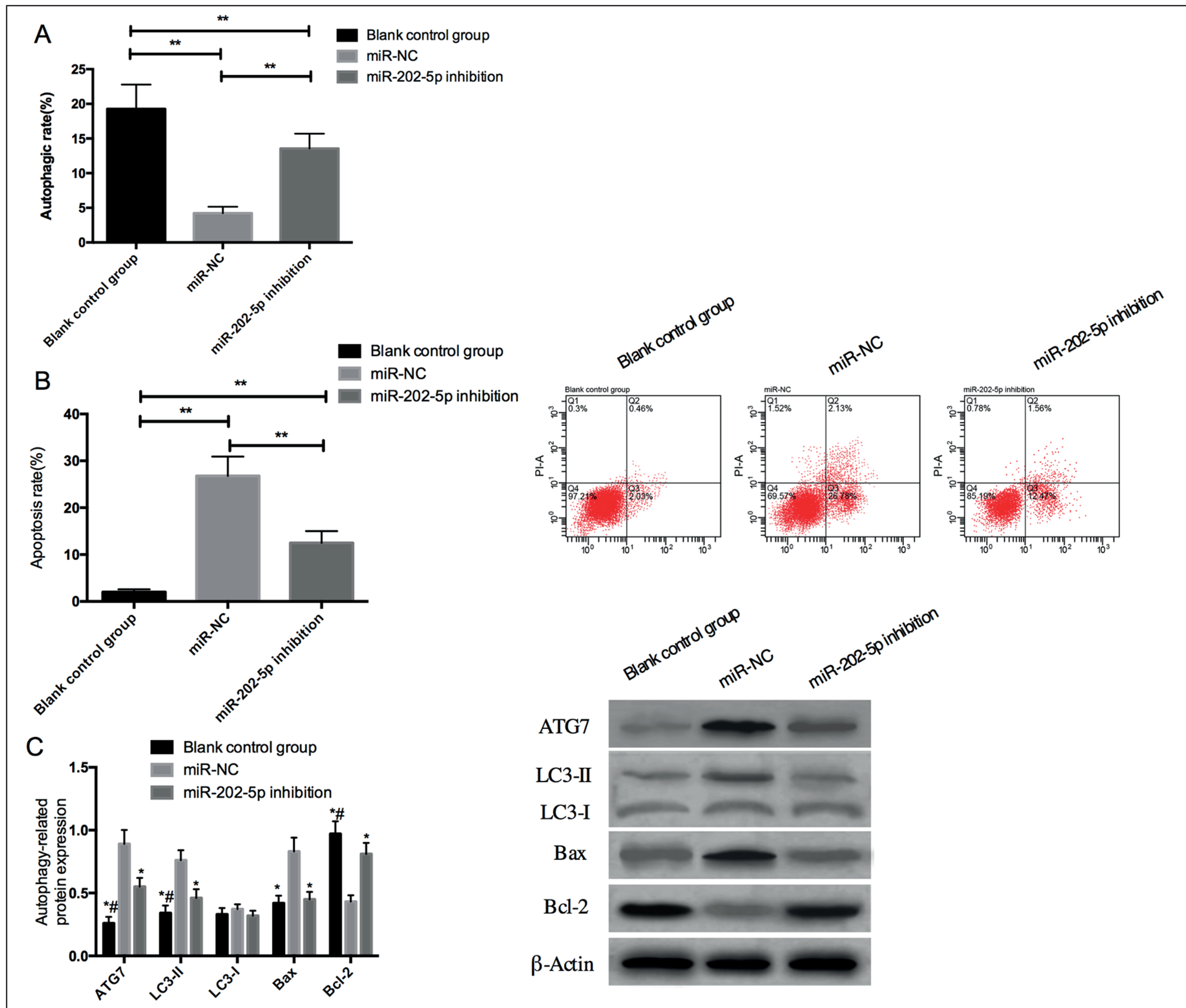

Figure 2. Effect of miR-202-5p overexpression on autophagy and apoptosis of degenerative NP cells. **A,** Compared with the miR-NC group, the autophagy rate in the miR-202-5p inhibition group significantly increased. **B,** Compared with the miR-NC group, the apoptosis rate in the miR-202-5p inhibition group significantly decreased. **C,** Compared with the miR-NC group, ATG7 expression, LC3-II/LC3-I ratio and anti-apoptotic protein Bcl-2 of NP cells in the miR-202-5p inhibition group were significantly upregulated. Pro-apoptotic proteins Bax was significantly downregulated. **Indicates $p<0.05$.

it plays an important role in the formation of autophagosomes[17,18]. In addition, studies have shown that ATG7 as an E1 sample enzyme can promote the binding of ATG12 and ATG5. Once ATG7 is absent, it can lead to tumors and some other diseases[19]. Zhu et al[20] have used ATG7 as a regulatory target for autophagy and achieved good results. In our study, we also found that the expression of ATG7 was downregulated in the degenerative NP tissues. It was also found that the expression of miR-202-5p and ATG7 was negatively correlated in NP tissues. This leds us to speculate that the degenerative disc disease was related to the downregulation of ATG7 regulated by miR-202-5p. At the same time, we also found that the expression of LC3-II was also significantly increased in the degenerative NP cells. This reminds that autophagy plays an important role in the pathogenesis of IDD. This is consistent with our previous research results. Subsequently, in order to further validate our hypothesis, we isolated the normal NP cells and degenerative NP cells and found that the expression trends of miR-202-5p and ATG7 in degenerative NP cells were consistent with those in the degenerative NP tissues. In order to explore the effects of miR-202-5p and ATG7 on degenerative NP cells, we per-

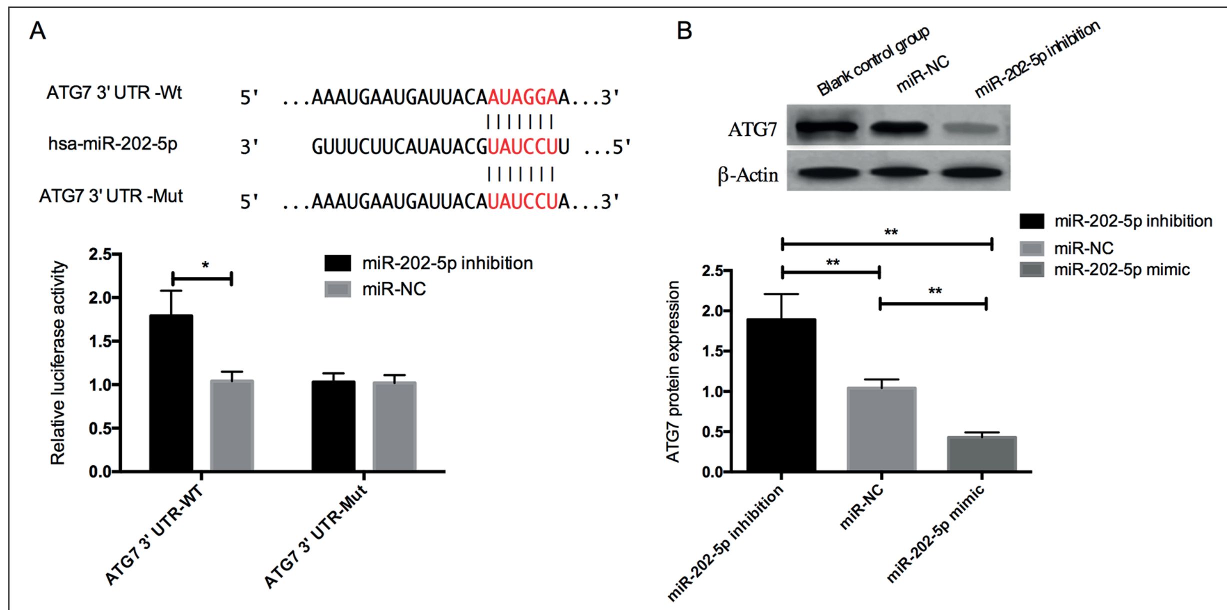

Figure 3. ATG7 was a direct target gene of miR-202-5p. **A,** There were binding sites between miR-202-5p and ATG7. Relative Luciferase activity – Dual-Luciferase reporter assay. **B,** The expression of ATG7 protein in NP cells after transfection.

formed low expression of miR-202-5p and the overexpression of ATG7 in the degenerative NP cells, respectively. The results showed that, when the expression of miR-202-5p was downregulated in the degenerative NP cells, the expression of ATG7 in cells significantly increased and significantly promoted autophagy in degenerative NP cells. In addition, the expression of lC3-2 in NP cells was also significantly higher, and the upregulation of ATG7 also had the same effect, separately. This indicates that miR-202-5p may have an effect on the autophagy of degenerative NP cells by regulating the expression of ATG7. We verified the targeting relationship between miR-202-5p and ATG7 by Dual-Luciferase reporter. However, Wang et al[21] indicated that miR-210 can affect the autophagy of degenerative NP cells by regulating AGT7, which indicates that AGT7 can affect the autophagy of cells by regulation of miRNA. This was also similar to our conclusion. In our study, it is worth noting that it was first discovered that miR-202-5p can affect autophagy of cells by regulating the expression of ATG7 in the degenerative NP cells. Furthermore, we observed that when autophagy was significantly promoted, the apoptosis of the degenerative NP cells was inhibited. Meanwhile, we also found that pro-apoptotic proteins Bax was significantly downregulated and the anti-apoptotic protein Bcl-2 was significantly upregulated. This reminds us that miR-202-5p can protect the apoptosis of the degenerative NP cells by inducing the expression of ATG7, which may be achieved by regulating Bax/Bcl-2. Liu et al[22] have pointed out that although the abnormal increase of autophagy may promote cell apoptosis under certain regulation, autophagy has cell protective effect in most cases. In addition, Li et al[23] indicated that autophagy could be induced to reduce the apoptosis of spinal cord neurons after spinal cord injury, thus promoting the recovery of nerve function. It also indicated that the increase of autophagy could inhibit apoptosis. No previous studies have reported the effect of miR-202-5p on cell apoptosis, so it is unclear whether miR-202-5p inhibits cell apoptosis through autophagy or other ways to affect the apoptosis of degenerate NP cells. Zhao et al[24] have found that miR-129-5p can induce autophagy of NP cells by targeting Beclin-1 and inhibit the apoptosis of NP cells by inducing autophagy of NP cells. This was also similar to our conclusion. However, there is an inconsistency with this study. There was no further speculation and exploration of the mechanism between ATG7 and apoptosis. For example, the ATG7/Bax or ATG7/P62 pathway has been further explored, and this point needs to be further clarified in the future.

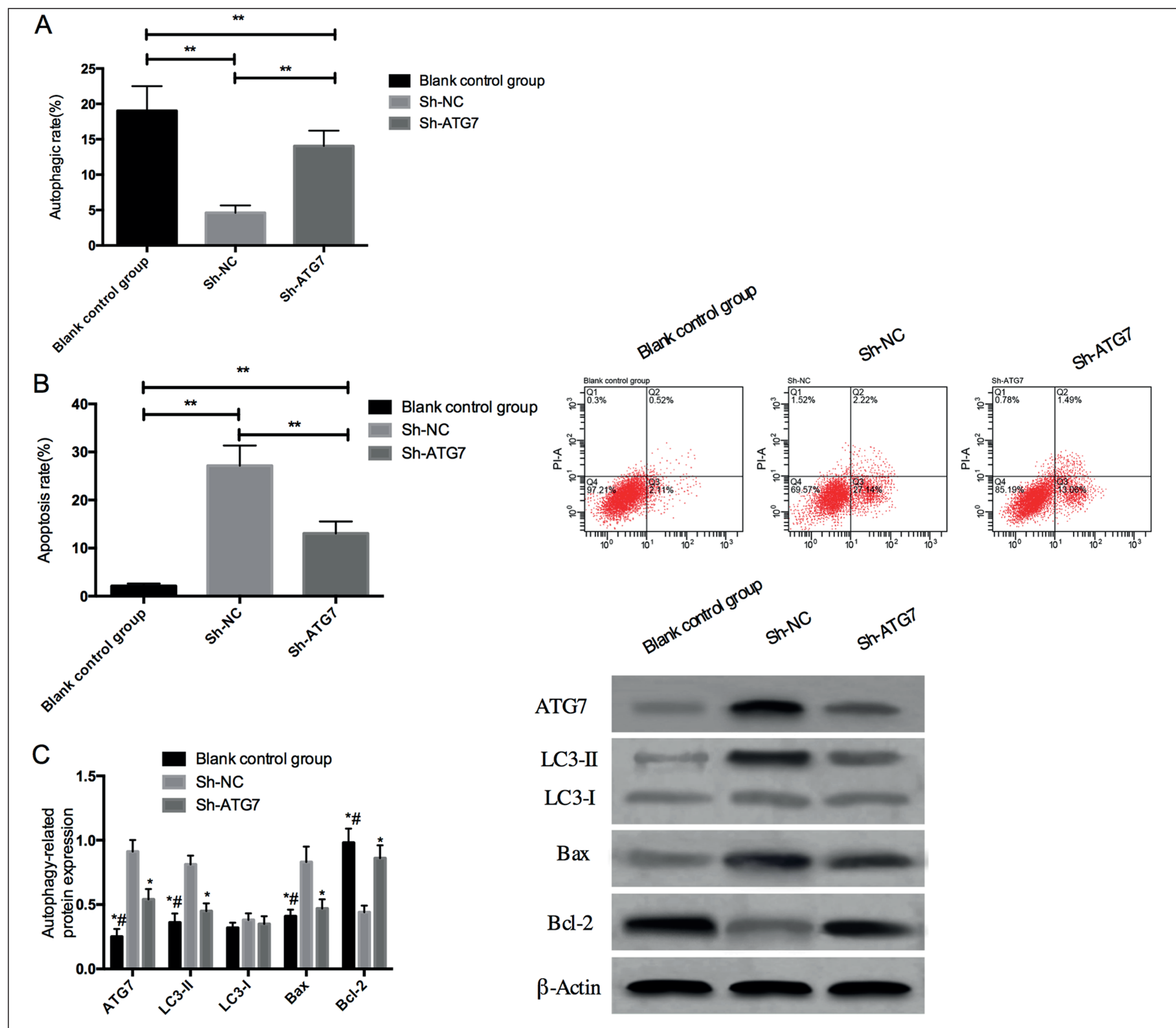

Figure 4. Effect of ATG7 on autophagy and apoptosis of degenerative NP cells. **A**, Compared with the Sh-NC group, the autophagy rate of Sh-ATG7 significantly increased. **B,** Compared with the Sh-NC group, the apoptosis rate of the Sh-ATG7 group significantly decreased. **C,** Compared with the Sh-NC group, the expression of ATG7 and the ratio of LC3-II/LC3-I and anti-apoptotic protein Bcl-2 in the NP cells of Sh-ATG7 group were significantly upregulated, and the pro-apoptotic protein Bax was significantly downregulated. **Indicates $p<0.05$.

Conclusions

MiR-202-5p can inhibit the autophagy of NP cells and promote the apoptosis of NP cells by regulating ATG7. When miR-202-5p was inhibited, it can effectively promote autophagy of NP cells and inhibit apoptosis of NP cells, which may be a new target direction for the treatment of intervertebral disc degenerative.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Acknowledgements

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