

LncRNA-p21 promotes chondrocyte apoptosis in osteoarthritis by acting as a sponge for miR-451

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Abstract. Osteoarthritis (OA) is the most common type of arthritis, and remains to be social and medical challenge. Thus, identifying novel molecular targets is important for the prevention and treatment of OA. Long noncoding RNAs (lncRNAs) have been reported to modulate various biological and pathological processes. The aim of the present study was to investigate the role of lncRNA-p21 in OA and its underlying mechanism, in order to better understand the development of OA and its treatment. Chondrocytes were isolated from cartilage samples obtained from OA and normal patients. Chondrocytes were transfected with microRNA (miRNA/miR)-451 mimics, miR-451 inhibitor, pcDNA3.1(+)-p21 or small interfering RNA-p21. Flow cytometry was performed to analyze cell apoptosis and reverse transcription-quantitative polymerase chain reaction was conducted to detect the expression of mRNAs and miRNAs. Cell Counting Kit-8 assay was performed to detect cell viability. The results revealed that the level of lncRNA-p21 was significantly upregulated in OA cartilage when compared with the normal cartilage. Silencing of lncRNA-p21 increased cell viability and inhibited the apoptosis rate of chondrocytes in OA, while lncRNA-p21 overexpression decreased cell viability and increased the apoptosis rate of chondrocytes in OA. Overexpression of lncRNA-p21 suppressed the expression of miR-451 while the silencing of lncRNA-p21 reversed this effect. MiR-451 inhibitor effectively inhibited the upregulatory effect of si-p21 on miR-451. The increased cell viability and decreased apoptosis rate induced by lncRNA-p21 silencing was abolished by the miR-451 inhibitor. MiR-451 mimic effectively increased the down-regulatory effect of pcDNA3.1-lncRNA-p21 on miR-451. The decreased cell viability and increased apoptosis rate induced

by the overexpression of lncRNA-p21 was abolished by the miR-451 mimic. Investigation into the underlying mechanism revealed that lncRNA-p21 interacted with miRNA-451. In addition, lncRNA-p21 negatively regulated the expression of miR-451. Furthermore, lncRNA-p21 promoted the apoptosis of chondrocytes in OA by acting as a sponge for miR-451. Thus, lncRNA-p21 was proposed as a promising target for the treatment of OA.

Introduction

Osteoarthritis (OA) is reported to afflict nearly 250 million people in the whole world and is the most common arthritis (1,2). Still, OA remains a critical challenge socially and medically due to the lack of optimal therapeutic methods (3). Till now, important advances have been made in the prevention and treatment of OA (4,5). The specific etiology of OA is complicated and unclear where multiple intertwining factors may take part in, for example, aging, trauma, obesity and heredity predisposition (6,7). The basic features of OA includes degradation of cartilage and synovitis which lead to pain, stiffness and abnormal joint structure (8). It remains undefined currently although the pathogenesis of OA has been paid much attention to. However, increasing evidence has identified the critical role of chondrocyte in OA initiation and progression (9). It has been reported that chondroapoptosis and chondrosenescence could be the two specific hallmarks of OA (10,11). A growing body of evidence reveals that chondrocyte apoptosis plays a key role in the destruction of cartilage (11). Therefore, identification of the underlying mechanisms of chondroapoptosis may provide a promising therapy for the management of OA.

Long noncoding RNAs (lncRNAs) refer to a kind of non-protein coding, longer than 200 nucleotides transcripts (12). In recent years, lncRNAs have been reported to modulate various biological and pathological processes, such as cell cycle, cell apoptosis, epigenetics and multiple cancers (13-15). Interestingly, lncRNAs also participate in the progression of OA (14). For example, the overexpression of lncRNA-CIR increases the expression of matrix-degrading enzymes and contributes to extracellular matrix degradation in OA (16). LncRNA plasmacytoma variant translocation 1 (PVT1) promotes the apoptosis of chondrocytes in OA by acting as a sponge for miR-488-3p (17). LncRNA-p21 has been

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reported to play roles in malignant and benign diseases. It is indicated that lncRNA-p21 could affect the growth of breast cancer cells by regulating the G1/S checkpoint and modulating energy metabolism of neoplasm cells (18,19). In osteosarcoma, lncRNA-p21 inhibits the proliferation of osteosarcoma cells by regulating miR-130b/PTEN/AKT signaling pathway (20).

Chondrocytes, the only kind of cell in articular cartilage, are important for the maintenance of the cartilage homeostasis. Apoptosis is a physiological process, and the purpose is to remove harmful, damaged or unwanted cells (21). Recent report has demonstrated that the apoptosis of chondrocytes is related to the development and progression of OA (22). Several molecules considered to be markers of chondrocyte apoptosis, such as bcl-2, bax, and so on (23). In addition, inflammation process of chondrocytes also affects cell survival and take parts in maintaining cell homeostasis (24).

Till now, the role of lncRNA-p21 in OA remains unclear. According to reported papers, we assume it play an important role in OA. Therefore, the current study is designed to investigate the role of lncRNA-p21 in OA and the underlying mechanism. The aim of the current study is to investigate the role of lncRNA-p21 in OA and its underlying mechanism which helps to better understand the development of OA and its treatment.

Materials and methods

Chondrocyte culture. A total of 20 OA articular cartilages were collected from patients during the surgery of total knee arthroplasty and 20 normal articular cartilages were obtained from non-OA patients with femoral neck fracture from Second Affiliated Hospital, Zhejiang University School of Medicine. The diagnosis of OA followed radiographic images and the American College of Rheumatology criteria. All experiments were completely approved by the Ethics Committee of Second Affiliated Hospital (Zhejiang University School of Medicine, Hangzhou, China). All patients signed informed consent forms. The cartilage samples were cut into small pieces and predigested in trypsin (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) for 10 min and the digested for 16 h with type II collagenase (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) in DMEM with 10% FBS. Then the suspension was strained with nylon cell strainer (100 μ m) and washed with Dulbecco's modified Eagle's medium (DMEM) (Invitrogen; Thermo Fisher Scientific, Inc.). The isolated chondrocytes were cultured in DMEM supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) at 37°C with 5% CO₂ as reported (25).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from cartilages (directly from cartilage samples) and chondrocytes (isolated chondrocytes) with TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Quantitation of RNAs was performed on Prism 7500 real-time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) using SYBR Premix Ex Taq (Takara Bio, Inc., Shiga, Japan). The results were analyzed using the 2^{- $\Delta\Delta$ C_q} method as reported (26). The primes used are listed below: lncRNA-p21: Forward, 5'-TGTTGCATT GTTGCATCATC-3'; reverse, 5'-TTTCTTCCAGTGGTGAGT

GG-3'. miR-451: forward, 5'-TAGCAAGAGAACCATTAC CA-3'; reverse, 5'-GAACATGTCTGCGTATCTC-3'.

Transfection. Chondrocytes were transfected with miR-451 mimics, miR-451 inhibitor, pcDNA3.1(+)-p21 or si-p21 (GenePharma, Shanghai, China) with Lipofectamine 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) when chondrocytes reached 70% confluence. Cells were harvested 48 h after transfection.

Cell viability assay. CCK-8 assay was performed to detect cell viability according to the manufacturer's protocol (27). Chondrocytes were seeded in 96-well plates after transfection. DMEM (100 μ l) and CCK-8 reagent (10 μ l, Dojindo, Kumamoto, Japan) were added to 96-well plates. After incubation at 37°C for one hour, the absorbance was detected with spectrophotometer at 450 nm and cell viability was calculated. The experiment was conducted in triplicate.

Flow cytometry analysis. The apoptosis rate of cells was measured by Annexin V-FITC Apoptosis Detection kit (BD Biosciences, Franklin Lakes, NJ, USA) as reported (28). Briefly speaking, cells were harvested 48 h after transfection and resuspended in 1X binding buffer after washed with cold PBS for twice. Then 5 μ l PI and 5 μ l FITC were added and incubated for 15 min at room temperature in the dark. The apoptotic cells were detected by flow cytometry. The FlowJo 7.6 software was used to analyze flow cytometry data. Each experiment was conducted in triplicate.

Western blot analysis. Western blot analysis was done as reported (27). Chondrocytes were harvested after transfection for 48 h and lysed with RIPA (Beyotime, Shanghai, China) supplemented. Protein concentration was quantified with the BCA kit (Beyotime) and 30 μ g of protein was loaded to SDS-PAGE gels for electrophoresis and transferred to PVDF membranes. The membranes were blocked in 5% fat-free milk for one hour at room temperature and incubated in primary antibodies at 4°C overnight. Then membranes were incubated with horse radish peroxidases (HRP)-conjugated secondary antibodies (1:4,000; Cell Signaling Technology, Inc., Danvers, MA, USA) for one hour at room temperature and detected with an ECL detection system (Thermo Fisher Scientific, Inc.). Anti-Bcl-2 antibody, anti-Bax antibody and anti-GAPDH antibody were purchased from Cell Signaling Technology, Inc. All primary antibodies were monoclonal and at a dilution of 1:1,000. The relative expression levels of proteins were normalized with GAPDH. All experiments were repeated for three times.

Statistical analysis. Data were expressed as mean \pm standard deviation. Statistical analysis was performed using GraphPad Prism 6.0 (GraphPad Software Inc., La Jolla, CA, USA). Student's t-test or analysis of variance (ANOVA) was used to conduct statistical comparisons after performing tests to verify the normal distribution of the data and confirming that the variances were homogeneous. Student's t-test was used to determine the significant differences between two groups. ANOVA followed by Bonferroni post hoc test were used to determine the significant differences among multiple groups.

$P < 0.05$ was considered to indicate a statistically significant difference.

Results

The expression of lncRNA-p21 is upregulated in OA chondrocytes. To determine the potential role of lncRNA-p21 in OA, we compared the expression level of lncRNA-p21 in cartilage samples of OA patients and normal people. Results showed that the expression level of lncRNA-p21 were significantly upregulated in OA compared with the normal (Fig. 1).

Downregulation of lncRNA-p21 inhibits apoptosis of chondrocytes in OA. Chondrocytes apoptosis plays a critical role in cartilage degradation in OA. In order to investigate the function of lncRNA-p21 in chondrocytes apoptosis, si-lncRNA-p21 was transfected into chondrocytes of OA. RT-qPCR was performed to confirm the silencing effect (Fig. 2A). Results showed silencing of lncRNA-p21 increased cell viability and inhibited the apoptosis rate of chondrocytes in OA (Fig. 2B and C) compared with the control group. At the same time, the downregulation of Bax and upregulation of Bcl-2 induced by the silencing of lncRNA-p21 was identical to the flow cytometry analysis (Fig. 2D). These results showed that lncRNA-p21 promoted apoptosis of chondrocytes in OA.

Overexpression of lncRNA-p21 promotes the apoptosis of chondrocytes in normal cartilage. To confirm the role of lncRNA-p21 in chondrocyte apoptosis further, pcDNA3.1 (+)-p21 or negative control plasmid was transfected into normal chondrocytes isolated from normal cartilage. RT-qPCR was performed to confirm the overexpression effect (Fig. 3A). We found that lncRNA-p21 overexpression decreased cell viability and increased the apoptosis rate of chondrocytes in OA (Fig. 3B and C). Identically, lncRNA-p21 overexpression upregulated Bax and downregulated Bcl-2 (Fig. 3D). These results further confirmed the pro-apoptotic effect of lncRNA-p21 in chondrocytes.

lncRNA-p21 interacts with miR-451 in OA. Although it was indicated above that lncRNA-p21 regulate the chondrocyte apoptosis in OA, the underlying mechanism remains unclear. A growing studies indicated that lncRNAs exert their biological or pathological functions by interacting with miRNAs. We reasoned that lncRNA-p21 regulates chondrocyte apoptosis by interacting with miRNA. MiR-451 was reported to inhibit the proliferation and promote apoptosis of osteosarcoma. Whether miR-451 plays a role in OA is unknown. To investigate whether lncRNA-p21 could exert its function by acting as a sponge for miR-451, we transfected chondrocytes of OA with pcDNA3.1(+)-p21 or si-lncRNA-p21 and detected the expression of miR-451. Results showed that the overexpression of lncRNA-p21 suppressed the expression of miR-451 while the silencing of lncRNA-p21 reversed this effect (Fig. 4A). Then miR-451 mimic or negative control was transfected into chondrocytes of OA, the expression of lncRNA-p21 was suppressed by miR-451 overexpression (Fig. 4B). These results indicated that lncRNA-p21 functioned by interacting with miR-451.

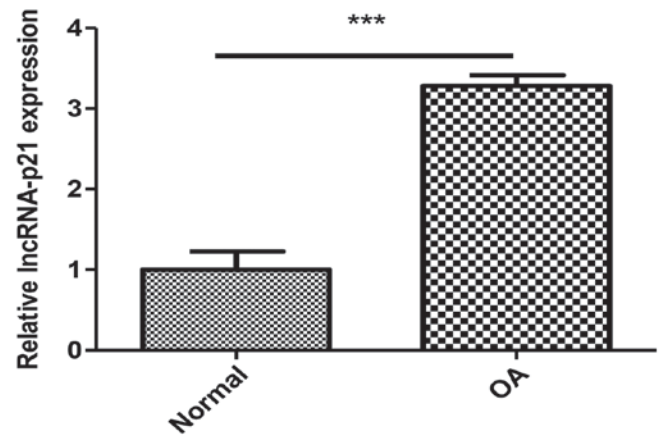


Figure 1. Expression of lncRNA-p21 in osteoarthritis and normal chondrocytes. Data are presented as the mean \pm standard deviation. The experiment was repeated for three times. *** $P < 0.001$, as indicated. OA, osteoarthritis; lncRNA, long noncoding RNA.

lncRNA-p21 promotes chondrocytes apoptosis through miR-451 in OA and normal. Given that lncRNA-p21 regulates the chondrocytes apoptosis in OA and interacts with miR-451, we reasoned that miR-451 might be involved in the regulation of chondrocytes apoptosis in OA. To investigate this speculation, miR-451 inhibitor was transfected into lncRNA-p21-silencing chondrocytes of OA. MiR-451 inhibitor effectively inhibited the upregulation effect of si-p21 on miR-451 (Fig. 5A). Interestingly, the increased cell viability and decreased apoptosis rate induced by lncRNA-p21 silence was abolished by the miR-451 inhibitor (Fig. 5B and C). Also, miR-451 inhibitor reversed the Bcl-2 upregulation and Bax downregulation effect exerted by lncRNA-p21 silence (Fig. 5D). To further investigate the role of miR-451 in normal chondrocytes, miR-451 mimic was transfected into lncRNA-p21 overexpression chondrocytes isolated from normal cartilage. MiR-451 mimic effectively increased the downregulation effect of pcDNA3.1-lncRNA-p21 on miR-451 (Fig. 5E). The decreased cell viability and increased apoptosis rate induced by overexpression of lncRNA-p21 was abolished by the miR-451 mimic (Fig. 5F and G). Meanwhile, miR-451 mimic reversed the Bcl-2 downregulation and Bax upregulation effect exerted by lncRNA-p21 overexpression (Fig. 5H). These results indicated that miR-451 was involved in lncRNA-p21-regulated chondrocytes apoptosis.

Discussion

OA is characterized by degradation of articular cartilage and often afflict the facets of various joints, such as knee, hip and elbow. With the progression of chronic inflammation, the degradation of articular and degeneration and deformation of joints occur. The chronic pain and regression of joints significantly reduce the quality of life of patients. In recent years, the biological roles including in regulating OA of miRNAs have been investigated extensively. However, lncRNAs, as novel and potentially valuable non-coding RNAs, are yet to be investigated (29). An increasing evidence supports that lncRNAs may regulate OA pathogenesis via competing endogenous RNA to act as sponges for miRNAs (30).

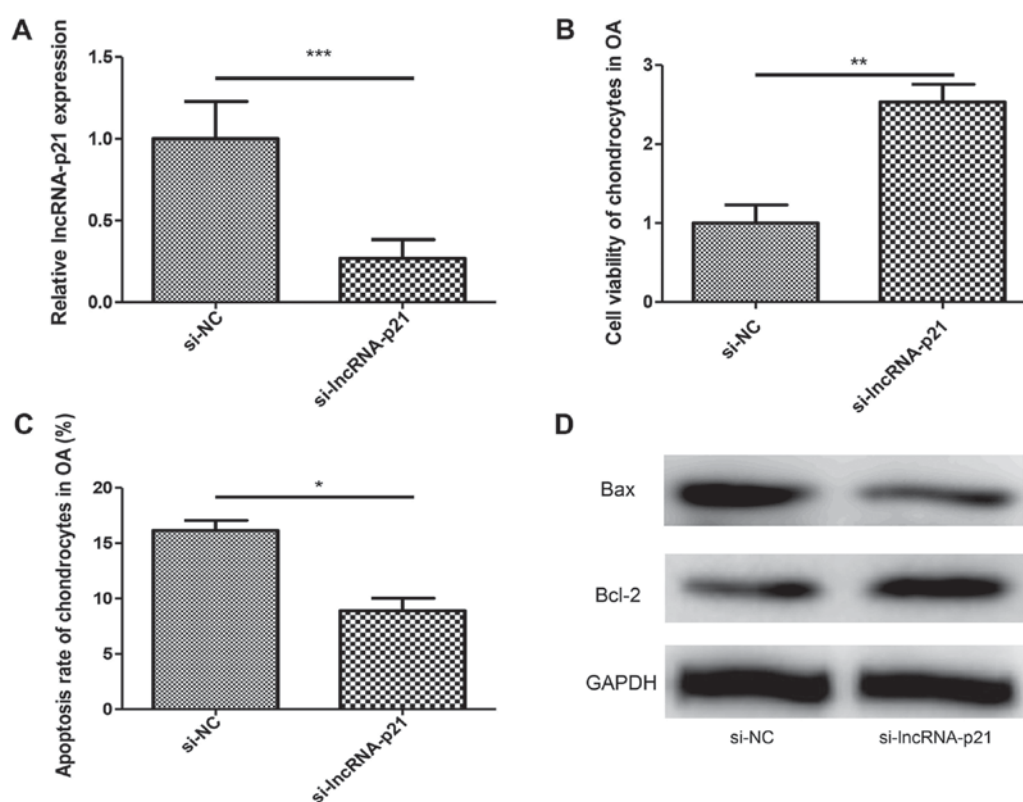


Figure 2. Silencing of lncRNA-p21 inhibits the apoptosis of chondrocytes in osteoarthritis. (A) Reverse transcription-quantitative polymerase chain reaction analysis was performed to confirm the silencing effect of lncRNA-p21. (B) The effect of lncRNA-p21 silencing on chondrocyte cell viability in osteoarthritis. (C) The effect of lncRNA-p21 silencing on chondrocyte apoptosis in osteoarthritis. (D) The expression of Bax and Bcl-2 was detected by western blotting. GAPDH and U6 were considered as internal controls for mRNA and microRNA. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$, as indicated. OA, osteoarthritis; lncRNA, long noncoding RNA; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein; NC, negative control; si-, small interfering RNA.

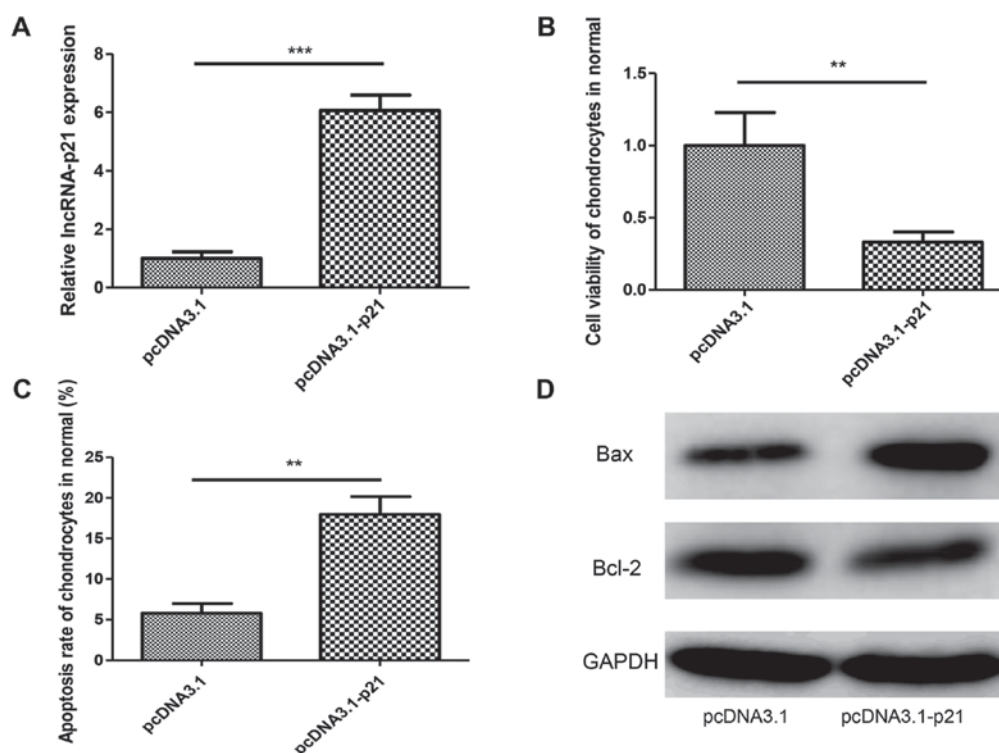


Figure 3. Overexpression of lncRNA-p21 promotes the apoptosis of normal chondrocytes. (A) Reverse transcription-quantitative polymerase chain reaction analysis was performed to confirm the overexpression effect of lncRNA-p21. (B) The effect of lncRNA-p21 overexpression on chondrocyte cell viability in osteoarthritis. (C) The effect of lncRNA-p21 overexpression on chondrocyte apoptosis in osteoarthritis. (D) The expression of Bax and Bcl-2 was detected by western blotting. GAPDH and U6 were considered as internal controls for mRNA and microRNA. ** $P < 0.01$ and *** $P < 0.001$, as indicated. lncRNA, long noncoding RNA; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein; NC, negative control; si-, small interfering RNA.

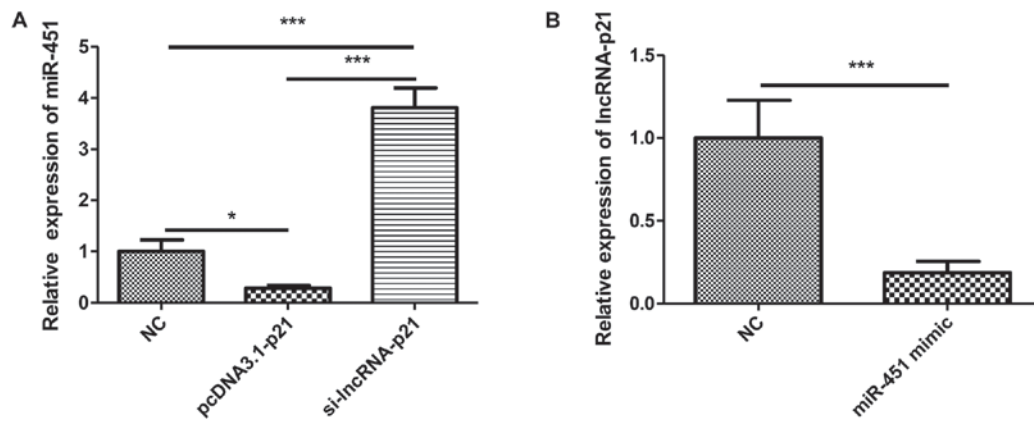


Figure 4. LncRNA-p21 interacts with miR-451 in osteoarthritis. (A) The effect of silencing or overexpressing LncRNA-p21 on the expression of miR-451. (B) The effect of miR-451 overexpression on LncRNA-p21 expression. * $P < 0.05$ and *** $P < 0.001$, as indicated. LncRNA, long noncoding RNA; miR, microRNA; NC, negative control; si-, small interfering RNA.

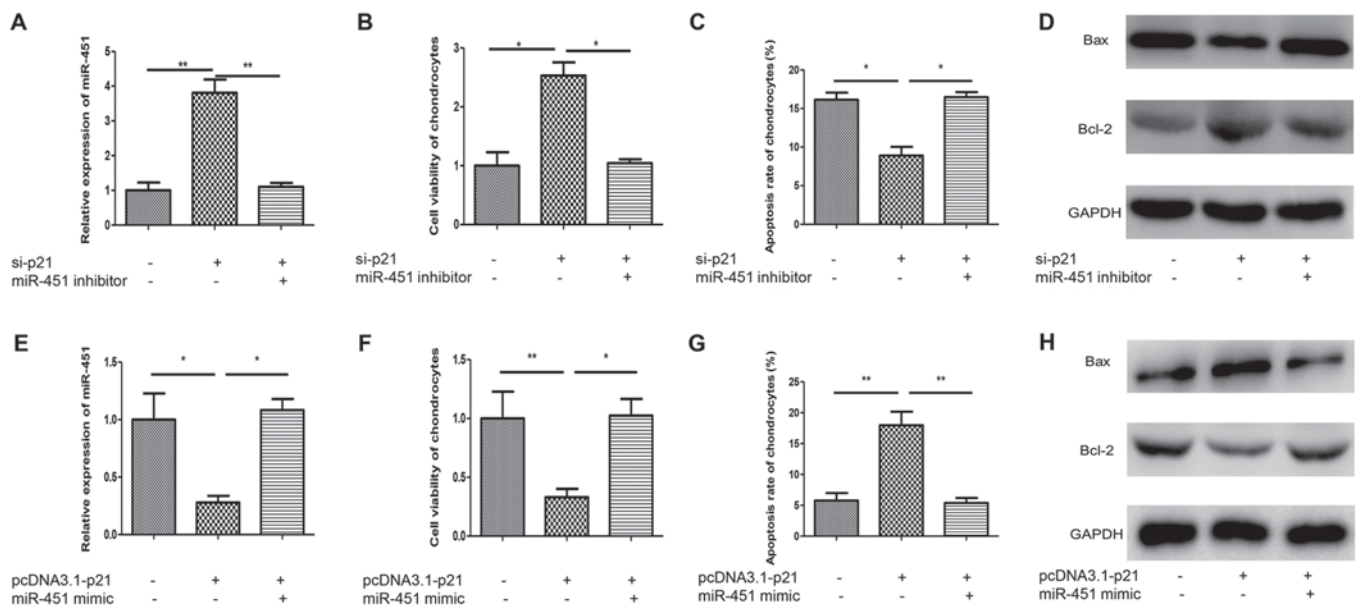


Figure 5. LncRNA-p21 promotes chondrocytes apoptosis in osteoarthritis via miR-451. (A) The effect of miR-451 inhibitor on the expression of miR-451. (B) The effect of miR-451 inhibitor on the increased cell viability induced by LncRNA-p21 silencing. (C) The effect of miR-451 inhibitor on the decrease of chondrocyte apoptosis induced by LncRNA-p21 silencing. (D) The protein expression levels of Bax and Bcl-2. (E) The effect of miR-451 mimic on the expression of miR-451. (F) The effect of miR-451 mimic on the decreased cell viability induced by LncRNA-p21 overexpression. (G) The effect of miR-451 mimic on the increase of chondrocyte apoptosis induced by LncRNA-p21 overexpression. (H) The protein expression levels of Bax and Bcl-2. GAPDH and U6 were considered as internal controls. * $P < 0.05$ and ** $P < 0.01$, as indicated. LncRNA, long noncoding RNA; miR, microRNA; NC, negative control; si-, small interfering RNA; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein; miR, microRNA.

Kang *et al* (31) reported that PCGM1, acting as a sponge for miR-770, promoted the proliferation of synoviocytes in OA. For another example, UFC1 stimulated chondrocytes proliferation in OA by interacting with miR-34a (32). In the current study, by loss of function and gain of function experiment, we found that the overexpression of LncRNA-p21 suppressed the expression of miR-451 while the silence of LncRNA-p21 upregulated the expression of miR-451. Further study indicated that miR-451 regulated the apoptosis of chondrocytes in OA and normal. Our results were in accordance with the previous published studies that lncRNAs possess the endogenous RNAs competing activities.

LncRNA-p21 have been reported to possess various biological functions in malignant tumors and non-tumor disorders.

In various tumors, LncRNA-p21 exhibits anti-cancer abilities and inhibits tumor cell viability. For example, LncRNA-p21 suppressed the proliferation of hepatocellular carcinoma cells by activating the endoplasmic reticulum stress, thereby alleviate sorafenib resistance (33). In prostate cancer, the overexpression of LncRNA-p21 promoted the apoptosis of cancer cells mediated by p53 (34). What's more, LncRNA-p21 downregulated the expression of β -catenin in colon cancer cells and glioma cells by which inhibiting the cell viability and enhancing radiotherapy effects (35,36). Han and Liu (20) reported that the overexpression of LncRNA-p21 effectively controlled proliferation of osteosarcoma cells by the activation of PTEN/pAKT signaling pathway. However, the role of LncRNA-p21 in OA remains unclear. Our study revealed that

lncRNA-p21 promoted the apoptosis of chondrocytes in OA via acting as a sponge for miR-451.

In conclusion, results from the study indicated that lncRNA-p21 promoted the apoptosis of chondrocytes in OA via acting as a sponge for miR-451. It opens up new horizons for the development and progression of OA. Meanwhile, it adds new interpretation of biological functions of lncRNAs, especially lncRNA-p21. We propose that lncRNA-p21 may be a promising molecular target for the therapy of OA.

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Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

LT and ZL were responsible for the conception, design and acquisition of data. JD and GZ were conducted the analysis and interpretation of data. LT was involved in drafting the manuscript and critically revising it for important intellectual content. ZL gave final approval of the version to be published.

Ethics approval and consent to participate

All experiments were approved by the Ethics Committee of The Second Affiliated Hospital, Zhejiang University School of Medicine (Zhejiang, China). All patients signed informed consent forms.

Patient consent for publication

All patients signed informed consent forms for the publication of any associated images/data in the manuscript.

Competing interests

The authors declare that they have no competing interests.

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