miR-183 as a prognostic marker in intervertebral disc degeneration and its role in the proliferation and apoptosis of nucleus pulposus

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Abstract

Background Accumulating evidence indicated that microRNAs are involved in the pathogenesis of intervertebral disc degeneration (IDD). The roles of miR-183 in IDD have not been elucidated. **Methods** A total number of 60 patients with IDD were included in this study, and degenerative nucleus pulposus (NP) tissues of the patients were collected. Total RNA was extracted from NP of patients, and the expression levels of miR-183 were examined using real-time quantitative PCR methods. Next, human NP cells were cultured, and transfected with miR-183 mimics and inhibitors, and the roles of miR-183 on the proliferation and apoptosis of cells were determined using MTT and flow cytometry methods; moreover, the expression of MMP9, Caspase-3, BAX, and BCL-2 were examined using RT-qPCR and western blot methods. **Results** Patients with lower miR-183 level has better prognosis compared with patients with higher miR-183 level; moreover, transfection of miR-183 inhibitors induced significant increase in the proliferation and marked decrease in the apoptosis of the degenerated human NP cells; moreover, transfection of miR-183 inhibitors induced significant decrease in the expression of MMP9, Caspase-3, BAX, and marked increase in the expression of BCL-2 on both mRNA and protein level. **Conclusion** miR-183 was up-regulated in IDD, and was negatively associated with the prognosis of patients; moreover, miR-183 might regulate the proliferation and apoptosis of NP cells.

Keywords: miR-183, prognosis, IDD, proliferation, apoptosis

Introduction

Intervertebral disc degeneration (IDD) is one of the major causes of the low back pain, which not only cause problems to the patient's life, but also bring great burden to the public health system. About 40% of people under the age of 30 have disc degeneration, and this number can reach to 90% among people over 50 (1-3). At current stage, the pathogenesis of intervertebral disc degeneration is still unclear. Different theories have been proposed to explain the mechanism of IDD, including the increased expression of proinflammatory cytokines, aberrant apoptosis of NP cells, etc. The in-depth study of the related mechanism of IDD can help scientists and physicians to establish novel effective therapies for the management of IDD.

miRNAs are a small non-coding RNA with the length of 20–22 nucleotides. In recent years, it have been proved that microRNAs (miRNAs) may participate in may biological events. miRNAs can bind to the 3'-UTR of their target mRNA and consequentially suppress the expression of the related genes. Based on data from previous studies, miRNAs were also involved in the pathogenesis of different diseases, for example cancer, renal failure, cardiovascular diseases, neuron diseases, autoimmune diseases and so on(4,5) .

The roles of miRNAs in bone diseases have also been discussed in previous studies (6-10). However, studies on the function of miRNAs in intervertebral disc diseases, including IDD, were relatively limited. It was reported by Lan et al. that miR-183 was abnormally up-regulated in the intervertebral disc of IDD patients (11), however, the underlying mechanism remains to be further investigate. In this study, the roles of miR-183 in the pathogenesis of IDD were explored. We investigated the relationship between the levels of miR-183 and the prognosis of the patients, and also explore the effect of miR-183 on the viability and apoptosis of human nucleus pulposus (NP) cells. Our data may provide a novel diagnostic marker and therapeutic for the treatment and prediction of the clinical outcome of patients with IDD.

Material and Methods

Patients

Between May 2014 and March 2016, a total number of 60 patients with degenerative disc disease (IDD) who undergo surgery in our hospital were enrolled in the study. Routine MRI scans of the lumbar spine have been performed on these patients, and the degree of disc degeneration was graded from T2-weighted images using a modified Pfirrmann classification. The degenerative lumbar nucleus pulposus (NP) of patients were collected from surgery. Six months after surgery, the Japanese Orthopaedic Association(JOA) Scores were recorded as the index of the prognosis of the patients. The study protocol was approved by the ethics committee of our hospital, and informed written consents were obtained from all participants.

Cell culture

Human NP cells were purchased from Procell Life Science&Technology Co.,Ltd. (Wuhan, China). Cells were maintained in DMEM medium with 10% fetal bovine serum (FBS) at 37 ℃ with 5% CO_2 supplied with 100 mg/mL streptomycin, 100 U/mL penicillin, and 1% NP cell growth supplement. Cells were passaged when reached confluence.

Cell transfection

NP cells were transfected with miR-183 inhibitor, miR-183 inhibitor negative control, miR-183 mimics or miR-183 mimic negative control oligo nucleotide (sythesized by GenePharma, Shanghai, China) with Lipofectamine® 3000 (Invitrogen/Thermo scientific, USA) following the manufacturer's instructions. The cells were maintained in culture medium for 48h and collected for further analysis.

Cell proliferation assay

MTT assay has been performed to examine the viability of cells with different treatment. Briefly, cells were cultured on 96 well plates, and 20ul MTT (purchased from Beyotime, Shanghai, China) was added to each well, then cells were incubated for 4h. The OD value at 490 was measured to determine the viability of the cells in each well.

Cell apoptosis assay

The apoptosis of the cells were determined by Annexin V/propidium iodide apoptosis detection kit (BD Biosciences, USA). Briefly, cells were doubled stained with Annexin V/propidium iodide 48h after transfection, and analyzed with BD FACSVerse flow cytometer (BD Biosciences, NJ, USA). The apoptosis rate of cells in each group was calculated.

Real-time quantitative RT-PCR

Total RNA was isolated from cells and tissues samples using TRIzol (Invitrogen, Thermo Fisher Scientific, Waltham, Massachusetts, USA), and the expression of miR-183 was examined by the Hairpin-it^{TM} miRNAs qPCR Quantitation Kit (GenePharma, Shanghai, China) according manufacturer's protocol, and U6 (RNU6B; GenePharma) was used as the internal control. Real-time quantitative PCR(qRT-PCR) was performed on an ABI 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) to determine the expressions of MMP9, Caspase-3, BAX, and BCL-2 with the following thermocycling profiles were: 95℃ for 30 sec, followed by 40 cycles of 95 ℃ for 5 sec and 60 ℃ for 30 sec. GAPDH was used as the internal control for evaluating the expression of the genes.

Western blot

Cells were lysed and the protein concentration was measured using BCA Protein Assay Kit (Beyotime, Shanghai, China). Then protein was separated by electrophoresis, and transferred to PVDF membranes; the membranes were blocked with 5% non-fat milk and incubated with primary antibodies (anti-MMP9, anti-Caspase-3, anti-BCL-2 and anti-BAX, and anti-β-actin, all purchased from Abcam, Cambridge, MA, USA) at 4℃ overnight; in day 2, the membranes were incubated with the secondary antibodies (Santa Cruz, CA, USA), and then incubated with enhanced chemiluminescent reagent (Beyotime, Shanghai, China). Finally, the signals were visulized using ChemiDoc™XRS+ imaging system (Bio-Rad, Hercules, CA, USA).

Statistical analysis

Statistical analysis was performed using SPSS 17.0 software. Data were presented as the means ± standard deviation, and the two independent sample T-test was performed to for comparison between two groups. Analysis of variance (ANOVA) was performed for comparison between multiple groups. P<0.05 was considered as significant difference.

Results

Lower expression of miR-183 in patients may indicate better prognosis of patients with IDD

First of all, we examined the expression of miR-183 in tissue samples of 60 patients with IDD, and divided patients into the miR-183 high expression group (n=33) and miR-183 low expression group (n=27), and the JOA score has been applied to evaluate the prognosis of patients. As shown in Figure 1, the JOA score of patients with lower miR-183 expression group was significantly higher than the high miR-183 expression group (p<0.01), suggesting that lower expression of miR-183 in patients may indicate better prognosis of patients with IDD.

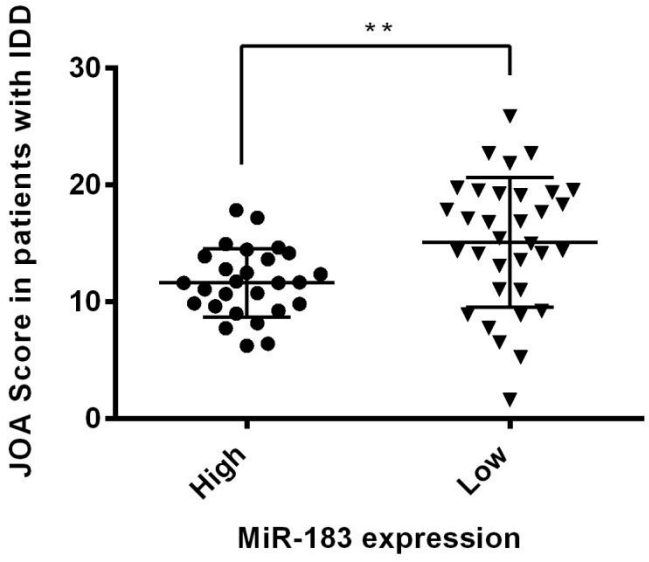

Figure 1. Lower expression of miR-183 in patients may indicate better prognosis of patients with IDD. **, $p < 0.01$.

miR-183 was up-regulated in degenerated NP cells compared with the normal NP cells in patients

Next, to further explore the roles of miR-183 in IDD, we isolated the degenerated NP cells, and compared the expression of miR-183 in normal and degenerated NP Cells using RT-qPCR methods. As expected, the expression of miR-183 was significantly up-regulated in degenerated NP cells compared with normal NP cells (Figure 2).

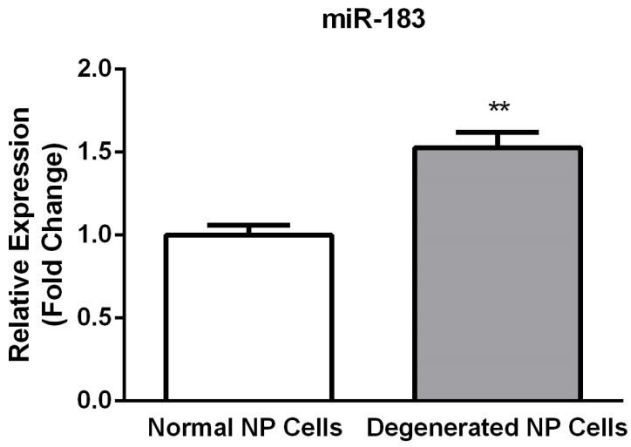

Figure 2 miR-183 was up-regulated in degenerated NP cells compared with the normal NP cells in patients. **, $p < 0.01$, vs. the Normal group

miR-183 can regulate the proliferation and apoptosis of degenerated NP cells

Furthermore, we transfected degenerated NP cells with miR-183 inhibitors, and explored the roles of miR-183 in the proliferation and apoptosis using MTT and flow cytometry methods. As shown in Figure 3, transient knockdown of miR-183 induced significant increase in the proliferation (Figure 3) and marked decrease in the apoptosis (Figure 4) of degenerated NP cells($p<0.01$).

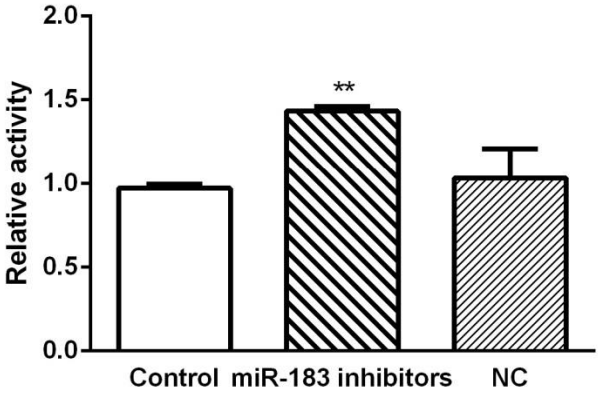

Figure 3 miR-183 can regulate the proliferation of degenerated NP cells.

**, $p < 0.01$, vs. the Contol group

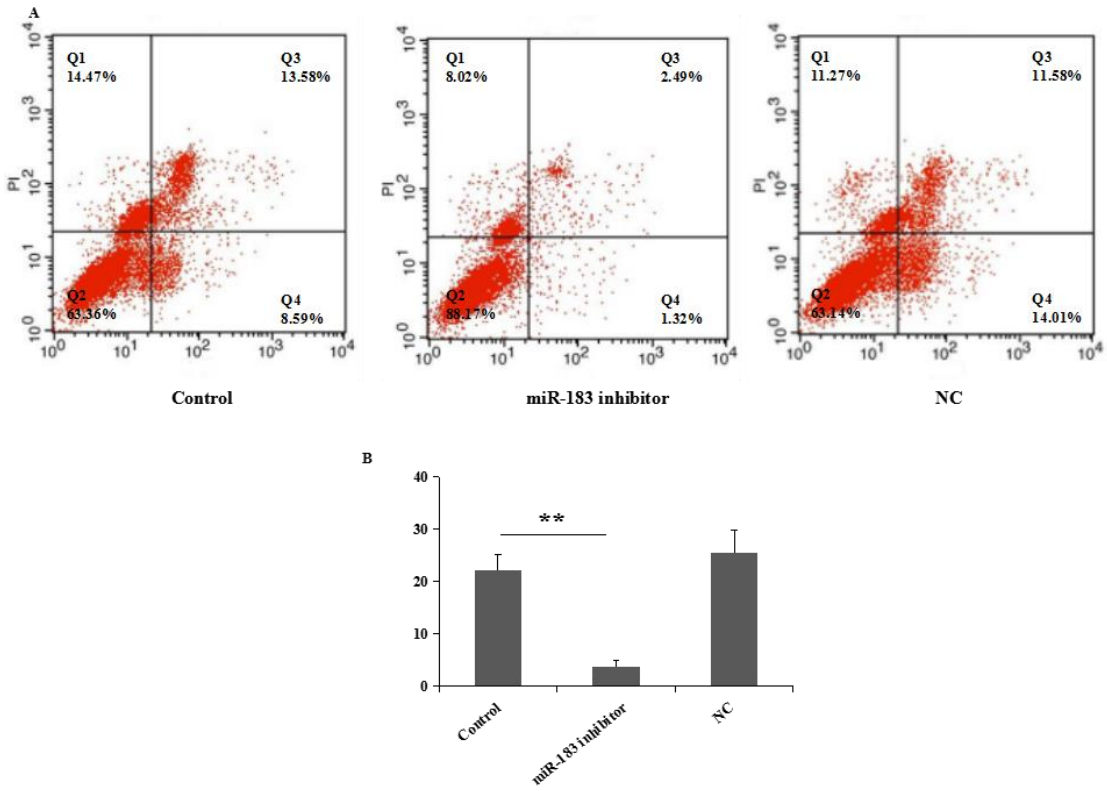

Figure 4 miR-183 can regulate the apoptosis of degenerated NP cells.

**, $p < 0.01$, vs. the Contol group

miR-183 can affect the expression of Bcl-2, Bax, Caspase-3 and MMP-9 in degenerated NP cells

Next, we performed RT-qPCR and western blot assays to examine the effect of miR-183 on the expression of some pro-apoptotic and anti-apoptotic factors. As

shown in Figure 5 and Figure 6, miR-183 inhibitors transfected have shown significant increase in the expression of Bcl-2 and decrease in the expression of Bax, Caspase-3 and MMP-9 on both mRNA and protein level.

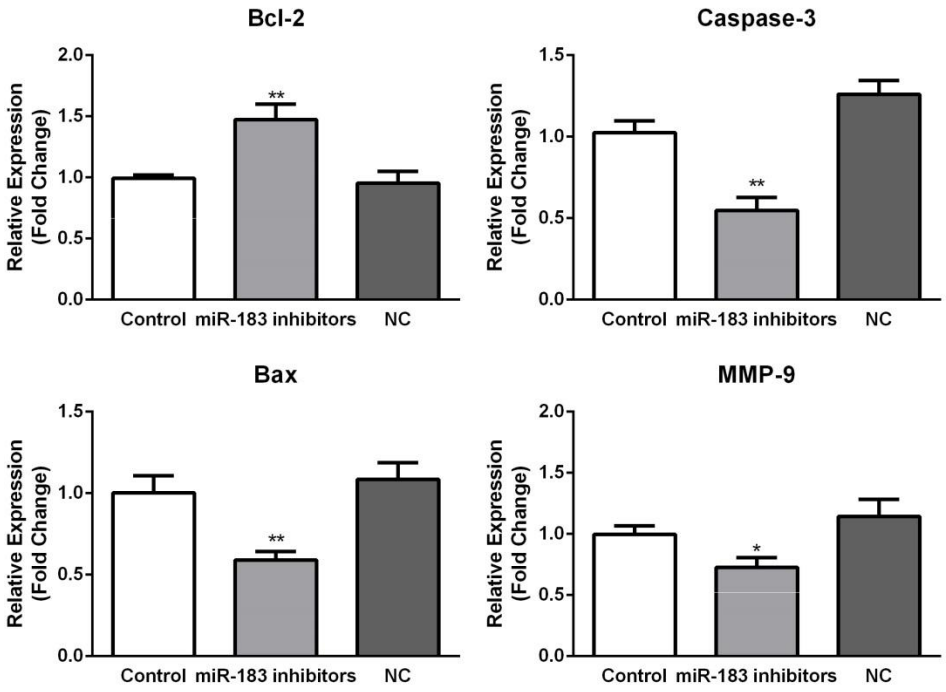

Figure 5. MiR-183 can affect the mRNA expression of Bcl-2, Bax, Caspase-3 and MMP-9 in degenerated NP cells. *, p <0.05; **, p < 0.01, vs. the Contol group

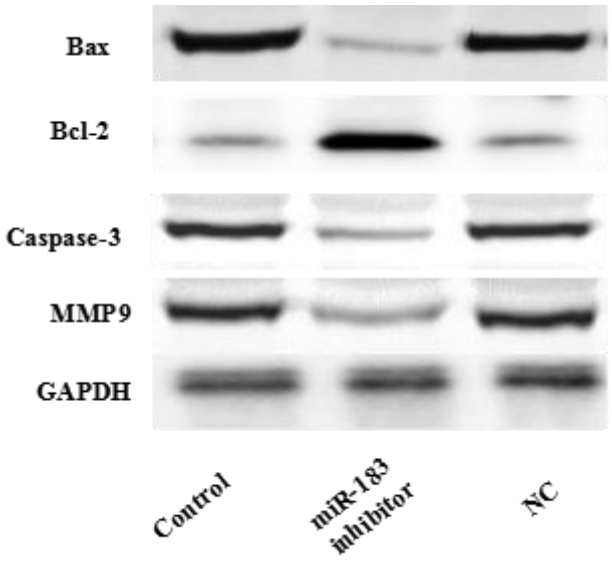

Figure 6. MiR-183 can affect the protein expression of Bcl-2, Bax, Caspase-3 and MMP-9 in degenerated NP cells.

Discussion

The roles of miRNAs in IDD have been discussed previously. It has been reported that miR-146a can alleviate the inflammatory condition of IDD through regulating the TRAF6/NF-κB signaling pathway in intervertebral disc cells (9); moreover, miR-15a was involved in the degeneration of intervertebral disc via directly targeting MAP3K9 (12); miR-155 has been identified as one of the down-regulated miRNAs in IDD, and miR-155 can regulate the proliferation and apoptosis of NP cells through targeting FADD (13); miR-138-5p was markedly up-regulated in degenerative NP tissues, and overexpression of miR-138-5p can promote TNF-α-induced apoptosis in IDD through PTEN/PI3K/Akt signaling pathway (14); furthermore, down-regulation of miR-27b has been proved to participate in the mechanism of loss of type II collagen in IDD through targeting matrix metalloproteinase 13 (MMP13) (15). In Lan's work, they performed sequencing analysis and discovered that miR-183 was up-regulated in tissue samples of patients with IDD (11), however, the specific mechanism is still unclear. In the present study, we examined the expression of miR-183 in tissue samples of 60 patients with IDD, and discovered for the first time that the JOA score of patients with lower miR-183 expression group was significantly higher than the high miR-183 expression group ($p<0.01$), these results indicated that lower expression of miR-183 in patients may indicate better prognosis; moreover, the expression of miR-183 was significantly up-regulated in degenerated NP cells compared with normal NP ($p<0.01$), which was consistent with Lan's finding; furthermore, transient knockdown of miR-183 in degenerated NP cells induced significant increase in the proliferation and decrease in the apoptosis of NP cells ($p<0.05$). Taken together, these results indicated that miR-183 was up-regulated in IDD, and it can regulate the proliferation and apoptosis of NP cells.

Some previous studies indicated that the aberrant degeneration and apoptosis of NP cells were often accompanied with the changes of the expression of apoptosis

related proteins e.g. the anti-apoptotic protein Bcl-2 and the pro-apoptotic protein Bax, Caspase-3 and MMP-9 (16,17). To further explore the roles of miR-183 on the expression of these apoptosis related proteins, transfected degenerated NP cells with miR-183 inhibitors, and the expressions of Bcl-2, Bax, Caspase-3 and MMP-9 were examined using RT-qPCR and western blot methods. Our results indicate that transfection of miR-183 inhibitors induced significant increase in the expression of Bcl-2 and decrease in the expression of Bax, Caspase-3 and MMP-9 on both mRNA and protein level, suggesting that miR-183 can regulate the regulate the proliferation and apoptosis of NP cells through affecting the expression of Bax, Bcl-2 and caspase-3 pathway.

Our study has some limitations. First, we only included tissue samples from patients but not healthy controls. This is mainly due to the ethical issues, it is impossible to obtain the degenerative disc from healthy people; second, we did not perform animal study, and the roles of miR-183 in IDD should be further investigate on animal models in future.

In conclusion, we proved that lower expression of miR-183 may indicate better prognosis of patient with IDD, and miR-183 can regulate the proliferation and apoptosis of NP cells. Our results indicated that miR-183 has the potential to become the prognostic marker and therapeutic target for the treatment of IDD.

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