Downregulation of microRNA-125a is involved in intervertebral disc degeneration by targeting pro-apoptotic Bcl-2 antagonist killer 1

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ARTICLE INFO	ABSTRACT
	Objective(s): To investigate the role of the microRNA-125a (miR-125a) and BAK1 in intervertebral disc degeneration (IDD). *Materials and Methods:* Degenerative lumbar nucleus pulposus (NP) tissues were obtained from 193 patients who underwent resection, and normal controls consisted of normal NP tissues from 32 patients with traumatic lumbar fracture in our hospital. All patients were graded according to the Pfirrmann criteria. QRT-PCR was used to detect the expression of miR-125a and BAK1, and apoptosis of NP tissues detected by TUNEL staining. After isolation of non- degenerative and degenerative nucleus pulposus cells (NPCs), the targeting relationship between miR-125a and BAK1 was verified by dual luciferase reporter gene assay. Flow cytometry was determined the NPCs apoptosis, and Western blot to measure the expressions of BAK1, Caspase-3, Bax and Bcl-2. *Results:* MiR-125a was reduced while BAK1 was elevated in IDD patients with the increase of Pfirrmann grade. Besides, miRNA-125a was negatively correlated to the NPCs apoptosis, while BAK1 mRNA was positively correlated to cell apoptosis. Additionally, BAK1 is the target gene of miRNA-125a. When transfection of miR-125a mimics *in vitro*, the apoptosis of NPCs were inhibited, with the down-regulation of BAK1, Caspase-3, and Bax, and the upregulation of Bcl-2 in NPCs. In addition, siBAK1 can reverse the pro-apoptosis function of miR-125a inhibitors in NPCs. *Conclusion:* miRNA-125a may regulate the apoptosis status of the NPCs by inhibiting the expression of its target gene BAK1, which provided a potential strategy for further development of IDD therapies.

Introduction

Intervertebral disc degeneration (IDD), a predominately contributor to neck or low back pain, as well as disc herniation, is credited as a normal process of disc aging, but occurs accelerated at an unexpected rate, ultimately leading to a variety of common diseases, such as cervical spondylosis, lumbar disc herniation (LDH), discogenic low back pain, degenerative spinal stenosis, and cervical and lumbar instability (1, 2). Nowadays, the incidence rate of IDD has went up year by year with the change of life and working styles and the trend of aging populations (3, 4), seriously affecting the human health and imposing a considerable economic burden on the society (5). Although IDD could be precipitated by a wide range of physiological and pathological factors, such as genetics (5), mechanical load (6), the reduction of functional cells caused by excessive apoptosis (7), degradation of extracellular matrix (8), and so on,

the etiology and mechanism of IDD remain not completely understood and need further study.

MicroRNAs (miRNAs), are non-coding endogenous small RNAs with the molecular length of 19 ~ 24 bp, implicated in the regulation of the expression of complementary mRNA targets (9). MiRNAs can not only involve in the regulation of cell proliferation, apoptosis, death and differentiation (10), but also play roles in nerve degeneration and tissue regeneration (11, 12). Many previous studies reported that various miRNAs were very active in IDD. For example, Liu *et al.* found the upregulated miR-27a may target PI3K to initiate the apoptosis of the nucleus pulposus cells (NPCs) *in vitro* (13). Besides, the overexpression of miR-21 could inhibit PTEN (phosphatase and tensin homolog) gene expression by targeting the 3'UTR, thus promoting the NPCs proliferasztion via the activation of AKT signaling, as suggested by Liu *et al* (14). MiR-125a, located at 19q13, is a kind of

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miRNA belonging to the miR-125 family (15). Accumulating evidence reported that miR-125a can exert important functions in a number of different types of diseases, such as cancers (16) cardiovascular diseases (17), immune response and autoimmune diseases (18), and greatly affect the proliferation and apoptosis of cells by regulating apoptosis related genes (17, 19). A previous study revealed that miR-125b, also a member of the miR-125 family, was obviously elevated in temozolomide (TMZ)-resistant cells, and repression of miR-125b can modulate the expression of its target gene BAK1, whereby enhancing TMZ-induced cytotoxicity and apoptosis while reducing the resistance to TMZ in glioblastoma stem cells (20). Besides, the downregulation of miR-125b can promote the apoptosis of chronic myeloid leukemia cells, and inhibit cell proliferation via targeting BAK1 (21). Meanwhile, BAK1, the Bcl-2 homologous antagonist/killer, is widely accepted as a critical pro-apoptotic regulator participated in various cellular activities, which may contribute to autoimmune diseases when overexpressed (PMID: 20978950). After retrieval on target gene prediction websites, we also found that BAK1 might be one of the potential target genes of miR-125a, but there are few researches about their impact on IDD.

Therefore, this study aims to explore the impact of the expression levels of miR-125a and its target gene BAK1 on IDD, and we also conducted an *in vitro* experiment to verify the relationship between miR-125a and the apoptosis of NPCs, to provide a new therapeutic target for the treatment of IDD.

Materials and Methods

Ethics statement

The study was approved by the Ethics Committee of Affiliated Shanxi Provincial People's Hospital, and all patients were well informed of the obtaining of specimens and signed an informed consent prior to study.

Subjects of study

From December 2014 to December 2016, we collected degenerative lumbar nucleus pulposus (NP) speci - mens from 193 patients who underwent surgical discectomy in the Department of Orthopedics in Affiliated Shanxi Provincial People's Hospital, including 125 males and 68 females. All patients received a routine MRI examination of the lumbar spine, and the degree of disc degeneration was assessed with the criteria of Pfirrmann grading (22): 71 cases were in grade Ⅲ, 64 cases in grade Ⅳ, and 58 cases in grade Ⅴ. During the same period, 32 patients with traumatic lumbar fracture who underwent surgical treatment in our hospital without imaging features of IDD were selected as the normal controls, including 22 males and 10 females. All specimens in this group were newly obtained during the operation and in Pfirrmann grade Ⅱ.

Expression levels of miRNA-125a and BAK1 detected by quantitative reverse transcription-PCR (qRT-PCR)

Lumbar intervertebral disc tissues were obtained for total RNA extraction by Trizol (Invitrogen Inc., Carlsbad, CA, USA). Reverse Transcription Kit (Hangzhou Bioer Technology Co, Ltd.) was used for the reverse transcription of RNA to cDNA and the 7500 quantitative PCR instrument (ABI Company, Oyster Bay, NY) was used for qRT-PCR. PCR reaction procedures in this study were as follows: pre-denaturation for 5 min at 94 ℃, then 40 cycles of denaturation for 30 sec at 94 ℃, annealing for 30 sec at 58 ℃, and extending for 20 sec at 72 ℃. The sequences of PCR primers were designed and synthesized by Sangon Biotech (Shanghai) Co. Ltd (Table 1). The relative expression levels of target genes were calculated by using $2^{-\triangle\triangle Ct}$.

Cell apoptosis of NP tissues detected by terminal deoxynucleotidyl transferase-mediated dUTP- biotin nick end labelling (TUNEL) staining

Tissues were made into paraffin sections, followed by routine dewaxing, hydration in gradient ethanol, proteinase K digestion, and water bath for 30 min at 37 ℃. Next, tissue sections were added with 0.3% H_2O_2 in methanol and placed at room temperature for 10 min, followed by washing with tris-buffered (Tris) solution for 2×3 min, the adding of biotinylated anti-digoxin antibody (MK2362, Wuhan Yide Biological Technology Co Ltd) for 60 min in water bath at, 37 ℃. After washing with TRIS-buffered solution for 3×3 min, sections were treated with SABC (BOSTER Biological Technology Co, Ltd) for 30 min in water bath at 37 ℃ and counter stained for 15-30 s with hematoxylin (Sangon Biotech (Shanghai) Co, Ltd), and finally mounting with neutral balsam. The nucleus of TUNEL-positive (apoptotic) cells appeared a brown or brownish-yellow colour under an optical micros-cope. Five visual fields were randomly selected to observe apoptotic cells under a light microscope at a magnification of 200. The apoptotic index (AI) was calculated as number of apoptotic cells /total number of cells × 100 (23).

Table 1. Primer sequences for quantitative reverse transcription-PCR

		Primer sequences
miRNA-125a	Forward	5'- GCCTCCCTGAGACCCTTTA-3'
	Reverse	5'-GTGTCGTGGAGTCGGCA-3'
U6	Forward	5'- CTCGCTTCGGCAGCACA-3'
	Reverse	5'-AACGCTTCACGAATTTGCGT-3'
BAK1	Forward	5'-AGGAACAGGAGGCTGAAGG-3'
	Reverse	5'-TAGCGTCGGTTGATGTCG-3'
β-actin	Forward	5'-CTCCATCCTGGCCTCGCTGT-3'
	Reverse	5'-GCTGTCACCTTCACCGTTCC-3'

Isolation and culture of primary nucleus pulposus cells (NPCs)

The specimen obtained during surgery was placed in high glucose Dulbecco's Modified Eagle Medium (DMEM) containing 2% penicillin/ streptomycin/ fungizone (PSF), incubated overnight at 37 °C to verify no bacterial growth and washed with phosphate buffered saline (PBS) for several times. The NP samples were diced to 1 mm^3 prior to to being digested with adequate 5% type Ⅱ collagenase (Invitrogen Inc, Carlsbad, CA, USA)for 8~12 hr in a 37 °C cell incubator with 5% CO_2. After complete digestion, cell suspensions were collected by adding an equal volume of medium to the digestive solution. The cell filter of 40 μm was used for filtrate collection. After centrifugation for 6.5 min at 1200 rpm, the supernatant was discarded; the complete culture medium was re-suspended; and cells were collected into a 25 cm^2 cell culture flask for culture in a 37 °C cell incubator with 5% CO_2 and 95% humidity. The growth of cells was observed under a microscope and culture medium was replaced every three days (24).

Dual luciferase reporter gene assay

The target gene of miR-125a was predicted by using the website http://www.microrna.org. In the Gene Bank, the 3'-UTR region of the human BAK1 mRNA sequence was linked with miR-125a, so two complementary sequences can be synthesized and inserted into the restriction sites Xba I and Fse I to construct the BAK1 plasmids of wild type and mutant type. The experiment was divided into four groups: miR-125a NC + BAK1-WT group, miR-125a mimics + BAK1-WT group, miR-125a NC + BAK1-MUT group, and miR-125a mimics + BAK1-MUT group. NPCs in the logarithmic growth phase were obtained and inoculated onto 24-well plate, followed by Lipofectamine 2000 (Invitrogen Inc., Carlsbad, CA, USA) transfection, and the detection of luciferase activity by Dual Luciferase Reporter Gene Assay Kit (Promega Corporation, Madison, WI, USA).

Grouping and transfection of NPCs

Normal and degenerative NPCs were obtained and inoculated onto 24-well plate with the cell density of 5×10^5 cells/well. After inoculation into the DMEM/F12 (1:1) culture medium (Gibco Company (Grand Island, NY, USA), cells would grow to confluence of 85% in 24 hr, which would be transfected according to the instructions of Lipofectamine 2000 (Invitrogen Inc, Carlsbad, CA, USA). Cells were divided into the following six groups: Normal group (normal NPCs), Mock group (degenerative NPCs without transfection), NC group (degenerative NPCs transfected with miR-125a negative control sequence), miR-125a mimics group (degenerative NPCs transfected with miR-125a mimics sequence), miR-125a inhibitors group (degenerative NPCs transfected with miR-125a inhibitors sequence), miR-125a inhibitors+ siBAK1

group (degenerative NPCs co-transfected with miR-125a inhibitors sequence and siBAK1). Based on the data in Genebank, we obtained BAK gene sequences. And we constructed the specific BAK1 siRNA expression plasmid according to the design principles of siRNA. Other sequences needed in the experiment were synthesized by Shanghai GenePharma Co, Ltd.

NPCs apoptosis detected by flow cytometry

Cells collected were centrifuged for 10 min at 1000 rpm. After discarding of the supernatant, cells were washed with PBS for 2 times and 70% ice-ethanol was added to the precipitate for cell fixing. Next, cells were washed for 2 times with PBS before adjusting cell density to 1×10^6/ml. Then 5 μl of Annexiv-FITC (Nanjing Genechem Co, Ltd) was added to the final concentration of 1 g/l before 30 min of warm bath at 37 °C in darkness. After that, propidium iodide (PI)(Nanjing Genechem Co, Ltd) was added to the final concentration of 5 μg/ml. A 350-mesh nylon filter membrane was used to remove cell clumps. After staining for 30 min in darkness, cells were detected by a flow cytometer (BD Company, USA) under the excitation wavelength of 488 nm. FITC fluorescence was detected by a passband filter under the wavelength of 515 nm while PI was detected by a filter with the wavelength over 560 nm.

Protein expression detected by Western blot

Proteins were extracted from cells of each group and their concentration was determined by BCA Kit (Biyuntian Biotech Company,China) according to instructions. The protein extracts were added with loading buffer and boiled for 10 min at 95 °C. Each well was loaded with 30 μg of samples. Then, 10% polyacrylamide gel electrophoresis (PAGE) was used to isolate proteins before polyvinylidene fluoride (PVDF) (Millipore, USA) transmembrane and 1 hr of closing with 5% bovine serum albumin (BSA) at room temperature. Later, primary antibodies BAK1 (ab104124, Abcam Company), caspase-3 (ab13586, Abcam Company), Bax (ab32503, Abcam Company), Bcl-2 (ab32124, Abcam Company), and β-actin (ab8226, Abcam Company) were added respectively for overnight incubation at 4 °C. Later, after washing with TBST (Tris-buffered saline with Tween 20) for 5 min×3 times, horseradish peroxidase- conjugated IgG secondary antibodies (Jackson Labs) were added for 1 hr of incubation at room temperature. Finally, the proteins were visualized by the chemilu -minescence reagent and analyzed Bio-rad Gel Dol EZ imager (GEL DOC EZ IMAGER, Bio-rad, California, USA). Grey value analysis of the target band was analyzed using software image J. We took β-actin as the internal reference gene.

Statistical method

All data were analyzed with the statistical software
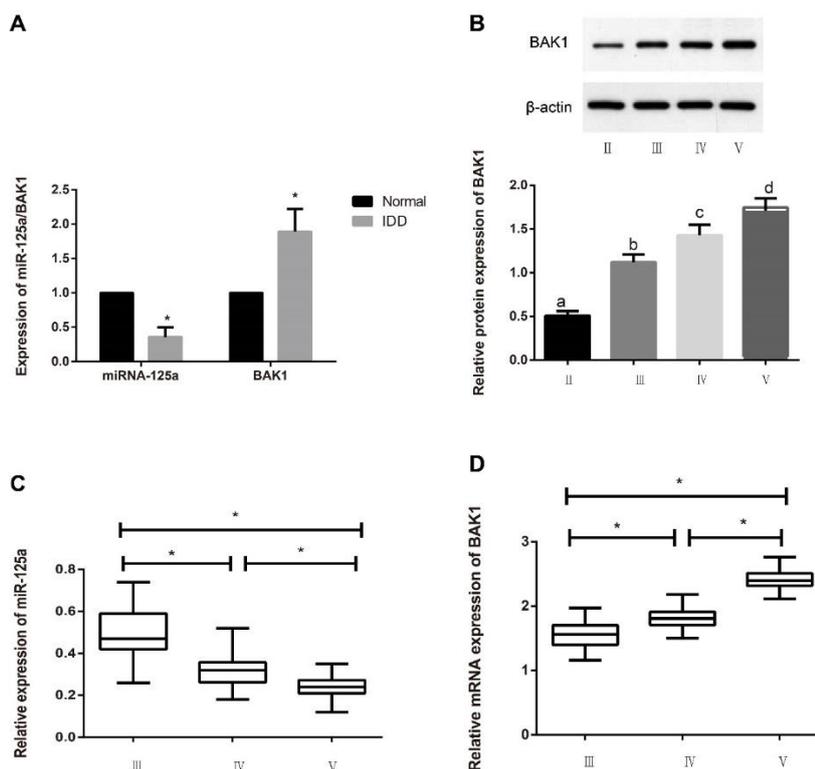
Figure 1. Expression levels of microRNA-125a and pro-apoptotic Bcl-2 antagonist killer 1 in patients with intervertebral disc degeneration and normal controls
A: The expression levels of miRNA-125a and BAK1 in patients with IDD and normal controls; *, $P<0.05$ compared with normal controls. B: The relative protein expression levels of BAK1 in IDD patients with different degree of the Pfirrmann and normal controls. Different letters indicate statistical significance ($P < 0.05$), whereas the same letter means no significantly different ($P>0.05$). C: Comparison of the expression levels of miRNA-125a in IDD patients with different degree of the Pfirrmann; *, $P<0.05$. D: Comparison of the relative mRNA expression levels of BAK1 in IDD patients with different degree of the Pfirrmann

package SPSS 21.0 (SPSS Inc, Chicago, IL, USA). Measurement data were presented by mean ± standard deviation ($\bar{x}$ ± s), and difference between two groups was analyzed using t-test, while comparison among multiple groups was conducted by one-way ANOVA. Besides, enumeration data were presented by frequency and tested by Chi-square test (χ^2). Pearson's correlation coefficient was used to establish associations. $P<0.05$ was considered statistically different.

Results

Expression levels of miRNA-125a and BAK1 in patients with IDD and normal controls

Compared with normal controls, patients with IDD had a significantly reduction in the expression level of miRNA-125a and an apparently elevation in the expression level of BAK1 mRNA (all $P<0.05$) (Figure 1A). Moreover, the expression of miRNA-125a showed a downward trend while the expression of BAK1 mRNA showed a completely opposite trend with the severity degree of disc degeneration in patients with IDD (all $P<0.05$) (Figure 1C, D). In addition, Western blot also confirmed that the relative protein expression of BAK1 was gradually enhanced with the severity grading of the Pfirrmann (all $P<0.05$, Figure 1B).

Comparison of the apoptosis between degenerative and normal NP tissues

As shown in Figure 2A and Figure 2B by using TUNEL staining, there were very few apoptotic cells, and only a few light-yellow colored cells in the normal controls; and while, patients with IDD had appreciably more apoptotic cells than normal controls with the increase of the Pfirrmann grade for IDD degree (all $P<0.05$). According to the correlation analysis, we found that the apoptotic index (AI) was negatively correlated to the expression of miRNA-125a (r=-0.742, $P<0.001$), but positively correlated to the mRNA level of BAK1 (r=0.869, $P<0.001$, Figure 2C, 2D).

BAK1 is the target gene of miRNA-125a

Using the website http://www.microrna.org, miR-125a was predicted to target the 3'-UTR of the BAK1 gene and the direct binding site of miR-125a to the BAK1 target sequence is shown in Figure 3A. Then, we applied dual luciferase reporter gene technology to verify the targeting relationship between miR-125a and BAK1, and found that transfection with miR-125a mimics had no significant effect on mutant BAK1 3'UTR (MUT) ($P>0.05$); however, the luciferase signal intensity of the wild type BAK1 3'UTR (WT) reporter
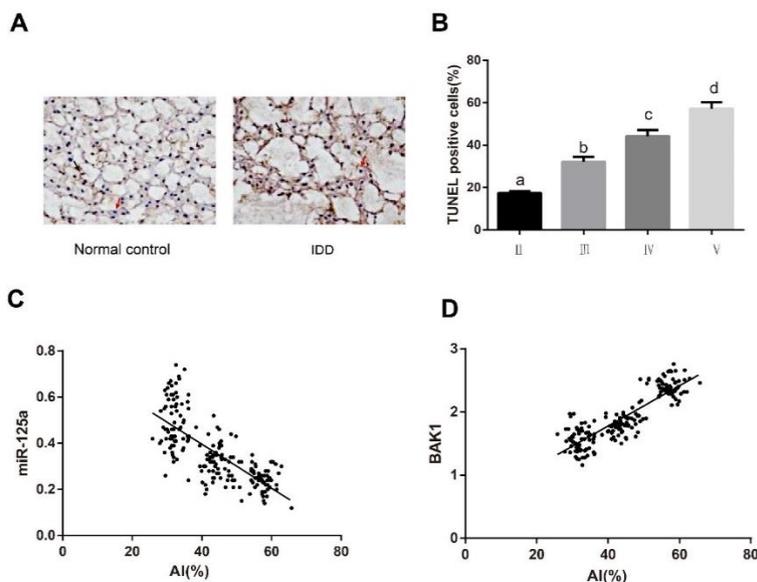
Figure 2. Cell apoptosis of nucleus pulposus tissues from patients with intervertebral disc degeneration and normal controls detected by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling staining
A: The cell apoptosis of NP tissues from patients with IDD and normal controls detected by TUNEL staining. Black arrows indicate TUNEL-positive (apoptotic) cells, red arrows indicate healthy cells. B: Comparison of the apoptotic index (AI) in NP tissues from IDD patients with different Pfirrmann grading and normal controls. Different letters indicate statistical significance ($P<0.05$), whereas the same letter means no significantly different ($P>0.05$). C: The correlation analysis of miRNA-125a expression and cell apoptosis. D: The correlation analysis of the mRNA level of BAK1 and cell apoptosis
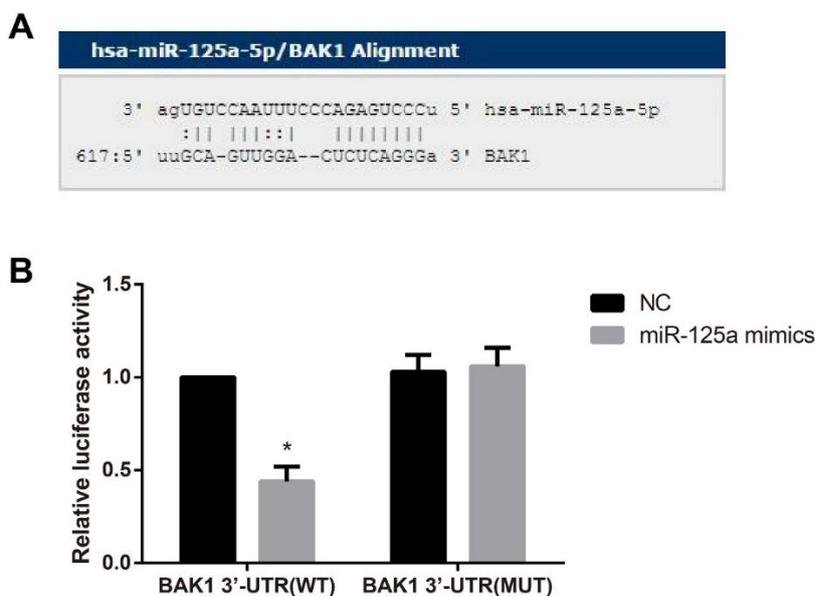
Figure 3. Pro-apoptotic Bcl-2 antagonist killer 1 is the target gene for microRNA-125a
A: miR-125a targeted to the 3'-UTR of the BAK1 mRNA predicted by microRNA.org. B: Results of luciferase reporter gene assay; *, $P<0.05$ compared with NC group

gene vector was obviously reduced ($P<0.05$) (Figure 3B), which suggested that BAK1 was the direct target gene of miR-125a.

Effect of miR-125a on the apoptosis rate of NPCs

As illuminated in Figure 4A, NPCs in the Mock and NC groups showed a remarkably elevated apoptosis rate compared to Normal group (both $P<0.05$). In

comparison with the Mock group, the miR-125a mimics group had the dramatically decreased apoptosis rate and the miR-125a inhibitors group had the notably increased cell apoptosis rate (both $P<0.05$), but there was not any differences from NC group ($P>0.05$). Furthermore, the apoptosis rate of NPCs in the miR-125a inhibitors + siBAK1 group was statistically lower than the miR-125a inhibitors group
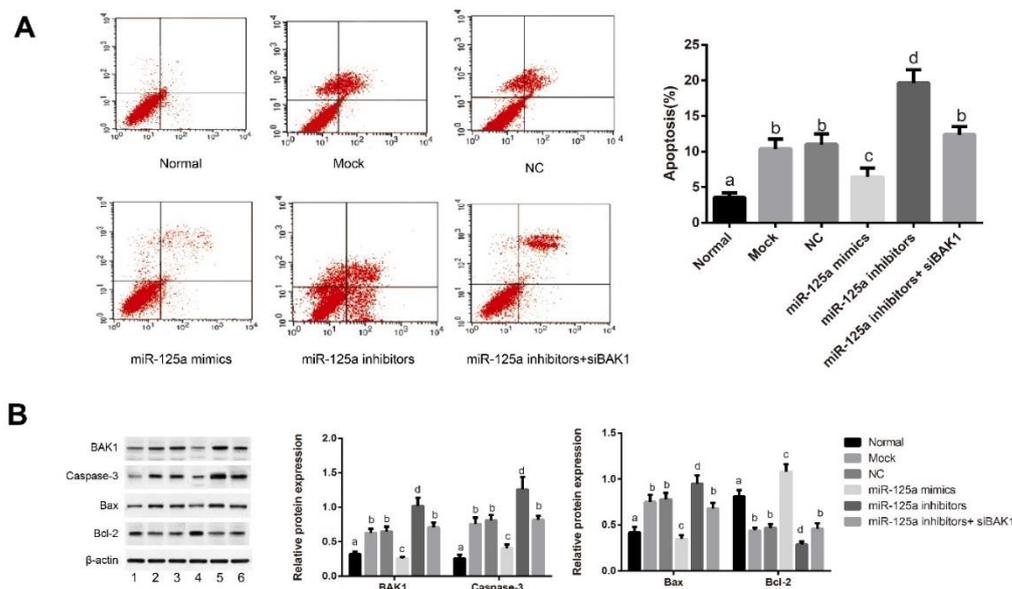
Figure 4. Effect of microRNA-125a on the apoptosis rate of nucleus pulposus cells
A: The apoptosis rate of NPCs in each group was detected by flow cytometry. B: The relative protein expressions of BAK1, Caspase-3, Bax and Bcl-2 in each group were detected by Western blot. 1, Normal group, 2, Mock group, 3, NC group, 4, miR-125a mimics group, 5, miR-125a inhibitors group , 6, miR-125a inhibitors + siBAK1 group; Different letters indicate statistical significance (*P*<0.05), whereas the same letter means no significantly different (*P*>0.05)

(*P*<0.05). In addition, the relative protein expressions of BAK1, Caspase-3, and Bax were markedly elevated, but Bcl-2 was obviously reduced in the NPCs from Mock group and the NC group when compared with NPCs in the Normal group (all *P*<0.05). Specifically, the expressions of BAK1, Caspase-3, and Bax were lower in the miR-125a mimics group, but higher in the miR-125a inhibitors group; however, Bcl-2 expression was higher in the miR-125a mimics group while lower in the the miR-125a inhibitors group, as compared with Mock group (all *P*<0.05). When compared with the miR-125a inhibitors group, a clearly decrease the protein expression levels of BAK1, Caspase-3, and Bax, as well as a remarkably increased Bcl-2 expression were detected in the NPCs from the miR-125a inhibitors+siBAK1 group (all *P*<0.05, Figure 4B).

Discussion

Our study found the downregulation of miRNA-125a and upregulation of BAK1 mRNA and protein in patients with IDD by performing qRT-PCR and Western blot. Actually, previous studies have presented the aberrant downregulation of miRNA-125a in various types of tumors, such as gastric, lung, and breast tumors (25-27). To be more specific, in breast cancer patients who carried with the T allele of rs12976445 within the pre-miR-125a gene had impaired miRNA processing and lowered the expression levels of miR-125a, consequently exhibiting a poor prognosis (28). More importantly, this polymorphism also remarkably declined the miR-125a expressions to be significantly related to the apoptosis status of NPCs, which was clinically served as a promising therapeutic bio -

marker in patients with IDD, as suggested by JF *et al* (29). From this point of view, the decreased expression of miR-125a in our study might also be attributed to its gene polymorphisms, which in turn meant that the overexpression of miR-125a may play a therapeutic role in IDD to some extent. On the other hand, the protein BAK1 functions to induce apoptosis, and localizes to the mitochondria, which was at the center of apoptosis modulation (30, 31). Generally, BAK1, as the molecular "gateway" to mitochondrial damage during the apoptosis, was activated in response to cell stress or injury, contributing to inducing an efficient release of apoptogenic factors from mitochondria (32). For example, BAK1 was reported by Falah *et al.* to be a useful target in patients with age-related hearing impairment, which was obviously enhanced, as well as the BAK1/Bcl2 ratio (33). Similarly, we also observed the upregulation of pro-apoptosis gene BAKI in IDD. Moreover, we demonstrated that, both miRNA-125a and BAK1 expressions would change more dramatically according to the Pearson correlation analysis with the increase of the Pfirrmann grade, indicating that the abnormal expressions of miR-125a and BAK1 were closely related to the occurrence and progression of IDD.

Meanwhile, TUNEL staining was conducted in our study to revealed that the apoptotic index was significantly increased in NP in degenerate discs, and the higher the Pfirrmann grade was, the more obvious the apoptosis would be, which was consistent with the findings of previous studies (34, 35). As recorded by previous documents, the cell population in degenerative NP tissues was gradually decreased, and the cell

apoptosis has been credited as a crucial contributing factor to reduce the cell number and promote the degeneration process (36-38). In our study, the increased apoptotic index in degenerative NP tissues was negatively correlated to the reduction of miR-125a, but was positively relevant to the elevation of BAK1 mRNA, showing that the abnormal expression of miR-125a and BAK1 may regulate the cell apoptosis of degenerative NP tissues. Additionally, the target gene prediction website pointed out that miR-125a could directly bound to the predicted 3'-UTR target sites of BAK1, and luciferase experiments also confirmed that BAK1 is the target gene of miR-125a. This provided a possibility that miR-125a may exert a critical function in the cell apoptosis of degenerative NPCs via targeting BAK1.

In support of this possibility, we performed the cell experiments *in vitro* by isolating NPCs via transfection with different plasmid vectors. The data demonstrated in this study showed an obviously lowered apoptosis rate in the miR-125a mimics group, but an apparently increased apoptosis rate in the miR-125a inhibitors group, indicating that overexpression of miR-125a can inhibit the apoptosis of degenerative NPCs. However, when transfected with the miR-125a inhibitors and the siRNA of BAK1, the cell apoptosis rate was clearly reduced, which suggested that siBAK1 can reverse the pro-apoptosis role of miR-125a inhibitors in NPCs. Furthermore, by detecting apoptosis-related proteins, we discovered that overexpression of miR-125a can not only significantly downregulate the protein expressions of BAK1, Caspase-3, and Bax, but also effectively enhance the production of Bcl-2; but inhibiting miR-125a had completely opposite effects, which can be reversed by siBAK1. It is clear that both the pro-apoptotic protein Bax and the anti-apoptotic protein Bcl-2 are the members of Bcl-2 family, and BAK1 could interact with Bax to regulate the mitochondrial outer membrane permeabilisation during apoptosis (39). When cells under stress or injury, complex interactions between the proteins of Bcl-2 family would occur and the signal would transmit to the mitochondrial outer membrane to activate BAK1 (40). The activated BAK1 accelerated the opening of voltage-dependent anion channel on the mitochondrial membrane, whereby resulting in changes in membrane potential and the release of apoptosis factors, like cytochrome C into cytoplasm, to further activate Caspases (particularly, Caspase-3) (41), which in turn accelerate the cell apoptosis (42). As such, these findings supported the notion that miR-125a can inhibit the target gene of BAK1 to modulate the balance of anti- and pro-apoptotic proteins, thereby achieving the possibility of delaying the apoptosis of degenerative NPCs.

Conclusion

In summary, our study exhibited the down-regulation of miR-125a and the upregulation of BAK1 in patients with IDD, and overexpression of miR-125a may alleviate the apoptosis of degenerative NPCs by targeting inhibition of BAK1, which hinted that specific regulation of miR-125a might become a novel therapeutic strategy for the management of IDD.

Acknowledgment

Conflict of interest

The authors declare that they have no competing interests.

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