Abstract. Legg-Calvé-Perthes disease (LCPD) is a pediatric form of femoral head osteonecrosis with unknown etiology. MicroRNAs (miRs) have been revealed to serve an important role in LCPD. MiR-214 serves an important role in chondrogenesis. The aim of the present study was to investigate the potential role of miR-214 in LCPD and the underlying mechanisms. The expression levels of miR-214 and B-cell lymphoma 2 (Bcl-2)-associated X protein (Bax) in dexamethasone (DEX)-treated TC28 cells, and the femoral head cartilage tissues, serum and primary chondrocytes of patients with LCPD, and healthy individuals were determined via reverse transcription quantitative polymerase chain reaction and western blot analysis. A luciferase reporter assay was conducted to investigate the association between miR-214 and Bax, while cell viability was determined via an MTT assay, and flow cytometry was performed to investigate cell apoptosis. The results revealed that miR-214 was downregulated and Bax was upregulated in DEX-treated TC28 cells and tissues obtained from patients with LCPD. MiR-214 was demonstrated to directly target Bax and negatively regulate its expression. DEX administration significantly suppressed cell proliferation, promoted apoptosis and decreased the Bcl-2/Bax ratio in TC28 cells; overexpression of miR-214 induced opposing effects, which were reversed by Bax overexpression. In conclusion, the results indicated that miR-214 and Bax may be potential therapeutic targets for the future clinical treatment of LCPD.

Introduction

Legg-Calvé-Perthes disease (LCPD) is a pediatric form of femoral head osteonecrosis that mainly affects children between the ages of 2-12 years; LCPD is characterized by painful synovitis in the knees, avascular necrosis of the femoral head, and deformities of the femoral head and acetabulum (1-3). The disease typically leads to deformities, physical dysfunction and lifelong disability in children (4,5). At present, the main therapy for LCPD is surgical treatment; however, long-term (1.5-2 months) use of a brace is required to improve the state of the hip, resulting in a certain degree of limited hip joint activity (6,7). In addition, due to the incomplete development of the nervous system and low tolerance to pain, children often experience strong reactions towards treatment, resulting in poor cooperation, which may affect postoperative recovery (8). Therefore, high quality care is particularly important. Nurses should be familiar with the aforementioned characteristics of children, and take appropriate measures to prevent complications and promote postoperative rehabilitation.

Various studies have indicated that the pathogenesis of LCPD is due to the uncoupling of bone metabolism (9,10); however, the exact pathophysiology of this disease remains unknown. Recent studies demonstrated increased levels of pro-apoptotic factors in patients with LCPD, suggesting that apoptotic processes may promote the development of LCPD (9,11); however, further investigation is required to determine the potential molecular mechanisms of apoptosis in LCPD.

MicroRNAs (miRNAs/miRs) are small noncoding RNAs (19-25 nucleotides) that can regulate the expression of numerous target genes via binding to their 3’ untranslated regions (3’-UTRs) (12,13). miRNAs regulate a variety of cellular functions, including proliferation, apoptosis, differentiation and metastasis (14,15). The aberrant expression of miRNAs can lead to cellular and tissue disorders. It has been reported that miRNAs serve important roles in chondrogenesis and LCPD. Luo et al (16) revealed that miR-206 promoted cell apoptosis in LCPD via downregulation of SRY-box 9. Furthermore, it was demonstrated that overexpression of miR-214 in vitro and in vivo negatively regulated chondrocyte differentiation, possibly by targeting activating transcription factor 4 (ATF4) (17). Additionally, Wang et al (18) revealed that miR-214 functions as a tumor suppressor in cervical
cancer by inhibiting cell proliferation and invasion, and promoting apoptosis. Collectively, these studies suggest that miR-214 may serve important roles in regulating cell growth and apoptosis, and that apoptotic processes may be involved in the pathogenesis of LCPD (9,11).

To the best of our knowledge, the role of miR-214 in LCPD has not been investigated. Therefore, the present study aimed to determine whether miR-214 may be involved in the development and progression of LCPD via the regulation of apoptosis. It was demonstrated that miR-214 was downregulated in cartilage, serum and chondrocytes from patients with LCPD, whereas B-cell lymphoma 2 (Bcl-2)-associated X protein (Bax) expression was upregulated. Furthermore, it was revealed that Bax was a target gene of miR-214; this miRNA increased the viability of the TC28 human chondrocyte cell line and inhibited apoptosis via downregulation of Bax. The results indicated that miR-214 may function as a predictive biomarker and potential therapeutic target for the treatment of LCPD.

Materials and methods

Clinical samples. All patients signed informed consent forms prior to the study, and the study received approval from the Institutional Ethics Committee of Nanjing Children's Hospital Affiliated to Nanjing Medical University (Nanjing, China). Human femoral head cartilage tissue was isolated from patients with LCPD (n=20, <14 years old, male 12, female 8) and healthy volunteers (n=20, <14 years old, 12 male, 8 female) from December 2015 to October 2017. LCPD was diagnosed on the basis of ultrasonographic examination and magnetic resonance imaging. Patients with other diseases, such as primary osteoarthritis, anklyosing spondylitis, systemic lupus erythematosus and inflammatory diseases, were excluded. Peripheral venous blood samples were drawn from all patients in the morning prior to surgery, collected into two 4.5-ml Vacutainer sodium citrate anticoagulant tubes (BD Biosciences, Franklin Lakes, NJ, USA) and stored at -80˚C until use. Peripheral venous blood samples were centrifuged at 1,000 x g for 10 min at 4˚C to obtain serum.

Chondrocyte isolation and culture. Chondrocytes were isolated from LCPD and healthy control femoral head cartilage tissues via collagenase digestion of cartilage, and cultured in monolayer as described previously (19). Cells of the first passage were used in the experiments of the study. The human cartilage cell line TC28 (immortalized human primary juvenile costal chondrocytes) was purchased from the American Type Culture Collection (Manassas, VA, USA). The two cell lines were propagated as monolayers maintained in Dulbecco's Modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% heat-inactivated fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 mg/ml streptomycin at 37˚C in a humidified atmosphere of 5% CO2.

TC28 cells were treated with 0.01 mmol dexamethasone (DEX) (cat. no. k1419; Guangzhou Kafen Biological Technology Co., Ltd., Guangzhou, China) at 37˚C for 2 h to establish an in vitro model of LCPD. Cells without any treatment were used as control. Then, the levels of miR-214 and Bax expression were evaluated to characterize the effects of DEX on TC28 cells.

Plasmids, oligonucleotides and transfection. miR-214 mimic (5'CCUCACAAUUAUGAUUUU-3') and mimic control (5'-ACAGGUGACUAACUGGGUU-3') were purchased from Guangzhou RiboBio Co., Ltd. (Guangzhou, China). The sequence of Bax was inserted into pcDNA3.1 (Invitrogen; Thermo Fisher Scientific, Inc.); empty vector (control-plasmid) was used as the control. TC28 cells were seeded in 6-well plates (1x106 cells/well), cultured for 24 h and transfected with 100 nM miR-214 mimic, 100 nM mimic control, 1 µg control-plasmid, 1 µg Bax-plasmid or 100 nM miR-214 mimic + 1 µg Bax-plasmid using Lipofectamine® 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols. The transfection efficiency was determined 48 h later.

RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from serum, cartilage tissue or cells using TRizol® reagent (Invitrogen; Thermo Fisher Scientific); 1 µg of total RNA was reverse transcribed to cDNA using a PrimeScript RT Reagent kit (Takara Bio, Inc., Otsu, Japan) according to the manufacturer's protocols. Reaction conditions for reverse transcription were: 50˚C for 5 min and 80˚C for 2 min. An miRNA-specific TaqMan MiRNA Assay kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) was used for the detection of miRNA expression according to the manufacturer's protocols. SYBR Premix Ex Taq (Takara Bio, Inc.) was used to analyze Bax mRNA expression. U6 small nuclear RNA (U6) and GAPDH expression were used as an internal control for miR-214 and Bax, respectively. The sequences of primers were as follows: miR-214, forward 5'-AGCATATA CACCAAGCACAGAC-3', reverse, 5'−AAAGTGGTTTCTT TCACTCTCTCAC-3'; Bax, forward 5'-GGCCCCACAGCT CGAGCAGA-3', reverse, 5'-GCCACGTTGCGTCCCAA AGT-3'; GAPDH, forward 5'-TGAACGGGAAGCTCAC TGG-3', reverse, 3'-TCCACACCTGTGCTGTGA-5'; and U6, forward 5'-CGCTTTGCGCAGACATAC-3' and reverse, 5'-AAATATGGAACGCTTCAAG-3'. RT-qPCR data were analyzed with an ABI 7900 Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). Amplification conditions for qPCR were as follows: 5 min at 95˚C, followed by 35 cycles at 95˚C for 15 sec, 40 sec at 55˚C, and 72˚C for 1 min. The relative expression levels were calculated using the 2−ΔΔcq method (20).

Dual-luciferase reporter assay. The bioinformatics tool miRBase (http://www.mirbase.org) was used to predict the potential targets of miR-214. The results indicated that Bax was a potential target of miR-214. To confirm this prediction, a dual-luciferase reporter assay was performed. A wild type (WT-Bax) and mutant (MUT-Bax) 3'-untranslated regions of Bax were cloned into a pmiR-RB-Report™ dual luciferase reporter gene plasmid vector (Guangzhou RiboBio Co., Ltd., Guangzhou, China). Then, the WT-Bax or MUT-Bax vectors, the miR-214 mimic or mimic control, and the pRL-TK Renilla luciferase reporter (Promega Corporation,
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MADISON, WISCONSIN — Co-transfected into TC28 cells using Lipofectamine® 2000. TC28 cells were collected 48 h following transfection and luciferase activity was analyzed using dual-luciferase assay system (Promega Corporation, Madison, WI, USA). Firefly luciferase activity was normalized to that of Renilla.

Western blot assays. Proteins from cells, serum and tissues were extracted using radiolabeled immunoprecipitation assay buffer (Beyotime Institute of Biotechnology, Haimen, China) with Protease/Phosphatase Inhibitor Cocktail (Cell Signaling Technology, Inc., Danvers, MA, USA). Protein concentrations were determined using a bicinchoninic acid assay kit (Pierce; Thermo Fisher Scientific, Inc.). Equal amounts of protein samples (30 µg/lane) were separated via 12% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Merck KGaA, Darmstadt, Germany). The membranes were blocked in 5% non-fat dry milk at room temperature for 1 h and incubated with primary antibodies overnight at 4°C. The primary antibodies used were: Anti-Bax (1:2,000; ab32503, Abcam, Cambridge, UK), anti-Bcl-2 (1:2,000; ab196495, Abcam) and anti-GAPDH (1:5,000; ab9485, Abcam). Membranes were then incubated with a goat anti-rabbit immunoglobulin G conjugated with horseradish peroxidase (cat. no. ab7090; 1:2,000; Abcam) at room temperature for 3 h. All bands were visualized using enhanced chemiluminescence western blotting detection kits (Merck KGaA). ImageJ 1.38X software (National Institutes of Health, Bethesda, MD, USA) was used to quantify protein expression.

MTT assay. Cell viability was determined by an MTT assay. Following treatment with DEX and transfection with mir-214 mimic, mimic-control, or mir-214 mimic + Bax-plasmid for 48 h, TC28 cells (1x10^4 cells/well) were seeded in 96-well plates and cultured for 24 h. Subsequently, 20 µl MTT solution (0.5 mg/ml; Sigma-Aldrich; Merck KGaA) was added to each well and the plates were further incubated at 37°C for 4 h. The medium in each well was discarded, and 150 µl dimethyl sulfoxide was added for 30 min. The absorbance at 570 nm was measured using a FLUOstar® Omega Microplate Reader (BMG Labtech GmbH, Ortenberg, Germany).

Flow cytometry analysis. An Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) Apoptosis Detection kit [cat. no. 70-AP101-100; MultiSciences (Lianke) Biotech, Co., Ltd., Hangzhou, China] was used to evaluate the apoptotic rate of TC28 cells. Following 48 h since transfection, TC28 cells were collected, washed with PBS and suspended with 5 µl Annexin V-FITC and 5 µl PI for 30 min in the dark at room temperature. A flow cytometer was used to analyze cell apoptosis. And the cell apoptotic rate (early apoptosis and late apoptosis in the right quadrant) was determined using FlowJo software version 7.6.1 (FlowJo LLC, Ashland, OR, USA).

Statistical analysis. Each experiment was repeated three times. Statistical analysis was performed using SPSS 18.0 (SPSS, Inc., Chicago, IL, USA). Data are presented as the mean ± standard deviation of at least three independent experiments. Differences between groups were analyzed by Student's t-tests or one-way analysis of variance with Tukey's post hoc tests. P<0.05 was considered to indicate a statistically significant difference.

Results

Expression of miR-214 is decreased in human LCPD cartilage, serum and chondrocytes. To determine miR-214 expression in human LCPD cartilage, serum and chondrocytes, samples were collected from 20 patients with LCPD and 20 healthy individuals. As presented in Fig. 1A and B, compared with the healthy group, miR-214 expression was significantly downregulated in femoral head cartilage and serum samples from patients with LCPD compared with the healthy controls. In addition, human primary chondrocytes were isolated from femoral head cartilage tissues from patients with LCPD and healthy controls. The results of RT-qPCR revealed that miR-214 expression was significantly downregulated in human primary chondrocytes from patients with LCPD compared with healthy controls (Fig. 1C). Furthermore, the expression levels of miR-214 were significantly downregulated in DEX-treated TC28 cells compared with untreated control cells (Fig. 1D).

Bax is a direct target of miR-214. The bioinformatics tool miRBase (http://www.mirbase.org) was used to predict the potential targets of miR-214. Bioinformatics analyses indicated that Bax was a potential target of miR-214 (Fig. 2A). Subsequently, to determine whether miR-214 directly modulates Bax expression via interactions with potential binding sites, a luciferase reporter assay was performed using TC28 cells transfected with vectors harboring the WT or MUT 3'-UTR of Bax, in the presence or absence of the miR-214 mimic or mimic control. As presented in Fig. 2B, compared with co-transfection with Bax-WT and mimic control, the luciferase activity was significantly decreased following co-transfection with Bax-WT and miR-214 mimic, while Bax-MUT did not . The results indicated that Bax was a target of miR-214.

Expression of Bax is increased in human LCPD cartilage, serum and chondrocytes. The expression of Bax was determined in human LCPD cartilage, serum and chondrocytes. As presented in Fig. 3A-C, compared with the healthy control group, the expression level of Bax mRNA was significantly upregulated in femoral head cartilage, serum and primary chondrocytes of patients with LCPD. Meanwhile, compared with the healthy control group, the levels of Bax protein appear to be higher in the femoral head cartilage, serum and primary chondrocytes of patients with LCPD. Furthermore, TC28 cells treated with DEX exhibited significantly elevated Bax mRNA expression compared with untreated control cells and seemingly higher Bax protein levels as well (Fig. 3D).

miR-214 negatively regulates Bax expression in TC28 cells. To further investigate the effects of miR-214 on Bax expression, TC28 cells were transfected with mimic control, miR-214 mimic, control-plasmid, Bax-plasmid, or miR-214 mimic + Bax-plasmid for 48 h. As presented in Fig. 4A, miR-214 mimic significantly increased the expression levels...
Figure 1. Expression of miR-214 in LCPD. The expression levels of miR-214 in LCPD were detected via reverse transcription-quantitative polymerase chain reaction. Relative miR-214 expression in (A) femoral head cartilage, (B) serum and (C) chondrocytes from patients with LCPD or healthy controls. (D) Relative miR-214 expression in TC28 cells treated with or without DEX. Data are presented as the mean ± standard deviation. **P<0.01 vs. healthy or untreated control groups. DEX, dexamethasone; LCPD, Legg-Calvé-Perthes disease; miR, microRNA.

Figure 2. Bax is a target of miR-214. (A) Interaction between miR-214 and 3'-UTR of Bax was predicted using miRBase. (B) Luciferase activity of reporter plasmids containing the WT or MUT Bax 3'-UTR is presented. All data are presented as the mean ± standard deviation of three independent experiments. **P<0.01 vs. mimic control group. Bax, B-cell lymphoma 2-associated X protein; miR, microRNA; MUT, mutated; UTR, untranslated region; WT, wild-type.
of miR-214 in TC28 cells compared with the control group (Fig. 4A). In addition, TC28 cells transfected with the Bax-plasmid exhibited significantly increased mRNA expression of Bax (Fig. 4B) and seemingly higher Bax protein levels as well (Fig. 4C). Compared with the mimic control group, transfection with miR-214 mimic significantly decreased the mRNA expression of Bax in TC28 cells, which was reversed by Bax overexpression (Fig. 4D). The results of western blot assay suggested that miR-214 mimic decreased the protein levels of Bax in TC28 cells, which may have been reversed by Bax overexpression (Fig. 4E).

miR-214 targets Bax to regulate chondrocyte viability and apoptosis. MTT assays were performed to detect the viability of TC28 cells, and flow cytometry was conducted to detect TC28 cell apoptosis. As presented in Figs. 5 and 6, compared with the control group, DEX treatment significantly reduced viability of TC28 cells and promoted apoptosis. Conversely, overexpression of miR-214 significantly increased cell viability and inhibited cell apoptosis compared with DEX treatment alone; these effects were reversed by Bax overexpression. Additionally, the protein expression of Bax and Bcl-2 was determined, and the Bcl-2/Bax ratio was calculated. As

Figure 3. Expression of Bax in LCPD. Protein and mRNA expression of Bax in (A) femoral head cartilage, (B) serum and (C) chondrocytes from patients with LCPD or healthy controls. (D) Protein and mRNA expression of Bax in TC28 cells treated with or without DEX. Data are presented as the mean ± standard deviation. **P<0.01 vs. healthy or untreated control groups. Bax, B-cell lymphoma 2-associated X protein; DEX, dexamethasone; LCPD, Legg-Calvé-Perthes disease.
presented in Fig. 7, compared with the control, the protein expression of Bax increased following DEX treatment, while that of Bcl-2 decreased and the Bcl-2/Bax ratio was reduced. These effects were inhibited by miR-214 overexpression and promoted by Bax overexpression.

Discussion

LCPD is an idiopathic osteonecrosis of the immature femoral head, in which the supply of blood in the capital femoral epiphysis is interrupted, resulting in osteonecrosis and cartilage necrosis, leading to gradual malformation of the femoral head and subsequent degenerative osteoarthritis (21,22). Recently, miRNAs were identified as important regulators of numerous diseases, including cancer, autoimmune diseases, inflammation and infertility (23). For example, miR-214 inhibits cervical cancer cell proliferation and invasion, and facilitates apoptosis via regulating the expression of mechanistic target of rapamycin (18). A recent study demonstrated that miR-214 suppresses the osteogenic differentiation of human periodontal ligament stem cells by targeting a TF4 (24). In addition, overexpression of miR-214 exerts a negative role in chondrogenesis by affecting chondrocyte differentiation (17). In the present study, it was revealed that miR-214 expression was significantly decreased in patients with LCPD compared with healthy controls. Additionally, treatment of Tc28 cells with 0.01 mmol DEX significantly decreased the expression of miR-214. Collectively, the results indicated that miR-214 was downregulated in patients with LCPD and DEX-treated Tc28 cells, suggesting that miR-214 may serve an important role in the development of LCPD.

Calder et al (25) reported that the processes leading to the death of femoral head cells in patients with femoral head necrosis involves an increased rate of apoptosis rather than bone cell necrosis alone. Furthermore, Zhang et al (26) revealed that chondrocyte apoptosis in the femoral head is induced by glucocorticoids in broilers. Additionally, miR-206 contributes to the progression of steroid-induced avascular necrosis of the femoral head by inducing osteoblast apoptosis via the suppression of programmed cell death 4 (27). A notable finding of the present study was that Bax was a direct target gene of miR-214. Bax is a pro-apoptotic member of the Bcl-2 family of proteins, and serves an important role in the mitochondrial apoptotic pathway; Bax migrates to the mitochondrial membrane during...
apoptosis (28). As a downstream target gene of p53, Bax is required for p53-dependent apoptosis in certain systems (29). It has been reported that p53 can directly activate Bax without active transcription (30). Pagliara et al (31) recently reported that, independent of p53 status, activated p21 can induce Bax translocation to the mitochondria, which in turn, increases the mitochondrial membrane permeability, leading to cytochrome c release and caspase pathway activation. Of note, significantly increased levels of Bax expression were reported in patients with LCPD, along with a significantly elevated Bax/Bcl-2 ratio (9). Consistent with Srzentić et al (9), the results of the present study revealed that the levels of Bax protein and mRNA expression in the cartilage, serum and chondrocytes of LCPD patients were significantly enhanced compared with the healthy control. DEX treatment also significantly increased Bax expression in TC28 cells. Then, to investigate the association between miR-214 and Bax, TC28 cells were transfected with miR-214 mimic. The results revealed that overexpression of miR-214 significantly decreased the levels of Bax expression. Furthermore, it was demonstrated that DEX treatment significantly decreased TC28 cell viability, promoted apoptosis and reduced the Bcl-2/Bax ratio, whereas miR-214 mimic exhibited opposing effects. Additionally, the effects of miR-214 upregulation on TC28 cells were eliminated by Bax overexpression. Collectively, the results suggested that miR-214 and Bax dysregulation may be involved in LCPD.

Figure 6. Effects of miR-214 on TC28 cell apoptosis. Following treatment with DEX and transfection with mimic-control, miR-214 mimic or miR-214 mimic + B-cell lymphoma 2-associated X protein-plasmid for 48 h, TC28 cell apoptosis was analyzed via flow cytometry. Data are presented as the mean ± standard deviation. **P<0.01 vs. control group; ##P<0.01 vs. DEX group; *P<0.05 vs. mimic group. DEX, dexamethasone; FITC, fluorescein isothiocyanate; miR, microRNA; PI, propidium iodide.

Figure 7. Effects of miR-214 on Bcl-2/Bax ratio in TC28 cells. Following treatment with DEX and transfection with mimic-control, miR-214 mimic or miR-214 mimic + Bax-plasmid for 48 h, (A) the protein expression levels of Bcl-2 and Bax were determined via western blotting, and (B) the Bcl-2/Bax ratio was calculated. Data are presented as the mean ± standard deviation. ***P<0.001 vs. control group; ##P<0.01 vs. DEX group; &&P<0.01 vs. mimic group. Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein; DEX, dexamethasone; miR, microRNA.
In conclusion, the present study revealed that miR-214 was downregulated and Bax was upregulated in the cartilage, serum and chondrocytes of patients with LCPD, and DEX-treated TC28 cells. miR-214 promoted chondrocyte viability and decreased apoptosis via downregulation of Bax. The present study indicated that miR-214 may function as a reliable biomarker and potential therapeutic target in the treatment of LCPD.

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Availability of data and materials

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

Authors' contributions

HZ, XQ and YL contributed to study design, data collection, statistical analysis, data interpretation and manuscript preparation. WL, XS and YT contributed to statistical analysis and literature search. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

All patients signed informed consent forms prior to the study, and the study received approval from the institutional ethics committee of the Children's Hospital Affiliated to Nanjing Medical University (Nanjing, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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