miRNA-155 expression and role in pathogenesis in spinal tuberculosis-induced intervertebral disc destruction

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Abstract. The current study aimed to investigate microRNA-155 (miR-155) expression in spinal tuberculosis-induced intervertebral disc destruction and its regulatory role in disease pathogenesis. A total of 26 patients with intervertebral disc destruction induced by spinal tuberculosis and 31 healthy individuals were included. Reverse transcription-quantitative polymerase chain reactions, western blot analysis and ELISA were performed to detect mRNA and protein expression levels. A bioinformatics analysis was applied to predict the upstream regulator of matrix metalloproteinase (MMP)13, which was confirmed by dual-luciferase reporter assay. Compared with the control group, mRNA and protein expression levels of MMP13 were significantly increased in the intervertebral disc of patients with spinal tuberculosis. However, miR-155 expression in the intervertebral disc of patients with spinal tuberculosis was significantly decreased compared with the control group. Dual-luciferase reporter assays suggested that miR-155 bound to the 3'-untranslated region of MMP13 to regulate gene expression. In primary annulus fibrosus cells, upregulated miR-155 expression significantly decreased MMP13 expression in the cells and culture supernatant, whereas it increased type II collagen expression. Upregulated MMP13 expression in the intervertebral disc in patients with spinal tuberculosis may be correlated with downregulated miR-155 expression. miR-155 may regulate expression levels of associated proteins in the intervertebral disc via modulating MMP13 expression, which contributes to the disease pathogenesis. The results of the current study may provide the theoretical basis for the diagnosis and treatment of disc damages caused by spinal tuberculosis.

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Introduction

Spinal tuberculosis is the most common type of osteoarticular tuberculosis accounting for ~75% of cases and typically induces the destruction of the vertebral body and intervertebral disc, and further leads to loss of spinal stability and compression of the spinal cord, resulting in paraplegia and kyphosis (1). Destruction and disability of the human body induced by spinal tuberculosis are more severe compared with other types of bone and extrapulmonary tuberculosis (1). A previous study has assessed the mechanism of spinal tuberculosis-induced damages in the vertebral body and intervertebral disc (2). It has been demonstrated that matrix metalloproteinases (MMPs) are closely associated with the degeneration of intervertebral discs (3). A previous study has revealed that MMPs serve important roles in the disruption of intervertebral discs caused by spinal tuberculosis (4).

MMPs are a group of endopeptidases that share high structural homology and can degrade extracellular matrix (ECM) proteins, usually containing metal ions, including zinc and calcium (5). Under physiological conditions, the normal intervertebral disc is located between upper and lower endplates, which consists of the outer annulus fibrosus (AF) and the inner jelly-like nucleus pulposus (NP), which is rich in proteoglycan (6). Normal intervertebral discs are composed of a large number of ECMs and a small number of cells, with cells accounting for 1% of the disc volume. NP cells synthesize type II collagen and fibroblasts synthesize type I and II collagen (7). MMP13 is derived from chondrocytes, the former of which mainly degrades type II collagen and aggrecan, therefore serving a dual role in matrix ECM degradation (8).

Along with in-depth analyses of microRNAs (miRNAs or miRs), certain studies have suggested that upregulation of miRNAs can inhibit MMP13 expression (9,10). miRNAs are associated with regulating differentiation and maturation of

immune cells, including T cells and B cells (11); osteogenic differentiation of stromal cells (12); expression of inflammatory cytokines and immune responses (13); host-virus interactions (14); and the sugar metabolism in the body (15). miR-155 has been predicted as an upstream regulator for MMP13, which serves an important role in the regulation of the immune system, inflammation and apoptosis in the body (16). However, there are few studies concerning the expression of miR-155 in the intervertebral disc in patients with spinal tuberculosis and its association with MMP13 in the disease pathogenesis.

The aim of the present study was to investigate the role of MMP13 in the pathogenesis of spinal tuberculosis, and MMP13 mRNA and protein expression were measured in patients with spinal tuberculosis-induced intervertebral disc destruction. The association between miR-155 and MMP13 was further analyzed. This present study may provide the theoretical basis for the diagnosis and treatment of disc damages caused by spinal tuberculosis.

Materials and methods

Study subjects. A total of 26 patients, 10 males and 16 females, aged 22-62 years (43.6+18.6 years) with spinal tuberculosis-induced intervertebral disc destruction were included in the current study. Patients were admitted to the First Affiliated Hospital of Guangxi Medical University (Nanning, China) between August 2015 and June 2017. A total of 31 healthy subjects, 13 males and 18 females, aged 20-60 years (41.8+15.8 years) were included as control. Subjects had no medical history of treatments with hormones, Traditional Chinese Medicine, radio- or chemotherapy. Patients from the control group were admitted to Guangxi Liuzhou Workers Hospital (Liuzhou, China), between August 2015 and June 2017, and were diagnosed by two independent pathologists. Written and informed consent was obtained from every patient and the study was approved by the local Ethics Review Boardof the First Affiliated Hospital of Guangxi Medical University (Nanning, China).

Sample preparation. A total of 10-15 ml peripheral venous blood was drawn from the subjects and stored at 4˚C for 1-2 h. Peripheral blood serum was obtained by density gradient centrifugation. Samples were then centrifuged at a speed of 400 x g for 10 min at 4˚C, following which the upper serum was collected and stored at -70˚C.

Intervertebral disc tissues and blood samples were harvested from the 26 patients with spinal tuberculosis-induced intervertebral disc destruction via surgery. Corresponding tissue and blood were removed from the 31 healthy controls with fresh disc trauma as control. Tissue was transferred to a mortar filled with liquid nitrogen, ground into a powder, collected into 1.5-ml tubes and stored in liquid nitrogen.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). MMP13 mRNA expression levels were detected by RT-qPCR. Total RNA was extracted from patient tissue or serum with TRIzol (cat. no. 10606ES60; Equitech-Bio, Inc., Kerrville, TX, USA). RT was performed to obtain a cDNA template using the TIANScriptII cDNA first strand synthesis kit (cat. no. KR107; Tiangen Biotech Co., Ltd., Beijing, China) according to the manufacturer's protocol. qPCR was performed with the Super Real Pre mix (SYBR Green; FP204; Tiangen Biotech Co., Ltd.) on the PCR-iQ5 system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The primer sequences used for qPCR were as follows: MMP13, forward 5'-TCAGGAAACCAGGTCTGGAG-3' and reverse 5'-TCACCAATTCCTGGGAAGTCT-3'; and β-actin, forward 5'-CTAAGTCATAGTCCGCCTAGAAGCA-3' and reverse 5'-TGGCACCCAGCACAATGAA-3'. The 25-μl PCR system consisted of 2 μl cDNA, 10 μl qRT-PCR-mix, 0.5 μl each primer and 7 μl ddH_2O. Reaction conditions were as follows: 95˚C for 2 min; 95˚C for 30 sec, 55˚C for 30 sec, 72˚C for 1 min, 40 cycles; 72˚C for 10 min. β-actin was used as control.

For the detection of miR-155 expression, miRNA was isolated from patient tissue and serum using the miRcute miRNA isolation kit (Tiangen Biotech Co., Ltd.) and qPCR was performed using the miRcute miRNA kit (FP401; Tiangen Biotech Co., Ltd.) in accordance with the manufacturers protocol. The primer sequences were as follows: miR-155, forward 5'-GTGCTGCAAACCAGGAAGG-3' and reverse 5'-CTGGTTGAATCATTGAAGATGG-3'; and U6, forward 5'-GCTTCGGCAGCACATATACTAAAAT-3' and reverse 5'-CGCTTCACGAATTTGCGTGTCAT-3'. The reaction conditions were as follows: 95˚C for 5 min; 95˚C for 15 sec, 60˚C for 30 sec, 72˚C for 10 sec, 40 cycles. U6 was used as control. Expression levels of target genes in mRNA and miRNA were calculated using the $2^{-\Delta\Delta Cq}$ method (17).

Bioinformatics analysis. The upstream regulatory miRNA of MMP13 was predicted using a bioinformatics analysis, performed with mirwalk3.0 (http://129.206.7.150/) and miRecords (http://c1.accurascience.com/miRecords/) software.

Isolation, culturing, and transfection of annulus fibrosus cells of the intervertebral disc. Harvested intervertebral disc tissue was carefully isolated and cut into pieces (tissues were pulverized upon collection and stored liquid nitrogen). Following digestion with 0.25% trypsin (Gibco; Thermo Fisher Scientific, Inc.) at 37˚C for 40 min, tissues were washed and digested with 0.025% type II collagenase (Invitrogen; Thermo Fisher Scientific, Inc.) at 37˚C for 4 h. Following filtering (aperture, 100 μm; 160 mesh) and centrifugation (at a speed of 500 x g, at 4˚C for 5 min), the supernatant was discarded. Cells were seeded in culture dishes ($1x10^5$ cells/dish) and grown at 37˚C and 5% CO_2 for 3 weeks in Dulbecco's modified Eagle's medium (DMEM)/F12 medium (Thermo Fisher Scientific, Inc.), containing 15% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.). Culture medium was changed twice weekly. For transfection, cells were plated in the 24-well plates ($3x10^5$ cells/well) and cultured at 37˚C in antibiotic-free DMEM/F12 containing 10% FBS. At 70% confluence, transfection was performed. Plasmid/siRNA/agomiR (1 μg/μl) a nd 1 μl lipo2000 (cat. no. 11668019; Invitrogen; Thermo Fisher Scientific, Inc.) was added to 50 μl OptiMemi medium (cat. no. 31985062; Thermo Fisher Scientific, Inc.). Following 20 min at room temperature, the mixture was added to the cells in the wells and incubated at 37˚C for 6 h. DMEM/F12

was then replaced with fresh medium containing 10% FBS. Following 48 h (at 37˚C), mRNA and protein were isolated from the cells. The cell culture supernatant was kept for further analysis.

Western blot analysis. Cells (*annulus fibrosus* cells) were lysed with lysis (cat. no. P0013B; Beyotime Institute of Biotechnology, Haimen, China). Total protein concentration was determined using a bicinchoninic acid assay (RTP7102; Real-TimesBiotechnology Co., Ltd., Beijing, China). A total of 20 μg protein was separated on 10% SDS-PAGE gels and transferred onto a polyvinylidene dilfuoride membrane. Membranes were blocked with 5% non-fat milk at room temperature for 1 h and then incubated with rabbit anti-human anti-MMP13 (dilution, 1:1,000; ab39012; Abcam, Cambridge, MA, USA), rabbit anti-human anti-type II collagen (dilution, 1:1,000; ab34712; Abcam) and rabbit anti-human anti-β-actin (dilution, 1:5,000; ab129348; Abcam) primary antibodies at 4˚C overnight. Membranes were then incubated with goat anti-rabbit horseradish peroxidase-conjugated polyclonal secondary antibody (dilution, 1:3,000; ab6721; Abcam) at room temperature for 1 h. Protein was visualized using an enhanced chemiluminescence kit (ab65623; Abcam) and protein bands were imaged and analyzed using Image Lab software (Version 3.0; Bio-Rad Laboratories, Inc.). β-actin was used as control. Molecular weights were as follows: MMP-13, 60 kDa; type II collagen, 142 kDa; and β-actin, 43 kDa.

Dual-luciferase reporter assay. Wild-type and mutant MMP13 seed regions for miR-155 were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China) with *Spe*I and *Hind*III restriction sites. Wild-type and mutant DNA fragments were cloned into the pMIR-REPORT luciferase reporter plasmids (Ambion; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Mutant 3'-untranslated region (UTR) served as control (NC, normal control group, transfected with the blank plasmid; wild-type, the group containing the wild-type fragment; and mutant, the group containing the mutant fragment; All groups were stimulated with agomir-155). A total of 0.8 μg plasmid containing wild-type and mutant 3'-UTR were transfected into the 293 cells (Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China) with the Lipofectamine® 2000 (cat. no. 11668019; Invitrogen; Thermo Fisher Scientific, Inc.) for 24 h, followed by a transfection with 100 nM agomiR-155 (5'-UUAAUGCUAAUCGUGAUAGGGGUU-3'; Sangon Biotech Co., Ltd.) for 24 h. Cells were lysed and luciferase activity was detected using the Dual-Luciferase Reporter® kit (cat. no. E1980; PromegaCorporation, Madison, WI, USA) on the GloMax 20/20 luminometer. *Renilla* was used as internal reference.

ELISA. MMP13 levels in cell culture supernatants were detected using an MMP13 ELISA kit (ab100605; Abcam) according to the manufacturer's instructions. Cell culture supernatants were collected by centrifugation (500 x g at 4˚C for 10 min) and 10 μl sample was added to the wells of the 96-well plate, followed by the addition of 40 μl dilution solution (as provided by the kit). Standards at indicated concentrations (50 μl; provided by the kit) were added to the standard wells. With an exception of the blank well, 100 μl horseradish peroxidase-conjugated detection antibody (provided by the kit) was added into the standard and sample wells. The plate was sealed and incubated at 37˚C for 1 h. Following washing, substrates A and B (50 μl each) were added to each well and the plate was incubated at 37˚C for 15 min. A total of 50 μl stop solution was added into each well and the OD at 450 nm was detected within 15 min at 37˚C, using the Thermo Fisher Multiskan FC microplate reader (Thermo Fisher Scientific, Inc).

Statistical analysis. Data are presented as mean ± standard deviation. SPSS 18.0 software (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. Following a normality test, one-way analysis of variance was performed for multiple comparisons, followed by a least significant difference or a Student-Newman-Keuls post-hoc test for the homogenous variance, or a Tamhane's T2 or Dunnett's T3 post-hoc test for heterogeneous variance. $P<0.05$ was considered to indicate a statistically significant difference.

Results

MMP13 expression in patients with spinal tuberculosis. MMP13 mRNA and protein expression in the intervertebral disc of patients with spinal tuberculosis were first investigated by RT-qPCR and western blot analysis, respectively. Results suggested that, compared with the control group, MMP13 mRNA and protein expression in the intervertebral disc were significantly increased in patients with spinal tuberculosis ($P<0.01$; Fig. 1). To investigate MMP13 mRNA and protein levels in the serum of patients with spinal tuberculosis, RT-qPCR and ELISA were performed, respectively. The results suggested that, compared with the control group, serum MMP13 mRNA and protein levels were significantly increased in patients with spinal tuberculosis ($P<0.05$; Fig. 2). These results demonstrated that MMP13 may serve a regulatory role in the pathogenesis of spinal tuberculosis-induced intervertebral disc destruction.

miR-155 expression in patients with spinal tuberculosis. Based on the bioinformatics analysis, miR-155 was predicted as an upstream regulator for MMP13 (Fig. 3). To investigate miR-155 expression in the intervertebral disc and levels in the serum in patients with spinal tuberculosis, RT-qPCR was performed. The results suggested that compared with the control group, miR-155 expression in all samples was significantly decreased in patients with spinal tuberculosis ($P<0.05$; Fig. 4). Combined with the data on MMP13 expression in patients with spinal tuberculosis, the results suggested that miR-155 may serve a regulatory role in the spinal tuberculosis-induced intervertebral disc destruction and potentially negatively regulates the transcription levels of the target gene MMP13.

miR-155 and MMP13 interaction. To confirm interactions between miR-155 and MMP13, a dual-luciferase reporter assay was performed. The results suggested that compared with the negative control (NC) group (transfected with the blank control), luciferase activity was significantly reduced for cells cotransfected with agomiR-155 and pMIR-REPORT plasmids ($P<0.01$), whereas no significant difference in luciferase activity was observed in the mutant group (Fig. 5). The results

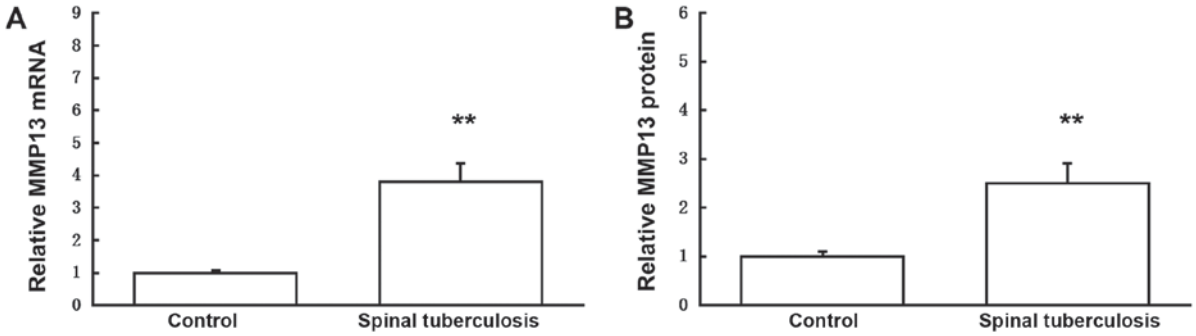

Figure 1. MMP13 expression in the intervertebral disc of patients with spinal tuberculosis. MMP13 (A) mRNA and (B) protein expression were detected using reverse transcription-quantitative polymerase chain reaction and western blot analysis, respectively. β-actin was used as a control. **P<0.01 vs. the control group. MMP13, matrix metalloproteinase 13.

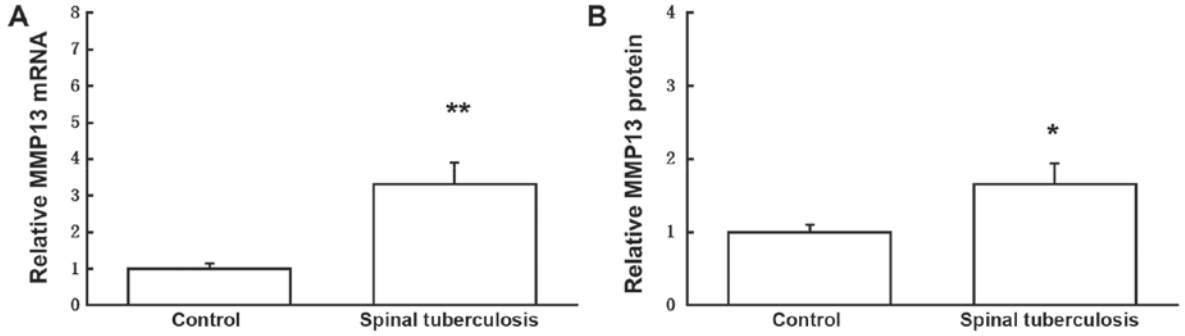

Figure 2. MMP13 serum levels in patients with spinal tuberculosis. MMP13 (A) mRNA and (B) protein levels in the serum of patients with spinal tuberculosis were detected using reverse transcription-quantitative polymerase chain reaction and western blot analysis, respectively. β-actin was used as a control. *P<0.05 and **P<0.01 vs. the control group. MMP13, matrix metalloproteinase 13.

suggested that miR-155 may directly interact with MMP13 to regulate gene expression.

Effects of agomiR-155 on primary annulus fibrosus cells. It has been demonstrated that agomir-155 significantly upregualtes miR-155 levels in cells (18). To further investigate interactions between miR-155 and MMP13, the effects of agomiR-155 transfection on primarily cultured annulus fibrosus cells were analyzed. The results suggested that following transfection with agomiR-155, MMP13 protein expression in the fibroblasts and MMP13 levels in the cell culture supernatant were significantly reduced compared with the NC (P<0.01; Fig. 6). Corresponding expression levels of type II collagen were significantly upregulated compared with the NC (P<0.05; Fig. 6C). The results suggested that upregulated miR-155 expression may regulate MMP13 and affect type II collagen expression.

Discussion

In the current study, MMP13 expression in patients with spinal tuberculosis-induced intervertebral disc was investigated and expression levels of miR-155, an upstream regulator, were analyzed. The biological functions of miR-155 and MMP13 were studied *in vitro*. The molecular mechanism through which miR-155 may regulate protein expression affecting patients with spinal tuberculosis-induced intervertebral disc destruction was also analyzed. Herein, a negative regulatory association between miR-155 and MMP-13 expression was proposed for the first time. Following the reduced miR-155 expression in the spinal tuberculosis tissue, the regulation of MMP13 expression was reduced, resulting in changes in type II collagen expression.

The majority of the ECM of the NP is composed of water, type II collagen (20%) and proteoglycan (50%, mainly aggrecan) (11). Other minor components include types III, IV, IX and XI collagen and small proteoglycans (11). The majority of the intervertebral disc is formed of ECM, including a variety of collagen. At ≥10 years, majority of cells disappear and adult NP cells represent a type of cartilage-like cells (19). Chondrocytes are characterized by the expression of type II collagen (19). Type II collagen is one of the most important collagen components in the intervertebral disc, also known as cartilage collagen, present mainly in the cartilage and disc nucleus (20). Sive *et al* (21) described *in situ* hybridization experiments that confirmed an increasing degree of degeneration associated with decreasing type II collagen expression in NP cells.

Under physiological and pathological conditions, MMPs participate in various biological processes, including embryonic and neovascular formation, wound healing, and pathogenesis of cardiovascular diseases and cancers (21). ECMs account for ≥95% of the intervertebral disc tissue, whereas other tissues rarely contain ECM levels this high (7). Due to the structural characteristics of the intervertebral disc, MMPs may serve an important role in the pathogenesis and

Figure 3. Bioinformatics analysis predicts miR155 as an upstream regulator of MMP13. MMP13, matrix metalloproteinase 13; miR, microRNA.

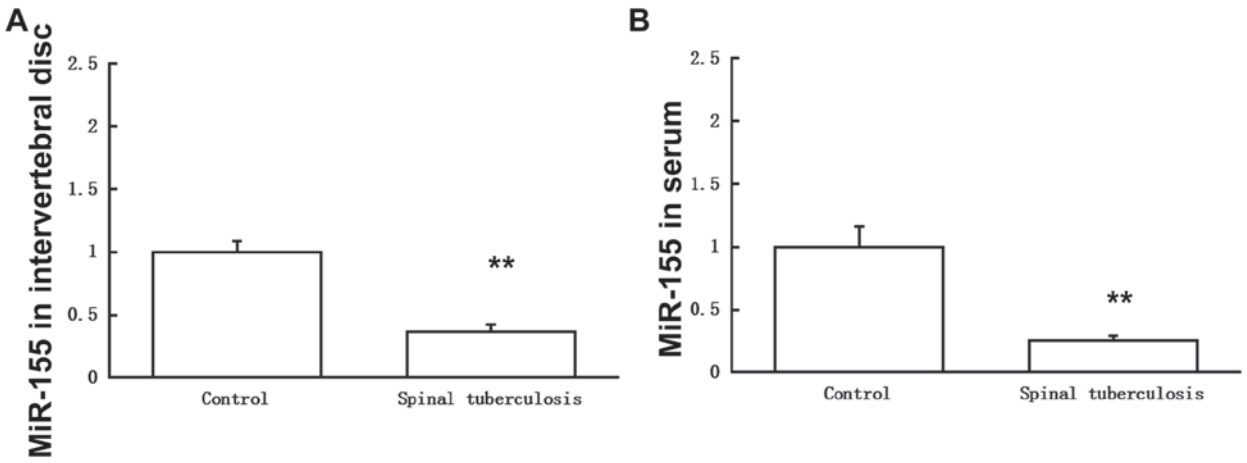

Figure 4. miR-155 expression in patients with spinal tuberculosis. miR-155 levels in (A) the intervertebral disc and (B) the serum of patients with spinal tuberculosis were detected using reverse transcription-quantitative polymerase chain reaction. U6 was used as a control. **P<0.01 vs. the control group. miR, microRNA.

development of spinal tuberculosis-induced intervertebral disc destruction (7). It has been suggested that MMP13 is expressed in the intervertebral disc in animals and humans (22,23). Anderson *et al* (24) established a rabbit model for intervertebral disc degeneration by damaging the annulus fibrosus and reported that MMP13 levels in the intervertebral disc are elevated (3-4 times). Roberts *et al* (25) compared patients with degenerated and normal intervertebral disc using immunohistochemical methods and demonstrated that compared with patients with normal intervertebral disc, MMP13 is significantly increased in patients with degenerated disc. In the current study, intervertebral disc lesions in patients with spinal tuberculosis were investigated and, in accordance with the previous findings, the results suggested that MMP13 expression was significantly upregulated in tissues of patients with spinal tuberculosis-induced intervertebral disc destruction, suggesting that MMP13 may serve an important role in disease pathogenesis.

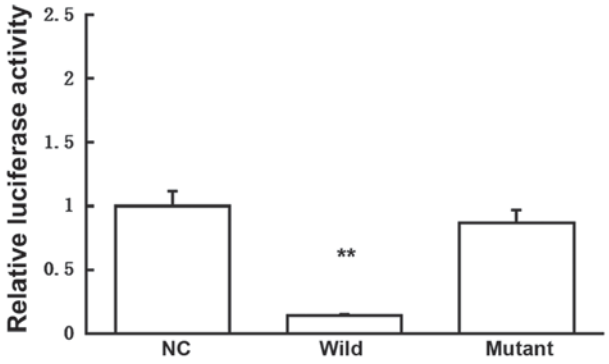

Figure 5. Analysis of the interaction between miR-155 and MMP13. Dual-luciferase reporter assay confirmed an interaction between miR-155 and MMP13 (NC, normal control group; wild, wild-type fragment; mutant, mutant fragment). **P<0.01 vs. NC. miR, microRNA; MMP13, matrix metalloproteinase 13; NC, negative control.

Regulation of mRNA transcription and expression is a complex process involving multiple factors. To investigate MMP13 upstream regulators, the present study focused on recently discovered endogenous, small, non-coding miRNAs, which exerted negative regulatory roles on target mRNAs to inhibit gene translation (26-28). These miRNAs are important regulators in normal development, physiological and pathological processes (29,30). Additionally, several miRNAs have been recognized as the biomarkers for various diseases (29,30). Based on a bioinformatics analysis, an upstream regulator of MMP13 was predicted and the results suggested miR-155. Wang *et al* (31) have reported that downregulated miR-155 expression may be the underlying mechanism through which Fas mediates cell apoptosis in degenerated NP cells. In addition, it has been demonstrated that in mouse models of liver cancer, miR-155 expression and nuclear factor-kB activity are significantly elevated (32). In estrogen-receptor positive breast cancer cells, miR-155 has been revealed to activate the mitogen-activated protein kinase signaling pathway to regulate transcription levels of cytokines (33). Additionally, miR-155 has been demonstrated to inhibit mothers against decapentaplegic homolog 2 expression to influence cellular responses to transforming growth factor-β (34). In the current study, the results suggested that miR-155 expression was significantly downregulated in patients with spinal tuberculosis-induced intervertebral disc destruction

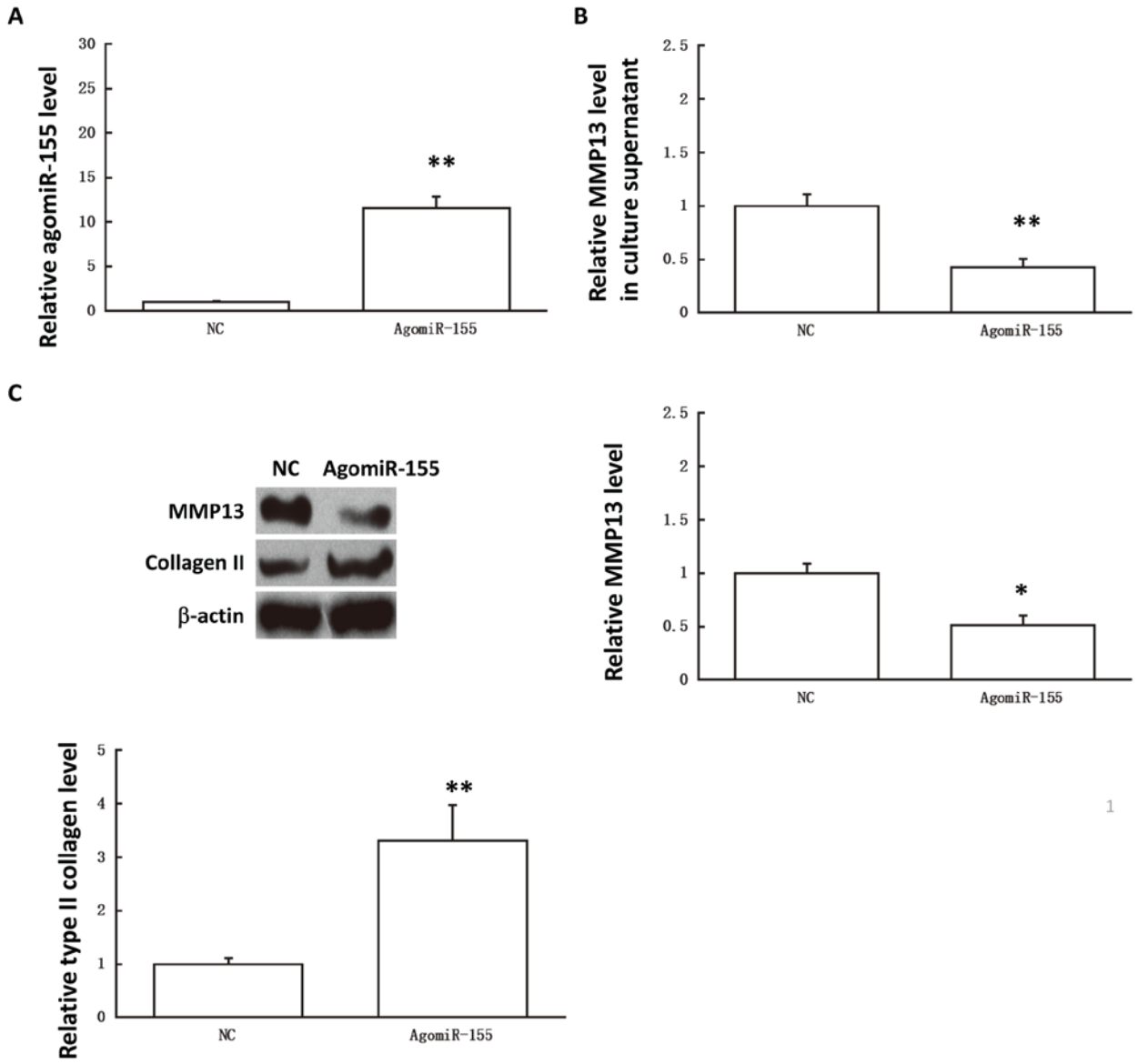

Figure 6. Effects of agomiR-155 transfection on primary annulus fibrosus cells. (A) Levels of miR-155 in primary annulus fibrosus cells transfected with agomiR-155 and an NC with reverse transcription quantitative polymerase chain reaction. (B) MMP13 levels in culture supernatant detected using ELISA. (C) MMP13 and type II collagen protein expression detected using western blot analysis. *P<0.05 and **P<0.01 vs. NC. miR, microRNA; MMP13, matrix metalloproteinase 13; NC, negative control.

compared with the normal control group. Due to various roles of miR-155 in regulating the body immune system, inflammation and cellular apoptosis (15), it is speculated that in intervertebral disc destruction influenced by spinal tuberculosis, miR-155 may affect the release and biological function of MMP13 in the intervertebral disc. Results from the dual-luciferase reporter assay suggested that MMP13 was a direct target for miR-155. Additionally, in the primary culture of annulus fibrosus cells, which were transfected with agomiR-155, MMP13 levels in the cells and cultured supernatant were significantly reduced, whereas type II collagen expression was significantly elevated. These results suggested that miR-155 negatively regulates MMP13 expression to influence the intracellular expression of type II collagen and further inducing lesions in intervertebral discs.

A limitation of the present study is the limited sample size and it should only be regarded as a preliminary study. Further in-depth analyses are required to address the roles of further MMPs, including MMP-8 and MMP-9. Furthermore, a majority of the current study focused on the regulatory association between miR-155 and MMP-13, whereas tissue inhibitors of metalloproteinases were not investigated, which should be considered as a focus of further studies. In addition, MMP-13 and/or miR-155 expression may be studied in patients with spondylitis to complete the presented conclusions.

In conclusion, the results suggested that there was negative association between miR-155 and MMP13. In patients with spinal tuberculosis-induced intervertebral disc destruction, miR-155 expression was decreased, affecting its target MMP13 by downregulating expression and further influencing type

II collagen expression. These findings may contribute to the understanding of the roles of miR-155 and MMP13 in spinal tuberculosis-induced intervertebral disc destruction.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

CY designed the current study, performed the experiments, and prepared the manuscript. ZS, JH, RW, GY, and DZ contributed to the data collection and analysis, and manuscript preparation. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Written and informed consent was obtained from every patient and the study was approved by the local Ethics Review Board of the First Affiliated Hospital of Guangxi Medical University (Nanning, China).

Patient consent for publication

Written and informed consent was obtained from every patient.

Competing interests

The authors declare that they have no competing interests.

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