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Research paper

Dysregulated miR-127-5p contributes to type II collagen degradation by targeting matrix metalloproteinase-13 in human intervertebral disc degeneration

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ABSTRACT

Background: Intervertebral disc degeneration (IDD) is a chronic disease associated with the degradation of extracellular matrix (ECM). Matrix metalloproteinase (MMP)-13 is a major enzyme that mediates the degradation of ECM components. MMP-13 has been predicted to be a potential target of miR-127-5p. However, the exact function of miR-127-5p in IDD is still unclear.
Objective: We designed this study to evaluate the correlation between miR-127-5p level and the degeneration of human intervertebral discs and explore the potential mechanisms.
Methods: miR-127-5p levels and MMP-13 mRNA levels were detected by quantitative real-time polymerase chain reaction (qPCR). To determine whether MMP-13 is a target of miR-127-5p, dual luciferase reporter assays were performed. miR-127-5p mimic and miR-127-5p inhibitor were used to overexpress or downregulate miR-127-5p expression in human NP cells, respectively. Small interfering RNA (siRNA) was used to knock down MMP-13 expression in human NP cells. Type II collagen expression in human NP cells was detected by qPCR, western blotting, and immunofluorescence staining.
Results: We confirmed that miR-127-5p was significantly downregulated in nucleus pulposus (NP) tissue of degenerative discs and its expression was inversely correlated with MMP-13 mRNA levels. We reveal that MMP-13 may act as a target of miR-127-5p. Expression of miR-127-5p was inversely correlated with type II collagen expression in human NP cells. Moreover, suppression of MMP-13 expression by siRNA blocked downstream signaling and increased type II collagen expression.
Conclusion: Dysregulated miR-127-5p contributed to the degradation of type II collagen by targeting MMP-13 in human IDD. Our findings highlight that miR-127-5p may serve as a new therapeutic target in IDD.

1. Introduction

Intervertebral disc degeneration (IDD) is a very common degenerative disease affecting over 90% of individuals aged over 50 years, and is usually associated with low back pain (LBP) [1,2]; in addition, over 90% of all spinal surgical procedures performed are related to IDD [3]. IDD is associated with an imbalance of the metabolism of the extracellular matrix (ECM), which is mediated by matrix metalloproteinases (MMPs), such as MMP-13 [4–9].

MicroRNAs (miRNAs) were proven to be critical molecules in the pathogenesis of IDD [10–16]. By binding to the 3′-untranslated region (3′-UTR) of mRNAs, miRNAs play roles in regulating the expression of the target gene [17]. Various biological processes, including cell proliferation, differentiation, and apoptosis, as well as inflammatory diseases and organ development, were demonstrated to be associated with miRNAs [17–21]. miR-127-5p has

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been studied in liver cancer [22,23] and osteoarthritis [24,25].

IDD is characterized by a loss of ECM, particularly type II collagen and aggrecan [6,9,26–28]. Various miRNAs are involved in the proliferation and apoptosis of nucleus pulposus (NP) cells, and the degeneration of NP tissues [10,11,14,15,29–32]. A recent microarray analysis showed that miR-127-5p is substantially downregulated in NP tissues of patients with IDD compared with healthy human NP tissues [15]; however, the specific functions and regulatory mechanisms of miR-127-5p in NP tissues are unclear.

MMP-13 has been predicted to be a potential target of miR-127-5p. Therefore, in this study, we evaluated whether miR-127-5p could downregulate type II collagen expression by targeting MMP-13, thereby contributing to IDD.

2. Materials and methods

2.1. Sample collection

All participants enrolled in this study were in-patients of Union Hospital, Tongji Medical College, Huazhong University of Science and Technology. The study was approved by the institutional ethics review board of Tongji Medical College, with written informed consent obtained from each participant.

Between January 2016 and June 2016, 20 degenerative NP tissue specimens were collected from 20 patients with IDD undergoing lumbar discectomy (mean age, 52.9 ± 14.5 years; range, 30–73 years). Additionally, 10 relatively normal NP tissue specimens were obtained from 10 patients with idiopathic scoliosis undergoing spinal fusion (mean age, 22.6 ± 11.3 years; range, 11–47 years) as controls.

Magnetic resonance imaging (MRI) scanning of the lumbar spine was performed routinely prior to surgery to determine the grade of disc degeneration based on T2-weighted images, as described by Pfirrmann et al. [33]. Relative normal discs (10 discs) from patients with idiopathic scoliosis were classified as grade II, and degenerative discs from patients with IDD were classified as grade III (10 discs), IV (5 discs), or V (5 discs). 5 NP tissue specimens of grade III were isolated and cultured for the subsequent experiments, and all the other tissue specimens were used for RNA isolation for the subsequent experiments.

2.2. Cell culture

After washing in phosphate-buffered saline (PBS), the NP tissue specimens were minced into pieces and then enzymatically digested with 0.2% collagenase II (Sigma, USA) and 0.25% trypsin (Gibco, USA) for 3 h. The NP cells were filtered and washed in PBS, then resuspended in DMEM/F12 (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA), 100 μg/mL streptomycin, and 100 U/mL penicillin. Cells were incubated in a 5% CO_2 (vol/vol) environment at 37 °C, and were passaged twice or three times for use in subsequent experiments.

2.3. Cell transfection

miR-127-5p mimic and miR-127-5p inhibitor (GenePharma, China) were used to overexpress or downregulate miR-127-5p expression in NP cells. Mimic control and inhibitor control (GenePharma, China) were transfected as controls. NP cells were transfected with siMMP-13 (Biofavor, China) to knock down MMP-13 expression. All cell transfection assays were performed with Lipofectamine 2000 (Invitrogen, USA), according to the protocol of the manufacturer. Three replicate transfections were performed.

Table 1
List of primers used in quantitative real-time polymerase chain reaction (qPCR).

Name	Primer	Sequence (5′–3′)	Product size (bp)
Type II collagen	Forward	AGAACTGGTGGAGCAGCAAGA	142
	Reverse	AGCAGGCGTAGGAAGGTCAT	
MMP-13	Forward	CCCAACCCTAAACATCCAA	147
	Reverse	AAACAGCTCCGCATCAACC	
GAPDH	Forward	TCAAGAAGGTGGTGAAGCAGG	115
	Reverse	TCAAAGGTGGAGGAGTGGGT	

2.4. Dual luciferase reporter assays

An MMP-13 3′-UTR-Luc vector (Genscript, China) with wild-type (WT) or mutant (MUT) binding sites of miR-127-5p was constructed. Vectors were cotransfected with miR-127 mimic/mimic control (GenePharma, China) with Lipofectamine 2000 (Invitrogen, USA). After 48 h, NP cells were harvested and assayed with a dual luciferase reporter assay system (Promega, USA). The luciferase values of firefly and Renilla were compared, and presented as firefly/Renilla luciferase ratios. Three replicate transfections were performed.

2.5. RNA isolation, and quantitative real-time polymerase chain reaction (qPCR)

Total cellular RNA was extracted from cultured NP cells with TRIzol (Invitrogen, USA) and was reverse transcribed into cDNA with TaqMan miRNA Reverse Transcription Kit (Applied Biosystems, USA).

qPCR was performed on a 7900 Real-time system (Applied Biosystems, USA). The reaction mixture contained 4 μL of cDNA, 0.4 μL of forward primer, 0.4 μL of reverse primer, 10 μL of SYBR Green/Fluorescein qPCR Master Mix (2X), and 5.2 μL of H2O, with a total volume of 20 μL. The reaction conditions were as follows: 95 °C for 10 min, followed by 40 cycles at 95 °C for 30 s and 60 °C for 30 s. The comparative Ct ($2^{-\Delta\Delta Ct}$) method was used to determine the relative amounts of transcripts. GAPDH mRNA expression was used to normalize quantification data for other genes. All primers were designed and produced by Tsingke Biotech Co., Ltd. (Beijing, China) and their sequences are shown in Table 1.

2.6. Protein isolation and western blotting

The total cellular proteins were extracted from cultured NP cells with RIPA lysis buffer (Beyotime, China), and the concentration was measured using the BCA protein assay (Beyotime, China). Proteins were separated by 10% SDS-PAGE, and then electrotransferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Germany). After blocking with TBST (10 mM Tris, pH 8.0, 150 mM NaCl, and 0.1% Tween 20) containing 5% nonfat dried milk for 2 h at 25 °C, the membranes were incubated with specific antibodies against type II collagen (1:1000; Abcam, UK), MMP-13 (1:1000; Abcam, UK) or GAPDH (1:1000; Abcam, UK) overnight at 4 °C. After washing in TBST, the membranes were then incubated with HRP-conjugated goat anti-rabbit antibodies (Boster, China), diluted 1:50,000, for 2 h at 37 °C. The protein bands were visualized using an ECL kit (Thermo, USA) and analyzed quantitatively using BandScan software (BioMarin Pharmaceutical Inc., UK). GAPDH was used to normalize the results of other proteins.

2.7. Immunofluorescence staining

NP cells were seeded on coverslips in 24-well plates for culture for 48 h. After removal of the medium and three washes with PBS,