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MiR-21 promotes ECM degradation through inhibiting autophagy via the PTEN/akt/mTOR signaling pathway in human degenerated NP cells

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ABSTRACT

Intervertebral disc degeneration (IDD) is the most common cause leading to low back pain, a highly prevalent, costly and crippling condition worldwide. Overexpression of miR-21 has been shown to promote proliferation of nucleus pulposus (NP) cells. However, it remains unclear whether miR-21 can promote the degradation of type II collagen (Col II) and aggrecan, two main extracellular matrix components within the disc. Here, the miRNA microassay assay identified 29 differentially expressed miRNAs in NP tissues from IDD patients compared with healthy controls. Following qRT-PCR validation, miR-21 expression was significantly upregulated in degenerated NP tissues, and showed a positive correlation with disc degeneration grade. Through gain-of-function and loss-of-function studies in human NP cells, miR-21 was shown to inhibit autophagy and then upregulate the expression of matrix metalloproteinase (MMP)-3 and MMP-9, leading to increased degradation of Col II and aggrecan. Mechanistically, phosphatase and tensin homolog (PTEN) was identified as a direct target of miR-21, and activated PTEN/ Akt/mammalian target of rapamycin (mTOR) signaling pathway was involved in miR-21-induced autophagy inhibition and Col II and aggrecan breakdown. Taken together, these results suggest that miR-21 contributes to Col II and aggrecan catabolism by inhibiting autophagy via the PTEN/Akt/mTOR signaling pathway in human NP cells.

1. Introduction

Low back pain (LBP), a predominant public health problem, severely impacts the quality of life and results in a heavy burden of socioeconomy. Epidemiological studies have demonstrated that approximately 84% of population will encounter LBP at some period in their lifetime. [1]. Despite the etiology of LBP is multifactorial, intervertebral disc (IVD) degeneration (IDD) has been regarded as the most common cause. Both type II collagen (Col II) and aggrecan are the most important components of extracellular matrix (ECM) within the disc. Decreased contents of these two proteins is the major characteristic of IDD. Matrix metalloproteinases (MMPs), a large family including a number of members, are the main enzymes that degrade Col II and aggrecan. Both MMP-3 and MMP-9 belong to the basic members of the MMP family. Recent studies have demonstrated higher expression of both MMPs in degenerated IVD tissue than in healthy controls [2,3]. Current therapies can merely relieve the clinical symptoms of IDD patients and do not target the underlying pathological alternations within the disc. Thus, a comprehensive understanding for regulation of MMP-3 and MMP-9 expression is essential to develop biologically-induced disc repair and improve the prognosis of LBP patients.

MicroRNAs (miRNAs) are a kind of small and noncoding RNA molecules. They negatively regulate gene expression via interaction with the 3'-untranslated region (UTR) of target mRNAs [4]. It is now well established that miRNAs participate in almost all biological processes [5,6]. Studies from our group and others have revealed a close link of dysregulated miRNAs to IDD [7,8]. As one of the well characterized miRNAs, upregulated miR-21 has been frequently reported in the vast majority of tumors, thereby acting as an oncogene. [9,10]. Recently, two studies have reported that transfection of human nucleus pulposus (NP) cells with miR-21 mimic leads to increased proliferation, revealing miR-21 as a potential contributor to IDD [11,12].

Autophagy is thought to be a homeostatic process, in which organelles or cytosolic proteins are encapsulated by autophagosomes and then fuse with lysosomes for degradation, thereby maintaining cellular homeostasis and integrity [13,14]. Recently, it is suggested that the number of autophagosomes in human degenerated NP cells is lower than that in healthy controls [15]. Moreover, autophagy activation has

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been shown to downregulate MMP-3 expression in human and rat NP cells exposed to tumor necrosis factor-α (TNF-α), suggesting a protective role of autophagy in controlling ECM cleavage [16,17]. The activated Akt/mammalian target of rapamycin (mTOR) pathway induces strong inhibition of autophagy [18,19]. As the inhibitor of the Akt/mTOR pathway, phosphatase and tensin homolog (PTEN) is also a direct target of miR-21 [20,21]. It has been demonstrated that miR-21 overexpression protects against autophagy by activating the Akt/mTOR pathway via silencing of PTEN in multiple types of cells, including hepatocellular carcinoma cells [22] and breast cancer cells [23]. However, it remains unclear whether miR-21 also regulates autophagy and ECM metabolism through activation of PTEN/Akt/mTOR pathway in human degenerated NP cells.

In the present study, we found that miR-21 was dramatically elevated in human degenerated NP tissues when compared with healthy controls, and positively correlated with disc degeneration grade. What more, miR-21 promoted Col II and aggrecan loss by inhibiting autophagy via the PTEN/Akt/mTOR signaling pathway in human degenerative NP cells. These data provide an insight into the mechanistic link between miR-21 – mediated autophagy and the process of IDD.

2. Materials and methods

2.1. Patients and NP tissues sources

A total of 65 IVD samples as IDD group were obtained from 65 patients (35 males, 30 females; mean age: 54.6 yr; age range: 45–67 yr) with IDD undergoing discectomy. In addition, sex-matched normal IVD tissue samples as normal control (NC) group were donated by 45 patients (24 males, 21 females; mean age: 20.4 yr; age range: 17–22 yr) with lumbar vertebral fracture (LVF) who underwent anterior discectomy (Fig. 1). All IVD tissue samples were obtained under sterile condition within 30 min during operation. All subjects underwent routine lumbar MRI scan before operation. The grade of IDD was evaluated by T2-weighted images according to the Pfirrmann classification [24]. This research was approved by the Ethics Committee of the First Affiliated Hospital of University of South China. We complied with the Declaration of Helsinki [25]. Moreover, all patients written informed consent before operation.

2.2. Antibodies and reagents

Rabbit polyclonal antibodies against Col II, aggrecan, MMP-3, MMP-9, Beclin-1, LC-3, SQSTM1/p62, PTEN, Akt, phosphorylated Akt (p-Akt) (Ser2448), mTOR, phosphorylated mTOR (p-mTOR) (Ser2448), glyceraldehyde phosphate dehydrogenase (GAPDH) and horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG were obtained from Cell Signaling Technology (CST, USA). MiR-21 mimic/mimic control and its inhibitor/inhibitor control were provided by Ribobio (Guangzhou, China). Rap, 3-MA, Tep, MK-2206 and Tem were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.3. Isolation, culture of human degenerated NP cells

The degenerated NP specimens were washed using phosphate-buffered saline (PBS) twice, followed by cutting into 1 mm^3 of small fragments using an ophthalmic scissor. These fragments were then digested in 0.25% trypsin solution (Sigma, USA) for 30 min, followed by treatment with 0.2% type II collagenase (Sigma, USA) at 37 °C for 4 h. The obtained cells were resuspended in Dulbecco's modified Eagle medium (DMEM)/F12 (Gibco, USA) containing 15% fetal bovine serum (FBS; Biolnd, Israel) and 1% penicillin/streptomycin (Gibco, USA) in a humidified atmosphere with 5% CO_2 at 37 °C. After reaching at least 80% confluence, cells were digested by 0.25% trypsin solution and continue culture. These cells were then collected at the second generation, and were used for all *in vitro* experiments.

2.4. Cell transfection and treatment

The cells were seeded at 1×10^5–1×10^6 cells/well into 6-well dishes before transfection, and then 2 ml of complete tissues culture medium was added to each well. After around 85% confluence, cells were transfected with a final concentration of 20 nM miR-21 mimic/mimic control and 40 nM miR-21 inhibitor/inhibitor control using Lipofectamine 2000 (Invitrogen, USA). Subsequently, Opti-MEM I medium was replaced with a fresh DMEM/F12 medium containing 10% FBS. Cells were incubated for 24 h and then subjected to assays. In addition, to interfere with autophagy and PTEN/Akt/mTOR pathway, cells were treated with Rap (10 nM, autophagy activator), 3-MA (10 mM, autophagy inhibitor), Tep (10 μM, PTEN activator), MK-2206 (10 μM, Akt inhibitor) or Tem (10 μM, mTOR inhibitor) for 6 h before transfection.

2.5. RNA isolation and quantitative real-time PCR (qRT-PCR)

Total RNAs from NP tissues or cells were extracted utilizing TRIzol (Invitrogen, USA) and RNeasy mini kit (Qiagen, USA) according to the manufacturers' instructions. The purity and concentration of RNAs was

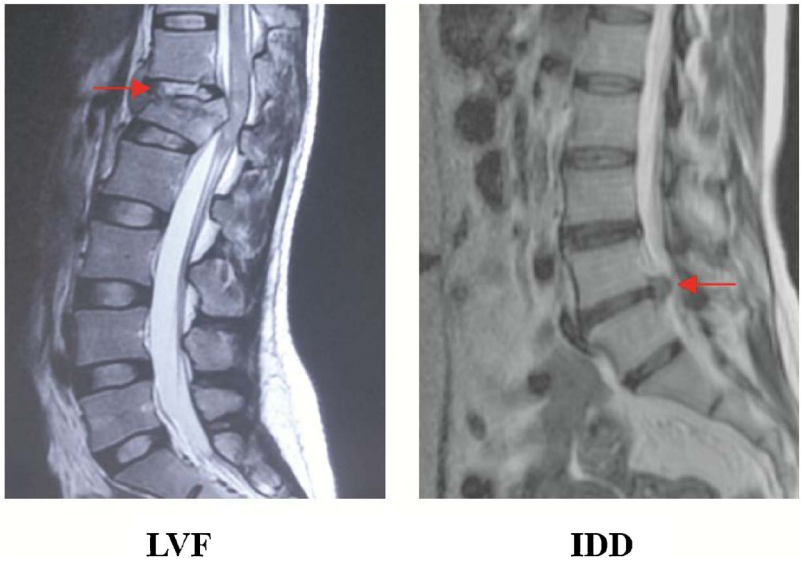

Fig. 1. Representative MRI scan of spine with LVF and IDD. Featured left is a LVF patient undergoing anterior discectomy. Featured right is an IDD patient who underwent discectomy. The red arrow displays the location of impaired IVD.