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Original article

Elevated expression of microRNA-30b in osteoarthritis and its role in ERG regulation of chondrocyte

Lisong Li[a,1], Cao Yang[b,1], Xianzhe Liu[b,1], Shuhua Yang[b], Shunan Ye[b], Jie Jia[b], Wei Liu[b], Yukun Zhang[b,*]

[a] Department of Orthopedics, the First Affiliated Hospital of Soochow University, Suzhou, Jiangsu 215006, China
[b] Department of Orthopedics, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430022, China

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ABSTRACT

ERG (ETS-related gene) belongs to the ETS family of transcription factors, and has been recently reported to contribute to homeostatic balance in skeleton cell plasticity. MicroRNA-30 (miR-30) family is also demonstrated to play a role in controlling chondrocyte differentiation. The current study investigated the miR-30b and ERG expression in articular cartilage of osteoarthritis (OA) patients. A total of 20 subjects, with 10 OA patients and 10 healthy participants, were included in this study. Human chondrosarcoma cell line SW1353 was used to explore the relationship of miR-30b and ERG *in vitro*. In OA patients, a significant increase of miR-30b and a decrease of ERG were observed in articular cartilage compared with Normal ones. MiR-30b mimic down-regulated the ERG mRNA and protein expression levels, while miR-30b inhibitor up-regulated ERG expression. In addition, miR-30b mimic also decreased the mRNA expression of COL2a and aggrecan, while miR-30b inhibitor had the opposite effect. Luciferase reporter assay confirmed that miR-30b targeted ERG. In conclusion, miR-30b was involved in the process of OA, and it probably functioned through its target gene ERG.

1. Introduction

Osteoarthritis (OA) is a chronic and degenerative joint disease and a leading cause of pain and disability in the elderly. Entire synovial joint, encompassing the cartilage, synovium, and underlying bone are involved in the pathophysiology of OA, with characterizing by cartilage degradation, narrowing of joint space, osteophytes formation and remodeling of subchondral bone [1]. The cells in each of these tissues have independent capacities to initiate and respond to injury in the joint, ultimately resulting in degeneration of cartilage. The decreased collagen, type II, alpha 1 (encoded by COL2a1) and degradation of aggrecan are a significant event in early-stage OA [2].

ERG (ETS-related gene) belongs to the ETS family of transcription factors, which is present in a wide variety of functions including the regulation of cellular differentiation, cell cycle control, cell migration, cell proliferation, apoptosis and angiogenesis [3]. The alteration of ETS family members are observed in synovial specimens from OA patients [4]. The emerging evidences have demonstrated that ERG expressed higher in superficial zone of articular cartilage compared with chondrocyte cultures, and contributes to the homeostatic balance in skeleton cell plasticity [5,6]. However, there have been very few studies of ERG in OA, and the potential regulatory mechanism is still unknown.

MicroRNAs (miRNAs) are a type small non-coding RNAs, binding to the 3′-UTR of target genes, to be a critical regulator of gene expression in various physiological processes [7]. The role of miRNAs in chondrogenesis and osteoarthritis was established [8]. miR-30 is commonly reported in cancers [9], glomerular diseases [10] and myocardial hypertrophy [11]. Recently, miR-30 family members have been found to be key negative regulators in osteoblast differentiation [12]. In addition, they also be indicated highly expressed in tracheal chondrocytes and play regulatory roles in controlling chondrocyte differentiation [13]. Our preliminary work showed that ERG may be a potential target gene of miR-30b using the mirSVR predicted target site scoring method. In this study, we investigated the expression levels of miR-30b and ERG in articular cartilage of OA patients and their potential mechanism in chondrosarcoma cells.

* Corresponding author at: Department of Orthopedics, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Jiefang Road, No. 1277, Wuhan 430022, China. Fax: +86 27 85351627.
E-mail address: zhangyukuncom@126.com (Y. Zhang).
[1] These authors contributed equally to this work.

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2. Materials and methods

2.1. Subjects and specimen collection

All the participants included in this study were from Wuhan Union Hospital. This experiment was approved by the ethics committee of the Wuhan Union Hospital of Huazhong University of Science and Technology. The written informed consents were obtained from all subjects. Normal articular cartilage was obtained from individuals undergoing surgical amputation ($n = 10$, 43 ± 16 years, female/male = 4/6), who has no history of joint disease, and OA articular cartilage from patients undergoing total knee arthroplasty ($n = 10$, 57 ± 18 years, female/male = 2/8). The diagnostic criteria of OA was according to American College of Rheumatology Subcommittee on Osteoarthritis Guidelines [14].

2.2. Cell culture

Human chondrosarcoma cell line SW1353 was purchased from ATCC (American Type Culture Collection, USA). SW1353 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM), which supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin at 37 °C in humidified air with 5% CO_2. Subconfulent cells were passaged using 0.25% trypsin-EDTA.

2.3. RNA isolation and real-time PCR

The total RNA from articular cartilage or SW1353 cells was extracted by TRIzol method with TRIzol reagent (Invitrogen, USA) according to the protocol of manufacture. After the detection of RNA concentration using spectrophotometry, RNA was reverse transcribed with a PrimeScript™ RT Master Mix (TakaRa, China) according to manufacturer's instructions. Real-time PCR was performed to quantify the miR-30b, ERG, COL2 and aggrecan expression levels. All the primers were designed and produced by Shanghai Generay Biotech Co., Ltd. (Shanghai, China). The real-time PCR was conducted using One Step SYBR® PrimeScript™ RT-PCR Kit (TakaRa, China). U6 served as the reference gene to normalize the expression of miR-30b, and β-actin served as the reference gene to normalize the expression of ERG, COL2 and aggrecan. The relative expression levels of target genes were calculated by the $2^{-\Delta\Delta Ct}$ method.

2.4. Transfection

MiR-30b mimic and miR-30b inhibitor were used to over-express and down-regulate miR-30b expression of cells, respectively. MiR-30b mimic, miR-30b inhibitor and their negative control (NC) were produced by Shanghai GenePharma Co., Ltd. (Shanghai, China). The mimic, inhibitor and NC were transfected into cells using Lipofectamine2000 (Invitrogen, USA) according to the protocol of manufacture.

2.5. Western blot

For Western blot analysis of protein expression, cultured SW1353 were lysed with RIPA buffer (Sigma–Aldrich, USA) on ice, and protein concentrations of supernatant were measured by the Enhanced BCA Protein Assay Kit (Beyotime, China). Equal amounts of protein were separated with 10% SDS-PAGE and transferred to PVDF membranes (Millipore, Germany). After blocking with TBST containing 5% non-fat milk, the membranes were incubated with primary antibodies against mouse ERG (1:1500, Cell Signaling Technologies, USA), aggrecan (1:1000, Cell Signaling Technologies, USA), Col2a (1:1500, Cell Signaling Technologies, USA) and β-actin (1:1000; Cell Signaling Technologies, USA) overnight at 4 °C. Secondary antibodies conjugated to horseradish peroxidase (HRP) were then co-incubated with membranes at room temperature for 1 h. The signals were visualized using the enhanced chemiluminescent (ECL) substrate.

2.6. Luciferase reporter assay

The ERG was predicted to be a potential target of miR-30b using the microRNA.org database (http://www.microrna.org/microrna/home.do). The results is shown in Fig. 3. Luciferase reporter assay was used to evaluate this prediction. In brief, 3′-UTR fragments of ERG were amplified by PCR method and inserted downstream of the pGL3 promoter (RiboBio Co., Ltd, China). The constructs were co-transfected with a luciferase normalization control into SW1353 cells using Lipofectamine2000 (Invitrogen, USA) according to the protocol of manufacture. After 24 h, cell lysates were prepared and subjected to luciferase assays using the Dual-Luciferase® Reporter Assay System (Promega, USA).

2.7. Immunofluorescent staining

Type II collagen is a critical component for cartilage structure. Immunofluorescence was used to determine protein type II collagen in SW1353 cells with the treatment of miR-30b mimic or miR-30b inhibitor. After the transfection of mimic or inhibitor, the cells were harvested and washed with PBS for three times, and blocked with 3% BSA for 1 h. Then cells were incubated with a mouse monoclonal anti-type II collagen (1:50, Cell Signaling Technologies, USA) overnight at 4 °C. After the incubation, the samples were washed with PBS for three times. Alexa Fluor

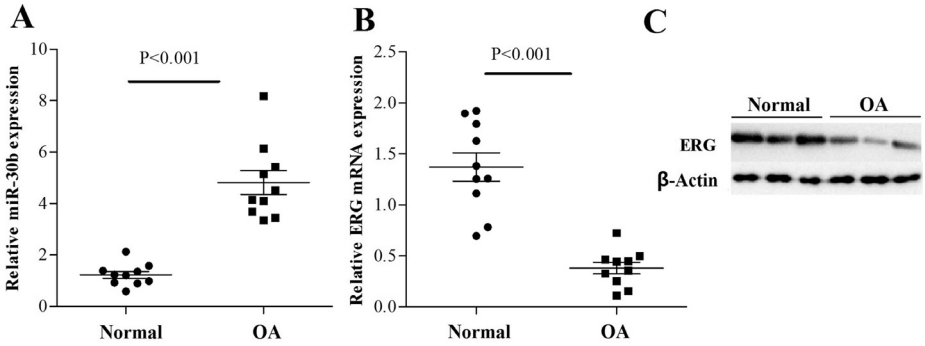

Fig. 1. miR-30b level was elevated and ERG levels were decreased in articular cartilage of osteoarthritis (OA) patients. The scatter plots represent Means with SD. The expression levels of miR-30b (A), ERG mRNA (B) and ERG protein (C) were determined.