European Review for Medical and Pharmacological Sciences 2018; 22: 2966-2972

LncRNA FAS-AS1 promotes the degradation of extracellular matrix of cartilage in osteoarthritis

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Abstract. – OBJECTIVE: To investigate the expression of long non-coding RNA (lncRNA) FAS-AS1 in osteoarthritis cartilage and to explore its effect on articular cartilage cells.

PATIENTS AND METHODS: A total of 20 tissue samples of primary knee joint osteoarthritis and 20 tissue samples of knee joint cartilage after traumatic amputation were collected. Fluorescence quantitative polymerase chain reaction (PCR) was performed to detect the expression of FAS-AS1, MMP1, MMP13, and COL2A1 in cartilage. FAS-AS1 small interfering RNA (siRNA) was transfected to chondrocytes transiently to observe its effects on proliferation, apoptosis of chondrocytes, and the expressions of MMP1, MMP13, and COL2A1.

RESULTS: The expressions of FAS-AS1, MMP1, and MMP13 in osteoarthritis tissues increased significantly, while COL2A1 presented a low expression. Reducing the expression of FAS-AS1 inhibited cell apoptosis and promote cell proliferation. Additionally, *in vitro* experiments showed that low expression of FAS-AS1 decreased the expressions of MMP1 and MMP13, but increased the expression of COL2A1.

CONCLUSIONS: The expression of FAS-AS1 was increased in osteoarthritis, and FAS-AS1 could be involved in the development of the disease by regulating the proliferation, apoptosis of chondrocytes and promoting the degradation of extracellular matrix.

Key Words:
LncRNA, Cartilage, Osteoarthritis, Extracellular matrix.

Introduction

Osteoarthritis (OA) is a chronic degenerative joint disease, the incidence of which may increase with age, and it's also a common joint disease in the middle-aged and elderly people. It has been reported[1-3] that OA is characterized by insufficiency of cartilage matrix synthesis, degeneration of the articular cartilage, formation of the osteophyte, and synovitis. The pathogenesis of OA involves multiple physiological and pathological processes of the body, including the dysfunction of articular cartilage anabolism, catabolism and the degradation of extracellular matrix, which are key factors in promoting the development of OA. However, the exact underlying mechanism of OA is still not clear[4].

Long non-coding RNAs (lncRNA) are defined as transcripts with a length between 200-100000 nucleotides and its biological origin is extensive. Unlike short non-coding RNA, lncRNA is less conserved in the primary sequence, while is more conserved in the secondary and tertiary structures[5-7]. Scholars have shown that epigenetic modification of lncRNA plays an essential role in OA. For example, Kim et al[8] found that the expression of HOTTIP in the articular cartilage of OA is up-regulated while the expression of HoxA13 is downregulated, which inhibits the synthesis of integrin α1 and results in the destruction of articular cartilage. Su et al[9] revealed that when compared with normal cartilage tissue, the expression of lncRNA MEG3 in the articular cartilage of OA is significantly downregulated.

FAS-ASl, as an antisense lncRNA located on the antisense strand of intron 1 of Fas gene, is high expressed in tissues such as the heart, placenta, liver, muscles and the pancreas in normal adults. However, FAS-ASl is low expressed in kidney, and even not expressed in the brain and lung tissue[10]. The expression of FAS-ASl in peripheral blood and lymphoma cells in B cell lymphoma patients is significantly lower than that in normal people. Moreover, the low expression of FAS-ASl in B cell lymphoma is negatively correlated with the mRNA expression of the soluble receptor (sFas) of the host gene Fas[11]. However, few studies investigated the expression of FAS-ASl and its effect on extracellular matrix in OA.

Therefore, the primary purpose of this study was to evaluate the expression of FAS-ASl in OA and its effect on extracellular matrix.

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Patients and Methods

Clinical Information

A total of 20 cartilaginous tissue samples of primary knee osteoarthritis patients who underwent joint replacement and 20 cartilaginous tissue samples of the knee joint of patients with posttraumatic amputation were collected from January 2013 to June 2017 in the Department of Orthopedics. All the specimens were approved by the Ethics Committee of First People's Hospital of Changzhou, and the informed consent of all patients was obtained. 20 patients with primary knee osteoarthritis included 12 males and 8 females, whose average age was 52 years (38-73 years); and 20 patients after traumatic amputation included 16 males and 4 females, whose average age was 43 years (25-57 years).

Total RNA Extraction and Fluorescence Quantitative Polymerase Chain Reaction (PCR)

We used TRIzol kit (Invitrogen, Carlsbad, CA, USA) to extract total RNA of the lung cancer tissues and the corresponding normal tissues, and an ultraviolet spectrophotometer was used to detect the concentration of RNA. According to the instruction of PrimeScript™ RT Master Mix (Eurofins MWG Operon, Ebersberg, Germany) kit to synthesize complementary Deoxyribose Nucleic Acid (cDNA) with the following reaction conditions: 40°C for 6 min and 65°C for 25 min, and the cDNA products were stored at -80°C for fluorescence quantitative PCR. The reaction system of quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) was 20 μL, including 2 μL of the cDNA product, 0.4 μL of the 50 × ROX reference dye, 10 μL of the SYBR qPCR Mix (TaKaRa, Otsu, Shiga, Japan), 0.8 μL of the upstream primer and 0.8 μL of the downstream primer, with additional RNase water to 20 μL. The reaction conditions were: first pre-denaturation at 95°C for 1 min, then 95°C for 30s and 60°C for 40s, totally 40 cycles. 3 separate wells were performed at each experiment, and all the samples were repeated for 3 times. A relative quantitative method was used and the relative expression of the target genes was represented by -ΔCt and $2^{-\Delta\Delta Ct}$. All the operations should be performed on ice. The primer sequence was shown in Table I.

Cell Culture

The knee joint was cut off under sterile condition for collecting articular cartilage. Then, the collected tissue was cut into segments with 1-2 mm^3 in size. At room temperature, 0.2% trypsin and 0.2% II collagenase were used to digest for 30 min and 2 h, respectively. Finally, the acquired cells were cultured in Dulbecco's modified Eagle medium (DMEM)/F12 solution, with 10% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin added (Gibco, Rockville, MD, USA). Primary cell monolayer culture was performed.

Cell Transfection

The cells were seeded into the 6-well plates, cultured for 24 h, and then, transfected when the cell density reached 70%. The FAS-AS1 interference group (si-FAS-AS1) and the negative transfection group (si-Control) were established, respectively. After mixing Lipofecamine™2000 (Invitrogen, Carlsbad, CA, USA) with si-FAS-AS1 or si-Control (the final concentration was 100 nmol/L), the mixture was added to the cells and incubated for 6 h, and then, the complete medium was used for culture for 24 h, after which the cells were collected for subsequent experiments.

IL-1 Treatment

The cells were seeded into the 6-well plates and incubated in serum-free medium for 24h, then 10 ng/mL IL-1 was added, and the cells were collected for experiments after incubation for 0, 2, 4, 6, 12 h.

Cell Proliferation Assay

After transfection, the cells were collected and reseeded into the 96-well plates at 1,000 per well

Table I. Primers used in this study.

Gene	Sense primer	Antisense primer
COL2A1	CGAGGCAGACAGTACCTTGA	TGCTCTCGATCTGGTTGTTC
MMP13	TGAGAGTCATGCCAACAAATTC	CAGCCACGCATAGTCATGTAGA
MMP1	AAAATTACACGCCAGATTTGCC	GTTGCTGCTTGCAGTAACCTT
FAS-AS1	GGTGTGACATTACTCCAGAGTTG	AGGGCCAAGTCCAACTCCTT
GAPDH	TCCATGACAACTTTGGTATCG	TGTAGCCAAATTCGTTGTCA

and cultured for 0, 24, 48, 72h. Cell counting kit-8 (CCK-8, TaKaRa, Otsu, Shiga, Japan) solution was added into each well at the final concentration of 10% and incubated for 1h without light. Then, the microplate reader was used to measure the absorbance (OD) values at 450 wavelengths.

Flow Cytometry Analyses of Apoptosis

The cells in the logarithmic growth period were collected and digested. The cells were washed twice with pre-cooled phosphate-buffered saline (PBS), and 1 mL of combined buffer solution was used for resuspension to make the density of the cells at 1×10^6/mL. 100 μL of cell suspension was added into a 5 mL flow tube, followed by adding 10 μL of Annexin-V-FITC staining and 10 μL Propidium Iodide (PI) staining. Then, they were incubated at room temperature for 15 min without exposure to light. Subsequently, the rate of apoptosis was analyzed by flow cytometry. All the assays were performed for three independent experiments.

Western Blotting

The total protein of the transfected cells was extracted and the concentration of the protein was determined by a bicinchoninic acid (BCA) kit (Thermo Fisher Scientific, Waltham, MA, USA). 20 μg of total protein was separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) under denaturing conditions and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Membranes were blocked in 5% non-fat milk and then incubated with anti-CLDN1 at 4°C overnight, and Tris-buffered saline and Tween 20 (TBST) was used to wash the membranes. Then the membranes were incubated with the secondary antibody at room temperature for 2 h and washed with TBS-T. Protein bands were visualized by using the enhanced chemiluminescence (ECL) Western blotting detection reagents. The results were compared to the gray value of reference for semi-quantitative analysis.

Statistical Analysis

We used statistical product and service solutions (SPSS) 17. 0 software (SPSS Inc., Chicago, IL, USA) for all the statistical analysis. All the data were represented as $\bar{x} \pm s$. The comparison between groups was done using One-way ANOVA test followed by Post-Hoc test (Least Significant Difference). $p<0.05$ was considered statistically significant.

Results

Expression and Function of FAS-AS1 in OA

Compared with the normal tissues, the expression of FAS-AS1 in the cartilage tissue of OA was significantly increased (Figure 1A), which could be reduced markedly by transiently transfecting siRNA (Figure 1B). Low expression of FAS-AS1 could remarkably promote cell proliferation and this ability could be maintained for at least 72 h (Figure 1C). The results of flow cytometry illustrated that low expression of FAS-AS1 could inhibit cell apoptosis (Figure 1D), which was also demonstrated by low expression of Bax and high expression of Bcl-2. All the above results suggested that high expression of FAS-AS1 might be involved in the development of OA by regulating cell proliferation and apoptosis.

Expression of Extracellular Matrix-Related Genes

Compared with the normal tissues, the expressions of MMP1 and MMP13 in the cartilage tissue of OA increased significantly, while COL2A1 presented a low expression (Figure 2A). IL-1 was reported as an important cytokine in the development of OA; therefore, we explored its effects on the expression of FAS-AS1, MMP1, MMP13, and COL2A1[12]. After adding IL-1, the expressions of MMP1 and MMP13 were high and the expression of COL2A1 was inhibited both in the mRNA (Figure 2B) and the protein level (Figure 2C). While, as the treatment time prolonged, the expression level of FAS-AS1 increased (Figure 2D). These findings demonstrated that the expressions of FAS-AS1, MMP1, and MMP13 are increased during the process of OA, while the COL2A1 is decreased with the stimulation of IL-1, which could play an important role in the occurrence of OA.

The Regulatory Effect of FAS-AS1 on Extracellular Matrix-Related Genes

Through transient transfection of siRNA, the expression of FAS-AS1 in chondrocytes was significantly reduced. At the same time, the expressions of MMP1 and MMP13 were inhibited while COL2A1 showed a high expression, which was validated both in the mRNA (Figure 3A) and the protein level (Figure 3B). All the findings indicated that FAS-AS1 might participate in the occurrence of OA by regulating the expressions of MMP1, MMP13, and COL2A1.

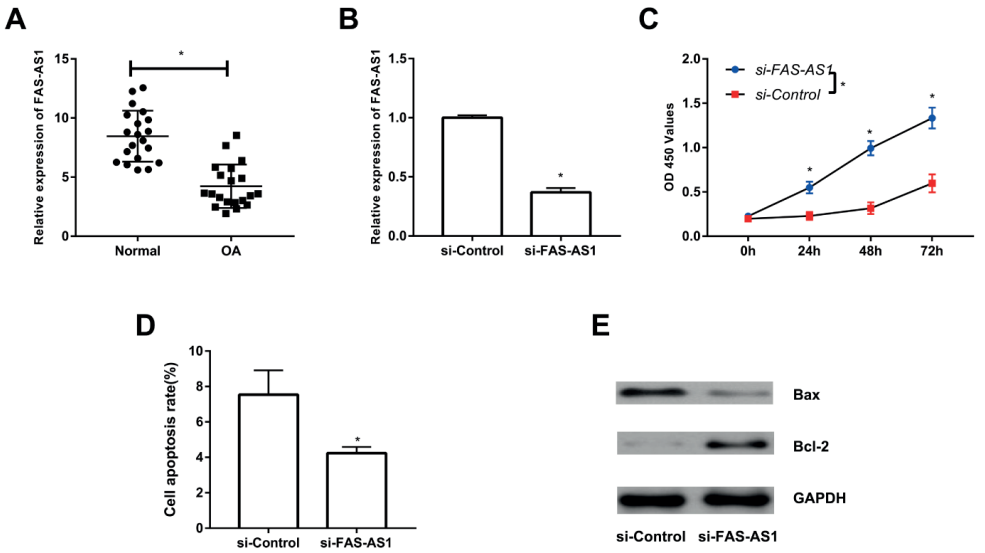

Figure 1. The expression and function of FAS-AS1 in OA. ***A,*** The expression of FAS-AS1 in OA detected by qPCR. Gene expression was represented as ΔCt. *B,* The expression in the cartilage tissue after transfection of si-FAS-AS1. ***C,*** The effect of transfection of si-FAS-AS1 on cell proliferation. ***D,*** The effect of transfection of si-FAS-AS1 on apoptosis. ***E,*** The expression of Bcl-2 and Bax after transfection of si-FAS-AS1. *$p<0.05$.

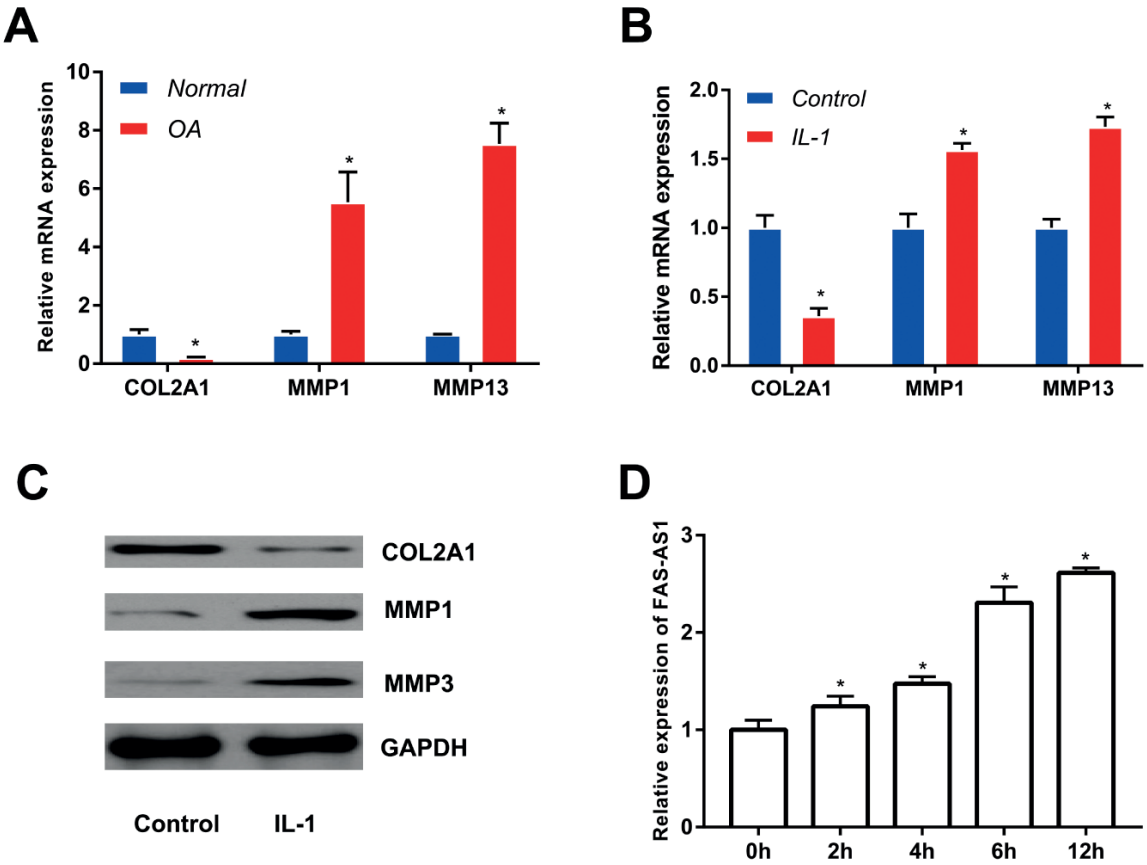

Figure 2. The changes of gene expression after IL-1 treatment. ***A,*** The expression of MMP1, MMP13 and COL2A1 in OA detected by qPCR. Gene expression was represented as ΔCt. ***B,*** The changes of the mRNA expressions of MMP1, MMP13, and COL2A1 12 hours after IL-1 treatment. ***C,*** The changes of the protein expression of MMP1, MMP13 and COL2A1 12 h after IL-1 treatment. ***D,*** The changes of FAS-AS1 expression over time after IL-1 treatment. The results were compared with those at 0 h after the treatment. *$p<0.05$.

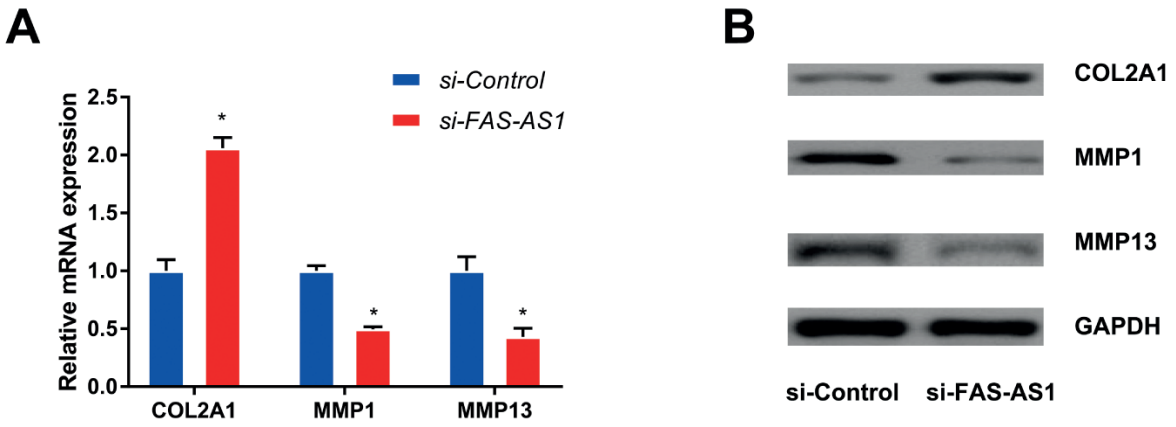

Figure 3. MMP1, MMP13 and COL2A1 were regulated by FAS-AS1. ***A,*** The changes of the mRNA expressions of MMP1, MMP13, and COL2A1 after the transfection of si-FAS-AS1. ***B,*** The changes of the protein expressions of MMP1, MMP13, and COL2A1 after the transfection of si-FAS-AS1.

Discussion

OA is known as a common joint and degenerative disease in middle-aged and elderly people. It is characterized by the degeneration of cartilage structure and function with most of the lesions located in tissues around the joint, which may eventually lead to joint pain and functional activity limitation[13]. The outcomes and prognosis of OA are not satisfactory. For example, the most commonly used non-steroidal anti-inflammatory and analgesic drugs may improve the life quality only through slowing pain and inflammation, and intraarticular injection of hormones or hyaluronic acid is only suitable for symptomatic relief of acute exacerbation of OA[14,15], while articular cavity irrigation could only improve the symptoms of knee osteoarthritis[16]. Therefore, the exploration of the pathogenesis of OA may provide a detailed theoretical basis for the treatment of OA.

MMP family plays a crucial role in the pathological process of OA. It was reported that the MMP family is a family of proteases widely distributed in human body whose main function may be degradation of extracellular matrix components. It is also considered as one of the basic factors of physiological reconstruction and pathological destruction[17]. In OA, MMP is expressed by chondrocytes, synovial cells, and osteoclasts. It also greatly involves in the hydrolysis of cartilage matrix macromolecules, including collagen type II and polysaccharide, and eventually destroys the integrity of the structure and function of the articular cartilage extracellular matrix. MMP1 is one kind of collagenase, one of the rate-limiting enzymes in the degradation of cartilage matrix and the most characteristic II collagen with the most content in the degradation of cartilage matrix. It could degrade collagen in the three-helix region of the collagen fiber and induce the activation of other members of the MMP family, eventually leads to an important role of MMA in the development of OA[18,19]. MMP13, also called collagenase 3, is a member of the MMP family that has recently been discovered, which is mainly expressed in connective tissues. Under normal circumstances, normal articular cartilage almost contains no MMP13 or at a very low level. Only during the osteogenesis of cartilage, MMP13 is secreted to degrade cartilage matrix for bone formation. Meanwhile, MMP13 is reported as a powerful protease, which has a certain degradation effect on collagen and non-collagenous substances in the extracellular matrix, especially for II collagen. Therefore, MMP13 is considered as the most effective protease for cracking type U collagen[20]. Recent studies have confirmed that not only overexpression of MMP-13 is found in damaged cartilage, but also its expression in synovial tissues, calcified cartilage, and subchondral bone is significantly upregulated[21]. In the present study, the expressions of MMP1 and MMP13 were downregulated after the treatment of si-FAS-AS1, suggesting that FAS-AS1 could be involved in the occurrence of OA by regulating extracellular matrix.

Schwab et al[22] showed that IL-l is an important inflammatory medium in the pathogenesis of OA and may induce the increase of MMPs in the articular cartilage. IL-1 includes two subtypes, IL-1α and IL-1β, the latter one is the main subtype of extracellular IL-1, which can be produced by

a variety of cells, including chondrocytes, synovial cells, macrophages and others. The expression of IL-1β receptor of the cartilage cells in OA is higher than that of normal chondrocytes. Some studies revealed that IL-1β could stimulate the cartilage cells, fibroblasts, and synovial cells to secrete prostacyclin (PGE2), collagenase and MMP, thereby accelerating the degradation of cartilage matrix protein polysaccharide and type II collagen, reducing the synthesis of proteoglycan and type II collagen, inhibiting the mRNA expression of type II procollagen, destroying the environment surrounding the cartilage cells, and promoting apoptosis of chondrocytes[12]. Therefore, IL-1 is considered one of the most important inflammatory factors in the development and progression of OA. Our research illustrated that the gradually increased expression of FAS-AS1 with the treatment of IL-1 was positively correlated with the expression trend of MMP1 and MMP13.

In cytology, a certain degree of proliferation activity, the appearance of apoptosis and cell hypertrophy swelling may represent the main pathological phenotypes of chondrocytes. The chondrocyte is the only type of cells in articular cartilage with a very low density of about 14000/mm^3, which is also an active component of articular cartilage metabolism. Once osteoarthritis occurs, the cartilage matrix may be destroyed and the chondrocytes may proliferate and gather together; however, the newborn matrix is less precipitated[23]. Apoptosis of chondrocytes is a vital pathological change in the progression of osteoarthritis and it mainly occurs in the sites with more matrix destruction[24,25]. Our study revealed that the proliferation ability of chondrocytes was enhanced while the apoptosis of chondrocytes was inhibited after the downregulation of FAS-AS1.

Conclusions

We showed that with the stimulation of IL-1, the expression of FAS-AS1 was increased, which could decrease the expression of COL2A1 by promoting the expressions of MMP1 and MMP13 and could reduce the related extracellular matrix involved in the occurrence of OA.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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