Abstract. – OBJECTIVE: Osteoarthritis (OA) is one of the most common chronic joint diseases, caused by lesions in articular cartilage and synovial membranes. Synovitis is a major characteristic of OA, due to the proliferation of synoviocytes. Long noncoding RNAs (lncRNAs) have implicated to play an important role in many different diseases. The aim of this study was to verify the role of lncRNA gastric cancer-associated transcript 3 (GACAT3) in osteoarthritis.

MATERIALS AND METHODS: We utilized the qRT-PCR to detect the expression of lncRNA GACAT3 in osteoarthritis synoviocytes (OAS) and normal synoviocytes (NS). The cell proliferation in NS and OAS after transfection with lncRNA-NC or lncRNA-GACAT3 was detected. The cell cycle and apoptosis rate in NS and OAS were measured by the Flow cytometry analysis. Western blot was used to analyze the possible related mechanism that GACAT3 regulated the cells proliferation in osteoarthritis.

RESULTS: We found that GACAT3 expression was significantly increased in OAS compared with NS. GACAT3 expression was decreased in OAS after transfection with siRNA and the cell proliferation in OAS after transfection with siRNA was significantly inhibited. The cell cycle was arrested in G0/G1 phase and the apoptosis rate was increased in OAS after transfection with siRNA. Moreover, GACAT3 could impact the proliferation of OAS by interleukin-6/signal transducer and activator of transcription-3 (IL-6/STAT3) signaling pathway.

CONCLUSIONS: In this study, we found that lncRNA GACAT3 was closely related to the osteoarthritis. GACAT3 may be involved in the development and progression of osteoarthritis and become a potential target for treating.

Key Words: GACAT3, Osteoarthritis, Synoviocytes, IL-6/STAT3 signaling pathway.

Introduction

Osteoarthritis (OA) is a common chronic joint disease, leading to degradation of articular cartilage and disability. Most researches on OA have paid attention to recover the function of chondrocytes, but the effect on treatment was not ideal. Recently, scatter reports indicated that synovitis is the major characteristic of OA and that, reducing the number of osteoarthritis synoviocytes (OAS), is a key factor for curing the disease. Activated OAS could secrete some cytokines such as interleukin-1, tumor necrosis factor α, and interleukin-6, resulting in the damage on bone and cartilage. Limiting the proliferation of OAS has turned to a focus on treating osteoarthritis. Long noncoding RNAs (lncRNAs) are an emerging class of molecules, with a length than around 200 nucleotides (nt). They play important roles in different diseases including tumors, and could regulate various physiological and pathological activities; indeed, abnormal expression of lncRNAs causes in proliferation, migration and invasion in tumor. At the present, accumulating evidence showed that lncRNAs involved in proliferation, apoptosis and inflammatory response in osteoarthritis. Zhang et al reported that IncRNA-UFC1 facilitates proliferation and inhibits apoptosis in a miR-34a-dependent manner in OA chondrocytes. Li et al found that that lncRNA-cartilage injury-related/miR-27/matrix metalloproteinase-13 axis involved in the degradation of the extracellular matrix of chondrocyte in OA. Kang et al indicated that IncRNA-prostate cancer gene expression marker 1 (PCGEM1) acts as sponge IncRNA for miR-770, which regulates proliferation/apoptosis and autophagy. These results showed that lncRNAs could regulate the biological behavior in osteoarthritis but the detailed mechanisms of lncRNAs still need to be explored.

Gastric cancer-associated transcript 3 (GACAT3) is a novel IncRNA, which was previously named AC130710. Nowadays it has been officially named as GACAT3 by the HUGO Gene Nomenclature Committee. In recent years, there have been more and more researches on IncRNA GACAT3. Our study aims at investigating whether IncRNA GACAT3 is related to the osteoarthritis.
In our study, we first detect the expression of GACAT3 in the normal synoviocyte and osteoarthritis synoviocytes. Then, the effect of GACAT3 on the proliferation of two cell lines was also investigated. Finally, we investigated the probable mechanism of GACAT3 in impacting activity of OAS.

**Materials and Methods**

**Cell Culture and Treatment**

Osteoarthritis synoviocytes (OAS) and normal synoviocytes (NS) were obtained from the Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China). All the cells were maintained under the recommended conditions; they were cultured with 9 ml complete culture medium and incubated at 37°C in a humidified environment with 5% CO₂. Two days later, the medium was reclaimed to remove supernatant cells and it was replaced with fresh medium. The culture dishes were observed closely for 48 hours to monitor the state of adherent cells. The cells were overspread in a monolayer culture.

**RNA Extraction and Real-Time Quantitative PCR Assays**

Total RNAs were severally extracted from cells using a TRIzol kit (Invitrogen, Carlsbad, CA, USA) according to manufacturer’s instructions. The concentration of RNA was detected and the RNA solution was stored at -80°C for further use. Then, cDNA was obtained by reverse transcription using the TaKaRa Reverse Transcriptase kit (TaKaRa, Otsu, Shiga, Japan). The expression level of GACAT3 in NS and OAS was detected and quantified using Real-time qPCR with SYBR Premix Ex Taq (TaKaRa, Otsu, Shiga, Japan). GAPDH was used as an endogenous control.

**Cell Proliferation Assay**

Cell proliferation was evaluated by the CCK-8 assay. The cells were plated in 96-well plates at density of 103 per well with 200 ul cell suspension. Respectively, 10 μL CCK-8 solution (Dojindo Laboratories, Tokyo, Japan) were added to each well and the plate was kept for 2 hours at 37°C. Then, they were detected in absorbance at 450 nm. All the experiments were repeated 3 times.

**Caspase-3 Activity Assays**

We used the Caspase-3 Colorimetric Activity Assay Kit (Millipore, Billerica, MA, USA) to detect the apoptosis radio of cells via the Standard Assay Instructions.

**Cell Cycle Analysis and Apoptosis Analysis**

NS and OAS were seeded into six-well plates with a concentration of 3×105 cells/well. After that, cells were collected with low-speed centrifugation (1200 rpm, 5 min) at 4°C and cell pellets were re-suspended in 1 ml of PBS solution, settled with 75% of ice-cold alcohol and stored at -20°C for 48 h. Before the analysis of flow cytometry (FCM), cells were lysed, centrifuged and re-suspended in propidium iodide (PI, Sigma-Aldrich, St. Louis, MO, USA) staining buffer with 50 μl/ml of PI and 250 μl/ml of RNase A. Lastly, the cell mixture was detected cell cycle and stained with 5 μL of annexin V-FITC and detected apoptosis by fluorescence activated cell sorting (FACS) technique (Fullerton, CA, USA) incubating for 30 mins at 4°C avoiding light. All the experiments were repeated 3 times.

**Plasmid Transfection**

The cells were seeded into 6-well plates at 60-80% confluence and placed in a fresh culture medium without fetal bovine serum (FBS) 2 hours before transfection. Plasmids were transfected into cells with lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) for 8 hours following the manufacturer’s protocol. Respectively, 1 ug of plasmid and 1 ul of lipofectamine 2000 were added into 250 μl of medium without fetal bovine serum (FBS) and incubated for 5-10 minutes. Diluted plasmid and lipofectamine 2000 were mixed and incubated for 20-30 minutes. Finally, the plasmid-lipid complex was added to the cells.

**Western Blot**

The cells were put in lysis buffering the presence of aprotinin, leupeptin, phenylmethanesulfonyl fluoride (PMSF, Sigma-Aldrich, St. Louis, MO, USA) and phosphatase inhibitor mix II and III (Sigma-Aldrich, St. Louis, MO, USA). 10% resolving gel and 5% stacking gel were used for immune-blotting experiments. Then, loading prepared samples in the gel and electrophoresis were performed through the stacking gel at 60 V for 30 min and through the resolving gel at 110 V for 80 min. The membrane was dealt with bovine serum albumin (BSA, HyClone, South-Logan, UT, USA) for 1 h, incubated at 4°C in a primary antibody overnight and developed and imaged using a gel documentation system (Bio-Rad, Hercules, CA, USA). Finally, the secondary antibody was used to incubate it for 1 hour at room temperature and the results were emerged.
**Statistical Analysis**

All the data were expressed as mean±SD (standard deviation) and all the statistical analysis was performed using the software Graphpad 6 (GraphPad, La Jolla, CA, USA). The Student’s t-test or one-way ANOVA followed by Student-Newman-Keuls post-hoc test was used to analyze the data to assess whether there was significant difference in each group. If p-value<0.05, we considered it as statistically significant.

**Results**

**GACAT3 was Highly Expressed in the OAS and Increased the Proliferation of Cells**

To explore the role of IncRNA GACAT3 in OA, we detected the expression of GACAT3 in OAS and OS by qRT-PCR. The result showed that the expression of GACAT3 was highly expressed in OAS, compared with the NS. Furthermore, we examined the proliferation of cell lines and we found that OAS had a much greater proliferation rate compared to NS (Figure 1A-B). These data suggested that increased expression of GACAT3 may be responsible for the progression of OS; however, the mechanism remains unclear.

**Alter the Expression of GACAT3 Influences the Proliferation of NS and OAS**

To confirm whether GACAT3 indeed exerts an influence on proliferation of synoviocytes, GACAT3 was overexpressed in NS and down-regulated GACAT3 expression in OAS; their varieties of proliferation were observed. The results showed that the overexpression of GACAT3 in NS increased the proliferation of cells and that the knockdown of the expression of GACAT3 inhibited the proliferation in OAS (Figure 2). This indicated that changing the expression of GACAT3 may affect the proliferation in synoviocytes.

**The Cell Cycle was Arrested and the Apoptosis Rate was Increased in OAS after the Expression of GACAT3 was Knocked Down**

In order to investigate the apoptosis rate in NS and OAS, we used the Caspase-3 to detect the cells and found that the activity of Caspase-3 was decreased after the expression of GACAT3 was overexpressed in NS and that the Caspase-3 was increased after the expression of GACAT3 was knockdown in OAS (Figure 3A-B). The results indicated that the expression of GACAT3 was related to the apoptosis of cells. Furthermore, the cell cycle distribution and apoptosis rate were measured in NS and OAS after the expression of GACAT3 was altered. Flow cytometry analysis was carried out to study the proliferation mechanism of GACAT3 in OA. These data indicated that the cell percentage in G0/G1 phase was significantly decreased and cell percentage in S-phase was significantly increased and reduced cell apoptosis in NS after GACAT3 was overexpressed. Furthermore, the cell percentage in G0/G1 phase was significantly increased and cell percentage in S-phase was significantly decreased and prompted cell apoptosis in OAS after GACAT3 was knockdown (Figure 3C-D). The results demonstrated that GACAT3 regulated the cell prolifera-
GACAT3 promoted proliferation of osteoarthritis synoviocytes by IL-6/STAT3 signaling pathway

In a previous study, we had known that GACAT3 could regulate the proliferation in OA by changing cell cycle; however, the molecular mechanism on it remained unclear. Recently, some scatters reported that GACAT3 involves in various biological processes of tumors via interleukin-6/signal transducer and activator of transcription-3 (IL-6/STAT3) signaling pathway (10). We aimed to detect whether lncRNA GACAT3 could promote the proliferation ability of OAS by IL-6/STAT3 signaling pathway. We used the IL-6 to simulate the expression of GACAT3 and STAT3. The results showed that IL-6 induced a strong activation of GACAT3 expression in NS by PCR (Figure 4B) and the activation of STAT3 expression by IL-6 stimulation was also confirmed by PCR and Western blot (Figure 4A-B). Then, we further wanted to confirm whether GACAT3 is a downstream target of the IL-6/STAT3 signaling pathway. We found that overexpression of STAT3 in NS could improve the expression of GACAT3, and that knockdown of STAT3 in OAS could reduce the expression of GACAT3 (Figure 4C-D). Meanwhile, a combination of siRNA, STAT3, and IL-6 treatment was executed, showing that the decreased GACAT3 level was observed in STAT3 siRNA cells even with the IL-6 treatment, demonstrating that STAT3 is a key protein for GACAT3 expression (Figure 4E). These results were as same as the previous reports. Moreover, we transfected the small interfering plasmid of STAT3 into the NS, in which GACAT3 was overexpressed, and over-expression plasmid of STAT3 into OAS, in which GACAT3 was knockdown (Figure 4F-G). The results showed that the proliferation ability was decreased in NS and covered in OAS by CCK-8 assay. It was demonstrated that GACAT3 could regulate cell proliferation ability in OA by IL-6/STAT3 signaling pathway.

Discussion

Osteoarthritis (OA) is featured by morphological and quantitative alterations in articular cartilage and synovial membranes, whose main pathological characteristic is caused to the activated synovial fibroblasts. A large number of osteoarthritis synoviocytes could secrete cytokines...
to destroy the structure of bone and cartilage\textsuperscript{11-13}. Previous studies put the focus of research on the recovering the function of chondrocytes, which is easier to conquer; however, the efficiency of treatment is dissatisfactory. How to reduce the number of activated synoviocytes may be a promising therapeutic for osteoarthritis.

Recently, many studies have showed that lncRNAs have closely related in regulating different processes of diseases, including cell growth, angiogenesis and tumorigenesis. For example, Yu et al\textsuperscript{14} found that lncRNA HULC silencing suppresses endothelial cell specific molecule-1 (ESM-1) mediated proliferation, adhesion, migration, invasion and angiogenesis through the cell cycle and anoikis regulation in the glioma U87MG and U251 cell lines. They also found that lncRNA highly up-regulated in liver cancer (HULC) is an oncogene in the phosphatidylinositol 3-kinase/protein kinase B/mammalian target of rapamycin (PI3K/Akt/mTOR) signaling pathway and up-regulates the expression of ESM-1. Accumulating evidence suggested that lncRNAs influenced various biological activities, containing OA. Su et al\textsuperscript{15} found that human lncRNA maternally expressed gene 3 (MEG3) is significantly downregulated in OA patients compared with normal cartilage sample. Song et al\textsuperscript{16} indicated that lncRNA growth arrest-specific 5 (GAS5) was up-regulated in OA chondrocytes compared with non-OA and normal chondrocytes.

In our study, we wanted to know whether lncRNA GACAT3 was related to the osteoarthritis and its probable mechanism. We found that lncRNA GACAT3 was highly expressed in the OAS, compared with NS, by qRT-PCR assay and the proliferation speed was higher in OAS, compared with NS, via CCK-8 assay. According to the analysis of lncRNA GACAT3 expression, we suggested that lncRNA GACAT3 was closely correlated with OA. Then, we overexpressed GACAT3 in NS improving the cell proliferation and knocked down GACAT3 in OAS that decre-
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ased cell proliferation. This result demonstrated that GACAT3 has a close relationship with the cell proliferation.

Cell proliferation and apoptosis are dynamic balancing processes. To further verify how the cell apoptosis impact the proliferation of OAS, we found that cell percentage in G0/G1 phase was significantly decreased; cell percentage in S-phase was significantly increased, and reduced cell apoptosis in NS after GACAT3 was overexpressed. Moreover, the cell percentage in G0/G1 phase was significantly increased and cell percentage in S-phase was significantly decreased and prompted cell apoptosis in OAS after GACAT3 was knocked down. These findings demonstrated that cell apoptosis was an essential part. Above results suggest that apoptosis can influence the cell proliferation, but its detail mechanism still remain unclear. IL-6/STAT3 signaling pathway is an important biological activities direction, involving in various biological processes and including cell proliferation, apoptosis, migration, invasion, and drug resistance, which are closely related to tumor occurrence, progression, metastasis and treatment. Li et al found that miR-155 regulates lymphoma cell proliferation and apoptosis through targeting suppressor of cytokine signaling 3/Janus kinase-STAT3 signaling pathway. According to that, we investigated whether expression of GACAT3 was increased after overexpressed STAT3 in NS and its expression was reduced after knocked down STAT3 in OAS. What’s more, we found that the proliferation of NS, whose expression of GACAT3 was overexpressed, was reduced after we transfected the small interfering plasmid of STAT3 into it. These data suggested that GACAT3 could influence the progression of OA by IL-6/STAT3 signaling pathway.

Conclusions

We firstly found that IncRNA GACAT3 was highly expressed in OAS, and closely related with proliferation of OAS. We also found that IncRNA GACAT3 may regulate cell apoptosis to promote proliferation of OAS via IL-6/STAT3 signaling pathway. These results indicated that IncRNA GACAT3 may be a potential target for OA treatment in future.
Conflict of Interest
The Authors declare that they have no conflict of interest.

References


