LncRNA GACAT3 contributes to osteoarthritis progression by suppressing growth and inducing apoptosis of chondrocytes through miR-195/TGF-β/Smad5 axis

Type
Research paper

Keywords
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Long non-coding RNAs (lncRNAs) were identified as an important regulator involved in the pathogenesis of osteoarthritis (OA). We aimed to evaluate whether lncRNA GACAT3 regulate OA progression by miR-195/TGF-β/Smad5 axis.

Material and methods
Expression levels in tissue or chondrocytes were detected by RT-qPCR and Western blot. Effects of GACAT3 on cell viability, proliferation, apoptosis were evaluated. Targeted interactions of GACAT3, miR-195 and Smad5 were confirmed by dual luciferase reporter gene assay and biotin-coupled miRNA capture assay. Transfected or non-transfected cells were treated with TGF-β1 to verify role of TGF-β in mechanisms of OA progression.

Results
GACAT3 was overexpressed in OA tissues and associated with OA severity. After GACAT3 overexpression, the ability of cell viability and proliferation as well as proliferation-related genes was inhibited with enhanced level of apoptosis-related genes and Smad5. miR-195-5p was negatively targeted by GACAT3 and reversed effects caused by GACAT3. miR-195-5p negatively targeted Smad5. In the presence of TGF-β1, miR-195-5p mimics inhibited activation of Smad1/5, Smad5 and Smad2 compared with negative control. Immunofluorescence showed that miR-195-5p inhibited Smad1/5 activation and transfer to nucleus. In the presence of TGF-β1, GACAT3 facilitated the activation of TGF-β signaling. In clinical sample analysis, GACAT3 was positively correlated with Smad5 expression in OA patients.

Conclusions
We confirmed the up-regulation of GACAT3 in OA patients, and found that GACAT3 regulated the chondrocytes phenotypes by miR-195/TGF-β/Smad5 axis and in turn contributed to OA progression. Findings indicated that GACAT3 may act as a novel therapeutic target for controlling OA progression.
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**Keywords:** osteoarthritis, long non-coding RNA GACAT3, microRNA, osteoarthritis pathogenesis.

**Abbreviations:** IncRNAs, long non-coding RNAs; OA, osteoarthritis; GACAT3, gastric cancer-associated transcript 3; GAS5, growth arrest-specific 5; PVT1, plasmacytoma variant translocation 1; UFC1, ubiquitin-fold modifier conjugating enzyme 1; TGF-β, Transforming growth factor-β; EV, empty vector; Brdu, Bromodeoxyuridine; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PVDF.

**Introduction**

Osteoarthritis (OA) is the most frequent form of joint disorder worldwide and is mainly characterized by the degradation of articular cartilage [1,2]. At present, it has become a leading cause of pain, loss of function, and disability in adults [3]. The pain would influence the overall physical condition, especially older adults [4]. Even in child, joint
dysfunction affects the functional characteristics of the lower limb, and causes the pain and discomfort [5]. The burden of disease caused by OA is projected to increase as the population ages [6]. However, the etiology and pathogenesis of OA has not been clarified and, meanwhile, there is no cure for OA [7]. Recently, multiple studied showed that chondrocyte death plays a key role in the cartilage degradation [8,9]. Thus, we assumed that prevent of chondrocyte apoptosis may have the potential to prevent OA development.

Excitingly, long non-coding RNAs (lncRNAs) were identified as an important regulator involved in the pathogenesis of OA [10], such as gastric cancer-associated transcript 3 (GACAT3) [11], growth arrest-specific 5 (GAS5) [12], plasmacytoma variant translocation 1 (PVT1) [13] and ubiquitin-fold modifier conjugating enzyme 1 (UFC1) [14]. Among them, GACAT3 is a novel IncRNA, which was found to act as a competing endogenous RNA (ceRNAs) and alleviate apoptosis [15]. Notably, GACAT3 was up-regulated in OA tissue and synoviocytes [11]. Thus, its role in pathological mechanisms of OA is worthy to be discussed. Micro RNAs (miRNAs) have a crucial role in various diseases, and identified as the noninvasive biomarker for disease development [16,17]. Among them, miR-195 as the endogenous regulator is thought to play critical functions in the pathogenesis of OA [18]. Many lncRNAs were demonstrated to function as ceRNAs to participate in various biological functions by sponging miR-195 [19-21]. However, the relationship between GACAT3 and miR-195 has not been confirmed. Additionally, Johannes et al. reported that miR-195-5p
interferes with the BMP/Smad-pathway in a dose-dependent manner, suggesting Smad5 may be a potential target of miR-195 [22]. Transforming growth factor-β (TGF-β) has previously identified as a powerful tool to prevent or repair cartilage damage [23]. Moreover, it is reported that inhibition of TGF-β signaling in mesenchymal stem cells could attenuate osteoarthritis [24]. Despite the fact that so many genes have been reported to be involved in the pathologic process of OA, the molecular mechanisms are not fully understood.

Combining these published evidences, we proposed an assumption that GACAT3 may regulate the progression of OA by miR-195/TGF-β/Smad5 axis. Thus, the present study aimed to evaluate whether IncRNA GACAT3 regulate OA progression by miR-195/TGF-β/Smad5 axis.

Materials and Methods

Human samples

Forty osteoarthritic cartilage tissue samples were obtained from knee cartilage of 40 patients with OA in The First people’s Hospital Of Yichang. Patients aged 18 to 65 years with pathologically confirmed OA were eligible for this study. The OA patients were classified according to the Kellgren-Lawrence grading system [25]. Fifteen normal cartilage tissues were obtained from knee cartilage of 15 healthy volunteers with no previous history of OA. Tissue samples were immediately frozen with liquid nitrogen and stored in at -80°C until analyses. All procedures were carried out in
accordance with the Declaration of Helsinki. Ethical approval was granted by the Ethics Committee of The First people’s Hospital Of Yichang. Informed consent was obtained from each participant.

**Chondrocytes preparation and culture**

OA chondrocytes were originally isolated from knee articular cartilage tissues of OA patients. In brief, the cartilage tissues were cut to pieces and then incubated in Dulbecco’s modified Eagle’s medium (DMEM) supplementing with pronase and collagenase B. Cells suspension was centrifuged and washed thrice with phosphate buffered saline (PBS) solution supplementing with 100 U/ml penicillin and 100 μg/ml streptomycin. The isolated chondrocytes were cultured with 9 mL complete culture medium supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 units/mL streptomycin and then incubated at 37°C in a humidified incubator with 5% CO₂. After twice of subculture, cells were collected for following experiments.

**Cell transfection and siRNA interference**

The overexpression of GACAT3 in primary chondrocytes was induced by pcDNA3.1 plasmid carrying the GACAT3 cDNA insert (pcDNA3.1-GACAT3), using an empty vector (EV) as the negative control. The overexpression of miR-195-5p in primary chondrocytes was induced by transfection with miRNA mimics for miR-195-5p (miR-195-5p mimics), using empty vector as miRNA negative control (miRNA-ctrl). The used vectors including pcDNA3.1-GACAT3, miR-195-5p mimics and theirs
corresponding empty vectors (pcDNA3.1 and miRNA-ctrl) were all synthesized by GenePharma company (Shanghai, China). Lipofectamine 2000 Transfection Kit was used to perform the cell transfections and siRNA interference following the manufacturer’s instruction (Invitrogen, USA). Transfected cells were cultured in the 6-well plates. After cultivation for 48 h, cells were collected for subsequent analyses.

RNA isolation and RT-qPCR

Following the manufacturer’s protocol, total RNA from tissues or cells was prepared by TRIzol® reagent (Invitrogen, USA). Then, an amount of 1 mg total RNA was reverse transcribed into cDNA using Takara primeScript RT-PCR Kit (TaKaRa, China). Target mRNA expression level was quantified relative to β-actin (endogenous control) using SYBR® GreenER™ qPCR SuperMix kit (Invitrogen, USA). Finally, RT-qPCR assay was performed in triplicate on a Step One Plus RT-PCR system (Thermo Fisher, USA). Comparative cycle threshold (CT) ($2^{-\Delta\Delta CT}$) method was performed to calculate the date of mRNA expression level. The sequences of PCR primer were listed in Table 1.

Bromodeoxyuridine (BrdU) staining

Transfected cells were cultured in 96-well plates overnight. After BrdU (10 μg/mL) addition, cells were incubated for 1 h, and then immediately fixed in 4% paraformaldehyde for 10 min, followed by the anti-BrdU antibody staining. Finally, cells were counterstained with DAPI and visualized under a fluorescent microscope (Olympus, Tokyo, Japan). BrdU-positive cells were manually counted from ten random
Cell viability assay

Cell viability assay was determined by Cell Counting Kit-8 (CCK-8, Beyotime, China) following the manufacturer's introduction. Briefly, transfected cells ($10^4$-$10^5$ cells/well) were seeded in a 96-well plate with the culture medium and incubated in a CO$_2$ incubator at 37°C for 24 h. Then, CCK-8 solution (10 μL) was added to each well and incubated for another 2 h. Finally, measure the absorbance at 450 nm using a µQuant MQX200 microplate reader (Bio-Tek Instruments, USA).

Flow cytometry assay

Transfected cells were cultured in 96-well plates overnight. Cell apoptosis was determined using the Annexin-VFITC apoptosis kit (BD Biosciences, CA, USA), following the manufacturer's protocol. Cells were incubated with 5 μL FITC-annexin V and 0.1 μg propidium iodide (PI) at room temperature for 15 min. Apoptosis rate was analyzed through flow cytometry (BD FACSAnia™ Fusion, USA).

Western blot

The whole-cell lysates were isolated from cells in RIPA lysis buffer on ice for 30 min. Then, total protein concentration was detected by bicinchoninic acid method using BCA Protein Assay Kit (Beyotime, China). Equal amount of lysate (15 μg) was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and subsequently transferred onto polyvinylidene fluoride (PVDF) membrane (Millipore,
USA). PVDF membrane was incubated in skim milk for 2 h at 25°C and probed with specific primary antibodies against Smad5 (1/5000 dilution, ab40771, Abcam), p-Smad1/5 (1/1000 dilution, 9516S, CST), p-Smad2 (1/1000 dilution, 18338, CST), Smad2 (1/2000 dilution, ab40771, Abcam), c-Myc (1/2000 dilution, ab32072, Abcam), p15INK4B (1/1000 dilution, 36303S, CST), CDC25A (1/1000 dilution, ab156574, Abcam), cleaved caspase 3 (1/1000 dilution, ab2302, Abcam), cleaved PARP (1/1000 dilution, ab32064, Abcam), PCNA (1/1000 dilution, ab29, Abcam), Bim (1/1000 dilution, ab32158, Abcam), PDCD4 (1/1000 dilution, ab80590, Abcam), Bcl-2 (1/1000 dilution, ab32124, Abcam) and GAPDH (1/1000 dilution, ab8245, Abcam, USA). The GAPDH was used as an endogenous control. Next, the membrane was incubated with horseradish peroxidase (HRP)-linked goat anti-rabbit IgG secondary antibody (1/10000 dilution, ab97051, Abcam, USA) at 25°C for 2 h. Finally, the blots were visualized by the enhanced chemiluminescence. Band intensity of target proteins was determined by Quantity One software (Bio-Rad, USA).

**Dual luciferase reporter gene assay**

The transcript 3’UTR sequence of GACAT3 or Smad5 (WT group) was inserted into the pGL3-Luciferase vector (Promega, USA) to construct the wild-type plasmids GACAT3-WT and Smad5-WT. The mutant of GACAT3 or Smad5 constructed by mutating the core binding sequences (MUT group). Then the mutant-type plasmids GACAT3-MUT and Smad5-MUT was constructed in same way. Cells were co-transfected with miR-195-5p mimic or a negative control wild-type or mutant-type
plasmids using Lipofectamine 2000 Transfection Kit. After 24 h transfection, the luciferase activity was determined by dual-luciferase reporter assay system (Promega, USA) on a microplate reader.

**Biotin-coupled miRNA capture**

Biotinylated wild-type miR-195-5p (Bio-wt-195-5p), its mutant (Bio-mt-195-5p) or nonsense control (GenePharma, China) were transfected into chondrocytes using Lipofectamine RNAiMax (Life Technologies). Then, cells were incubated for 48 h. Streptavidin magnetic beads were washed in lysis buffer and blocked with yeast tRNA on a low speed rotator for 2 h at 4 °C. Next, cells were lysed, sonicated and incubated with blocked beads overnight at 4 °C. After streptavidin capture, bound RNAs were purified using the RNeasy Mini Kit (Qiagen) and the abundance of GACAT3 and Smad5 in bound fractions was quantified by qRT-PCR.

**Immunofluorescence**

Cells (70% confluence) were fixed with 4% paraformaldehyde at room temperature for 15 min and washed with PBS containing 1% BSA and 0.05% Tween-20. After blocking with 1% bovine serum albumin, simples were probed with primary antibodies pSmad1/5 (1/500 dilution, 9516S, CST) for 1 h, followed by incubation with secondary antibodies at room temperature for 30 min. DAPI was used for nuclear staining. Cells were visualized under a laser scanning microscope (Leica, Germany).

**Immunohistochemistry (IHC)**
Cartilage tissues from OA patients with different expression levels of GACAT3 were sectioned and fixed in 10% formalin for 48 h. Then, the tissues were embedded into paraffin and sliced to 5 μm sections. The IHC staining was performed according to the standard streptavidin-peroxidase protocol. Tissue sections were deparaffinized with xylene, rehydrated, unmasked epitope with sodium citrate buffer and immediately treated with 3% hydrogen peroxide for 10 min to block endogenous peroxidase. Subsequently, tissue sections were incubated with primary antibody anti-Smad5 (dilution 1:50; ab40771, Abcam, USA) overnight at 4°C, followed by incubation with secondary antibody and HRP-conjugated streptavidin. Finally, the immunoreactions were visualized using DAB (Dako, Copenhagen, Denmark) as chromogen. Images were obtained under the fluorescent microscopy (Olympus Co. Tokyo, Japan).

Statistical analysis

SPSS statistics 20.0 software (IBM SPSS Inc., Chicago, USA) was used to perform the statistical analysis. Each experiment was repeated at least three times. Results were expressed as the mean ± standard error of mean (SEM) of three separate experiments. Differences among groups were analyzed by Student's t-test and one way analysis of variance with followed by post hoc Bonferroni correction. Spearman correlation was performed to estimate the correlation. All statistical tests were two-sided, p<0.05 along with 95% confidence intervals (CI) was considered to be statistically significant.
Results

GACAT3 was highly expressed and associated with Kellgren-Lawrence classification in OA patients.

To explore the role of GACAT3 in OA pathology, the cartilage tissue was collected from OA patients (n=40) and healthy volunteers (n=15). Then, RT-qPCR was performed to detect the expression of GACAT3, and the result showed that GACAT3 was overexpressed in OA patients compared to the healthy volunteer (Fig. 1A).

Furthermore, we noticed that the expression level of GACAT3 is associated with Kellgren-Lawrence classification in OA patients. As shown in Fig.1B, the one-way ANOVA with Bonferroni correction showed the statistically difference of GACAT3 expression among OA patients with different Kellgren-Lawrence grade. The mean expression level of GACAT3 in OA patients with grade III (n=9) or IV (n=9) were respectively 5.31±1.03 and 5.62±1.30, which were all significantly higher than that in patients with grade I (n=11, 3.22±0.99) or II (n=11, 3.52±0.91) (III vs. I, $p=0.001$; III vs. II, $p=0.004$; IV vs. I or II, all $p<0.001$; Fig.1B). These results implicated that GACAT3 is associated with the severity of OA. Taken together, the results indicated that GACAT3 may be involved the OA pathology and promote the OA progression.

GACAT3 overexpression inhibited cell proliferation and promotes apoptosis of primary chondrocytes.

To verify the role of GACAT3 in OA pathology, the GACAT3 was up-regulated in
primary chondrocytes using cell transfection (Fig. 2A). CKK-8 assay and BrdU staining showed that transfected cells with GACAT3 overexpression had lower cell viability (Fig. 2B) and less Brdu-positive cells compared to the control (Fig. 2C and 2D). Conversely, the apoptosis rate of primary chondrocytes was increased by the up-regulation of GACAT3 (Fig. 2E and 2F), along with the up-regulation of pro-apoptotic proteins (cleaved caspase 3 and cleaved PAPR) and down-regulation of proliferation-related protein PCNA (Fig. 2G and 2H). Taken together, these above results indicated that GACAT3 inhibited cell proliferation and promotes apoptosis of primary chondrocytes.

GACAT3 negatively regulated miR-195-5p and enforced miR-195 expression attenuates the effects caused by GACAT3 overexpression.

To explore the downstream mechanism of GACAT3 in OA pathology, we investigated its potential targets. As shown in Fig. 3A, the 3’UTR of GACAT3 have consequential pairing with miR-195, thus, to verify the hypothesis that miR-195-5p also participates in the OA pathology, the expression of miR-195-5p were detected and found to be significantly down-regulated in pcDNA3.1-GACAT3 transfected cells (Fig. 3B). Dual luciferase reporter gene assay showed that the relative luciferase activity was significantly increased in cells co-transfected with mutant 3’ UTR of GACAT3 and miR-195-5p, indicating that miR-195-5p is a target of GACAT3(Fig. 3C). To further confirm the sponge effect between GACAT3 and miR-195-5p, we conducted biotin-coupled miRNA capture assay. The specific enrichment of GACAT3 in Bio-wt-miR-
195-5p group was higher than that in Bio-mt-miR-195-5p group, which proved that miR-195-5p can directly bind to GACAT3 (Fig. 3D). Furthermore, pcDNA3.1-GACAT3 and miR-195-5p mimics were co-transfected into primary chondrocytes, and then cell phenotypes were detected. We found that the GACAT3-induced inhibition of cell viability (Fig. 3E) and proliferation (Fig. 3F and 3G), as well as the enhancement of cell apoptosis (Fig. 3H and 3I) were remarkably reversed by miR-195-5p. Meanwhile, the miR-195-5p inhibited the expression of cleaved caspase 3 and cleaved PAPR, while promoted the expression of PCNA in co-transfected cells than negative control (Fig. 3J and 3K). Moreover, we observed that the expression levels of miR-195-5p in OA patients were significantly down-regulated compared to the healthy volunteer (Fig. 3L), and also negatively correlated with the expression levels of GACAT3 in OA patients (Fig. 3M), further confirming the in vitro findings. Taken together, these above results indicated that GACAT3 negatively regulated miR-195-5p and the enforced miR-195 expression could attenuate the effects caused by GACAT3 overexpression.

MiR-195-5p inhibited TGF-β signaling in primary chondrocytes by targeting Smad5.

Since bioinformatics analysis showed that miR-195-5p shares miRNA response elements with Smad5 (Fig. 4A), we next investigated whether miR-195-5p targets Smad5 by the luciferase reporter assay and biotin-coupled miRNA capture assay. As expected, we observed that the relative luciferase activity was significantly increased...
in cells co-transfected with mutant 3’ UTR of Smad5 and miR-195-5p (Fig. 4B), and
specific enrichment of Smad5 in Bio-wt-miR-195-5p group (Fig. 4C). Subsequently, primary chondrocytes transduced
with miR-195 mimics or miRNA negative control vector were respectively treated with
TGF-β1 or vehicle control for 24 h, western blot was performed to analyze the
downstream factors of TGF-β signaling. The results revealed that miR-195-5p inhibited
the Smad5 either in the presence of TGF-β1 or not (Fig. 4D and 4E). However, the
phosphorylation of Smad1/5 and Smad2 were inhibited only in the presence of TGF-β1 (Fig. 4D and 4E). Immunofluorescence staining showed that the miR-195-5p
inhibited Smad1/5 activation and transfer to the nucleus (Fig. 4F). Besides, spearman
correlation analysis verified the negative correlation between miR-195-5p and Smad5
(Fig. 4G). Taken together, these above results suggested that miR-195-5p negatively
regulated Smad5, and in turn, inhibited TGF-β signaling in primary chondrocytes.

GACAT3 mediated TGF-β signaling and downstream proliferation and
apoptosis related genes through miR-195/Smad5 axis.

As shown in Fig 5A-5C, GACAT3 up-regulated the expression of Smad5, but it was in
turn reversed by the overexpression of miR-195-5p. In addition, qRT-PCR showed that
GACAT3 suppressed the expression of c-myc, CDC25A and BCL-2, while promoted
the p15INK4B, Bim and PDCD4 (Fig. 5D). In the presence of TGF-β1, GACAT3 further
facilitated the phosphorylation of Smad1/5, expression of Smad5 and enhanced the
TGF-β-induced expression changes of these above proliferation and apoptosis related
genes (Fig. 5E and 5F). Meanwhile, in the presence of TGF-β1, GACAT3 further induced the phenotypic change, including the inhibition of cell growth and enhancement of apoptosis (Fig. 5G and 5I). Taken together, these above results suggested that GACAT3 mediates TGF-β signaling and downstream proliferation and apoptosis related genes through miR-195/Smad5 axis.

GACAT3 was positively correlated with Smad5 expression in OA patients.

In clinical samples, the correlation between expression of GACAT3 and Smad5 was analyzed using Spearman correlation analysis, showing a positive correlation (Fig. 6A). Samples with low, moderate or high expression levels of GACAT3 were collected for detection of Smad5 levels. The expression levels of Smad5 were increased with the GACAT3 expression levels (Fig. 6B-6D). Overall, we concluded that GACAT3 is positively correlated with Smad5 expression in OA patients.

Discussion

LncRNAs were reported to play a crucial part in the development of cartilage and have the potential to act as the novel therapeutic targets for controlling OA progression [26]. However, the functional mechanism of various lncRNAs is not fully understood yet. Thereby, the present study focused on the function of lncRNA GACAT3 in the progression of OA and evaluated the possible signaling pathway. This present study provided this potential mechanism of OA pathogenesis for the first time, in which lncRNA GACAT3 contributes to the OA progression by suppressing growth and
inducing apoptosis of chondrocytes via miR-195/TGF-β/Smad5 axis.

GACAT3 as a novel lncRNA is known to be differentially expressed in multiple tissues and be involved in various signaling pathways [27]. The GACAT3 was overexpressed in various tumor cells, and promotes cell proliferation and disease progression, including glioma, colorectal cancer, gastric cancer and breast cancer [28,29,15], indicating that targeting GACAT3 may be a potential therapy for controlling the tumor progression[30,31]. In this study, we verified the fact that GACAT3 was also significantly overexpressed in OA tissues, similar with the results from cancers [28,29,15]. And also, the expression levels of GACAT3 were found to be associated with the severity of OA in patients. Whereas, the majority of researches on GACAT3 were addressed in oncology. Recently, GACAT3 was observed to be overexpressed in OA synoviocytes recently and promote cell proliferation by IL-6/STAT3 signaling pathway [11]. Even so, this evidence alone is not sufficient to prove the role of GACAT3 in OA progression. Thus, we verified the effects of GACAT3 on cell phenotype of chondrocytes. As a consequence, the overexpression of GACAT3 inhibited the cell viability, proliferation and promotes apoptosis in vitro. The effects were also supported by the results of western blot analysis, in which GACAT3 suppressed the expression of PCNA while enhanced the cleaved caspase 3 and cleaved PAPR. These results were consistent with that from studies on tumours [32,27]. Thus, we speculated that GACAT3 was involved in the OA pathology, and meanwhile its up-regulation in chondrocytes may promote OA progression.
Recently, lncRNAs were identified in the biological processes of OA, including cartilage development, degeneration, and regeneration [33]. Plenty of evidence showed the functional interactions among lncRNAs and miRNA, in which lncRNAs could act as a miRNA sponge to regulate the pathogenesis of OA [34]. Numerous lncRNA-miRNA-mRNA networks were found to be related to pathogenesis of OA, such as lncRNA SNHG7/miR-34a-5p/SYVN1 [35] and lncRNA PVT1/miR-488-3p [36]. However, only a small number of lncRNAs were fully characterized. Through the target prediction, we found the targeted relationship between the GACAT3 and miR-195. Moreover, previously, Xia T. et al. have reported the potential association between GACAT3 and miR-195 [19]. Also, lncRNA TUG1 was demonstrated to promote OA-induced degradation of chondrocyte extracellular matrix via miR-195/MMP-13 axis [37]. Notably, it has been demonstrated that miR-195 was aberrantly expressed in OA chondrocytes [38]. Thus, we speculated that lncRNA GACAT3 may function as sponge for miR-195. Accordingly, miR-195 was selected as a potential target for GACAT3 to be explored. In current study, the luciferase reporter assay and biotin-coupled miRNA capture assay confirmed that GACAT3 directly targeted miR-195 in chondrocytes. And also, we found that the up-regulation of miR-195 reversed the cell inhibition caused by GACAT3 overexpression, which further supported our hypothesis that GACAT3 negatively regulated miR-195 in chondrocytes. Overall, these above evidences implicated that GACAT3 indeed acts as a ceRNA by sponging miR-195.

TGF-β is a kind of secreted pleiotropic factor that plays a critical role in the maintaining
homeostasis of both articular cartilage and subchondral bone [39]. The inhibition of endogenous TGF-β led to increased damage to cartilage [40]. Recently, Smad proteins such as Smad1, Smad2, Smad4, Smad5, and Smad8, are known to be the signal transducers of TGF-β signalling pathway [41]. Among them, Smad5 has been implicated as a downstream signal mediator for several bone morphogenetic proteins (BMPs) [42]. The miR-199a* regulates early chondrocyte differentiation by targeting to Smad1 [43]. The miR-133 inhibits BMP-induced osteogenesis by targeting to Runx2 and Smad5 [44]. The miR-146a is increased in OA and induces chondrocyte apoptosis by targeting Smad4 [45]. Thus, TGF-β signalling pathway and its downstream factor Smad5 were investigated in this study. As expected, we found that Smad5 is a direct target of miR-195, and negatively regulated by miR-195 in chondrocytes. Similar with our finding, a recent study demonstrated that BMP/Smad-pathway was regulated by miR-195 [22]. In addition, to further verify the role of TGF-β pathway in GACAT3-mediated OA progression, the TGF-β downstream Smad proteins were evaluated in the presence of TGF-β1. TGF-β1 binds to TGF-β1 receptors to signal through the Smad proteins [46]. In the presence of TGF-β1, we found that miR-195 inhibited the activation of Smad proteins and translocate into the nucleus, while GACAT3 promoted the activation of Smad proteins. Furthermore, the Smad proteins are activated by receptors and translocate into the nucleus, where they regulate transcription [47]. We preliminary summarized that miR-195 negatively regulated TGF-β signalling pathway and Smad5. Meanwhile, either in the presence of TGF-β1 or not, GACAT3
overexpression inhibited the expression levels of c-myc (pro-proliferative) [48], CDC25A (anti-apoptotic)[49], and Bcl-2 (anti-apoptotic) [50], but promote the p15^INK4B (anti-proliferative) [51], Bim (pro-apoptotic) [52] and PDCD4 (anti-proliferative) [53]. Given that all these proteins were downstream transcription factor for TGF-β signalling pathway, we could conclude that GACAT3 positively regulated TGF-β signalling pathway. Additionally, the correlation analysis of clinical samples provided additional evidence that GACAT3 is positively correlated with Smad5 expression in OA patients. Thus, collectively, we have tentatively proposed a possible mechanism for OA pathogenesis that the overexpression of GACAT3 inhibited the miR-195 expression and in turn up-regulated Smad5, leading to the growth inhibition and apoptosis enhancement of chondrocytes. Although we identified the role of novel identified lncRNA GACAT3 in OA progression, the results was mainly based on in vitro experiments. We verified our findings using the expression analysis of clinical samples, which may not be optimal. The lack of animal experiment verification may be the potential criticism of this study. Thus, a further verification in animal models remains needed.

**Conclusion**

In conclusion, the study confirmed the overexpression of GACAT3 in OA patients. We also found that GACAT3 inhibited cell proliferation and promoted apoptosis of chondrocytes. What’s more, we provided this potential mechanism of OA pathogenesis that GACAT3 contributes to the OA progression by miR-195/TGF-β/Smad5 axis. The
present study indicated that GACAT3 may act as a novel therapeutic target for controlling OA progression.

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Declaration of interest: The authors declare that they have no conflict of interest.

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Table 1. Sequences of PCR primer

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<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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Figure 1. GACAT3 was highly expressed and associated with Kellgren-Lawrence classification in OA patients. The expression of GACAT3 in healthy (n=15) and OA human cartilage tissues (n=40) was identified by qRT-PCR (A) and was positively correlated with Kellgren-Lawrence grade (B). ***p<0.001.

Figure 2. GACAT3 overexpression inhibited cell proliferation and promotes apoptosis of primary chondrocytes. A, primary chondrocytes were transduced with EV or GACAT3 expression vector, then relative expression of GACAT3 was evaluated by qRT-PCR. B, primary chondrocytes were transduced with EV or GACAT3 expression vector, then relative cell growth were evaluated by cell viability assay at day 0, 2, 4, 6. C-D, primary chondrocytes were transduced with EV or GACAT3 expression vector, after culturing with BrdU for 24 h, cells were stained with DAPI or BrdU antibody, represent images (C) and relative BrdU positive cells (D) were shown. E-F, primary chondrocytes were transduced with EV or GACAT3 expression vector, after culturing for 72 h, cells were stained with Annexin V or PI, then analyzed by flow cytometry. Annexin V and PI positive subsets (E) and percentages (F) were shown. G-
primary chondrocytes were transduced with EV or GACAT3 expression vector, after culturing for 72h, cells were collected for western blot (G). Relative protein normalized GAPDH were shown (H). PCNA is marker of cell proliferation. **p<0.01. Each experiment was repeated three times.

**Figure 3. GACAT3 negatively regulated miR-195 expression and enforced miR-195 expression attenuates the effects caused by GACAT3 overexpression.** A, miR-195 is a predicted target of GACAT3. B, primary chondrocytes were transduced with EV or GACAT3 expression vector, then expression of miR-195 was evaluated by qRT-PCR. C, 293T cells transduced with wild-type GACAT3 (wt GACAT3) or mutant GACAT3 (mt GACAT3) expression pMIR-REPORT vector were used for luciferase reporter assay. Cells were co-transfected with miR-195-5p or miR-ctrl expression vector as indicated. The putative binding sites of miR-195-5p family for wt GACAT3 or mt GACAT3 were shown. D, Relative expression of GACAT3 in the bound RNAs pulled down by biotinylated wild-type (wt) miR-195-5p, mutant (mt) miR-195-5p and negative control (NC) were evaluated by qRT-PCR. The putative binding sites of GACAT3 for wt or mt miR-195-5p were shown. E, primary chondrocytes were transduced with GACAT3 with or without miR-195 expression vector, then relative cell growth were evaluated by cell viability assay at day 0, 2, 4, 6. F-G, primary chondrocytes were transduced with GACAT3 with or without miR-195 expression vector, after culturing with BrdU for 24 h, cells were stained with DAPI or BrdU antibody, represent images (F) and relative BrdU positive cells (G) were shown. H-I,
primary chondrocytes were transduced with GACAT3 with or without miR-195 vector, after culturing for 72h, cells were stained with Annexin V or PI, then analyzed by flow cytometry. Annexin V and PI positive subsets (H) and percentages (I) were shown. J-K, primary chondrocytes were transduced with GACAT3 with or without miR-195 vector, after culturing for 72h, cells were collected for western blot (J). Relative protein normalized GAPDH were shown (K). PCNA is marker of cell proliferation. n.s., not significant; ***p<0.001. Each experiment was repeated three times.

Figure 4. MiR-195 inhibited TGF-β signaling in primary chondrocytes by targeting Smad5. A, Smad5 is a predicted target for miR-195-5p. B, 293T cells transduced with wild-type Smad5 (wt Smad5) or mutant Smad5 (mt Smad5) expression pMIR-REPORT vector were used for luciferase reporter assay. Cells were co-transfected with miR-195-5p or miR-ctrl expression vector as indicated. The putative binding sites of miR-195-5p family for wt Smad5 or mt Smad5 were shown. C, Relative expression of Smad5 in the bound RNAs pulled down by biotinylated wild-type (wt) miR-195-5p, mutant (mt) miR-195-5p and negative control (NC) were evaluated by qRT-PCR. The putative binding sites of Smad5 for wt or mt miR-195-5p were shown. D-E, primary chondrocytes were transduced with miR-195 or miR-ctrl expression vector, then treated with TGF-β or vehicle control for 24 h, cell lysates were collected for western blot analysis (D). Relative protein expression was shown (E). F, primary chondrocytes were transduced with miR-195 or miR-ctrl expression vector, then cells were stained with pSmad1/pSmad5 (green) and DAPI (Blue). Represent images were
shown. n.s., not significant; ***p<0.001. Each experiment was repeated three times.

Figure 5. GACAT3 mediated TGF-β signaling and downstream proliferation and apoptosis related genes through miR-195/Smad5 axis. A-B, primary chondrocytes were transduced with GACAT3, EV, miR-195 or miR-ctrl expression vector as indicated, then relative expression of Smad5 were evaluated by qRT-PCR (A), or collected cell lysates for western blot analysis (B, C). D, primary chondrocytes were transduced with EV or GACAT3 expression vector, then treated with TGF-b for 24 h. The expression of proliferation related genes MYC, INK4B and CDC25A or apoptosis related genes BIM, BCL-2 and PDCD4 were evaluated by qRT-PCR (D). E-F, primary chondrocytes were transduced with EV or GACAT3 expression vector, then treated with TGF-b or vehicle control for 24 h. Collected cell lysates for western blot analysis (E), and relative protein expression normalized to GAPDH were shown (F). ***p<0.001. Each experiment was repeated three times.

Fig 6. GACAT3 was positively correlated with Smad5 expression in OA patients. A, Smad5 expression and GACAT3 expression in 40 OA patients were evaluated by qRT-PCR and their correlation were shown. B, the protein expression of Smad5 in low or high GACAT3 expression OA patients were shown. 1#-10# represents ten OA pateints. C-D, OA human cartilage tissues with low, moderate or high GACAT3 expression were stained with Smad5, represent images (C) and relative Smad5 positive cells were shown (D). ***p<0.001.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<tbody>
<tr>
<td>GACAT3</td>
<td>5′-CTTCCGGAGCAGGCTCTGAGT-3′</td>
<td>5′-CTTCCCTGCAGAGACCAGT-3′</td>
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<td>Smad5</td>
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<td>5′-GCCTTTTCTGCCCATTTCTCT-3′</td>
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<tr>
<td>miRNA-195-5p</td>
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<td>β-actin</td>
<td>5′-ATTGCTGACGGATGCAAG-3′</td>
<td>5′-GCTGATCCACATCTGCTGAA-3′</td>
</tr>
</tbody>
</table>
Figure 1

(A) Relative expression of GACAT3 between Normal and OA groups.

(B) Relative expression of GACAT3 across different Kellgren-Lawrence grades (I, II, III, IV).

Legend:
- Normal OA
- Kellgren-Lawrence grade

Statistical significance indicated by asterisks: **p < 0.01, ***p < 0.001.
Figure 2

A. Relative expression of GACAT3

B. Relative cell growth

C. BrdU, DAPI, Merge

D. Relative BrdU positive cells

E. Annexin V

F. Apoptotic cells (%)

G. Western Blotting

H. Relative protein expression normalized to GAPDH

Figure 2
Figure 3
Figure 4

A

B

C

D

E

F

G

Preprint
Figure 5
Figure 6

(A) Scatter plot showing the relationship between Relative GACAT3 expression and Relative Smad5 expression. The equation $Y = 0.6812X + 1.646$ is displayed.

(B) Western blot analysis of Smad5 expression in Low GACAT3 and High GACAT3 conditions over time (1h, 2h, 3h, 4h, 5h, 6h, 7h, 8h, 9h, 10h). GAPDH is used as a loading control.

(C) Immunohistochemical staining for Smad5 in Low, Moderate, and High GACAT3 expression conditions.

(D) Bar graph showing Relative Smad5 positive cell counts labeled as Low, Moderate, and High. Statistical significance marked with triple asterisks.

Figure 6