MEDICAL SCIENCE MONITOR

CLINICAL RESEARCH

e-ISSN 1643-3750
© Med Sci Monit, 2019; 25: 8019-8024
DOI: 10.12659/MSM.917135

Received: 2019.04.22
Accepted: 2019.06.24
Published: 2019.10.26

Long Non-Coding RNA (LncRNA) CAIF is Downregulated in Osteoarthritis and Inhibits LPS-Induced Interleukin 6 (IL-6) Upregulation by Downregulation of MiR-1246

Authors' Contribution:
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Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Source of support: Shanghai Sports Science and Technology Takeoff Project (No. 16T017); the National Natural Youth Project of China (No. 81601412)

Background: Osteoarthritis (OA) affects about 40% of people older than 40 years of age, and the mechanism is not well understood. Long non-coding RNA (lncRNA) CAIF is a recently identified critical player in myocardial infarction, while its role in other human diseases is unclear. The present study aimed to investigate the role of CAIF in OA.

Material/Methods: Levels of CAIF in synovial fluid of OA patients (n=60) and healthy controls (n=60) were measuring by performing quantitative real-time polymerase chain reaction (qRT-PCR). MiR-1246 and interleukin (IL)-6 levels in synovial fluid were measured by performing qRT-PCR and enzyme-linked immunosorbent assay (ELISA), respectively. Cell apoptosis analysis was performed after CHON-001 cells were treated with 500 mg/mL lipopolysaccharide (LPS) for 24 hours.

Results: We found that CAIF in synovial fluid was downregulated in OA patients and inversely correlated with miR-1246 and IL-6. Downregulated CAIF distinguished OA patients from healthy controls. In cells of chondrogenic cell line CHON-001, CAIF overexpression mediated the inhibited expression of miR-1246 and secretion of IL-6, while miR-1246 overexpression reduced the effects of CAIF overexpression on IL-6 secretion. In addition, CAIF overexpression inhibited the apoptosis of CHON-001 cells under LPS treatment, while miR-1246 overexpression attenuated the effects of CAIF overexpression.

Conclusions: Therefore, CAIF may downregulate miR-1246 to improve OA.

MeSH Keywords: Apoptosis • Osteoarthritis • RNA, Long Noncoding

Full-text PDF: https://www.medscimonit.com/abstract/index/idArt/917135

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Background

Osteoarthritis (OA) is a common chronic joint disorder in clinical practices. Synovitis, local inflammation, and cartilage proteolytic degradation are the main characteristics of OA [1]. OA affects about 40% of people older than 40 years of age, and the incidence rate shows an increasing trend in recent years. With the progression of OA, joint stiffness and severe pain will develop [1,2]. Incidence of OA varies across different subtypes. It has been reported that symptomatic hip OA affects about 25% of people during their lifetime, and this percentage for symptomatic knee OA is as high as 44.7% [3,4]. At present, symptom control and pain reduction are the main therapeutic options for OA, while effective therapeutic strategies are lacking [5]. Therefore, a better understanding of the pathogenesis of OA is needed to improve the conditions of OA patients.

Inflammatory responses play pivotal roles in the development of OA [6]. Certain pro-inflammatory chemokines and cytokines, such as interleukin (IL)-6, promote the progression of OA by mediating a series of pathological changes, such as knee cartilage loss [7]. Therefore, inhibition of the production and secretion of those pro-inflammatory factors may contribute to the treatment of OA [8]. In a recent study, Wu et al. reported that miR-1246 can mediate the secretion of IL-6 after lipopolysaccharide (LPS)-induced inflammation [9]. It has been well established that IL-6 has pivotal functions in OA [10]. It is also known that miR-1246 can mediate the function of p53 signaling [11], which can be inactivated by lncRNA CAIF [12]. Previous studies suggest the potential interactions among CAIF, p53, and miR-1246. Based on these studies, we speculated that CAIF and miR-1246 might also participate in OA. This study was therefore carried out to verify this hypothesis.

Material and Methods

Research participants

This study enrolled 60 OA patients (38 males and 22 females, age range 30 to 65 years, 46.8±6.0 years) and 60 healthy volunteers (38 males and 22 females, age range 30 to 66 years, 46.4 ± 6.2 years). All patients and healthy volunteers were selected in Changhai Hospital from June 2016 to December 2018. The OA patients were selected from the 188 cases of OA cases admitted to the aforementioned hospital. Inclusion criteria of OA patients: 1) newly diagnosed OA cases; and 2) no therapies for any clinical disorders were performed during 3 months before admission. Exclusion criteria of OA patients: 1) patients complicated with other clinical disorders; 2) history of malignancies; 3) therapies have initiated. The 60 healthy volunteers were enrolled in the physiological healthy center of the aforementioned hospital to match the distributions of gender and age of OA patients. All participants were informed about the study details. They all provided written informed consent. The Ethics Committee of the aforementioned hospital approved this study.

Synovial fluid and cells

Before the initiation of any therapies, synovial fluid (1.5 mL) was extracted from the joint of patients and healthy volunteers. Synovial fluid was kept in liquid nitrogen before subsequent experiments.

Human chondrocyte cell line CHON-001 (ATCC, USA) was used. Cells were cultivated under conditions of 5% CO_2 and 37°C. Cell culture medium was Dulbecco's Modified Eagle's Medium (10% fetal bovine serum (FBS) and 0.1 mg/mL G-418).

Transient transfections

CAIF expression pcDNA3 vector and empty pcDNA3 vector were from GenePharma (Shanghai, China). Negative control miRNA and miR-1246 mimic were from Sigma-Aldrich (USA). Following the manufacturers' instructions, 10^5 CHON-001 cells were transfected with 10 nM CAIF expression pcDNA3 vector, 10 nM empty pcDNA3 vector (negative control, NC), 30 nM negative control miRNA (NC), or 30 nM miR-1246 mimic. Control (C) group included cells with no transfections. Transfected cells were collected at 24 hours post-transfections to performed subsequent assays.

Quantitative real-time polymerase chain reaction (qRT-PCR)

TRIzol reagent (Invitrogen, USA) was mixed with synovial fluid (1 ml TRIzol per 0.2 mL synovial fluid) and CHON-001 cells (1 mL TRIzol per 10^5 cells) to extract total RNAs. Following DNase I digestion, MMLV Reverse Transcriptase (Lucigen) and QuantiTect SYBR Green RT-PCR Kit (QIAGEN, Shanghai, China) were used to prepare cDNA through reverse transcriptions and make qPCR mixtures, respectively.

Extractions of miRNAs from synovial fluid and CHON-001 cells were performed using miRNeasy Mini Kit (QIAGEN). MiScript II RT Kit (QIANGEN) and miScript SYBR Green PCR Kit (QIANGEN) were used to perform reverse transcriptions and prepare qPCR mixtures, respectively.

Expression of CAIF and miR-1246 was detected using 18S rRNA and U6 as endogenous control, respectively. Three replicate wells were set for each experiment, and $2^{-\Delta\Delta CT}$ method was used to process all data.

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Table 1. Basic information of two groups of participants.

	OA	Control
Age (years)	46.8±6.0	46.4±6.2
Gender		
Males	38	38
Females	22	22
Affected area and/or source of synovial fluid		
Knee	37	37
Hip	23	23

OA – osteoarthritis.

Enzyme-linked immunosorbent assay (ELISA)

IL-6 in synovial fluid and cell culture medium was detected by performing ELISA. Human IL-6 Quantikine ELISA Kit (Cat. No. D6050, R&D Systems) was used. Levels of IL-6 in synovial fluid and cell culture medium were both expressed as pg/mL.

Cell apoptosis assay

CHON-001 cells were harvested at 24 hours after transfections. CHON-001 cells were dissolved in the aforementioned cell culture medium supplemented with LPS (500 ng/mL, Sigma-Aldrich, USA), followed by incubation at 37°C for 24 hours. After that, cells were collected and were subjected to digestion using 0.25% trypsin. Following staining with propidium iodide (PI) and Annexin V-FITC (Dojindo, Japan), apoptotic cells were detected by performing flow cytometry (NovoCyte Quanteon™ Flow Cytometer, ACEA Biosciences).

Statistical analysis

All experiments were performed in 3 biological replicates. The data were used to calculate mean values. GraphPad Prism 6 was used for all statistical analysis. In all cases, 0.85-0.95 statistical power was reached. Differences between the patient group and control group were explored using unpaired *t*-test. Differences among different cell groups were analyzed using ANOVA (one-way) and Tukey test. Correlations were analyzed by linear regression. Diagnostic analysis was performed using receiver operating characteristic (ROC) curve. $P<0.05$ was statistically significant.

Results

CAIF was downregulated in synovial fluid of OA patients and showed diagnostic potentials

See Table 1 for basic information of patient and control groups. Levels of CAIF in synovial fluid of OA patients (n=60) and healthy controls (n=60) were measuring by performing RT-qPCR. Levels of CAIF were compared between two groups by performing unpaired t test. It was observed that levels of CAIF in synovial fluid were significantly lower in OA patients than in healthy controls (Figure 1A, $P<0.05$). Diagnostic values of CAIF in synovial fluid for OA were analyzed by performing ROC curve. In the ROC curve analysis, OA patients were used as true positive cases and healthy volunteers were true negative cases. As shown in Figure 1B, the area under the curve (AUC) was 0.89 (95% confidence interval: 0.83–0.94; standard error: 0.028).

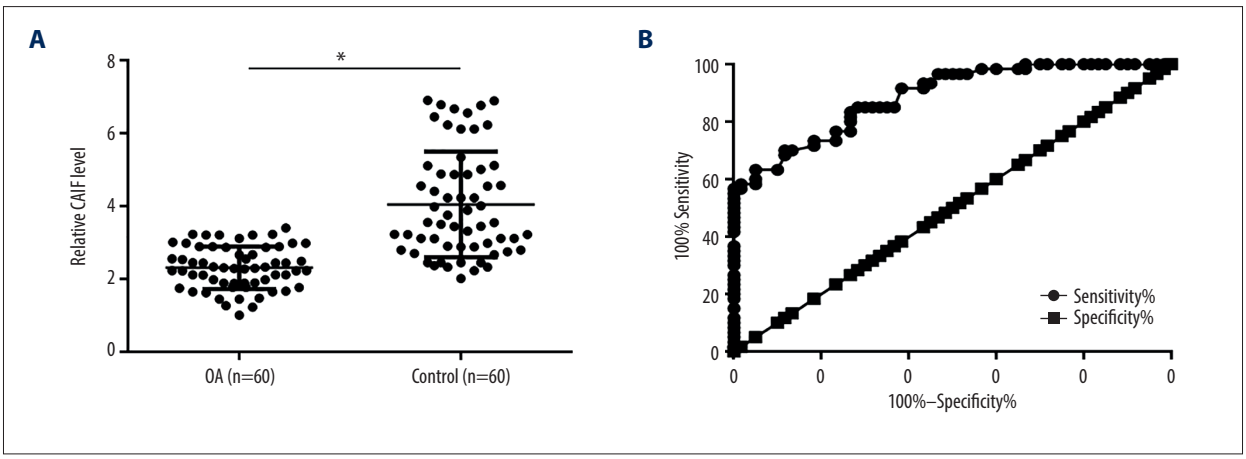

Figure 1. CAIF was downregulated in synovial fluid of OA patients and showed diagnostic potentials. (**A**) Levels of CAIF in synovial fluid measured by RT-qPCR and compared by unpaired *t*-test showed that levels of CAIF in synovial fluid were significantly lower in OA patients than in healthy controls (* $P<0.05$). (**B**) ROC curve analysis showed that decreased levels of CAIF distinguished OA patients from healthy controls. OA – osteoarthritis; qRT-PCR – quantitative real-time polymerase chain reaction; ROC – receiver operating characteristic.

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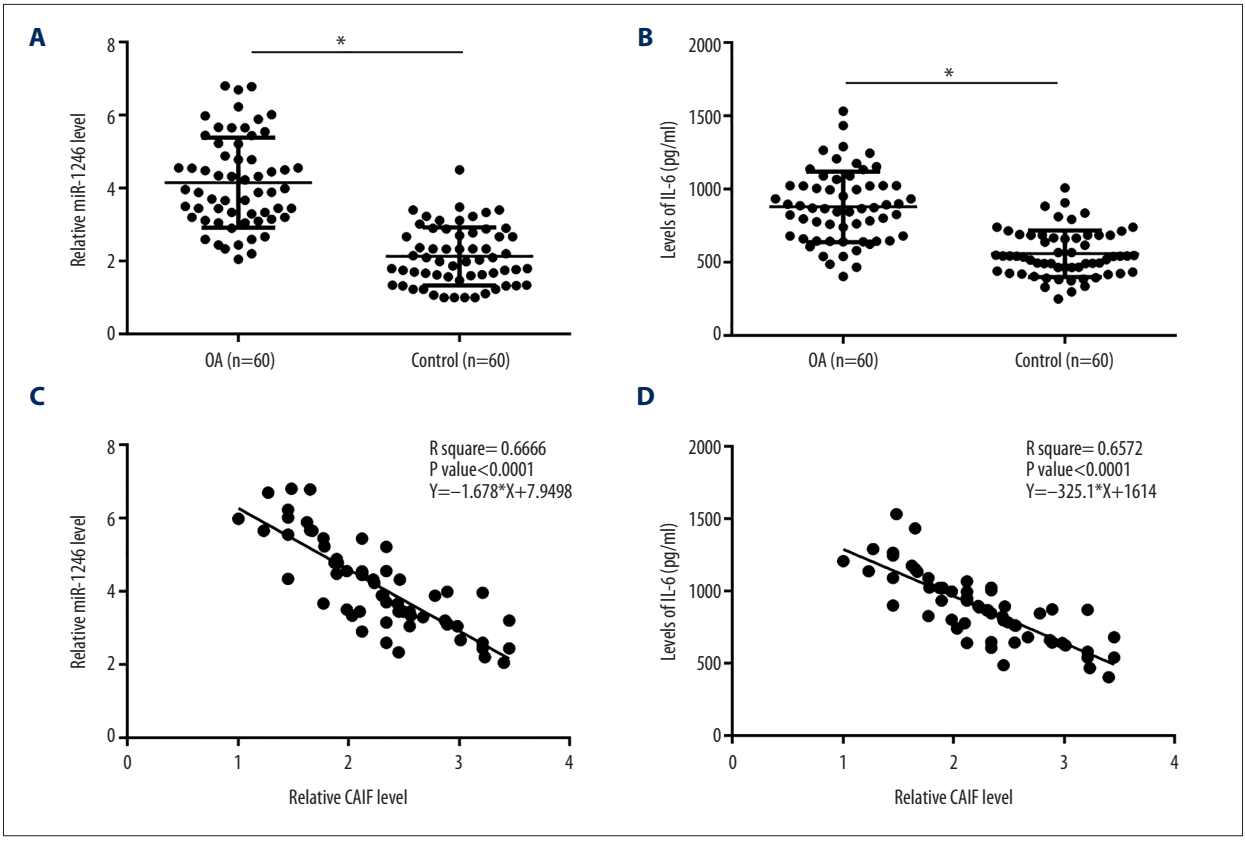

Figure 2. MiR-1246 and IL-6 were upregulated in OA and were inversely correlated with CAIF. Levels of miR-1246 and IL-6 in synovial fluid were measuring by qRT-qPCR and ELISA, respectively. Unpaired *t*-test showed that levels of (**A**) miR-1246 and (**B**) IL-6 in synovial fluid were significantly higher in OA patients than in healthy controls (* $P<0.05$). Linear regression showed that CAIF was inversely and significantly correlated with (**C**) miR-1246 and (**D**) IL-6 in OA patients. IL – interleukin; OA – osteoarthritis; qRT-PCR – quantitative real-time polymerase chain reaction; ELISA – enzyme-linked immunosorbent assay; ROC – receiver operating characteristic.

MiR-1246 and IL-6 were upregulated in OA and were inversely correlated with CAIF

MiR-1246 and IL-6 levels in synovial fluid of OA patients (n=60) and healthy controls (n = 60) were measuring by performing qRT-PCR and ELISA, respectively. Experimental data were compared by performing unpaired *t*-test. It was found that levels of miR-1246 (Figure 2A, $P<0.05$) and IL-6 (Figure 2B, $P<0.05$) in synovial fluid were significantly higher in OA patients than in healthy controls. Correlations between miR-1246/CAIF and IL-6/CAIF were analyzed by performing linear regression. It was found that CAIF was inversely and significantly correlated with miR-1246 (Figure 2C) and IL-6 (Figure 2D) in OA patients.

CAIF inhibited the secretion of IL-6 from CHON-001 cells by downregulating miR-1246

CAIF expression vector and miR-1246 mimic were transfected into CHON-001 cells. Compared to the two controls, C and NC, expression levels of CAIF and miR-1246 were significantly increased at 24 hours post-transfections (Figure 3A, $P<0.05$). Moreover, qRT-PCR results showed that, compared to the 2 controls, CAIF overexpression mediated the inhibited expression of miR-1246, while CAIF expression was not significantly affected by miR-1246 overexpression (Figure 3B, $P<0.05$). In addition, ELISA was performed to detect the levels of IL-6 in cell culture medium. Compared to the 2 controls, CAIF overexpression mediated suppressed the secretion of IL-6, while miR-1246 overexpression played an opposite role and reduced the effects of CAIF overexpression on IL-6 secretion (Figure 3C, $P<0.05$).

CAIF suppressed CHON-001 cell apoptosis through miR-1246

Cell apoptosis analysis was performed after CHON-001 cells were treated with 500 mg/mL LPS for 24 hours. Compared to the 2 controls, NC and C, CAIF overexpression led to reduced apoptotic rate of CHON-001 cells, while miR-1246 overexpression played an opposite role and attenuated the effects of CAIF overexpression (Figure 4, $P<0.05$).

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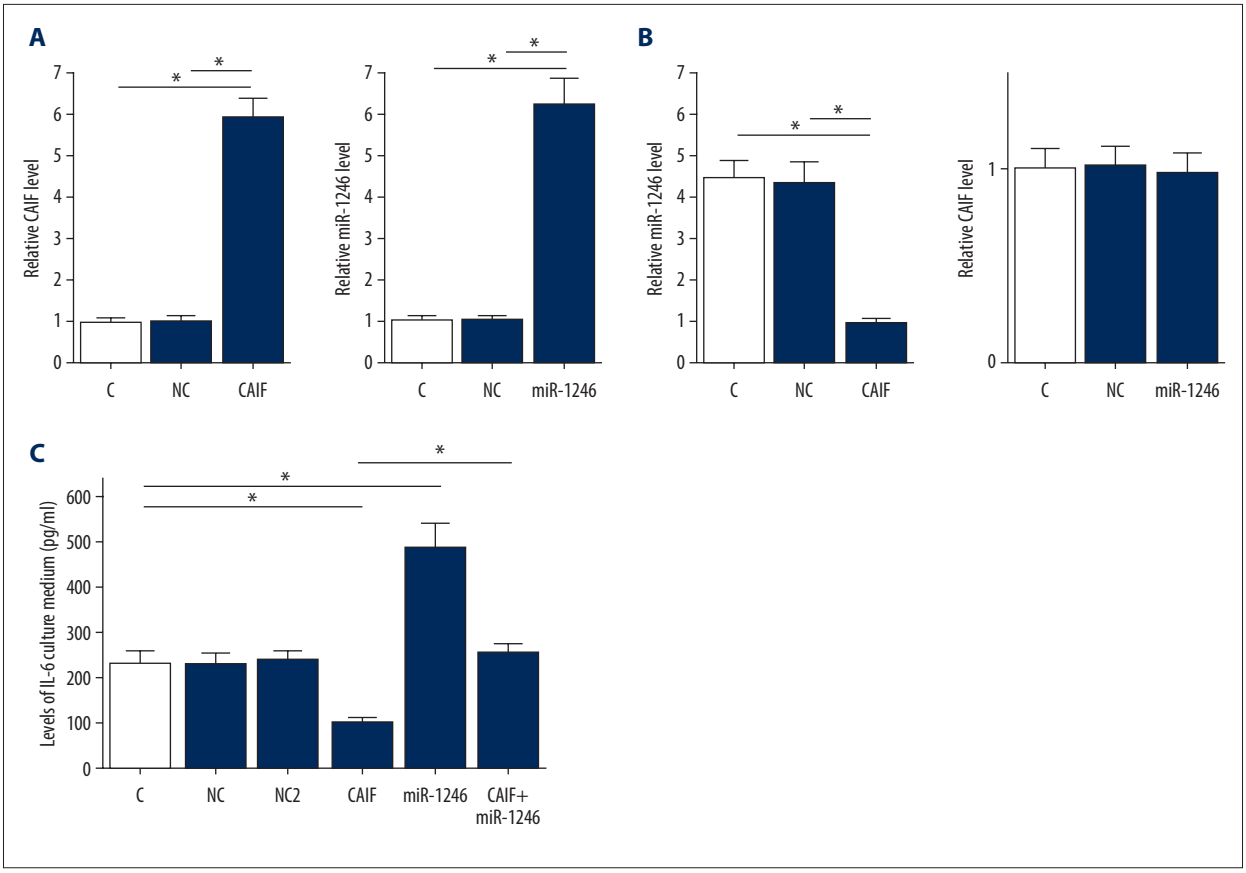

Figure 3. CAIF inhibited the secretion of IL-6 from CHON-001 cells by downregulating miR-1246. (**A**) Compared to the 2 controls C and NC, the expression levels of CAIF and miR-1246 were significantly increased at 24 hours after the transfection of CAIF expression vector and miR-1246 mimic. (**B**) qRT-PCR results analyzed by ANOVA (one-way) and Tukey test showed that, compared to the 2 controls, CAIF overexpression mediated the inhibited expression of miR-1246, while CAIF expression was not significantly affected by miR-1246 overexpression. (**C**) In addition, ELISA was performed to detect the levels of IL-6 in cell culture medium and experimental data were compared by performing ANOVA (one-way) and Tukey test. Compared to the 2 controls, CAIF overexpression mediated suppressed the secretion of IL-6, while miR-1246 overexpression played an opposite role and reduced the effects of CAIF overexpression on IL-6 secretion (* $P<0.05$). IL – interleukin; C – no transfection control; NC – transfection negative control; OA – osteoarthritis; qRT-PCR – quantitative real-time polymerase chain reaction; ELISA – enzyme-linked immunosorbent assay.

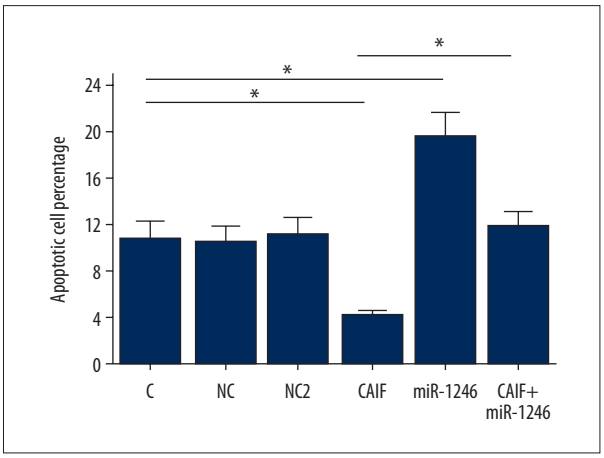

Figure 4. CAIF suppressed CHON-001 cell apoptosis through miR-1246. Cell apoptosis analysis data compared by ANOVA (one-way) and Tukey test showed that, comparing to the 2 controls NC and C, CAIF overexpression led to reduced apoptotic rate of CHON-001 cells, while miR-1246 overexpression played an opposite role and attenuated the effects of CAIF overexpression (* $P<0.05$). C – no transfection control; NC – transfection negative control.

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Discussion

This study mainly investigated the expression pattern and functionality of CAIF in OA. We found that CAIF was downregulated in OA and might inhibit the apoptosis of chondrocyte cell by inhibiting miR-1246.

A recent study reported CAIF as a novel critical inhibitor of myocardial infarction and the actions of CAIF in this pathological process were mediated by the inactivation of p53 [12]. The activation of p53 is involved in the pathogenesis of OA by inducing cell apoptosis [13]. Therefore, inhibition of p53 signaling contributes to the recovery of OA [14]. We therefore hypothesize that CAIF is likely involved in OA. In this study we showed that CAIF is downregulated in OA patients. We also observed the inhibited chondrocyte cell apoptosis under LPS treatment after CAIF overexpression. Our data suggest that CAIF is likely an inhibitor of OA.

It is worth noting that in this study we used LPS-treated chondrocyte cell as the cell model of OA. Chondrocyte cells are the only components identified in healthy cartilage. The main function of chondrocyte is to maintain and produce new cartilaginous matrix [15]. Chondrocyte apoptosis promotes the progression of osteoarthritis [16]. In a recent study it is reported that LPS was upregulated in OA and contributes to disease progression [11]. Therefore, the cell model used in this study was appropriate and data were solid.

MiR-1246 is a key mediator in LPS-induced inflammatory responses [9]. It is known that the expression of miR-1246 can be regulated by certain lncRNA, such as lncRNA MIAT [17]. In this study, we showed that CAIF was likely an upstream inhibitor of miR-1246 in chondrocyte cells. In addition, the downregulation of miR-1246 is involved in the regulation of chondrocyte cell apoptosis and IL-6 secretion from chondrocyte cells. Therefore, we identified a novel CAIF/miR-1246/IL-6 pathway in OA. MiR-1246 can mediate the function of p53 signaling [11], while p53 can be inactivated by lncRNA CAIF [12]. Therefore, p53 pathway-related factors might mediate the interaction between CAIF and miR-1246. This study provided new insights to the pathogenesis of OA. Future studies should focus on the clinical values of CAIF in the treatment and diagnosis of OA. It is expected that a novel CAIF-based therapeutic approach might be developed. However, this study was limited by the small sample size. In addition, we also failed to distinguish different types of OA (knee and hip) and different degrees of severity. Future studies are needed to resolve these problems. In addition, animal model experiments are expected to be develop based on established methods [18] to further confirm our conclusions under *in vivo* conditions.

Conclusions

CAIF was downregulated in OA; and CAIF overexpression might improve OA by inhibiting chondrocyte cell apoptosis and IL-6 secretion through the downregulation of miR-1246.

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