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ORIGINAL ARTICLE

LncRNA cardiac autophagy inhibitory factor is downregulated in rheumatoid arthritis and suppresses the apoptosis of fibroblast-like synoviocytes by promoting the maturation of miRNA-20a

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ABSTRACT

Objectives: In this study, we aimed to investigate the effects of LncRNA cardiac autophagy inhibitory factor (CAIF) and miR-20a on the apoptosis of synovial cells in rheumatoid arthritis (RA) and the regulatory mechanism.

Patients and methods: Between May 2018 and March 2020, a total of 62 RA patients (24 males, 38 females; mean age: 55.2±4.9 years; range, 42 to 68 years) and 62 controls (24 males, 38 females; mean age: 55.3±4.8 years; range, 41 to 68 years) were included in this study. Plasma samples were collected from all participants. The expression levels of CAIF, mature miR-20a, and miR-20a precursor in these plasma samples were determined by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Correlations were analyzed using linear regression analysis. Overexpression of CAIF was achieved in human fibroblast-like synoviocytes (HFLSs) and the expression levels of mature miR-20a and miR-20a precursor were determined using RT-qPCR. Cell apoptosis was analyzed by cell apoptosis assay.

Results: The CAIF was downregulated in RA and positively correlated with the expression of mature miR-20a. In HFLSs, LPS treatment resulted in downregulation of both CAIF and miR-20a in a dose-dependent manner. In HFLSs, overexpression of CAIF did not affect the expression of miR-20a precursor, but upregulated the expression of mature miR-20a. Cell apoptosis analysis showed that overexpression of CAIF and miR-20a inhibited the apoptosis of HFLSs induced by LPS. The combination of overexpression of CAIF and miR-20a showed a stronger effect.

Conclusion: The CAIF may suppress the apoptosis of HFLSs in RA by promoting the maturation of miR-20a.

Keywords: Cardiac autophagy inhibitory factor, human fibroblast-like synoviocytes, miR-20a, precursor, rheumatoid arthritis.

Rheumatoid arthritis (RA) is a long-term chronic autoimmune disorder that causes swelling, pain, and inflammatory responses in two or more joints.[1] Patients with RA are usually treated with biological disease-modifying antirheumatic drugs (DMARDs) and targeted synthetic DMARDs to slow the progression of RA.[2] However, there is still no cure for RA.[3,4] Therefore, preventative approaches and novel treatment strategies are needed. During the progression of RA, the synovium is in a state of continuous inflammation. Due to changes in proliferation and apoptosis processes, the number of fibroblast-like synoviocytes (FLS) and other immune cells (such as macrophages and lymphocytes) in the synovium has increased significantly, forming an inflammatory environment, thereby attracting more immune cells to reach the damaged part of the synovium and accelerate the destruction of joints.[5-7] Recent studies have shown that the resistance of FLS to apoptosis is part of the cause of

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synovial hyperplasia.[5-7] Many current treatments for RA are based on restoring the balance between synovial cell proliferation and synovial cell apoptosis.[8] Therefore, elucidating the mechanism that regulates FLS cell apoptosis is essential to promote the development of new therapies for RA.

Non-coding ribonucleic acid RNA (ncRNA), such as micro-RNA (miRNA) and long ncRNA (LncRNA), regulate gene expression to participate in human diseases.[9,10] Therefore, they are promising targets for targeted therapy. Studies have shown that overexpression of LncRNA cardiac autophagy inhibitor (CAIF) can inhibit the apoptosis of CHON-001 cells treated with LPS.[11] In addition, miR-20a has been shown to play anti-apoptotic and anti-inflammatory effects in LPS-induced HK-2 cells;[12] however, their respective roles in RA and whether there is any interaction are unclear. Therefore, in the present study, we aimed to investigate the effects of CAIF and miR-20a on the apoptosis of synovial cells in RA and the regulatory mechanism.

PATIENTS AND METHODS

Study design and study population

This retrospective study was conducted at the Second Affiliated Hospital of Fujian Medical University, Department of Rheumatology & Immunology between May 2018 and March 2020. A total of 62 RA patients (24 males, 38 females; mean age: 55.2±4.9 years; range, 42 to 68 years) and 62 controls (24 males, 38 females; mean age: 55.3±4.8 years; range, 41 to 68 years) were included in this study. All RA patients were diagnosed by experienced rheumatologists. According to the 1987 Rheumatoid Arthritis Classification, all patients met at least four out of the seven criteria, and criteria 1 through 4 were present for at least six weeks by the time of admission. Disease Activity Score in 28 Joints (DAS28) of RA patients ranged from 4.3 to 5.3 (mean score: 4.8±0.3). Disease duration of RA was 6.4±2.8 years. All patients were diagnosed for the first time and recurrent RA patients were excluded from this study. Among the 62 RA patients, anti-cyclic citrullinated peptide (anti-CCP) ab was detected in 53 cases and anti-extractable nuclear antigen (anti-ENA) ab was detected in six cases. According to the Steinbrocker staging system, 62 patients included 32 cases at Stage 3 and 30 cases at Stage 4. All patients were positive for rheumatoid factor (RF concentration higher than 14 IU/mL [positive] was used as the first criterion to enroll RA patients to avoid the enrollment of patients misdiagnosed as RA). All 62 controls received systemic physiological examination and all physiological functions were within the normal range. Controls with a history of ant type of arthritis were excluded. The mean serum C-reaktif protein (CRP) (mg/L) level in RNA patients was 31.5±19.4 copies/mL and was 1.11±1.42 copies/mL in controls. The control group in this study included patients with suspected joint synovium and patients with trauma, which were excluded after synovial biopsies. Synovial biopsy mainly detects synovial hyperplasia and inflammatory infiltrates by hematoxylin-eosin (H&E) staining, CD3 antibody, and CD68 antibody. Figure 1 shows a representative immunostaining image of RA.

Plasma samples, synovial tissues, and human fibroblast-like synoviocytes (HFLSs)

Prior to therapy, blood (3 mL) was drawn from all RA patients and controls. All blood samples were transferred to ethylenediaminetetraacetic acid (EDTA) tubes and centrifuged at 1,200 g at room temperature for 15 min to separate plasma samples.

Synovial tissues were collected from six RA patients and two controls through biopsy. Tissue samples were kept in liquid nitrogen prior to subsequent experiments. Two controls were with suspected joints synovial diseases, which were excluded after synovial biopsies.

The HFLSs (408-05A) cell line was purchased from Sigma-Aldrich was used. Synoviocyte Growth Medium (415-500, Sigma-Aldrich, St. Louis, MO; USA) was used to cultivate HFLSs at 37°C in a 5% CO_2 and 95% humidity incubator. In cases of lipopolysaccharide (LPS) treatment, HFLSs were cultivated in medium containing 0, 1, 2, 4, 6, and 8 µg/mL LPS (Sigma-Aldrich, St. Louis, MO; USA) for 48 h before the subsequent experiments.

(a)

Demographic and clinical characteristics of the RA patients (n=62) included in the study

	n	%	Mean±SD
Age (year)			55.2±4.9
Disease duration (year)			6.4±2.8
DAS28 CRP, score 0-10			4.8±0.3
Anti-CCP-positive	53	85	
Anti-ENA-positive	6	9	
Steinbrocker			
Stage 3	32	51	
Stage 4	30	48	
RF-positive	62	100	
Serum CRP (mg/L)			31.5±19.4

(b)

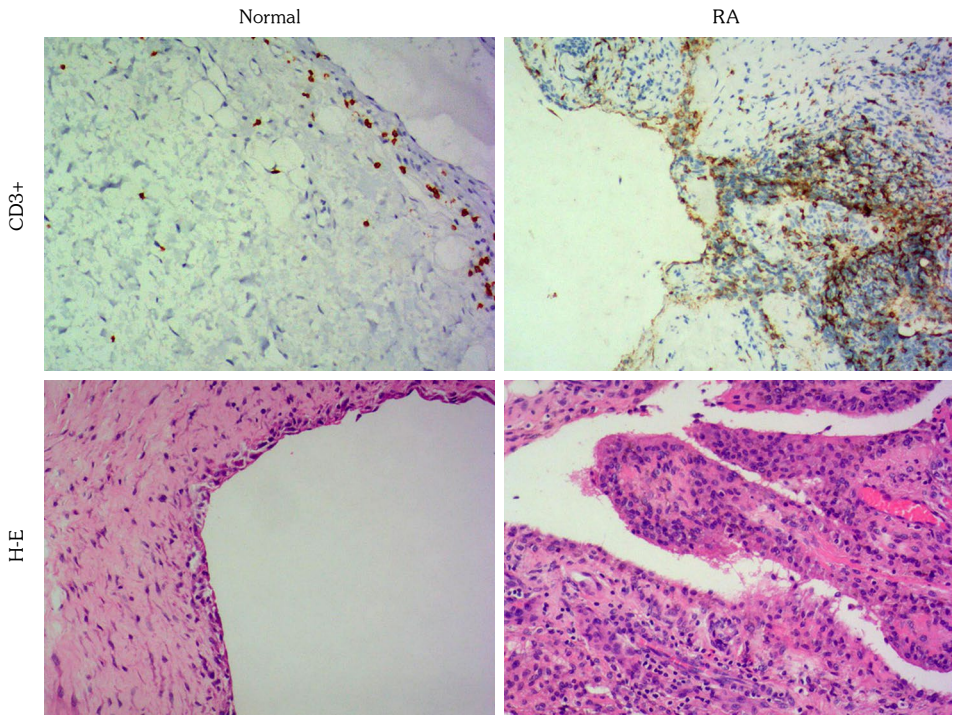

Figure 1. Patient data. **(a)** Demographic and clinical characteristics of the RA patients (n=62) included in the study. **(b)** The representative image of H&E staining and CD3 immunostaining.

RA: Rheumatoid arthritis; SD: Standard deviation; DAS28: Disease Activity Score in 28 Joints; CRP: C-reactive protein; ENA: Extractable nuclear antigen; RF: Rheumatoid factor.

Transient transfections

The vector expressing CAIF was constructed using pcDNA3.1 (Invitrogen, Carlsbad, CA, USA) as the backbone. Mimic of miR-20a and negative control (NC) miR-20a were purchased from Sigma-Aldrich, St. Louis, MO; USA). The HFLSs (10^8) were transfected with either 1 μg expression vector or 50 nM miRNA through transient transfections using lipofectamine 2000

(Invitrogen, Carlsbad, CA, USA). To perform NC experiments, the same number of cells was transfected with the same amount of empty vector or NC miRNA. Untransfected cells were cells without transfections. Cells were cultivated in fresh medium after transfections for 48 h before the subsequent experiments.

RNA isolations

The RNAzol reagent (Sigma-Aldrich, St. Louis, MO; USA) was used to isolate total RNAs from HFLSs, synovial tissues and plasma samples. Genomic deoxyribonucleic acid (DNA) removal was performed using DNase I (Invitrogen, Carlsbad, CA, USA) to digest all RNA samples at 37°C for 2 h. The RNA integrity was checked by 5% Urea-PAGE gel electrophoresis. The OD 260/280 ratios of all RNA samples were determined to reflect RNA purity.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) assay

Reverse transcriptions (RTs) were performed on all RNA samples with an OD value close to 2.0 (pure RNA) using SS-III-RT system (Invitrogen, Carlsbad, CA, USA) to prepare cDNA samples. The qPCRs were performed using SYBR Green Master Mix (Bio-Rad, CA, USA). The expression levels of CAIF were determined with 18S rRNA as the internal control. The expression of mature miR-20a and miR-20a precursor was determined by All-in-One™ miRNA qRT-PCR Reagent Kit (Genecopoeia, Guangzhou, China). To determine the expression of miR-20a, poly (A) was added to mature miRNAs and poly (T) was used as reverse primer in qPCRs. Forward and reverse primers used to determine miR-20a precursor expression were both sequence-specific primers. The U6 was used as the internal control for miR-20a. Three technical replicates were included in each qPCR and cycle threshold (Ct) values of target genes were normalized to internal controls using $2^{-\Delta\Delta CT}$ method.

Cell apoptosis analysis

Apoptosis of HFLSs with transfections was analyzed using the FITC Annexin V Apoptosis Detection Kit with PI (BioLegend, San Diego, CA, USA). Cells (9,000) were, then, transferred to each well of a six-well plate. Cells were cultivated in medium containing 8 μg/mL LPS for 48 h, followed by washing with ice-cold PBS. Following treatment with 0.25% trypsin, staining was performed with FITC Annexin V and PI for 15 min in dark. Flow cytometry was performed to analyze apoptotic cells.

Autophagy detection

Autophagy assay kit (ab139484) was used to analyze the autophagy of HFLSs following the manufacturer's instructions. Each experiment was performed using 10,000 cells in a 96-well plate. The fluorescence was analyzed using a Gemini XPS fluorescent plate reader (Abcam, Cambridge, England).

Statistical analysis

Statistical analysis was performed using the IBM SPSS version 22.0 software (IBM Corp., Armonk, NY, USA). Descriptive data were expressed in mean ± standard deviation (SD), median (min-max), and number and frequency, where applicable. Gene expression levels in plasma samples from both 62 RA patients and controls were compared using the Mann-Whitney U test. Differences among multiple transfection groups were compared using the analysis of variance (ANOVA) Tukey's test. Correlations were analyzed using the linear regression analysis. A p value of <0.05 was considered statistically significant.

RESULTS

Patient data

Demographic and clinical characteristics of the RA patients included in the study are shown in Figure 1a. In addition, synovial hyperplasia and inflammatory infiltrates were also evaluated by H&E staining and CD3 immunostaining. Figure 1B is the representative image of H&E staining and CD3 immunostaining.

The expression of CAIF, mature miR-20a and miR-20a precursor was altered in RA

The expression levels of CAIF, mature miR-20a and miR-20a precursor in plasma samples from both 62 RA patients and controls were determined by RT-qPCR. Compared to the control group, RA group exhibited significantly lower expression levels of CAIF (Figure 2a,

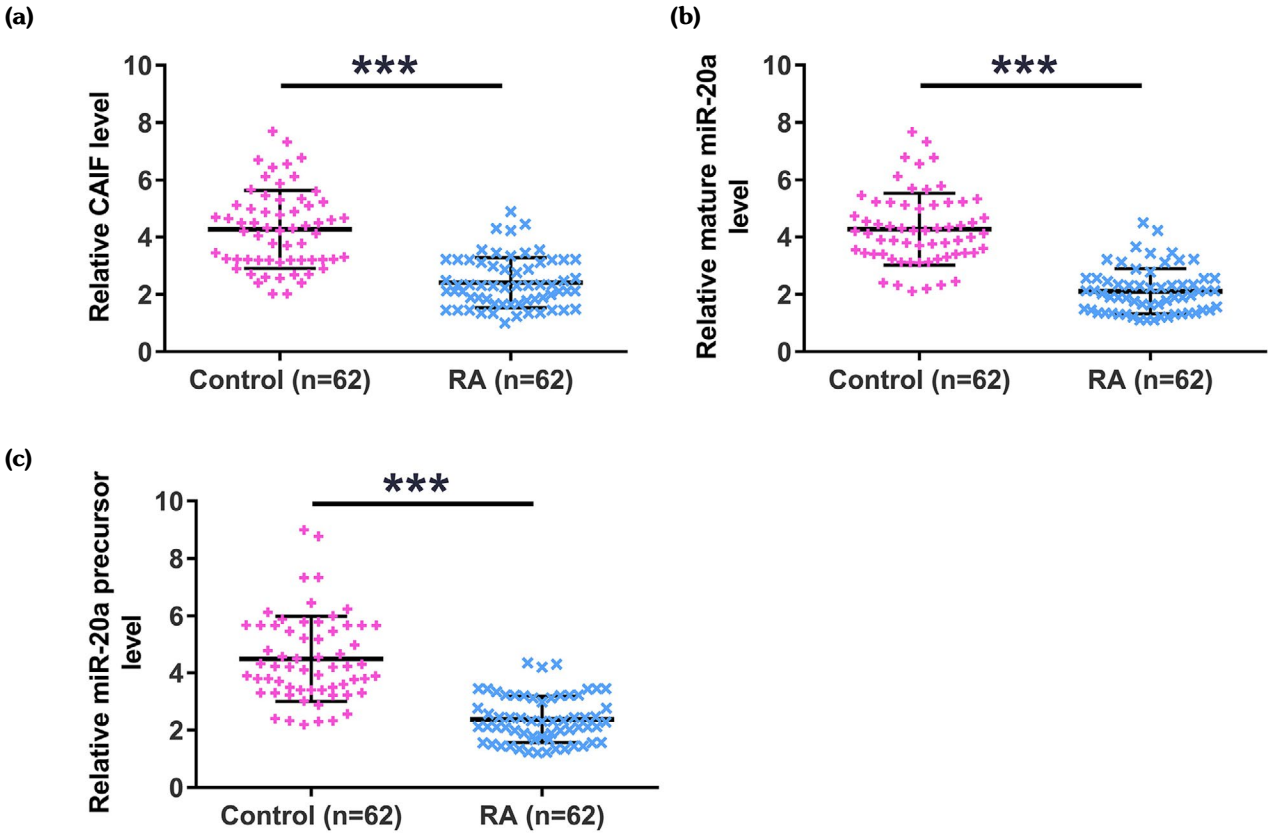

Figure 2. The expression of CAIF, mature miR-20a and miR-20a precursor was altered in RA. The expression of CAIF **(a)**, mature miR-20a **(b)** and miR-20a precursor **(c)** in plasma samples from both 62 RA patients and controls were determined by RT-qPCR. Mean values of three technical replicates were used to express gene expression levels.

CAIF: Cardiac autophagy inhibitor; RA: Rheumatoid arthritis; RT-qPCR: Reverse transcription-quantitative polymerase chain reaction; *** $p<0.001$.

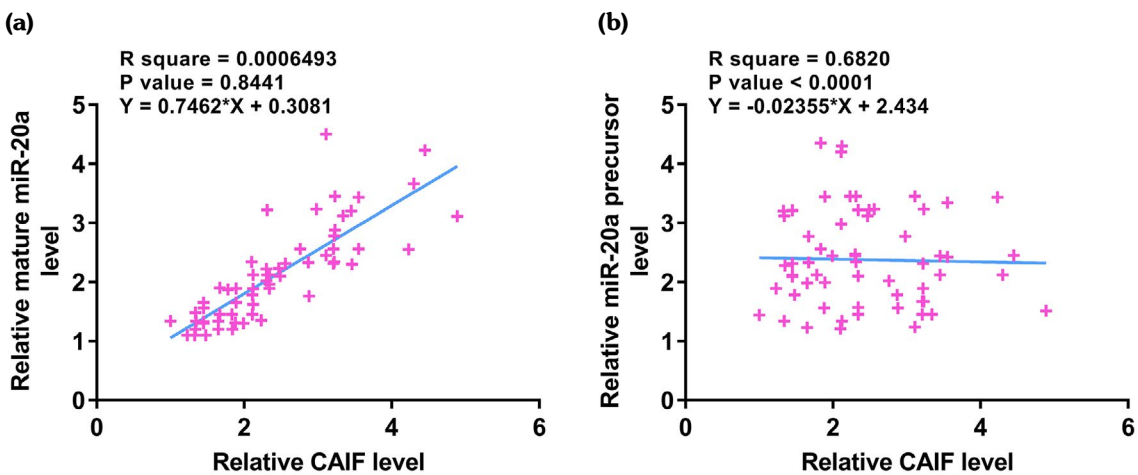

Figure 3. CAIF and mature miR-20a were positively correlated. Correlations between CAIF and mature miR-20a **(a)** or miR-20a precursor **(b)** across plasma samples from RA patients were analyzed by linear regression.

CAIF: Cardiac autophagy inhibitor; RA: Rheumatoid arthritis.

p<0.001), mature miR-20a (Figure 2b, p<0.001), and miR-20a precursor (Figure 2c, p<0.001). To further confirm the altered expression of these three factors in RA, synovial tissues from six patients and two controls were also included to perform RNA isolation and RT-qPCR. It was observed that RA synovial tissues showed significantly lower expression levels of CAIF (Supplemental Figure 1a, p<0.05), mature miR-20a (Supplemental Figure 1b, p<0.05) and miR-20a precursor (Supplemental Figure 1c, p<0.05) compared to that in the control group.

CAIF and mature miR-20a were positively correlated

Correlations between the expression of CAIF and mature miR-20a or miR-20a precursor were analyzed using the linear regression analysis. The CAIF and mature miR-20a were positively and significantly correlated across plasma samples from RA patients (Figure 3a). In contrast, no significant correlation was observed between CAIF and miR-20a precursor (Figure 3b). These results suggested that CAIF might affect the maturation of miR-20a.

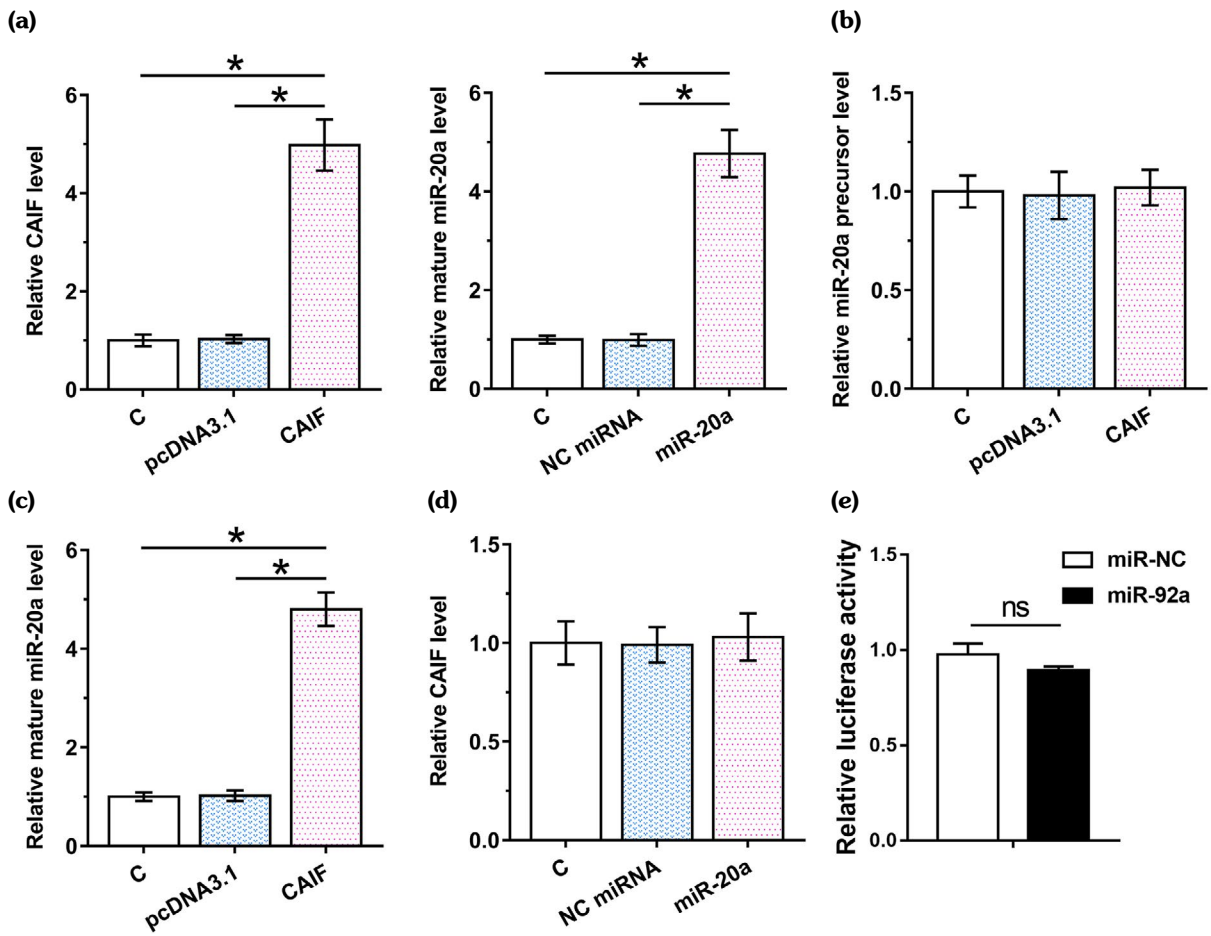

Figure 4. Overexpression of CAIF resulted in the upregulation of mature miR-20a in HFLSs. To test the hypothesis that CAIF may affect the maturation of miR-20a, HFLSs were transfected with CAIF expression vector or miR-20a mimic, and the transfections were confirmed by RT-qPCR **(a)**. The effects of CAIF overexpression on the expression of miR-20a precursor **(b)** and mature miR-20a **(c)**, and the effects of miR-20a overexpression on CAIF **(d)** were also analyzed by RT-qPCR. Luciferase assay **(e)** for luciferase activity of CAIF-reporter with miR-20a binding sites co-transfected with miR-20a mimics. n=3. Mean±SD values were used to express data of multiple transfection groups. C, control cells without transfections; pcDNA3.1, cells transfected with empty pcDNA3.1 vector; NC miRNA, cells transfected with NC miRNA.

CAIF: Cardiac autophagy inhibitor; HFLSs: Human fibroblast-like synoviocytes; RT-qPCR: Reverse transcription-quantitative polymerase chain reaction; * p<0.05.

Overexpression of CAIF resulted in upregulation of mature miR-20a in HFLSs

To test the hypothesis that CAIF may affect the maturation of miR-20a, the HFLSs were transfected with CAIF expression vector or miR-20a mimic, and the transfections were confirmed by RT-qPCR (Figure 4a, $p<0.05$). Overexpression of CAIF did not affect the expression of miR-20a precursor (Figure 4b), but upregulated the expression of mature miR-20a (Figure 4c, $p<0.05$). In contrast, overexpression of miR-20a did not affect the expression of CAIF (Figure 4d). Therefore, CAIF may promote the maturation of miR-20a in HFLSs. In addition, luciferase experiments showed that CAIF and miR-20a did not interact with each other (Figure 4e).

CAIF and miR-20a inhibited the apoptosis of HFLSs induced by LPS

The HFLSs were cultivated in medium containing 0, 1, 2, 4, 6, and 8 μg/mL LPS for 48 h, followed by the determination of the expression levels of CAIF and mature miR-20a by RT-qPCR. The LPS treatment resulted in downregulation of both CAIF (Figure 5a) and mature miR-20a (Figure 5b) in a dose-dependent manner ($p<0.05$). Cell apoptosis

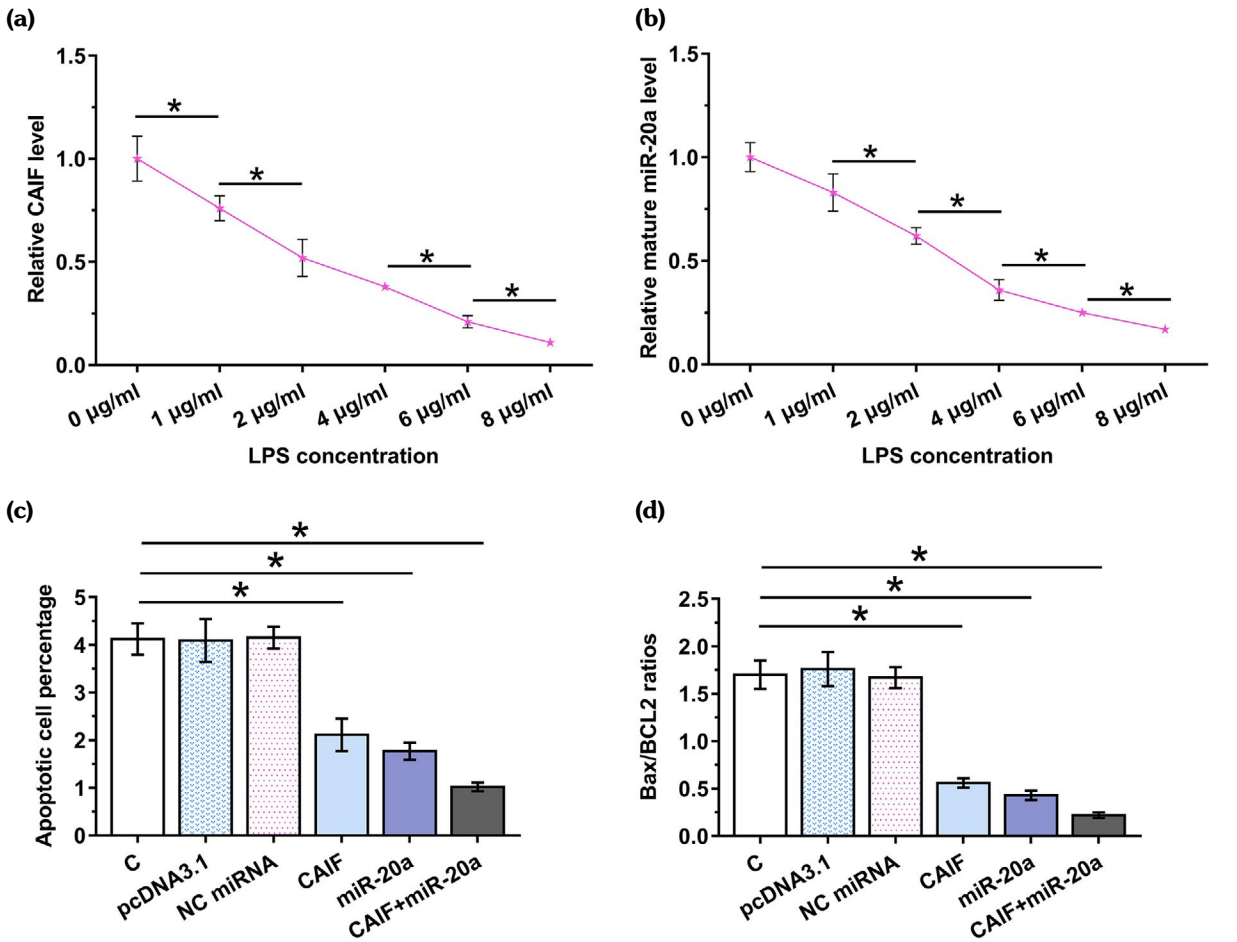

Figure 5. CAIF and miR-20a inhibited the apoptosis of HFLSs induced by LPS. HFLSs were cultivated in medium containing 0, 1, 2, 4, 6, and 8 μg/mL LPS for 48 h, followed by the determination of CAIF **(a)** and mature miR-20a **(b)** expression by RT-qPCR. The roles of CAIF and miR-20a overexpression in the apoptosis of HFLSs induced by LPS (8 μg/mL for 48 h) were analyzed by cell apoptosis assay **(c)**. Expression of Bax and BCL2 in each transfected groups was determined by RT-qPCR. Bax/BCL2 ratios of all groups were calculated **(d)**. Mean±SD values were used to express data of multiple transfection groups.

CAIF: Cardiac autophagy inhibitor; HFLSs: Human fibroblast-like synoviocytes; LPS: Lipopolysaccharide; RT-qPCR: Reverse transcription-quantitative polymerase chain reaction; * $p<0.05$.

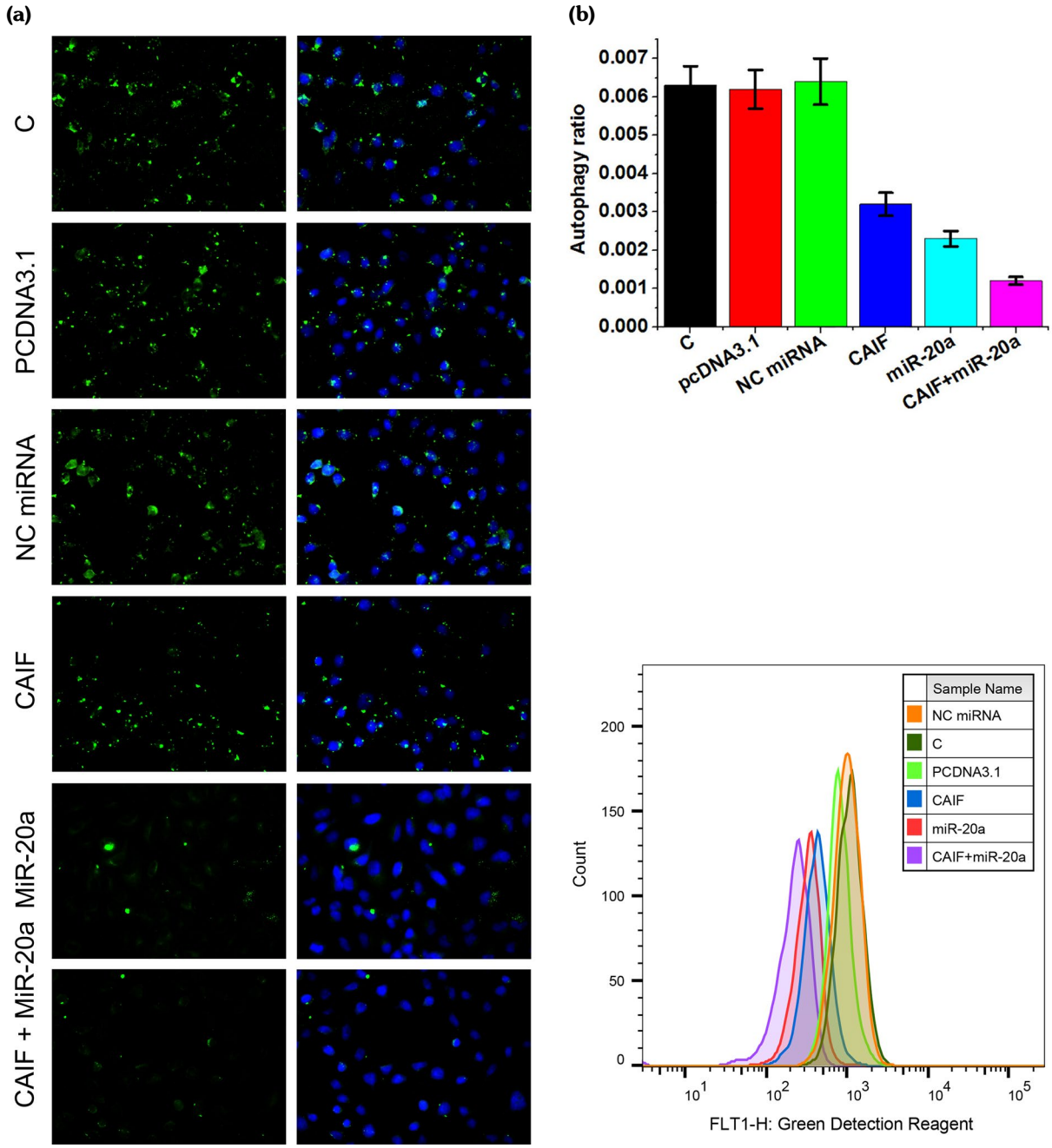

Figure 6. CAIF and miR-20a inhibited the autophagy of HFLSs. Autophagy analysis was performed to analyze the role of CAIF and miR-20a in the autophagy of HFLSs. Each experiment was performed using 10,000 ells in a 96-well plate. The fluorescence was analyzed using a Gemini XPS fluorescent plate reader. The normalized data represent the data of three biological replicates.

CAIF: Cardiac autophagy inhibitor; HFLSs: Human fibroblast-like synoviocytes; * $p<0.05$.

analysis showed that overexpression of CAIF and miR-20a inhibited the apoptosis of HFLSs induced by LPS. In addition, overexpression of both CAIF and miR-20a showed a stronger effect (Figure 5c, $p<0.05$). The expression levels of Bax and BCL2 in each transfected groups were determined by RT-qPCR. The Bax/BCL2 ratios of all groups were calculated. It was observed that the Bax/BCL2 ratios were consistent with the apoptotic rates (Figure 5d, $p<0.05$). It is

worth noting that overexpression of CAIF and miR-20a did not affect the apoptosis of HFLSs under normal conditions (without LPS, data not shown). Therefore, the functions of CAIF and miR-20a could be RA-specific.

CAIF and miR-20a inhibited the autophagy of HFLSs

Autophagy analysis was performed using an autophagy kit from Abcam (ab139484) to analyze the role of CAIF and miR-20a in the autophagy of HFLSs. Changes in autophagosome were analyzed by immunofluorescence. It was observed that CAIF and miR-20a overexpression resulted in decrease in autophagosomes. Compared to the single overexpression of CAIF or miR-20a, the combination of CAIF and miR-20a overexpression showed a stronger effect (Figure 6a, $p<0.05$), and the fluorescence activated cell sorting (FACS) results also confirm this (Figure 6b, $p<0.05$).

DISCUSSION

In the present study, we explored the interaction between CAIF and miR-20a in RA. We found that CAIF, mature miR-20a, and miR-20a precursor were downregulated in RA. In addition, CAIF was downregulated in RA and it could suppress the maturation of miR-20a to suppress LPS-induced cell apoptosis.

The CAIF plays critical roles in different human diseases, such as myocardial infarction and osteoarthritis.[11,13] In myocardial infarction, CAIF suppresses autophagy and inactivates p53-mediated myocardin transcription to suppress disease progression.[13] Another study reported that CAIF was downregulated in osteoarthritis and downregulated miR-1246 to suppress the upregulation of IL-6 induced by LPS.[11] In this study, downregulation of CAIF was also observed in RA and decreased apoptosis of HFLSs was detected after the upregulation of CAIF. Therefore, CAIF may play protective roles in RA by suppressing LPS-induced cell apoptosis.

The miR-20a has been characterized as a critical player in LPS-induced injuries.[12,14] It has been reported that miR-20a interacts with C-X-C motif chemokine ligand 12 (CXCL12) to regulate the nuclear factor kappa B (NF-κB) and extracellular signal-regulated kinases $^1/_2$ (ERK$^1/_2$) signaling, thereby suppressing LPS-induced injury in HK-2 cells.[12] In another study, miR-20a was reported to target thioredoxin-interacting protein (TXNIP) in adjuvant-induced arthritis fibroblast-like synoviocytes to suppress the expression of the NLR family pyrin domain containing 3 (NLRP3) inflammasome.[14] To the best of our knowledge, this study is the first to report the downregulation of miR-20a in RA and downregulation of miR-20a by LPS treatment in HFLSs. Moreover, decreased apoptosis of HFLSs induced by LPS was observed after overexpression of miR-20a. These findings suggest that miR-20a may suppress cell apoptosis in RA.

In this study, we demonstrated that CAIF might promote the maturation of miR-20a in HFLSs. In a recent study, colon cancer-associated transcript 2 gene (CCAT2) was reported to suppress the maturation of miR-145 by inhibiting the transportation of miR-145 precursor in colon cancer cells.[15] Similarly, we speculated that CAIF might promote the transportation of miR-20a to accelerate its maturation. However, further studies are needed to test our hypothesis.

Furthermore, studies have shown that the resistance of FLS to apoptosis is part of the cause of synovial hyperplasia.[5-7] Many current RA treatments are based on restoring the balance between synovial cell proliferation and synovial cell apoptosis.[8] Therefore, our research provides an experimental basis for promoting the development of new therapies for RA.

However, our studies were limited to the second affiliated hospital of Fujian Medical University, and there was no multicenter study, which may be subject to selection bias. Further studies with large sample sizes are still needed for validation.

In conclusion, CAIF and miR-20a were both downregulated in RA. The CAIF may promote the maturation of miR-20a to suppress the apoptosis of HFLSs induced by LPS. We believe that our findings would enhance the understanding of LncRNA regulation of miRNA and provide an experimental basis for promoting the development of new therapies for RA.

Ethics Committee Approval: The study protocol was approved by the Ethics Committee of the second affiliated hospital of Fujian Medical University (Date: 2018,

no: LCS18057). The study was conducted in accordance with the principles of the Declaration of Helsinki.

Patient Consent for Publication: A written informed consent was obtained from each participant.

Data Sharing Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Author Contributions: Study concepts, study design, literature research, experimental studies, manuscript preparation and editing: D.X.; Literature research, experiments work and manuscript writing: L.L., Z.C.

Conflict of Interest: The authors declared no conflicts of interest with respect to the authorship and/or publication of this article.

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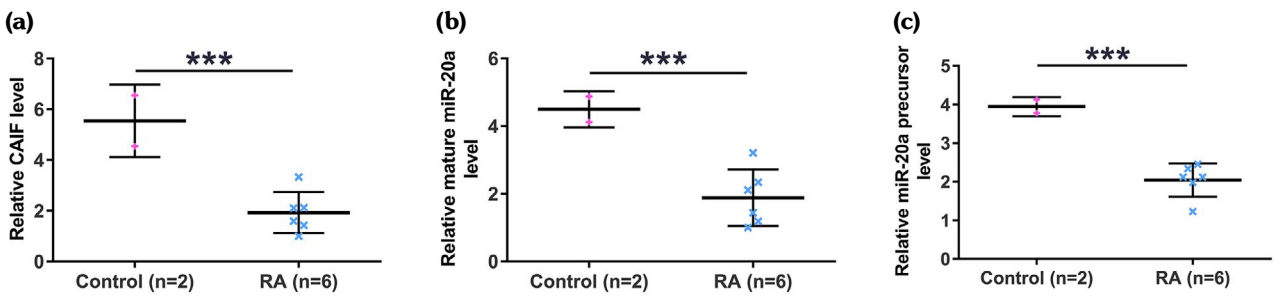

Supplemental Figure 1. The expression of CAIF, mature miR-20a and miR-20a precursor were altered in synovial tissues from RA patients. **(a)** Expression of CAIF, **(b)** mature miR-20a and **(c)** miR-20a precursor in synovial tissue samples from both 62 RA patients and controls was determined by RT-qPCR. Mean values of three technical replicates were used to express gene expression levels.

CAIF: Cardiac autophagy inhibitor; RA: Rheumatoid arthritis; RT-qPCR: Reverse transcription-quantitative polymerase chain reaction; * $p<0.05$.