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ORIGINAL ARTICLE

LncRNA GAS5 overexpression downregulates IL-18 and induces the apoptosis of fibroblast-like synoviocytes

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Abstract

Background Long non-coding RNA (lncRNA) growth arrest specific transcript 5 (GAS5) negatively regulates interleukin-18 (IL-18) in ovarian cancer, while IL-18 contributes to the development of rheumatoid arthritis (RA). Therefore, GAS5 may also participate in RA.

Methods GAS5 and IL-18 in plasma of RA patients ($n = 60$) and healthy controls ($n = 60$) were measured by RT-qPCR and ELISA, respectively. Linear regression was performed to analyze the correlations between plasma levels of IL-18 and GAS5 in both RA patients and healthy controls.

Results In the present study, we found that plasma GAS5 was downregulated, while IL-18 was upregulated in RA patients than in healthy controls. A significant and inverse correlation between GAS5 and IL-18 was found in RA patients but not in healthy controls. IL-18 treatment did not significantly alter the expression of GAS5 in fibroblast-like synoviocytes, while GAS5 overexpression led to the inhibited expression of IL-18. GAS5 overexpression also resulted in the promoted apoptosis of fibroblast-like synoviocytes.

Conclusions Therefore, GAS5 overexpression may improve RA by downregulating IL-18 and inducing the apoptosis of fibroblast-like synoviocytes.

Key points

- *The present study mainly showed that overexpression of GAS5 may assist the treatment of RA.*
- *The mechanism of GAS5 for the treatment of RA involves the downregulating inflammatory IL-18 and mediating the apoptosis of fibroblast-like synoviocytes.*
- *GAS5 and IL-8 were correlated in RA patients but not in healthy controls.*

Keywords Interleukin-18 · lncRNA GAS5 · Rheumatoid arthritis

Introduction

Rheumatoid arthritis (RA) is one of the most diagnosed autoimmune inflammatory diseases in clinical practices and is characterized by joint destruction (irreversible), abnormal immune response, and chronic inflammatory responses [1, 2]. Patients with RA are usually at a high risk of other severe diseases, such as polyangiitis, interstitial lung disease, and cardiovascular diseases, so clinical treatment of RA is always challenging [3]. RA affects about 1% of the population worldwide and is considered a heavy burden on public health [4]. To date, pathogenesis of RA still has not been fully elucidated and the unknown molecular pathogenesis of RA is the major challenge in its clinical treatment [5].

RA causes the inflamed and thickened synovium, which is thin under physiological conditions [6]. Fibroblast-like synovial cells (FLSs) are key effector cells in the thickening of synovium, and abnormal replication of FLSs contributes to the development and progression of RA [7]. Therefore, inhibition of FLS proliferation and inflammation and induction of FLS apoptosis may contribute to the treatment of RA [7]. It has been reported that the development of RA was

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accompanied by changes in expression levels of a large number of long (> 200 nt) non-coding RNAs (lncRNAs) [8] and regulation of lncRNA expression affects the behaviors of FLSs [9]. It has been reported that lncRNA arrest specific transcript 5 (GAS5) negatively regulates interleukin-18 (IL-18) in ovarian cancer [10], while IL-18 contributes to the development of RA [11], indicating the potential involvement of GAS5 in RA. Our study was therefore carried out to investigate the interaction between GAS5 and IL-18 in RA.

Materials and methods

Subjects

During the time period between January 2016 and July 2018, our study enrolled 60 patients with RA (34 males and 26 females, 31 to 68 years, 49.8 ± 5.9 years) and as well as 60 healthy controls (33 males and 27 females, 32 to 69 years, 49.5 ± 5.6 years) in China-Japan Union Hospital of Jilin University. RA patients were diagnosed according to the criteria established by American College of Rheumatology/European League [12]. Disease activity score (DAS) ranged from 4.1 to 5.5, with a mean of 4.8 ± 0.3. Disease duration ranged from 5.5 to 13.2 years, with a mean of 9.8 ± 2.4 years. Serum levels of C-reactive protein ranged from 9.6 to 35.5 ml/L, with a mean of 19.7 ± 2.4 mg/L. Inclusion criteria of the patient group: (a) no history of malignancies; (2) no therapies received within 3 months before admission; (3) first time diagnosis. Exclusion criteria: (1) patients complicated with other clinical complications; (2) patients without complete medical record. Controls were enrolled in the physical health examination center of the aforementioned hospital. All healthy controls showed normal physical functions, and they were selected to match the distributions of age and gender of the RA group. All patients and controls signed informed consent. The Ethics committee of China-Japan Union Hospital of Jilin University approved this study.

Plasma and human fibroblast-like synoviocytes

Fasting blood was extracted from each patient and healthy control before any therapies. Blood was transferred to EDTA tubes and centrifuged at 1200 g for 15 min to prepare plasma.

According to the procedures described by Lee et al. [13], plasma and human fibroblast-like synoviocytes (HFLSs) were isolated and cultivated. HFLSs were harvested from passage 5 to 7 for experiments. The only difference is that synovial biopsy was used to obtain synovial tissues instead of collecting these tissues during joint replacement surgery.

Enzyme-linked immunosorbent assay (ELISA)

IL-18 in plasma of both RA patients and healthy controls was detected by enzyme-linked immunosorbent assay (ELISA) using Human IL-18 ELISA Kit (ab215539, Abcam). The detection range was 62.5–4000 pg/ml. The intra-assay and inter-assay coefficients were 2.7% and 14.2%, respectively. Plasma levels of IL-18 were expressed as pg/ml.

RT-qPCR

RNAzol reagent (Sigma-Aldrich) was used to extract total RNA from plasma and HFLSs. Following reverse transcriptions performed using SuperScript III Reverse Transcriptase (Thermo Fisher Scientific), PCR reaction systems were prepared using Luna® Universal One-Step RT-qPCR Kit (NEB) with 18S rRNA as endogenous control to detect the expression of GAS5. Primer sequences were 5′-CCATGGATGACTTGCTTGG-3′ (forward) and 5′-GCATGCTTGCTTGTTGTGG-3′ (reverse) for GAS5; 5′-TCGACAGTCAGCCGCATCTTCT-3′ (forward) and 5′-CCAAATCCGTTGACTCCGACCT-3′ (reverse) for GAPDH. Cycling condition were 95 °C for 55 s followed by 40 cycles of 94 °C for 8 s, 56 °C for 10 s, and 72 °C for 35 s. PCR reactions were performed on QuantStudio 5 qPCR System (Thermo Fisher Scientific) This experiment as performed in triplicate manner, the and $2^{-\Delta\Delta CT}$ method was used to perform data normalizations.

Transient cell transfection

Lipofectamine 2000 reagent (Invitrogen, Thermo Fisher Scientific, Inc.) was used in this study to perform all transient cell transfections. GAS5-expression pcDNA3.1 vector was constructed by Sangon (Shanghai, China). Ten-nanometer vectors were used in all transfections. Two controls, including negative control (empty vector transfection) and control (non-transfection), were included in this experiment. Subsequent experiments were performed at 24 h after transfections.

Cell apoptosis assay

HFLSs were harvested at 24 h after transfection to prepare single-cell suspensions (3×10^4 cells/ml) using serum-free culture medium. Two-milliliter cell suspensions were added into each well of a 6-well plate. Cells were cultivated for 48 h, followed by 0.25% trypsin digestion. Finally, cells were subjected to Annexin V-FITC (Dojindo, Japan) and propidium iodide (PI) staining and flow cytometry was carried out to detect apoptotic cells.

Western blotting

RIPA lysis and extraction buffer (Thermo Fisher Scientific) was mixed with HFLSs at 24 h after transfection to extract total protein. Following denaturing, protein samples were subjected to electrophoresis using 10% SDS-PAGE gel. After gel transfer to PVDF membranes, membranes were blocked for 2 h at room temperature in 5% fat-free milk. After that, membranes were subjected to incubation with primary antibody of rabbit anti-human IL-18 (ab68435, 1:1400, Abcam) or (ab9485, 1:1400, Abcam) as well as secondary goat anti-rabbit IgG-HRP antibody (1:1500, MBS435036, MyBioSource). Signals were developed using ECL (Sigma-Aldrich, USA) and processed by ImageJ v1.46 software.

Statistical analysis

Three biological replicates were included in each experiment. Differences between RA patients and healthy controls were analyzed by an unpaired *t* test. Differences among different cell treatment groups were analyzed by ANOVA (one-way) combined with a Tukey test. Linear regression was performed to analyze the correlations between plasma levels of IL-18 and GAS5. Differences with $p < 0.05$ were statistically significant.

Results

Plasma GAS5 and IL-18 were dysregulated in RA patients comparing with healthy controls

GAS5 and IL-18 in plasma of RA patients ($n = 60$) and healthy controls ($n = 60$) were measured by RT-qPCR and ELISA, respectively. Plasma levels of GAS5 and IL-18 were compared between RA patients and healthy controls by performing an unpaired *t* test. Comparing with healthy controls, plasma GAS5 was significantly downregulated (Fig. 1A), while IL-18 was significantly upregulated (Fig. 1B) in RA patients ($p < 0.05$).

Plasma GAS5 and IL-18 were significantly and inversely correlated in RA patients

Linear regression was performed to analyze the correlations between plasma levels of IL-18 and GAS5 in both RA patients ($n = 60$) and healthy controls ($n = 60$). A significant and inverse correlation between GAS5 and IL-18 was found in RA patients (Fig. 2A). However, the correlation between plasma levels of IL-18 and GAS5 was not significant in healthy controls (Fig. 2B).

GAS5 overexpression led to downregulated IL-18 in HFLSs

To further investigate the interaction between GAS5 and IL-18, HFLSs were treated with IL-18 (Sigma-Aldrich) at doses of 0, 200, 500, and 1000 pg/ml for 24 h to detect the effects of IL-18 on GAS5. It was observed that IL-18 treatment had no significant effects on GAS5 expression (Fig. 3A). GAS5 expression vectors were transfected into HFLSs. Comparing with two controls (control, C; negative control, NC), expression levels of GAS5 were significantly increased at 24 h after transfection (Fig. 3B, $p < 0.05$). In addition, GAS5 overexpression led to the inhibited expression of IL-18 (Fig. 3C, $p < 0.05$).

GAS5 overexpression resulted in promoted apoptosis of HFLSs

Cell apoptosis data were compared among different cell transfection groups. Cell apoptosis assay showed that, comparing with two controls (control, C; negative control, NC), GAS5 overexpression resulted in the promoted apoptosis of HFLSs (Fig. 4, $p < 0.05$), indicating the involvement of GAS5 in regulating the apoptosis of HFLSs.

Discussion

LncRNA GAS5 regulates IL-18 in ovarian cancer [11], which contributes to the development of RA [10]. The present study

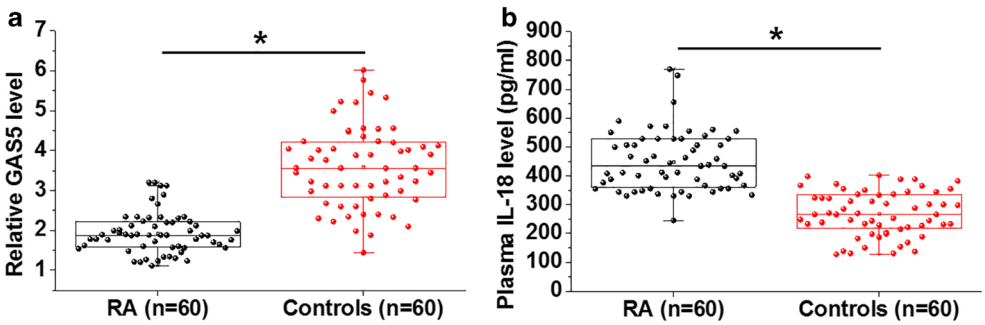

Fig. 1 Plasma GAS5 and IL-18 were dysregulated in RA patients comparing with healthy controls. RT-qPCR and ELISA showed that plasma GAS5 was significantly downregulated (A), while IL-18 was significantly upregulated (B) in RA patients comparing with healthy controls (*$p < 0.05$)

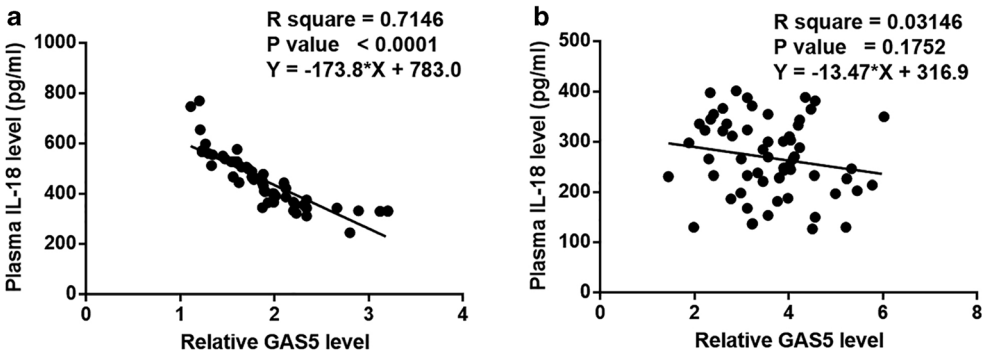

Fig. 2 Plasma GAS5 and IL-18 were significantly and inversely correlated in RA patients. Linear regression analysis revealed a significant and inverse correlation between GAS5 and IL-18 in RA patients (A) but not in healthy controls (B)

first reported the downregulation of GAS5 in RA and overexpression of GAS5 may contribute to the treatment of RA by downregulating IL-18 and promoting the apoptosis of HFLSs.

Inflammatory factors are critical players in the pathogenesis of RA [14]. Altered expression of certain inflammatory factors, such as IL-6, IL-17, and IL-15 mediates inflammatory responses in RA patients, thereby aggregating the conditions of disease [15, 16]. IL-18 was usually upregulated in RA patients and measurement of IL-18 levels assists the determination of disease activity [17]. It is known that the expression of IL-18 under pathological conditions can be regulated by certain lncRNAs [11, 18]. In a recent study, Li et al.

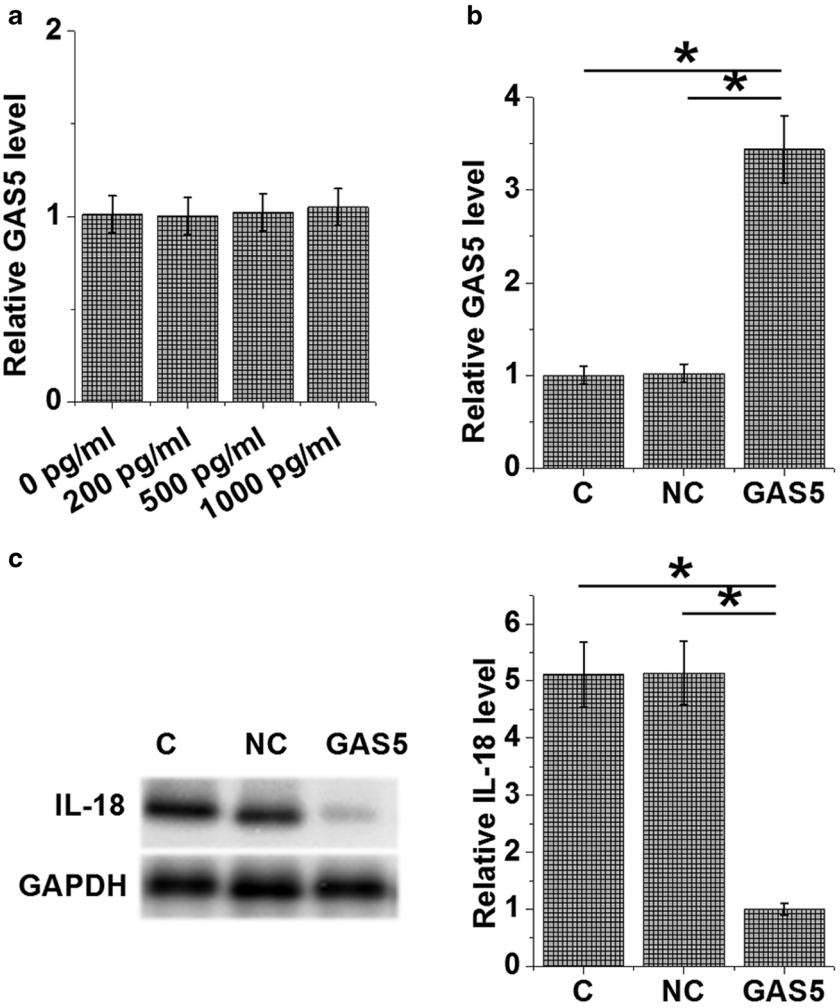

Fig. 3 GAS5 overexpression led to downregulated IL-18 in HFLSs. Treatment with IL-18 at doses of 0, 200, 500, and 1000 pg/ml for 24 h showed no significant effects on GAS5 expression (A). Comparing with two controls (control, C; negative control, NC), expression levels of GAS5 were significantly increased at 24 h after transfection (B). In addition, GAS5 overexpression led to the inhibited expression of IL-18 (C), (*$p < 0.05$)

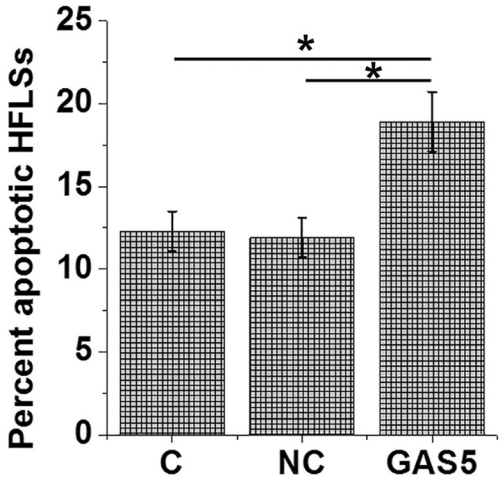

Fig. 4 GAS5 overexpression resulted in promoted apoptosis of HFLSs. Comparing with two controls (control, C; negative control, NC), cell apoptosis data showed that GAS5 overexpression resulted in the promoted apoptosis of HFLSs (*$p < 0.05$)

reported that GAS5 regulated the expression of IL-18 to suppress ovarian cancer [11]. In the present study, we first reported the inhibitory effects of GAS5 on IL-18 in HFLSs, which are critical effectors in RA [7]. Our data suggested that overexpression of GAS5 may improve RA by inhibiting IL-18.

LncRNA GAS5 is a well-characterized tumor-suppressive lncRNA in different types of cancer [11, 19]. GAS5 participate in cancer biology by affecting the behaviors of cancer cells, such as promoting cancer cell apoptosis [19]. In the present study, we showed that overexpression of GAS5 led to the increased apoptotic rate of HFLSs. Therefore, GAS5 may have similar functions in regulating cell behaviors in different types of diseases. However, the molecular mechanism is still unknown. Our experimental data revealed that the downregulation of IL-18 (consequence of GAS5 overexpression) has no enhancing effect on the apoptosis of HFLSs (data not shown). Therefore, GAS5 may also interact with other downstream effectors to affect the apoptosis of HFLSs. Our future studies will try to identify other factors that participate in GAS5-mediated apoptosis of HFLSs.

This study is limited by the small sample size and the lack of animal model experiments. Our future studies will try to include more patients and construct RA animal models to further confirm our conclusions. In addition, the mechanism of the regulation of IL-18 by GAS5 is unclear. Our preliminary experimental data revealed the potential involvement of GAS5 in epigenetics. Our future studies will investigate the effects of GAS5 in the regulation of the methylation of IL-18 gene.

Conclusion

In conclusion, GAS5 was downregulated in RA, and overexpression of GAS5 may improve RA by inducing the apoptosis of HFLSs and downregulating IL-18.

Authors' contribution Cuili Ma, Weigang Wang, and Ping Li designed the study; Cuili Ma, Weigang Wang, and Ping Li participated the experiments and analysis; Cuili Ma and Weigang Wang wrote the manuscript; Ping Li revised the manuscript.

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Data availability The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

Compliance with ethical standards

Disclosures None.

Ethics approval and consent to participate The present study was approved by the Ethics Committee of China-Japan Union Hospital of Jilin University. The research has been carried out in accordance with the World Medical Association Declaration of Helsinki. All patients and healthy volunteers provided written informed consent prior to their inclusion within the study.

Consent for publication Not applicable.

Abbreviations RA, rheumatoid arthritis; FLSs, fibroblast-like synovial cells; PI, propidium iodide

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