miR-483-3p promotes IL-33 production from fibroblast-like synoviocytes by regulating ERK signaling in rheumatoid arthritis

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Abstract

Our previous identified miR-483-3p to be highly expressed in synoviocytes from patients with rheumatoid arthritis (RA); however, its effects on inflammation of RA fibroblast-like synoviocytes (FLSs) have remained unclear. The expression of miR-483-3p and cytokines in RA FLSs was detected using quantitative real-time polymerase chain reaction. Enzyme-linked immunosorbent was conducted to determine interleukin (IL)-33 production from RA FLSs. Western blotting was employed to quantify the levels of p-ERK and total ERK. Overexpressed miR-483-3p significantly increased the mRNA and protein expression of IL-33, but not of IL-27 or IL-34, in RA FLSs, whereas miR-483-3p suppression showed the opposite effects. Furthermore, miR-483-3p upregulation activated the ERK signaling pathway. The ERK signaling inhibitor PD98059 partly reversed the elevation of IL-33 levels mediated by miR-483-3p overexpression. Our results reveal that miR-483-3p promotes IL-33 expression by regulating the ERK signaling pathway in RA FLSs. Thus, miR-483-3p may be a potential effective target for RA treatment.

Introduction

Rheumatoid arthritis (RA) is a chronic disease that affects several organs and tissues, predominantly the joints, in 0.5–1% of the population worldwide [1]. As one of the most common autoimmune diseases, RA is characterized by persistent synovitis, systemic inflammation, and autoantibody production [2]. The global burden of RA, measured by incidence, complications, and expenses, is substantial and constantly on the rise [3]. Due to the development of comprehensive treatment methodologies in the past decades, clinical outcomes of patients with RA have significantly improved [4]. However, some patients still fail to achieve total remission with persistent synovial inflammation.

Dysfunction of synovial tissues is the basis for the persistent inflammation and pannus formation in RA [5]. The normal synovium, a thin membrane lining the joint capsule, consists of two cell populations, namely fibroblast-like synoviocytes (FLSs), and macrophage-like synoviocytes. FLSs are critical in the pathogenesis of RA [6]. Distinct from other inflammatory arthritis, FLSs from patients with RA show aggressive tumor cell-like features, such as excessive proliferation, resistance to cell death, and inflammatory cytokine secretion [7]. Therefore, identifying novel potential target molecules in RA FLSs would be of great importance in this field.

MicroRNAs (miRNAs), defined as small non-coding RNAs 18–25 nt in length, modulate gene expression through degradation or translational repression of mRNA [8]. Accumulating evidence has indicated miRNAs to be associated with the occurrence and development of RA by regulating several physiological and pathological processes in RA FLSs, such as cell proliferation, migration, invasion, and inflammation [9, 10]. Our previous studies had identified miR-483-3p as a dysregulated miRNA in synovial tissues and FLSs from patients with RA [11]. Through gain- and loss-of-function assays, we could demonstrate the promotion of cell proliferation and suppression apoptosis by miR-483-3p in RA FLSs. However, the precise roles of miR-483-3p in the inflammation of RA FLSs are still unknown.
In this study, we demonstrated that miR-483-3p promoted the inflammatory cytokine secretion from FLSs by regulating the ERK signaling pathway in RA. Overall, we suggest miR-483-3p to potentially serve as a promising therapeutic target for patients with RA.

**Materials And Methods**

**Cell culture**

Healthy FLSs (HFLS) and immortalized RA FLSs (HFLS-RA) were purchased from the Jennio Biotech Co., Ltd. (Guangzhou, China). They were cultured in MEM (Corning, USA) and DMEM (Corning), respectively, at 37°C in a 5% CO2 incubator. LPS (1 µg/mL) and TNF-α (10 ng/mL) (Sigma-Aldrich, St.Louis, USA) were used to stimulate HFLS-RA cells, respectively.

**Cell Transfection**

miR-483-3p mimics, mimics negative control (NC), inhibitor and inhibitor NC were commercially obtained from GenePharma (Suzhou, China) and transfected into HFLS-RA using jetPRIME reagent (Polyplus, Illkirch, France) according to the manufacturer's instructions.

**Quantitative Real-time Polymerase Chain Reaction (Qrt-pcr)**

Total RNA isolation from HFLS and HFLS-RA, reverse transcription, and quantitation of target genes were conducted as described previously [11]. GAPDH and U6 were used as controls for IL-33 and miR-483-3p expression, respectively. The primers commercially obtained from Sangon Biotech (Shanghai, China) were as follows: 5'-AGACGGCAGGCGACCTT-3' (forward) and 5'-GGCTGACTGTGAACCTCCCTC-3' (reverse) for IL-27; 5'-GTGACGGGTGGATGTAAGAT-3' (forward) and 5'-AGCTCCACAGAGTGTTCCTTG-3' (reverse) for IL-33; 5'-AAACAAAGCTCCGTCTCAACTG-3' (forward) and 5'-GCCGCATACTGCAATGAGG-3' (reverse) for IL-34; 5'-ACAACCTTTGGTATCGTGGAAG-3' (forward) and 5'-GCCATCAGCCACAGGTTTC-3 (reverse) for GAPDH; 5'-TCACTCCTCCTCCGTCCTT-3' (forward) for miR-483-3p. Relative expression of genes was calculated using the $2^{-\Delta\Delta C_t}$ method.

**Enzyme-linked Immunosorbent Assay (Elisa)**

The cell culture supernatant from HFLS-RA was collected as described previously [12]. Concentration of IL-33 in the supernatants was detected using an ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

**Western Blotting**

HFLS-RA, under different treatments, were lysed with RIPA buffer (Solarbio, China) to obtain the total protein; a BCA kit (Solarbio) was used to quantify the levels of total protein. Approximately 30 µg of protein was separated through SDS-PAGE (Solarbio) and transferred to PVDF membranes. The membranes were then incubated with primary antibodies against ERK1/2 (1:1000) (Proteintech, China), p-
ERK1/2 (1:1000) (Proteintech) and GAPDH (1:5000) (Proteintech), and subsequently with secondary antibodies (Proteintech).

**Statistical analysis**

All experiments were conducted independently at least thrice. The Student’s t-test and one-way analysis of variance followed by Dunn’s multiple comparison post-hoc test were used to compare the significance of differences across two and multiple groups, respectively. GraphPad Prism (v.8.0, CA) was used to analyze the data. *P* < 0.05 was considered statistically significant.

**Results**

**miR-483-3p was highly expressed in RA FLSs**

As shown in Fig. 1a, HFLS-RA had a higher miR-483-3p expression than HFLS (*p* < 0.05). The expression of miR-483-3p was further increased in HFLS-RA after treatment with LPS (*p* < 0.01, Fig. 1b). Consistently, the expression of miR-483-3p was significantly increased in HFLS-RA after treatment with TNF-α (*p* < 0.01, Fig. 1c), suggesting dysregulated miR-483-3p might be involved in the pathogenesis of RA.

**Mir-483-3p Promoted Inflammation Of Ra Flss By Regulating IL-33**

In order to explore the role of miR-483-3p in inflammation, we first manipulated the expression of miR-483-3p in HFLS-RA. As shown in Fig. 2a, mimics treatment significantly increased miR-483-3p levels in HFLS-RA (*p* < 0.0001), while inhibitor treatment decreased the miR-483-3p levels (*p* < 0.0001). Next, we explored the role of miR-483-3p in inflammation by measuring the levels of cytokines, including IL-27, IL-33, and IL-34. qRT-PCR assays showed that IL-33 expression, but not IL-27 (*p* > 0.05, Fig. 2b) or IL-34 (*p* > 0.05, Fig. 2c) expression, was upregulated in the mimics group (*p* < 0.001, Fig. 2d) and downregulated in the inhibitor group (*p* < 0.05). Consistently, the secretion of IL-33 from HFLS-RA was increased in the mimics group (*p* < 0.0001, Fig. 2e) and decreased in the inhibitor group (*p* < 0.01) compared to that in the NC groups. These results indicated the promotion of RA FLSs inflammation to occur via IL-33 induction.

**Mir-483-3p Activated The Erk Signaling Pathway In Ra Flss**

Since ERK signaling activation is commonly involved in RA-related inflammation [13], we evaluated the role of miR-483-3p in ERK signaling. As shown in Fig. 3, the levels of p-ERK1/2 were significantly unregulated in the mimics group (*p* < 0.0001, Fig. 3a and c) and downregulated in the inhibitor group compared to that in the NC groups. However, the levels of total ERK showed no significant difference (*p* > 0.05, Fig. 3a and b). These results demonstrated the activation of ERK signaling was mediated by miR-483-3p in RA FLSs.

**miR-483-3p promoted IL-33 levels in RA FLSs by activating the ERK signaling pathway**

To confirm whether miR-483-3p regulated the inflammation of RA FLSs through ERK signaling, rescue assays were performed with PD98059, an ERK signaling pathway inhibitor. As shown in Fig. 4a-c,
PD98059 treatment significantly suppressed the levels of p-ERK1/2 (p < 0.001), although not of total ERK1/2 (p > 0.05), in HFLS-RA. Notably, PD98059 treatment significantly rescued the upregulation of IL-33, at both mRNA and protein levels, after being induced by miR-483-3p mimics (both p < 0.05, Fig. 4d and e), thereby suggesting that promotion of IL-33 levels in RA FLSs by miR-483-3p occurred via activation of the ERK signaling pathway.

**Discussion**

Persistent synovial inflammation is a unique hallmark of RA pathology, resulting in progression of the disease and, eventually, deformity of joints [14]. FLSs are important non-immune cells mainly located in the synovium, whose interaction with immune and non-immune cells lead to synovial hyperplasia, pannus formation, and of proinflammatory cytokine secretion in patients with RA [15].

Recent studies have suggested that miRNAs are involved in the inflammatory responses of RA FLSs. Najm A et al. had identified miR-17 as a low-expression miRNA that exerted anti-inflammatory effect by suppressing the IL-6 and IL-1β expression in RA FLSs [10]. Tsai MH et al. had demonstrated that miR-137 downregulated IL-6 and cyclooxygenase-II (COX-II) levels in RA FLSs [16]. Additionally, our previous study had reported the suppression of tumor necrosis factor (TNF)-α, IL-1β, IL-6, and metalloprotease (MMP)-9 levels in RA FLSs by miR-410-3p via NF-κB signaling [12]. Particularly, some novel ILs have been associated with miRNAs in RA. Yang et al. had demonstrated a positive correlation between miR-21 and IL-34 in patients with RA [17]; Figueiredo et al. had reported that miR-29b, miR-21, and miR-20b were involved to regulate the function of IL-27 in inflammation of RA [18]. Therefore, we planned to explore the association of miR-483-3p with these relatively novel ILs. In this study, we confirm that miR-483-3p promotes inflammation in RA FLSs by regulating the expression of IL-33, but not of IL-27 or IL-34.

IL-33 has been confirmed to be highly expressed in blood serum, synovial fluid, and FLSs of patients with RA [19–22]; additionally, it is associated with disease activity, autoantibody production, and response to drugs [23]. Wu S et al. had reported that IL-33 promotes proliferation and inhibits apoptosis of RA FLSs by regulating NF-κB signaling [24], suggesting that IL-33 plays its role as a proinflammatory cytokine in RA FLSs. Recent studies have found that IL-33 production might be promoted via ERK signaling pathway activation [25]. Furthermore, abnormal activation of the ERK signaling pathway has been reported to regulate cell proliferation, apoptosis, migration, invasion, and inflammation in RA [26, 27]. Therefore, we speculated that promotion of IL-33 levels by miR-483-3p could be mediated by ERK signaling pathway. In our study, we observed that overexpression of miR-483-3p activated the ERK signaling pathway in RA FLSs, and the ERK signaling inhibitor reversed the promotion of IL-33 production by miR-483-3p, hence confirming the promotion of IL-33 expression by miR-483-3p in RA FLSs to occur via ERK signaling pathway.

In conclusion, we found miR-483-3p to be highly expressed in RA FLSs, promoting IL-33 production in RA FLSs via the ERK signaling pathway. These findings together revealed miR-483-3p to play an important role in inflammation of RA FLSs, possibly representing an effective target for RA treatment.
Declarations

Ethics approval and consent to participate

This study was performed according to the recommendations of the Declaration of Helsinki and approved by the Ethics Committee of Shengjing Hospital of China Medical University (2019PS637K).

Consent for publication

Not Applicable.

Conflict of Interest

The authors declare that they have no conflict of interest.

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Authors' contributions

YW: conception and design of the research; KZ: acquisition of data and drafting the manuscript; KZ, TJ and SZ: analysis and interpretation of data; DW: statistical analysis; WF and YW: revision of manuscript for important intellectual content. All authors contributed to data analysis, drafting and revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

Acknowledgements

Not Applicable.

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**Figures**
miR-483-3p is highly expressed in RA FLSs (a) The expression of miR-483-3p in HFLS and HFLS-RA was measured using qRT-PCR. (b) The expression of miR-483-3p in HFLS-RA, with or without LPS (1 μg/mL) treatment for 24 h, was detected using qRT-PCR. (c) The expression of miR-483-3p in HFLS-RA, with or without TNF-α (10ng/mL) treatment for 24 h, was detected using qRT-PCR. *P < 0.05, **P < 0.01, with respect to the NC groups.
miR-483-3p promotes inflammation of RA FLSs by regulating IL-33 expression

(a) The expression of miR-483-3p in HFLS-RA after transfection with mimics, mimics NC, inhibitor and inhibitor NC for 48 h was identified using qRT-PCR. (b-d) Expression of IL-27 (b), IL-34 (c), and IL-33 (d) in HFLS-RA after transfection for 48 h was determined using qRT-PCR. (e) The concentration of IL-33 from cell culture supernatant of HFLS-RA after transfection for 48 h was quantified through ELISA. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, compared to the NC groups.

Figure 3

miR-483-3p activates the ERK signaling pathway in RA FLSs (a-c). The levels of total ERK (b) and p-ERK (c) in HFLS-RA after transfection for 72 h were determined through western blotting. ****P < 0.0001 compared to the NC groups.
Figure 4

miR-483-3p promotes IL-33 levels in RA FLSs by activating the ERK signaling pathway (a-c) The levels of total ERK (b) and p-ERK (c) in HFLS-RA after treatment with PD98059 (25 μmol/L) for 72 h, as determined through western blotting. (d) mRNA expression of IL-33 in HFLS-RA after transfection with mimics, with or with PD98059 treatment for 48 h, was determined using qRT-PCR. (e) The concentration of IL-33 from culture supernatant of HFLS-RA after transfection of mimics, with or without PD98059 treatment for 48 h, was determined using ELISA. *P < 0.05, **P < 0.01 and ***P < 0.001 compared to the NC groups.