Aberrant expression of miR-127 in rheumatoid arthritis and its related mechanism

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Abstract

Objective: This study aims to investigate the role and mechanism of miR-127 in rheumatoid arthritis (RA).

Methods: Totally 30 cases of RA patients were enrolled and 30 cases of healthy volunteers were enrolled as the control group. Peripheral blood samples and synovial tissues of the participators were collected, and RT-qPCR has been performed to detect the expression of miR-127; moreover, fibroblast-like synoviocytes (SFs) of patients with RA were cultured and stimulated with TNF-α, and transfected with miR-127 mimics, and MTT assay as well flow cytometry assay have been performed to examine the effect of miR-127 on the proliferation and apoptosis of SFs; furthermore, cells were collected, and RT-qPCR as well as western blot assays have been performed to examine the expression of Bcl-2, Bax and OPN; finally, dual luciferase assay was used to validate whether miR-127 directly target OPN.

Results: It was found that miR-127 was significantly decreased in blood and synovial tissues of RA patients, while OPN expression was significantly decreased in synovial tissues of patients with RA ($P<0.05$). Next, transfection of miR-127 mimics induced significant increase in the proliferation and marked decrease in the apoptosis of SFs isolated from patients with RA. Moreover, transfection of miR-127 mimics also induced significant decrease in the expression of OPN and Bax, and marked increase in the expression of Bcl-2. Finally, results of dual luciferase assay showed that

miR-127 can directly target OPN.

Conclusion: OPN was significantly increased in RA patients, which might be associated with the down-regulation of miR-127. miR-127 may promote the development and progression of RA through targeting OPN.

Key word: miR-127, rheumatoid arthritis, Osteopontin

Introduction

Rheumatoid arthritis (RA) is a common systemic autoimmune disorder characterized by symmetrical arthritis. RA can affect RA can affect different organs, including heart, kidney, artery, lung, nerve, and eyes (1). In recent years, the prevalence of RA has shown an increased tendency, and it ranks NO.1 in the autoimmunity-related connective tissue disease worldwide (2). The incidence rate of RA is about 0.5-1% worldwide (3). In clinics, RA can occur at any age, and popular age is between 40-60. Most RA patients are females, and the incidence rate of RA among females was 2-3 times higher than in males (4).

It has been discussed in many previous studies that miRNAs may participate in the development and progression of different diseases (5), and miR-127 has been observe to promote the chondrogenic differentiation of rat bone marrow mesenchymal stem cells (6). In osteosarcoma, miR-127 has been proved to inhibit the cell proliferation and invasion via targeting ITGA6 (7). Results of the previous studies indicated that miR-127 has been closely correlated with the development of the diseases. However, it was still unclear whether miR-127 also play a role in RA.

Osteopontin (OPN/SPP1) known as early activation factor 1 for lymphocyte, is a potential promoter among inflammatory cytokines (8). Large numbers of OPN surface receptors are expressed in synovial cell, which can be combined with OPN to induce signaling transduction and influence the adhesion and proliferation of synovial cells (9). In RA and juvenile idiopathic arthritis patients, OPN protein was significantly increased in synovium, and it was also significantly higher in articular cartilage of

osteoarthritis patients (10). OPN protein may induce the gradual degradation of articular cartilage (11). OPN protein in synovial fluid comes from synovium and cartilage.

In this study, we detected OPN expression and miR-127 expression both in mRNA level and in protein level in blood and joint fluid of RA patients. Then, the relationship between miR-127 and OPN was analyzed. The mechanisms underlying the role of miR-127 in the development and progression were discussed.

Material and methods

Patients

Blood samples were obtained from 30 patients at xxx Hospital between Dec. 2016 and Apr. 2017. All patients fulfilled the American College of Rheumatology (ACR) criteria for RA. A number of 30 healthy volunteers were enrolled as the control group. This study has been proved by the ethical committee of xxx Hospital. Synovial tissues of 10 patients with emergency trauma amputation were obtained as the control. This study has been proved by the ethical committee of xxx Hospital and the informed consent form was obtained from every patient.

Cell culture and transfection

Fibroblast-like synoviocytes (FS) were isolated from RA patients. Cells were cultured with RPMI 1640 medium with 10% fetal bovine, and treated with recombinant human TNF-α (20 ng/mL, R&D Systems, USA) at 37 oC and 5% CO2. miR-127 mimics and miR-127 mimics negative control (NC) were synthesized by GenePharma (Shanghai, China), and cell transfection was performed using Lipofectamine RNAi Max (Invitrogen, Thermo Fisher Scientific, Waltham, Massachusetts, USA). The effects of miR-127 mimics on cells were examined at 48h after transfection with different assays.

Cell proliferation analysis

The effect of miR-127 mimics on the proliferation of SFs was determined by MTT assay on 12, 24 and 48h using cell proliferation kit I (MTT) (Sigma-Aldrich, St.

Louis, MO, USA) according to manufacturer's protocol.

Cell apoptosis analysis

After transfection for 48h, SFs were double-stained with PI and Annexin V-FITC using PI/ Annexin V-FITC apoptosis detection kit(Sigma-Aldrich, St. Louis, MO, USA), and the apoptosis of the cells in different groups was examined on a BD FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA).

Real-time quantitative PCR

The total RNAs were extracted from cells and tissue samples using TRIzol (Invitrogen, Thermo Fisher Scientific, Waltham, Massachusetts, USA), and RT-qPCR was performed to examine the expression of miR-127 using Hairpin-itTM MicroRNAs Quantitation Kit (GenePharma, Shanghai, China), and U6 (RNU6B; GenePharma) has been applied for normalization. The expression of other genes was examined using SYBR® Fast qPCR Mix(Takara, Dalian, China), and GAPDH has been used for normalization. Real-time quantitative PCR has been conducted on an ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol.

Western blot

SFs were harvested at 48h and lysed by RIAP (Beyotime, Shanghai, China), and the concentration of the total protein was determined using BCA Protein Assay Kit (Beyotime, Shanghai, China). Then SDS-PAGE has been performed, and then proteins were transferred to PVDF membranes and blocked with 5% non-fat milk; next, the membranes were incubated with primary antibodies overnight at 4℃; in the following day, the membranes were incubated with HRP-conjugated secondary antibody (purchased from Beyotime, Shanghai, China), and treated with BeyoECL Plus(an enhanced chemiluminescent reagent, Beyotime, Shanghai, China). The signals were detected and photographed by ChemiDoc™XRS+ (Bio-Rad, Hercules, CA, USA).

Dual Luciferase reporter assay

Wild-type OPN 3'UTR (OPN-3'UTR) that contains the miR-210-3p binding site

and mutant OPN 3'UTR(OPN-MUT) were cloned into the p-MIR-reporter plasmid (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and transfected into 293 cells with miR-127 mimics or NC using Lipofectamine RNAi Max (Invitrogen, Thermo Fisher Scientific, Waltham, Massachusetts, USA). Cells were collected 48h after transfection, and the activities of the luciferases were detected by the Dual luciferase Reporter Assay Kit (Beyotime, Shanghai, China).

Statistical analysis

All statistical analysis was performed using SPSS 22.0. Data were presented as means ± standard deviation, and the differences between two groups were analyzed using t-test, and or the differences among multiple groups were analyzed using analysis of variance (ANOVA). $P<0.05$ has been set as significant difference.

Results

Decreased expression of miR-127 in serum and synovial tissues of patients with RA

First of all, the serum and synovial tissues of the RA patients and healthy controls were collected, and the expression of miR-127 in RA patients and healthy volunteers were compared. As shown in Figure 1, miR-127 was significantly down-regulated in both serum and synovial tissues of RA patients compared with healthy controls ($p<0.01$).

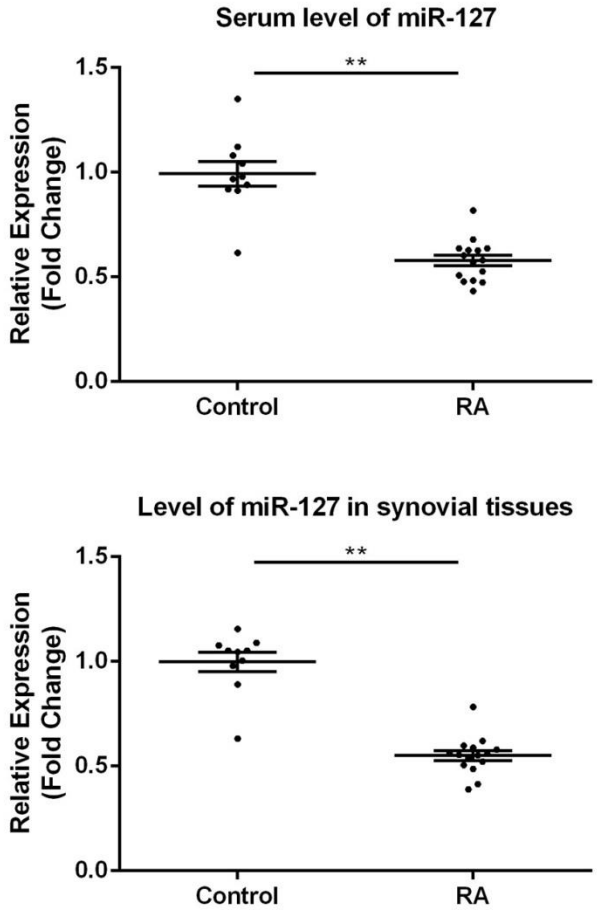

Figure 1. Expression of miR-127 in serum and synovial tissues of RA patients compared with healthy controls. $^{**}p<0.01$, compared with Control group

Effect of miR-127 on the proliferation of SFs

Next, SFs that isolated from RA patients were cultured and transfected with miR-127 mimics, and MTT assay has been performed to examine the effect of miR-127 on the proliferation of cells. It was observed that transfection of miR-127 mimics induced notably enhanced cell viability compared with the un-transfected cells and miR-127 mimics NC transfected cells (Figure 2, $p<0.05$) at 24 and 48h.

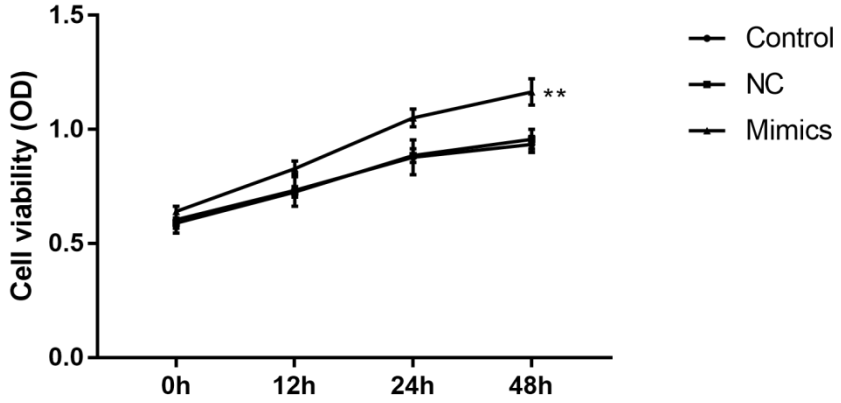

Figure 2. Effect of miR-127 on the proliferation of SFs.

** $p<0.05$, compared with NC group

Effect of miR-127 on the apoptosis of SFs

Furtuermore, flow cytometry assay has been performed to examine the effect of miR-127 on the apoptosis of the cells. As shown in Figure 3, 48h after transfection, miR-127 mimics induced significant decrease in the apoptosis of SFs compared with the un-transfected cells and miR-127 mimics NC transfected cells (Figure 3, $p<0.05$).

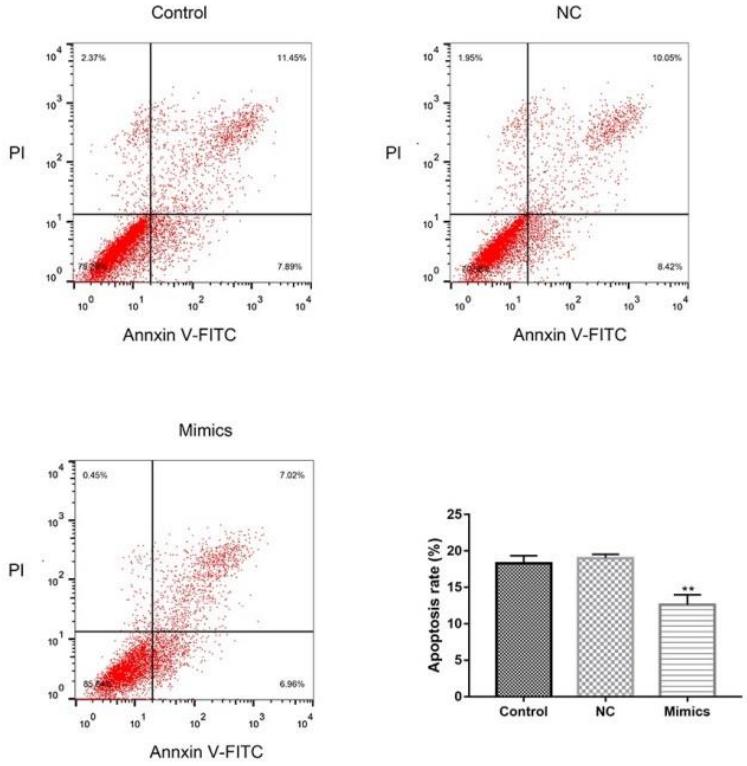

Figure 3. Effect of miR-127 on the apoptosis of SFs.

$^{**}P < 0.05$, compared with Control group

Effect of miR-127 on the expression of proliferation and apoptosis related proteins in SFs

To further investigate the anti-apoptotic effects of miR-127 in SFs, the effect of miR-127 on the expressions of apoptosis related proteins Bcl-2 and Bax in SFs were examined. As shown in Figure 4, miR-127 mimics induced significant increase in the anti-apoptotic protein Bcl-2 and marked decrease in the pro-apoptotic protein Bax in SFs compared with the un-transfected cells and miR-127 mimics NC transfected cells on both mRNA and protein levels (Figure 4, $p<0.05$).

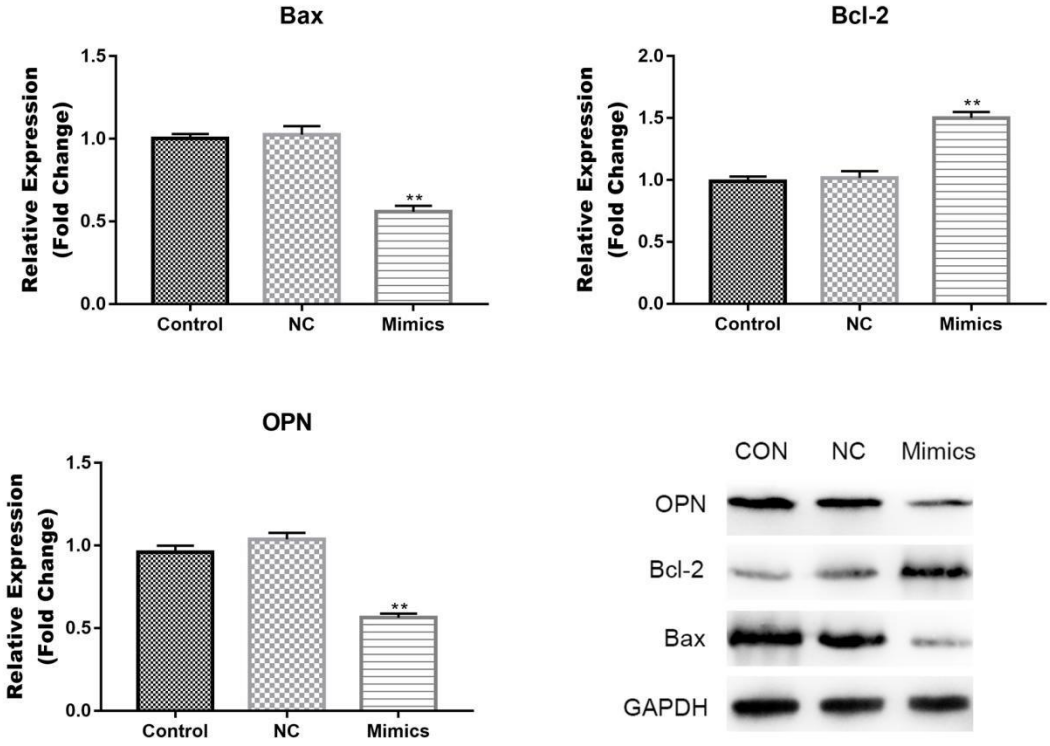

Figure 4. Effect of miR-127 on the expression of proliferation and apoptosis related proteins in SFs. $^{**}P < 0.05$, compared with Control group

OPN is a direct target of miR-127 in RA

Using online bioinformatic website, it has been predicted that OPN is a potential

target of miR-127. Next, dual luciferase reporter assay has been performed to validate the targeting relationship between miR-127 and OPN. As shown in figure 5, the activity of the luciferases in cells co-transfected with OPN WT 3'-UTR and miR-127 mimics were significantly decreased (P<0.05), while the activity of the luciferases in cells co-transfected with OPN WT 3'-UTR and miR-127 mimics has shown no significant difference between the control group (P>0.05). These results indicated that OPN is a direct target of miR-127.

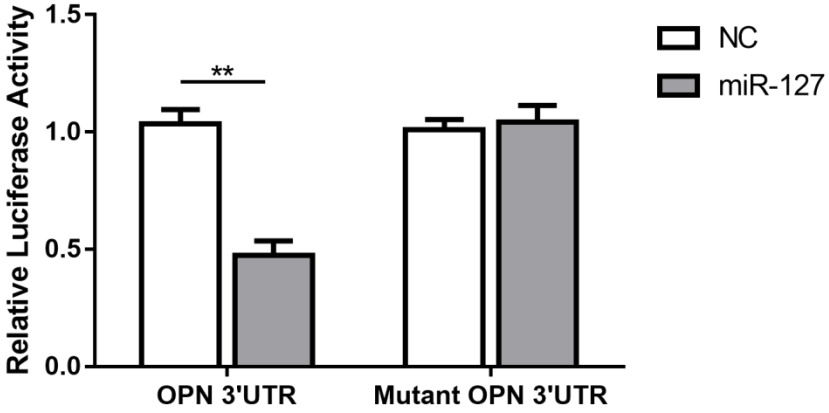

Figure 5. OPN is a direct target of miR-127 in RA.

** p<0.05, compared with NC group

miR-127 can regulate the proliferation and apoptosis through targeting OPN

Finally, to determine whether miR-127 was involved in the pathogenesis of RA through regulating the expression of OPN, a series of experiments have been performed. First, the expressions of OPN in the synovial tissues of patients and healthy controls were compared. It was observed that the expression of OPN was significantly up-regulated in RA (Figure 6, p<0.01); moreover, transfection of miR-127 mimics induced significant decrease in the expression of OPN in SFs (Figure 4, p<0.01).

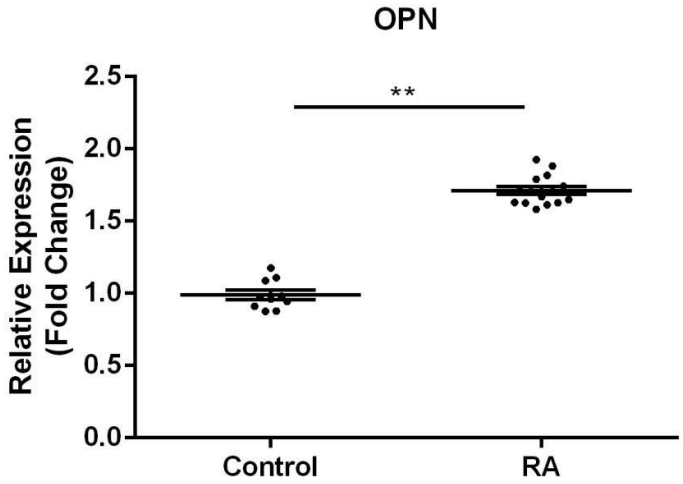

Figure 6. Expressions of OPN in the synovial tissues of patients and healthy controls.

** $P < 0.05$, compared with Control group

Discussion

In the present study, the roles of miR-127 in RA and the related mechanism have been discussed. We observed that miR-127 was down-regulated in RA, and miR-127 can miR-127 can regulate the proliferation and apoptosis through targeting OPN.

The role of miRNAs in RA has been discussed in many previous studies. Wang et al. proved that miR-522 can affect the expression of proinflammatory cytokines and MMPs through regulating the expression of SCOS3 in RA synovial fibroblasts (12); Du et al proved that miR-137 can affect the proliferation, migration and invasion of RA fibroblast-like synoviocytes (13); Wang et al proved that miR-548a-3p can regulate the inflammatory response in RA through TLR4/NF-κB signaling pathway (14). As a member of the miRNA family, miR-127 has been observed to be down-regulated in the cartilage of patients with OA compared with normal cartilage (15); however, studies on the roles of miR-127 in RA were still unclear. In the present study, we focused on the roles of miR-127 in RA and the related mechanism. We first reported that miR-127 was significantly up-regulated in both serum and synovial tissues of RA patients; moreover, transient over-expression of miR-127 induced significant increase in the proliferation and marked decrease in the apoptosis of the

SFs. Taken together, these results indicated that miR-127 may participate in the pathogenesis of RA through regulating the proliferation and apoptosis of the SFs.

It is well known that miRNAs exert their function through inhibiting the expression of its target genes. Using online bioinformatic website (targetscan), osteopontin (OPN) has been predicted as a target of miR-127. OPN was identified from tumor epithelial cells, and it has been proved to promote the adhesion of tumor cell adhesion, degradation of the extracellular matrix (ECM) and also the over-proliferation of the cancer cells (16, 17). In the case of joint diseases, OPN has been found to be up-regulated in the cartilage and synovium of patients with OA, and the levels of OPN has been associated with the occurrence and development of the disease. In the case of RA, Tsai et al. reported that OPN can inhibit the expression of miR-129-3p, and lead to the migration of monocytes in rheumatoid arthritis (18); Iwadate et al observed that plasma levels of osteopontin has been correlated with the bone resorption markers in patients with RA (19). In the present study, we observed that OPN was up-regulated in the synovial tissues of patients, which was consistent with previous finding; moreover, transfection of miR-127 mimics induced significant decrease in the expression of OPN in SFs. Taken together, these results indicated that miR-127 can regulate the proliferation and apoptosis of SFs through targeting OPN.

In conclusion, we proved for the first time that miR-127 can regulate the proliferation and apoptosis of SFs through targeting OPN. Our results have provided novel evidence for the application of miR-127 as a potential therapeutic for the treatment of RA.

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