MiR-20a regulates fibroblast-like synoviocyte proliferation and apoptosis in rheumatoid arthritis

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Abstract. – OBJECTIVE: STAT3 expression is elevated in the synovial tissue of patients with rheumatoid arthritis (RA). MiR-20a plays a role in mediating synovial inflammation in RA. Bioinformatics analysis has identified a binding site between miR-20 and the 3'UTR of STAT3 mRNA. This study aimed to investigate the role of miR-20a in the regulation of STAT3 expression and synovial cell proliferation as well as apoptosis.

PATIENTS AND METHODS: Synovial tissues were collected from RA patients and osteoarthritis (OA) patients to measure miR-20a, STAT3, p-STAT3, and Ki-67 expressions. Fibroblast-like synoviocytes (FLS) were treated with IL-17 (10 ng/ml) and then Ki-67 expression and cell cycle were evaluated by flow cytometry. The targeting relationship between miR-20a and STAT3 was assessed by dual luciferase reporter gene assay. FLS cells were divided into five groups: miR-NC, miR-20a mimic, si-NC, si-STAT3, and miR-20a mimic + si-STAT3 groups.

RESULTS: In RA patients, significantly lower MiR-20a expression, and substantially higher STAT3, p-STAT3, and Ki-67 expression were found in the synovial tissues compared with those in OA patients. IL-17A treatment markedly promoted FLS cell proliferation, inhibited cell apoptosis, reduced miR-20a expression, as well as upregulated levels of STAT3, p-STAT3, and Bcl-2. MiR-20a played a regulatory function on the expression of STAT3. MiR-20a mimic and/or si-STAT3 transfection apparently downregulated STAT3, p-STAT3, and Bcl-2 expression, attenuated IL-17A-induced cell proliferation promotive and enhanced cell apoptosis in FLS cells.

CONCLUSIONS: The expression of miR-20a was reduced in synovial tissue of RA patients with the increased level of STAT3. Downregulation of miR-20a promoted the expression of STAT3, p-STAT3, and Bcl-2, facilitated FLS cell proliferation, reduced apoptosis and, thereby, played a critical role in RA.

Key Words: miR-20a, STAT3, Bcl-2, RA, FLS.

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multiple autoimmune diseases, including multiple sclerosis and systemic lupus erythematosus. In addition, miR-20a was demonstrated to play a regulatory role in the inflammation of synovial cells in RA. Bioinformatics analysis has identified a binding site between miR-20 and the 3’-UTR of STAT3 mRNA. This study investigated the role of miR-20a in regulating STAT3 expression, as well as synovial cell proliferation and apoptosis.

Patients and Methods

Patients
We enrolled a total of 49 RA patients in Baise People’s Hospital between December 2015 and August 2016, including 19 males and 30 females with a mean age of 60.8 ± 12.9 years (range: 49-73 years). No patients received vitamin D, glucocorticoid, or immunosuppressor treatment before surgery. Twenty-one patients with osteoarthritis (OA) in Baise People’s Hospital during the same period were selected as controls. The synovial tissues and articular cavity synovia were collected.

Main Reagents and Materials
Dulbecco’s Modified Eagle Medium (DMEM) and fetal bovine serum (FBS) were purchased from Hyclone (South Logan, UT, USA). IL-17A cytokine and antibody were obtained from R&D systems (Minneapolis, MN, USA). PCR primers were synthesized by Generay (Shanghai, China). QuantiTect SYBR Green RT-PCR Kit was purchased from Qiagen (Valencia, CA, USA). STAT3 and p-STAT3 antibodies were purchased from Abcam (Cambridge, MA, USA). Goat anti-rabbit and mouse IgG secondary antibody were purchased from Biovector (Shanghai, China). MiR-20a mimic and negative control were obtained from GE Dharmacon (Lafayette, CO, USA). pIS2 luciferase reporter vector was purchased from Addgene (Middlesex, UK). Endonuclease SacI and XbaI were purchased from New England Biolabs (Shanghai, China). Dual-Luciferase® Reporter Assay System was provided by Promega (Madison, WI, USA). Annexin V-FITC/PI cell apoptosis detection kit was purchased from Solarbio (Beijing, China). IL-17A ELISA kit was purchased from RayBiotechnology (Norcross, GA, USA).

Immunofluorescence
The frozen section of synovial tissue was prepared to measure STAT3 expression. The tissue was fixed with formaldehyde and permeabilized by Triton X. After the section was blocked with BSA, it was incubated overnight with STAT3 monoclonal antibody (1:300 dilution). Then, the section was incubated with Alexa Flour 488 labeled secondary antibody with a dilution of 1:300. Next, the section was stained with DAPI and observed under an inverted microscope.

Flow Cytometry Detection of Ki-67 Expression
The synovial tissue was digested by type II collagenase and trypsin. The tissue was then incubated with PECy5 labeled Ki-67 at 4°C and was detected by flow cytometry.

FLS Cell Separation and Cultivation
The synovial tissue was digested by type II collagenase and 0.05% trypsin. Next, the cells were cultured in DMEM. The cells in the 4th generation were used for the experiment.

ELISA
The IL-17A level in articular cavity synovia was detected by ELISA. The plate was incubated sequentially with primary antibody and biotin labeled secondary antibody. After washing and development, the absorbance of each well was detected by a spectrometer at 450 nm.

IL-17A Treatment
FLS cells were seeded into a 6-well plate and treated with IL-17A (10 ng/ml) to simulate inflammation condition. The cells were then collected after 48 h treatment.

Luciferase Reporter Assay
The full-length fragment or mutant fragment of STAT3 3’-UTR was amplified by PCR. The product was treated with SacI and XbaI, and further cloned to pS2 luciferase vector. After sequencing, the plasmid with correct sequence was named as pS2-STAT3-wt and pS2-STAT3-3’-mut, respectively. pS2-STAT3-wt or pS2-STAT3-3’-mut was co-transfected to HEK293T cells using Lipofectamine 2000 together with miR-20a mimic or miR-NC. Luciferase activity was measured after 48 h by the kit.

FLS Cell Transfection and Grouping
FLS cells in the 4th generation were seeded into 10-cm dish and transfected using Lipofectamine...
2000 transfection reagent. FLS cells were divided into five groups, including miR-NC, miR-20a mimic, si-NC, si-STAT3, and miR-20a mimic + si-STAT3 groups. After 48 h of cultivation, the cells were treated with 10 ng/ml IL-17A for 48 h.

**qRT-PCR**
QuantiTect SYBR Green RT-PCR Kit was used for qRT-PCR detection. The primers used were as follows. STAT3F: 5′-ATCACGCCTTCTACAGACTGC-3′, STAT3R: 5′-CATCCTGGAGATTCTCTACCACT-3′; Bcl-2F: 5′-GGTGGGGTCATGTGTGTGG-3′, Bcl-2R: 5′-CGGTTCAGGTACTCAGTCATCC-3′; β-actinF: 5′-GAACCCTAAGGCCAAC-3′, and β-actinR: 5′-TGTCACGCACGATTTCC-3′.

**Western Blot**
Total protein was extracted and separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Then, the protein was transferred to polyvinylidene fluoride (PVDF) membrane and blocked with skim milk at room temperature for 60 min. Next, the membrane was sequentially incubated with primary antibody (STAT3, p-STAT3, Bcl-2, and β-actin at a dilution of 1:300, 1:200, 1:300, 1:300, and 1:600, respectively) and secondary antibody to a dilution of 1:5000. At last, the membrane was developed by ECL and scanned.

**Flow Cytometry Detection of Cell Apoptosis and Proliferation**
The cells were collected and incubated with 5 μl of Annexin V and 5 μl of PI. Then, the cells were tested on ACEA NovoCyte™ flow cytometry to evaluate cell apoptosis. The cells were collected and incubated with Ki-67 antibody at 4°C. Cell proliferation was detected by ACEA NovoCyte™ flow cytometry.

**Statistical Analysis**
All data analyses were performed by SPSS 18.0 software (IBM SPSS, Chicago, IL, USA). The measurement data were presented as mean ± standard deviation and compared by t-test. p<0.05 was considered statistically significant.

**Results**

**MiR20a Downregulated, while STAT3 Elevated in the Synovial Tissue of RA**
ELISA result showed that the level of IL-17A was significantly increased in the articular cavity of RA compared with that in OA (Figure 1A). qRT-PCR analysis demonstrated that miR-20a expression was declined, while STAT3 mRNA expression was enhanced in RA compared with that in OA (Figure 1B). Immunofluorescence analysis revealed that STAT3 protein level was markedly higher in the synovial tissue of RA than that in OA (Figure 1C). Western blot suggested that STAT3 and p-STAT3 protein expressions in RA were apparently higher than those in OA (Figure 1D). Flow cytometry exhibited that Ki-67 level was significantly higher in RA than that in OA, indicating the excessive proliferation of synovial tissue in RA patients (Figure 1E). Pearson correlation analysis showed that the level of IL-17A in the synovia was positively correlated with STAT3 mRNA expression, and negatively correlated with miR-20a expression (Table I).

**IL-17A Downregulated miR-20a and Elevated STAT3 Expressions in FLS Cells**
To study the role of IL-17A, flow cytometry detection was employed and revealed that IL-17A significantly enhanced Ki-67 expression, facilitated cell proliferation, and reduced cell apoptosis (Figure 2A and B). Moreover, qRT-PCR exhibited that IL-17A downregulated miR-20a expression and elevated STAT3 expression in FLS cells (Figure 2C). Western blot confirmed the finding and demonstrated that STAT3, p-STAT3, and Bcl-2 protein levels after IL-17A treatment were markedly higher compared with before treatment (Figure 2D). Bioinformatics analysis revealed the binding site between miR-20 and the 3’-UTR of STAT3 mRNA (Figure 2E), which was later confirmed by the reporter assay revealing that miR-20a mimic transfection significantly declined the relative luciferase activity in HEK293T cells after transfected with pIS2-STAT3-wt (Figure 2F), indicating the targeted regulatory effect of miR-20a on STAT3.

**Upregulation of miR-20a Inhibited STAT3 Expression, Attenuated Cell Proliferation, and Increased Cell Apoptosis**
To elucidate the function of miR-20a on the expression of STAT3, we used miR-20a mimic

<table>
<thead>
<tr>
<th>IL-17A</th>
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<th>p</th>
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<tr>
<td>miR-20a</td>
<td>-0.583</td>
<td>0.037</td>
</tr>
<tr>
<td>STAT3</td>
<td>0.615</td>
<td>0.031</td>
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and/or si-STAT3 transfection and found apparently reduced expressions of STAT3, p-STAT3, and Bcl-2 after treatment (Figure 3A). MiR-20 attenuated IL-17A’s effect on the promotion of cell proliferation (Table II), and enhanced cell apoptosis in FLS cells (Figure 3B).

**Discussion**

JAK-STAT signaling pathway can be mediated by various cytokines, including IL-6, TNF-α, IL-1β, IL-17, IFN-γ and plays a key role in regulating a variety of pathophysiological processes, such as cell proliferation, apoptosis, inflammation, and homeostasis. STAT3 is one of the most important members of STAT family and participates in promoting cell proliferation and inhibiting cell

Table II. Mean fluorescence intensity of Ki-67 expression in each group.

<table>
<thead>
<tr>
<th>Group</th>
<th>Ki-67 protein mean fluorescence intensity (n=3)</th>
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<tr>
<td>miR-NC</td>
<td>218.7±19.5</td>
</tr>
<tr>
<td>miR-20a mimic</td>
<td>31.5±2.4</td>
</tr>
<tr>
<td>si-NC</td>
<td>235.6±21.4</td>
</tr>
<tr>
<td>si-STAT3</td>
<td>19.8±1.1</td>
</tr>
<tr>
<td>miR-20a mimic+ si-STAT3</td>
<td>9.2±0.5</td>
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apoptosis through regulating Bcl-2, Cyclin D1, and VEGF. It has been shown that inflammatory cytokines-mediated FLS excessive proliferation and apoptosis blockage was an important pathophysiologic feature of RA. JAK-STAT3 signaling pathway may be involved in RA pathogenesis, considering its role in regulating cell proliferation and apoptosis. More importantly, it has been confirmed that STAT3 was abnormally upregulated in the synovial tissue of RA patients. On the contrary, attenuation of STAT3 activity led to alleviation of inflammation in RA, which further elucidates the role of JAK-STAT3 in RA. MiR-20a was downregulated in multiple autoimmune diseases, including multiple sclerosis and systemic lupus erythematosus. It has been shown that miR-20a played a regulatory role in mediating synovial cell inflammation in RA. Bioinformatics analysis has revealed a binding site between miR-20a and the 3’-UTR of STAT3 mRNA. This study investigated the role of miR-20a in regulating STAT3 expression, as well as synovial cell proliferation and apoptosis. Flow cytometry exhibited that Ki-67 level was signi-

Figure 2. IL-17A downregulated miR-20a and upregulated STAT3 expression in FLS cells. (A) flow cytometry detection of Ki-67 expression in FLS cells; (B) flow cytometry detection of FLS cell apoptosis; (C) qRT-PCR detection of miR-20a and STAT3 mRNA expression in FLS cells; (D) Western blot detection of protein expression in FLS cells; (E) the binding site between miR-20a and the 3’-UTR of STAT3 mRNA; (F) Luciferase reporter gene assay.

miR-20a 3’ guuggaCDUGAUACUGCUUGAAu 5’
STAT3 5’ cuuuupCAAUCCUUUCAUUCUu 3’
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Significantly higher in RA than in OA, indicating the role of excessive proliferation in the synovial tissue in RA pathogenesis. MiR-20a expression was declined, while STAT3 and p-STAT3 expressions were enhanced in RA, compared with that in OA. It suggested that p-STAT3 elevation might result from STAT3 upregulation and JAK-STAT3 signaling enhancement. Isomaki et al\(^6\) reported that STAT3 expression was significantly increased in T cells and monocytes from peripheral blood and synovia of RA. Lee et al\(^{19}\) found that the expression of STAT3 and p-STAT3 was enhanced in the synovia of RA compared with that of OA. Our results were similar to those in Isomaki et al\(^6\) and Lee et al\(^{19}\), which confirmed that STAT3 upregulation was associated with the pathogenesis of RA. Li et al\(^{12}\) showed that miR-20a level in FLS from rat RA model was markedly lower than the control. Our study found that miR-20a expression in synovial tissue of RA was lower than that in OA, indicating that miR-20a downregulation might be involved in the pathogenesis of RA, which was consistent with the study conducted by Li et al\(^{12}\). ELISA showed that IL-17A content was significantly increased in the articular cavity synovia of RA compared with that in OA. Tsai et al\(^{20}\) revealed that IL-17 content was abnormally elevated in the articular cavity synovia of RA. Pavlovic et al\(^{21}\) exhibited that IL-17 level was increased in the peripheral blood of RA. Hwang et al\(^{1}\) found that IL-17 expression abundance was significantly increased in the monocytes of synovia. Correlation analysis showed that IL-17A content in the synovia was positively correlated with STAT3 mRNA expression and negatively correlated with miR-20a expression in synovial tissue. It suggested that IL-17A might play a role in downregulating miR-20a, upregulating STAT3, and promoting RA. Flow cytometry detection revealed that IL-17A significantly enhanced Ki-67 expression, indicating that IL-17A facilitated cell proliferation. Lee et al\(^{19}\) demonstrated that IL-17 facilitated the proliferation of synovial cells from RA in vitro. Hashizume et al\(^{22}\) reported that FLS cell proliferation was markedly enhanced after treated by IL-17. We observed that IL-17A apparently accelerated FLS cell proliferation, which was consistent with the results obtained by Lee et al\(^{19}\) and Hashizume et al\(^{22}\). Moreover, IL-17A significantly downregulated miR-20a expression, while elevated STAT3, p-STAT3, and Bcl-2 levels in FLS cells. Philippe et al\(^{13}\) used LPS to treat FLS cells in vitro, and found that miR-20a expression was reduced in FLS cells, revealing that miR-20a might be involved in the pathogenesis of RA. In this study, miR-20a expression was markedly repressed in FLS cells after treated with IL-17A, which was consistent with Philippe et al\(^{13}\) findings. Lee et al\(^{19}\) demonstrated that IL-17 significantly facilitated STAT3 activation in FLS cells. Lee et al\(^{19}\) reported that IL-17 can upregulate Toll-like receptor 3 expression via promoting STAT3 activation. Also, the present study found the enhancement of IL-17 on STAT3 expression, of which miR-20a downregulation may be a reason of STAT3 elevation. Lee et al\(^{8}\) revealed that IL-17 can induce Bcl-2 expression in synovial cells. Dual luciferase reporter gene assay confirmed that miR-20a can bind to the 3′-UTR of STAT3 mRNA and regulate STAT3 expression. Both Kim et al\(^{23}\) and Lee et al\(^{8}\) suggested the role of STAT3 activation in upregulating Bcl-2 expression, accelerating FLS

Figure 3. Upregulation of miR-20a inhibited STAT3 expression, attenuated cell proliferation, and increased cell apoptosis. (A) Western blot detection of protein expression in FLS cells; (B) flow cytometry detection of FLS cell apoptosis.
cell proliferation, and antagonizing apoptosis. In this study, IL-17A-mediated Bcl-2 elevation may be achieved by enhancing STAT3 and p-STAT3, which was similar with Kim et al23 and Lee et al8 results. Besides, it has been demonstrated that miR-20a induced the apoptosis of HepG2 cells and subsequently reduced cell proliferation via the activation of caspase-8 and caspase-3. Therefore, we further explored the potential role of miR-20a on the proliferation and apoptosis of FLS cells. MiR-20a mimic and/or si-STAT3 transfection apparently downregulated STAT3, of miR-20a on the proliferation and apoptosis of FLS cells. MiR-20a induced the apoptosis of HepG2 cells and, thereby, played a critical role in RA.

Conclusions

Decreased level of miR-20a expression and increased STAT3 expression were found in synovial tissue of RA patients. Downregulation of miR-20a promoted STAT3, p-STAT3, and Bcl-2 levels, facilitated FLS cell proliferation, reduced apoptosis and, thereby, played a critical role in RA.

Conflict of interest

The authors declare no conflicts of interest.

References

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