miR-26a-5p Regulates Synovial Fibroblast Invasion in Patients with Rheumatoid Arthritis by Targeting Smad 1

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Background: We studied the expression and effect of miR-26a-5p in synovial fibroblast in patients with rheumatoid arthritis (RA).

Material/Methods: The synovial tissues of 55 RA patients with total knee arthroplasty performed from January 2016 to December 2016 were collected as the RA group, and 62 patients without RA history amputation or total knee arthroplasty served as the control group. The expressions of miR-26a-5p and Smad 1 mRNA in synovial fibroblast in patients with RA were detected by qPCR; The expression of Smad 1 and TGF-β1 protein in synovial tissue or synovial fibroblasts was detected by immunoblotting. Transwell assay was used to detect the invasive ability of synovial fibroblasts.

Results: The expression of miR-26a-5p and Smad 1 in synovial fibroblast in patients with RA were significantly higher than those in the control group (P<0.05). The expression of miR-26a-5p in synovial tissue of RA patients was positively correlated with the expression of Smad 1 mRNA (r=0.8982, P<0.001). The luciferase system showed that miR-26a-5p targeting synovial membrane FLS cells (P<0.05); the expression of MMP-1, MMP-3, MMP-13, and TGF-β1 protein and mRNA in the synovial FLS cells of RA patients was significantly decreased; and the expression of miR-26a-5p was significantly decreased in FLS cells with invasive ability.

Conclusions: miR-26a-5p is highly expressed in synovial tissue of patients with RA, and its high expression can improve the invasive ability of synovial fibroblasts by targeting Smad 1 gene and accelerating the progression of RA.

MeSH Keywords: Arthritis, Juvenile • MicroRNAs • Smad Proteins

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Background

Rheumatoid arthritis (RA) is a chronic systemic autoimmune disease with unknown etiology. The major clinical features are chronic inflammatory hyperplasia and hypertrophy of the synovium, as well as joint damage in the hands and feet [1]. With progression of RA, most patients develop bone injury, joint deformity, and even permanent disability, which brings economic burdens for the family and society [2,3]. Synovial fibroblasts are among the most important cells in synovial tissues, and the increase of their invasive ability is closely related to the genesis and progression of RA. Studies [4–6] have shown that synovial fibroblasts from RA patients can produce high levels of MMPs proteins to degrade all the components in the extracellular matrix, thereby enhancing the migration and invasive ability of synovial fibroblasts, and thus accelerating the progression of RA.

MicroRNAs are a class of non-coding single-stranded RNA molecules that participate in the regulation of cellular functions by modulating the transcription of target genes. In recent years, a number of studies have shown that the abnormal expression of microRNA is associated with the genesis and progression of many diseases [7–11] such as cancer, cardiovascular diseases, and RA. Ormseth et al. [12] showed that the expression level of miR-26a-5p was significantly up-regulated in the plasma of RA patients. However, the expression of miR-26a-5p in synovial tissues of RA patients and its role in the development and progression of RA have not been reported yet. Therefore, in the present study we investigated the expression of miR-26a-5p in synovial tissues of RA patients through qPCR and Western blot analysis, and explored the effect of its expression on the invasive ability of synovial FLS cells in RA patients.

Material and Methods

Clinical specimens

We collected RA synovial tissue specimens from 55 RA patients who received total knee replacement in our hospital from January 2016 to December 2016. There were 32 male patients and 23 female patients, ages 43–74 years old, with the average age of 59.3±8.7 years. X-ray examination showed 13 cases were Stage II, 26 cases were Stage III, and 16 cases were Stage IV. Specimens from 62 patients who underwent amputation (49 cases) or total knee replacement (13 cases of OA patients) were collected as controls. All patients in the control group had no history of RA; while there were 37 males and 25 females, ages 45–73 years old, with the average age of 60.4±7.2 years. Exclusion criteria were: other rheumatic diseases, autoimmune diseases, tumor, liver, and kidney insufficiency; pregnant or lactating, as well as mental disorders; and taking immunomodulatory drugs within the previous 6 months. All the patients enrolled in this study were informed of the study contents and signed the informed consent form. Our study was approved by the Ethics Committee of Wenzhou Central Hospital.

Table 1. qPCR primers.

| Gene         | Primer (5’-3’)                                      |
|--------------|----------------------------------------------------|
| has-miR-26a-5p | For: ACACCTCCAGTGTTTGAATGATCCAGGA                   |
|              | Rev: TGGTGTCGTGGAGTCG                                |
| U6           | For: CCCCCTCTTCAAGGGGTCT                           |
|              | Rev: AAGCCTCAGGAAATTTGCGT                          |
| Smad 1       | For: TTGTGTCGTGGGACTGCC                            |
|              | Rev: CCACAGCAAATCCCGGCCAAG                           |
| MMP-1        | For: CTCGATTTCATGGCCGAGCT                           |
|              | Rev: ATCCCTGTCATTCCAGGGT                           |
| MMP-3        | For: GGTCGCGCCGCCTGTGGG                            |
|              | Rev: GCACCTTATGGGAGCCGTG                            |
| MMP-13       | For: AGAGTATGGAGGCGTACG                             |
| TGF-β1       | For: TCTGTTGGGAGTGACAGGA                            |
|              | Rev: TTGTTGAGAGGGCAAGGAC                            |
| GAPDH        | For: AAGGGGCCCTGACAACCTTT                           |
|              | Rev: CCTCCCTTCCAGGGGTCT                            |

Experimental methods and reagents

Extraction of total RNA from synovial tissues and qPCR

Total RNA of liquid-nitrogen-preserved or fresh synovial tissues were extracted using the miRNeasy RNA isolation kit (Qiagen, USA). A Real-time Quantitative PCR Detecting System (qPCR) was used to detect the relative expressions of has-miR-26a-5p (U6 was used as control) and Smad 1 mRNAs (GAPDH was used as control) in different synovial tissues. The qPCR primers are shown in Table 1. Western blot detection of protein expressions

Total protein of cartilage tissues was extracted by tissue total protein extraction kit (Beyotime, China). SDS-PAGE was performed (90 V-30 min, 120 V-60 min), and the proteins were transferred to a wet PVDF membrane (400 mA-90 min), which was blocked with 5% no-fat milk at 37°C for 2 h, and incubated with primary antibodies of Smad1, TGF-β1 MMP-1, MMP-3, MMP-13, or GAPDH (1: 1000, Abcam, UK) at 4°C overnight. Then, the primary antibodies were removed, and the membrane was washed 3 times with TBST before incubating with...
goat anti-rabbit secondary antibodies (1: 3000, Abcam, UK) at 37°C for 2 h. The membrane was washed 3 times with TBST before image development. The band density of target proteins was normalized against GAPDH levels to be presented as the final results.

**Validation of miR-26a-5p target genes by luciferase reporter**

The binding site of miR-26a-5p on the 3'-UTR of Smad 1 gene was analyzed by searching on NCBI, miRanda, PicTar, and other web sites. The wild-type (WT) or mutated sequence (MUT) of the binding site on 3'-UTR was cloned into pmirGLO plasmid (Addgene, USA) that was double-digested by Sac I and Sal I. The constructed recombinant plasmid was co-transfected with miR-30a-5p-mimic or miR-30a-5p-NC into FLS cells, then the cells were collected 48 h after transfection and the luciferase fluorescence intensity was detected by a Dual-Luciferase system (Promega, China).

**Cell transfection**

miR-26a-5p-NC, miR-26a-5p-mimic, and miR-26a-5p-inhibitor (Shanghai Bioengineering Co., Ltd., China) were transfected into the isolated FLS cells according to the instructions of the purchased Lipofectamine RNAiMAX transfection kit (Invitrogen, USA). The detailed procedures were as follows: (1) 1 day before transfection, the isolated human FLS cells were plated into 6-well plates at the concentration of 1×10⁵ cells per well; (2) on the day of transfection, the Lipofectamine RNAiMAX transfection reagent was mixed evenly with opti-MEM culture medium and the synthesized miR-26a-5p-NC, miR-26a-5p-mimic, or miR-26a-5p-inhibitor, and the mixtures were incubated for 5–10 min at room temperature before they were added into the cell culture medium; (3) 72 h after the transfection, cells were digested with trypsin, rinsed once with PBS, and preserved for further experiments.

**Cell invasion assay**

Transwell assay was performed to detect the metastasis/invasive ability of cells. The Matrigel (Becton Dickinson Company, USA) stored at –20°C was melted overnight at 2°C to 8°C on ice. The Transwell plate (Corning, USA), while the lower chambers were loaded with DMEM culture medium supplemented with 20% FBS (Hyclone, USA). The Transwell plate was incubated for 24 h at 37°C. The culture medium was removed and the cells were rinsed twice with PBS before they were fixed with methanol for 30 min. Then, the membranes were dried and dyed with crystal violet for 20 min before the cells were observed and counted under an optical microscope (Leica Microsystems, German).

**Flow cytometry**

Collected FLS cell after different treatment. FLS cells was washed once by pre-cooled PBS solution at 4°C, then cells were added to 70% ethanol to fix overnight at 4°C. The following experiments were performed according to Annexin V-FITC/PI double-staining with Annexin V-FITC/PI Double Apoptosis Assay Kit (Shanghai Bioengineering, China), and finally by flow cytometry-MACSQuant® Analyzer 10 (Miltenyi Biotec, Germany).

**Statistical analysis method**

The quantitative data are expressed as mean ± standard deviation while the enumeration data are expressed in the form of percentage. Pearson correlation analysis was performed to evaluate the correlation between the expression levels of miR-26a-5p and Smad 1 mRNA in synovial tissues. The differences between groups of other data were measured by t test using SPSS 20.0 software, and P<0.05 was considered statistically significant.

**Results**

**miR-26a-5p and Smad 1 were highly expressed in RA synovial tissues**

Total RNA was extracted from the synovial tissues of RA patients or patients in the control group, and the relative expressions of miR-26a-5p and Smad 1 mRNA were detected by qPCR. The results showed that the relative expressions of miR-26a-5p and Smad 1 mRNA in the synovial tissues of RA patients were 7.03±1.97 and 0.58±0.16, respectively (P<0.05), as shown in Figure 1A, 1B. Total proteins were extracted from the synovial tissues of RA patients or patients in the control group, and Western blot was performed to detect the expression of Smad 1 protein in either of the groups. The results showed that the relative expression of Smad 1 protein in the synovial tissues of RA patients was 2.63±1.03 and 0.58±0.16, respectively (P<0.05), as shown in Figure 1A, 1B.
Pearson correlation analysis was used to compare the correlation between the expressions of miR-26a-5p and Smad 1 mRNA in the synovial tissues, and results showed that they were positively correlated in the synovial tissues of RA patients (r=0.8982, P<0.001), as shown in Figure 1E.

**Targeted regulation of Smad 1 gene expression by miR-26a-5p in FLS cells**

The target genes of miR-26a-5p were predicted by bioinformatics methods, and the results showed that there was a complementary sequence of miR-26a-5p at the 3'-UTR end of Smad 1 mRNA, as shown in Figure 2A. In order to verify that miR-26a-5p could regulate the expression of Smad 1 by binding to the 3'-UTR end of Smad 1, a luciferase gene reporter system was applied: transfection of miR-26a-5p-mimics significantly increased the luciferase activity of wild-type Smad 1 mRNA 3'-UTR (P<0.05), while transfection of miR-26a-5p-inhibitor significantly decreased the luciferase activity of wild-type Smad 1 mRNA 3'-UTR (P<0.05). In addition, we also transfected miR-26a-5p-NC, miR-26a-5p-mimics, or miR-26a-5p-inhibitors into synovial fibroblasts, and results showed that the expression of Smad 1 was significantly increased in the synovial fibroblasts transfected with miR-26a-5p-inhibitors (P<0.05), as shown in Figure 2B–2F.

**MiR-26a-5p regulated the expression of MMPs-related proteins**

MMPs proteins are a series of enzymes that degrade all the components of the extracellular matrix and therefore enhance cell migration and invasion. MMP-1, MMP-3, and MMP-13 are 3 MMPs proteins that have been shown to be highly expressed in RA synovial tissues and enhance the invasive ability of synovial fibroblasts in RA patients [13,14]. In this study, we found that the expression of MMP-1, MMP-3, and MMP-13 proteins and mRNA in synovial fibroblasts decreased significantly when the cells were transfected with miR-26a-5p-inhibitors or when the expression of Smad1 was suppressed (P<0.05), as shown in Figure 3.

**miR-26a-5p regulated the invasive ability of FLS cells through Smads/TGF-β signaling pathway**

At 72 h after transfection of miR-26a-5p-inhibitor and miR-26a-5p into FLS cells, there was no significant change in cell viability (Figure 4A), while the expressions of miR-26a-5p were significantly decreased/increased in FLS cells (Figure 4B). Smad1 and TGF-β1 proteins (Figure 4C, 4D) were significantly
**Figure 2.** Targeted regulation of Smad 1 gene expression by miR-26a-5p in synovial fibroblasts. (A) Bioinformatics prediction of the target site of miR-26a-5p on the 3'-UTR of Smad 1 mRNA; (B) Luciferase activity analysis of FLS cells co-transfected with miR-26a-5p-NC or miR-30a-5p-mimics and wild-type or mutated Smad 1 3'-UTR; (C) Expression of miR-26a-5p in FLS cells with different treatments; (D) Expression of Smad 1 mRNA in FLS cells with different treatments; (E, F) Expression of Smad 1 protein in FLS cells with different treatments. * Represents statistically significant differences compared to the miR-26a-5p-NC group, P<0.05.

**Figure 3.** miR-26a-5p positively regulated the expression of MMP-1, MMP-3, and MMP-13 proteins. (A) Western blot detection of MMP-1, MMP-3, and MMP-13 proteins; (B) Statistical comparison of the 3 proteins in different groups; (C) Comparison of mRNA expression of MMPs gene in different groups. * Represents statistically significant differences compared to the miR-26a-5p-NC group, P<0.05.
decreased in FLS cells after transfection of miR-26a-5p-inhibitor. Meanwhile, the invasive ability of FLS cells decreased by 21.4% (Figure 4E, 4F). After transfection of miR-26a-5p-mimic 72h, the invasiveness of FLS cells increased by 27.6% (Figure 4E, 4F).

Discussions

RA is a chronic and aggressive autoimmune disease, and the main clinical outcome is RA. For a long time, the academic community generally believed that T cells and other immune cells and their cytokines play a key role in the genesis and progression of RA [15–17]. In recent years, however, a number of studies on synovial fibroblasts isolated from RA patients have posed a strong challenge to this concept [18]. Scholars both in China and abroad have studied synovial fibroblasts from many aspects, such as signaling pathway [19], cell apoptosis [20], invasion, and migration [21], and the results showed that synovial fibroblasts play a leading role in the development of RA without dependence on any external factors [18].

miR-26a-5p is located on the human chromosome 3p22 and encodes a single-stranded RNA with 21 nucleotides. At present, there are only a few studies on its role in RA. Studies by Ormseth et al. [12] showed that the expression of miR-26a-5p in the plasma of RA patients was significantly increased. In this study, we found that the relative expression of miR-26a-5p and Smad 1 gene in the synovial tissues of RA patients were significantly higher than those in the control group (P<0.05), and that the expression of miR-26a-5p in the synovial tissues of RA patients was positively correlated with the expression of Smad 1 mRNA (r=0.8982, P<0.001). In addition, it was verified by the luciferase system that Smad 1 was the target gene of miR-26a-5p. Smad1 protein is an important member of the Smads cytoplasmic protein family, and is an important transmitter on the TGF-β/Smads signaling pathway. Researches by Chen et al. [22] pointed out that miR-345 could inhibit the proliferation, migration, and invasion of prostate cancer cells through targeted down-regulation of Smad 1 protein. Meanwhile, the study of Yong W et al. [23] in hepatocellular carcinoma also showed that inhibiting the expression of miR-345 could promote tumor growth, invasion, and migration.

Figure 4. miR-26a-5p regulated the invasive ability of FLS cells through Smads/TGF-β signaling pathway. (A) Enhanced or inhibited miR-21 expression showed no effect on FLS cell viability; (B) At 72 h after transfection of miR-26a-5p-inhibitor or miR-21-mimic, the expression of miR-26a-5p was significantly changed in FLS cells; (C, D) Inhibition of miR-26a-5p expression significantly reduced the expression of Smad 1 and TGF-β1 genes in FLS cells; (E, F) Enhanced or inhibited miR-21 expression significantly changed the colony number of FLS cells that invaded into the lower Transwell chamber. * Represents statistically significant differences compared to the miR-26a-5p-NC group, P<0.05.

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Smad 1 protein could restrain the invasion and migration of HCC cells. For RA, synovial fibroblasts can enhance the invasion/migration ability by regulating the expression of cell adhesion molecules as well as MMPs proteins, thus invading articular cartilage and destroying bone tissues. Xiong et al. [24] found that inhibition of miR-21 expression in the synovial fibroblasts of RA patients could inhibit the expression of MMPs proteins through the TGF-β1/Smad4/7 signaling pathway, thereby reducing the invasiveness of synovial fibroblasts. In combination with the results of this study, we hypothesized that the high expression of miR-26a-5p in synovial tissues of RA patients promotes the invasion and migration of synovial fibroblasts by targeting the up-regulation of Smad 1 protein, thus accelerating the progression of RA.

We also found that inhibition of miR-26a-5p expression and silencing of Smad 1 gene in synovial fibroblasts could both inhibit the expression of MMP-1, MMP-3, and MMP-13 genes in synovial FLS cells. MMPs proteins are a series of enzymes that degrade all the components of the extracellular matrix and therefore enhance cell migration and invasion. Among them, MMP-1, MMP-3, and MMP-13 are 3 highly expressed proteins in RA synovial tissues, which enhance the invasive ability of synovial fibroblasts in RA patients [13,14]. Combined with the results of our study, it can be concluded that the high expression of miR-26a-5p in RA synovial tissues may promote the expression of MMP-1, MMP-3, and MMP-13 proteins by targeting the expression of the Smad 1 gene, thus promoting the invasive ability of synovial fibroblasts.

Conclusions

Our further studies showed that inhibiting the expression of miR-26a-5p in synovial fibroblasts not only significantly reduced the expression of Smad 1 and TGF-β1 proteins, but also restrained the invasive ability of synovial fibroblasts. In conclusion, miR-26a-5p is highly expressed in the synovial tissues of RA patients, and its high expression promotes the invasive ability of synovial fibroblasts by targeting Smad 1 gene expression, thus accelerating the progression of RA. Further research is needed to elucidate the mechanism of the miR-26a-5p/Smad 1 signaling pathway, and the method for pathway identification should be based on attractor and crosstalk [25].

Conflict of interest

None

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