Am J Transl Res 2016;8(12):5512-5518
www.ajtr.org /ISSN:1943-8141/AJTR0035448

Original Article

MiR-19 suppresses fibroblast-like synoviocytes cytokine release by targeting toll like receptor 2 in rheumatoid arthritis

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Received July 10, 2016; Accepted November 12, 2016; Epub December 15, 2016; Published December 30, 2016

Abstract: Fibroblast-like synoviocytes (FLS) play an important role in the pathogenesis of rheumatoid arthritis (RA) through participating in joint tissue inflammation and joint damage. MicroRNAs are a kind of small non-coding RNAs that can regulate gene expression in the transcription level to affect cell behaviors. This study intended to investigate the expression of miR-19 in FLS from RA patients and related mechanism. A total of 126 RA patients were selected in this study. MiR-19 expression in FLS was detected by qRT-PCR. Toll like receptor 2 (TLR2) protein expression was tested by Western blot. MiR-19 target genes were confirmed by bioinformatics analysis and luciferase reporter assay. The impact of miR-19 on the expression of TLR2, interleukin 6 (IL-6), and matrix metalloproteinase 3 (MMP-3) in FLS were analyzed by cell transfection and Western blot. MiR-19 expression in FLS from RA patients was significantly downregulated compared with control ($P < 0.05$), while TLR2 level was increased ($P < 0.05$). Bioinformatics analysis and luciferase reporter assay confirmed that TLR2 was the target gene of miR-19. Transfection of miR-19 mimic or miR-19 inhibitor obviously suppressed or increased TLR2 expression, and reduced or promoted release of cytokines IL-6 and MMP-3 in FLS, respectively. In conclusion, MiR-19 expression was downregulated in FLS from RA patients, leading to increased TLR2 expression and enhanced cytokines release.

Keywords: Rheumatoid arthritis, fibroblast-like synoviocytes, miR-19, TLR2, cytokine

Introduction

Rheumatoid arthritis (RA) is an autoimmune disease with unknown etiology. Its pathological processes include synovial inflammation and the damage of bone tissue and adjacent cartilage [1]. Movement function is seriously affected in advanced RA patients due to bone tissue destruction and joint deformity [2]. At present, it was thought that RA was caused by the interaction of genetic factors with environmental factors. It is still lack of effective treatment method, resulting in high disability rate and poor prognosis [3]. Numerous studies found that fibroblast-like synoviocytes (FLS) play a critical role in RA occurrence and development [4].

It was showed that FLS participates in cell inflammation through synthesizing cytokines and chemokines, and also involves in the process of joint tissue damage in RA [5]. Toll like receptor (TLR) family protein is a kind of immune receptor protein expressed on immune cells and FLS [6]. TLR2 can promote inflammation by participating in the release of cytokines and matrix metalloproteinases (MMP) [7]. Meanwhile, it was found that TLR2 downregulation alleviated inflammation in mouse arthritis model [8]. The abovementioned results suggested that regulation of TLR expression in FLS was of great significance in RA treatment.

MicroRNAs are a kind of small non-coding RNA molecules with hairpin structure. They can regulate multiple cell behaviors by regulating target gene expression [9]. It was found that miR-19 may regulate TLR2 expression at posttranscriptional level to participate in inflammation [10]. Thus, this study aimed to explore the expression of miR-19 in FLS from RA and related mechanism.

Table 1. Primers sequences

Name	Sequence
miR-19-F	5' CATAGTTGCACTACAAGAAG 3'
miR-19-R	5' GCACAACTACATTCTTCTTG 3'
U6-F	5' CTCGCTTCGGCAGCACA 3'
U6-R	5' AACGCTTCACGAATTTGCGT 3'

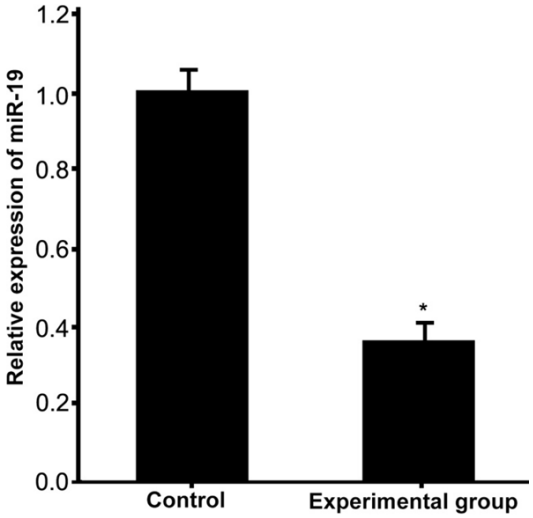

Figure 1. MiR-19 expression in FLS. Total RNA was extracted from FLS followed by analysis of the miR-19 expression by qRT-PCR. *P < 0.05, compared with control.

Materials and methods

Object of study

A total of 126 RA patients in the General Hospital of Jinan Military Command between Jun 2008 and Dec 2014 were enrolled, including 40 males and 86 females with a mean age of 56.3±10.2 (range: 36-72) years old. All subjects were diagnosed according to the criteria published by the American rheumatism association. Another 48 patients with osteoarthritis in our hospital at the same time were selected as controls. No statistical significance was observed on age and gender between these two groups. This research was approved by the ethics committee of the General Hospital of Jinan Military Command and all subjects had signed informed consents.

FLS separation and identification

Knee joint synovial tissue was extracted during the arthroscopic surgery. After removing the attached adipose tissue and cartilage, the synovial tissue was washed by sterile PBS and DMEM. Then the tissue was cut up and digested by collagenase. The cells were collected through low-speed centrifugation and cultured in DMEM containing 10% FBS for 12 h. Next, the cells were washed by sterile PBS to remove the unattached cells and further cultured to obtain primary FLS [11]. The primary FLS was identified by morphological observation and whole cell immunochemical staining. FLS cells were in fusiform with oval nuclei located in the center. Whole cell immune staining showed positive Vimentin and low or no expression of CD68.

qRT-PCR

Total RNA was extracted using RNAprep pure Tissue Kit (QIAGEN) for qRT-PCR analysis. The primers for miR-19 were designed according to the sequence (GeneBank: NR_029489). The primer sequence was listed in **Table 1**. PCR was performed using mirVanat qRT-PCR miRNA detection kit (Ambion), including 95°C for 3 min, followed by 40 cycles of 95°C for 15 s and 60°C for 30 s. U6 was selected as internal reference to calculate the relative level of miR-19 based on $2^{-\Delta\Delta Ct}$ method [12].

MiR-19 target prediction

The potential target of miR-19 was predicted using TargetScan Release 5.1 software (www.targetscan.org). Luciferase reporter assay was applied to test the potential target of miR-19.

Luciferase reporter assay

The primers for 3'-UTR of TLR2 were designed according to the sequence (GeneBank: NM_001318796). The primers sequence was as follows: 5'-AAAAAGCAGGCTTCCCATATTTAAGACCAGTCTTTGT-3' and 5'-AGAAAGCTGGGTTGTAAAGTTTAATAGGAAATACACAGC-3'. The 3'-UTR sequence of TLR2 mRNA was obtained by PCR amplification and inserted to the downstream of firefly luciferase gene coding region in pmirGLO to construct pmirGLO-TLR2 vector. Then the pmirGLO-TLR2 and pmirGLO vectors were transfected to HEK293 cells, together with miR-19 mimics using INTERFERin™ Polyplus transfection kit. HEK293 cells were purchased from the cell bank, Chinese academy of scienc-

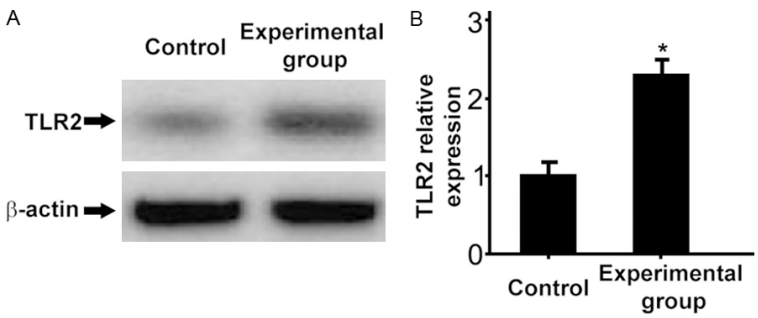

Figure 2. Western blot detection of TLR2 protein expression. Whole protein was extracted followed by analysis of the protein expression of TLR2 by western blot. A: Western blot detection. B: TLR2 relative expression. *P < 0.05, compared with control.

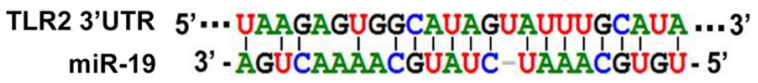

Figure 3. The homology between miR-19 and the 3'-UTR of TLR2.

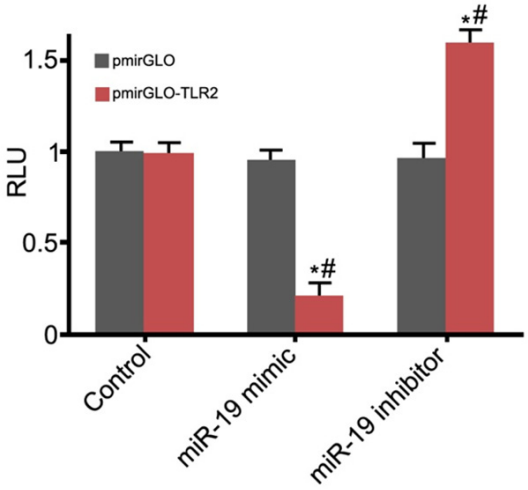

Figure 4. Luciferase reporter assay. The 3'-UTR sequence of TLR2 mRNA was obtained by PCR amplification and inserted to the downstream of firefly luciferase gene coding region in pmirGLO to construct pmirGLO-TLR2 vector followed by transfection to HEK293 cells for analysis of fluorescence intensity by MicroLumatPlus LB96V photometer. *P < 0.05, compared with control. #P < 0.05, compared with pmir-GLO.

es. HEK293 cells in logarithmic phase were digested and seeded in 96-well plate. After cultured for 24 h, cells were transfected using the kit according to the manual for 48 h. The fluorescence intensity was analyzed by dual luciferase report gene analysis system (Promega) and MicroLumatPlus LB96V photometer (Berthold) [13].

Cell transfection

To explore the impact of miR-19 on FLS, miR-19 mimics and inhibitor were designed according to the sequence of miR-19 and transfected to FLS.

Western blot

Total protein was extracted from FLS after 48 h cultivation and separated by SDS-PAGE. Then the protein was transferred to PVDF membrane and blocked with skim milk. Next, the membrane was sequentially incubated with primary antibody and HRP labeled secondary antibody (1:1000 dilution). After washed by PBST, the membrane was developed by DAB and the image was analyzed by gel imaging analysis system [14].

Data analysis

All data analysis was performed on SPSS 20.0 software. The data was depicted as mean ± standard deviation. Statistical significance between two treatment groups was analyzed by unpaired student t test. Statistical significance between multiple treatment groups was assessed by One-way ANOVA. A statistical significance was considered as $P < 0.05$.

Results

MiR-19 expression in FLS from RA

FLS were isolated to extract total RNA. qRT-PCR detection showed that compared with osteoarthritis patients, miR-19 expression was obviously declined in FLS from RA patients ($P < 0.05$) (**Figure 1**).

TLR2 expression in FLS

Western blot was applied to determine TLR2 protein level in FLS from RA patients (**Figure 2**). It was found that TLR2 expression in FLS from RA patients was significantly higher than that in osteoarthritis patients ($P < 0.05$).

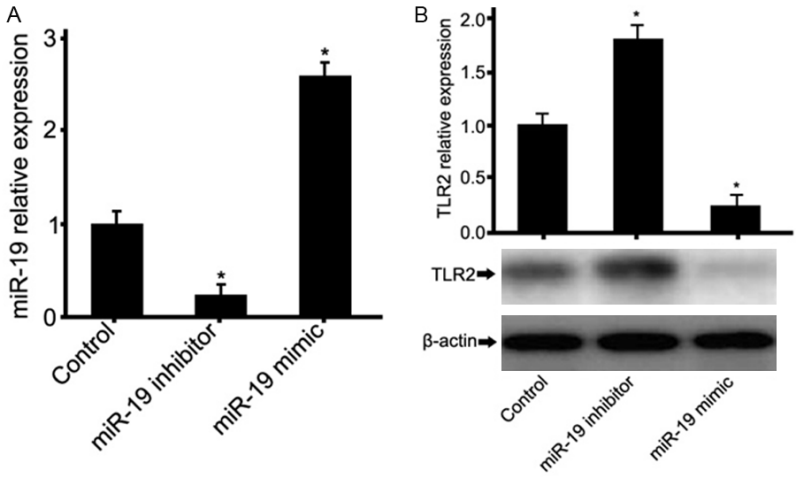

Figure 5. The impact of miR-19 on TLR2 expression. miR-19 mimics and inhibitor were designed and transfected to FLS followed by measuring the expression of miR-19 by qRT-PCR or the protein expression of TLR2 by western blot. A: Cell transfection. B: TLR2 expression. *P < 0.01, compared with control.

The relationship between miR-19 and TLR2 gene expression

TargetScan Release 5.1 software was adopted to predict the target gene of miR-19. It was showed that miR-19 sequence exhibited certain homology with the 3'-UTR of TLR2 mRNA (**Figure 3**), thus speculating that TLR2 gene may be the target gene of miR-19. Luciferase reporter gene system was constructed for verification (**Figure 4**). The fluorescence intensity was significantly decreased after miR-19 mimic transfection. On the contrary, the fluorescence intensity of HEK293 cells was obviously enhanced after miR-19 inhibitor transfection (P < 0.05), suggesting the 3'-UTR of TLR2 was the binding site of miR-19.

The impact of miR-19 expression on TLR2

MiR-19 mimic and inhibitor were used to manipulate miR-19 expression level in FLS. Total RNA was extracted after transfection to detect miR-19 relative expression (**Figure 5**). It was showed that miR-19 level was increased about 2.6 times after miR-19 mimic transfection (P < 0.05), while it was markedly reduced after miR-19 inhibitor transfection (P < 0.05).

Western blot was performed to detect TLR2 expression in FLS after transfection (**Figure 5**). It was demonstrated that TLR2 level was declined about 76% in FLS after miR-19 mimic transfection (P < 0.05), whereas it was upregulated about 1.8 times in FLS after miR-19 inhibitor transfection (P < 0.05).

Cytokine release

At 48 h after transfection, cytokines levels, including IL-6 and MMP-3, in FLS were evaluated by Western blot. β-actin was selected as internal control. It was presented that IL-6 and MMP-3 levels were obviously downregulated in FLS transfected with miR-19 mimic (P < 0.05), whereas they were significantly enhanced in FLS transfected by miR-19 inhibitor (P < 0.05) (**Figure 6**).

Discussion

TLR2 is a member of TLR family that plays an important role in nonspecific immunity. It was found that TLR2 elevation may promote inflammation by facilitating related cytokines release, including IL-1β and TNF [7]. This study revealed that TLR2 expression was significantly increased, while miR-19 level was reduced in FLS from RA patients, suggesting that miR-19 may be involved in the pathological process of RA.

MiRNAs are a type of small non-coding RNA with a length of 21-25 bp that is high conservative in evolution. They play key roles in regulating cell behaviors and metabolism [14]. In addition, many studies demonstrated that a variety of miRNAs including miR-23b and miR-19 were involved in the pathological process of several autoimmune diseases [15]. Collins considered that miR-19 can regulate SOCS3 expression to enhance JAK-STAT signaling pathway activity, so as to promote inflammation [16]. Our results showed miR-19 was downregulated in FLS from RA patients. Next, we confirmed miR-19 may exert its biological function through TLR2 upon bioinformatics analysis and luciferase reporter assay.

Research suggested that all of TLRs family proteins had a conservative sequence TIR in intracellular region, which can interact with various intracellular signal molecules, such as interleu-

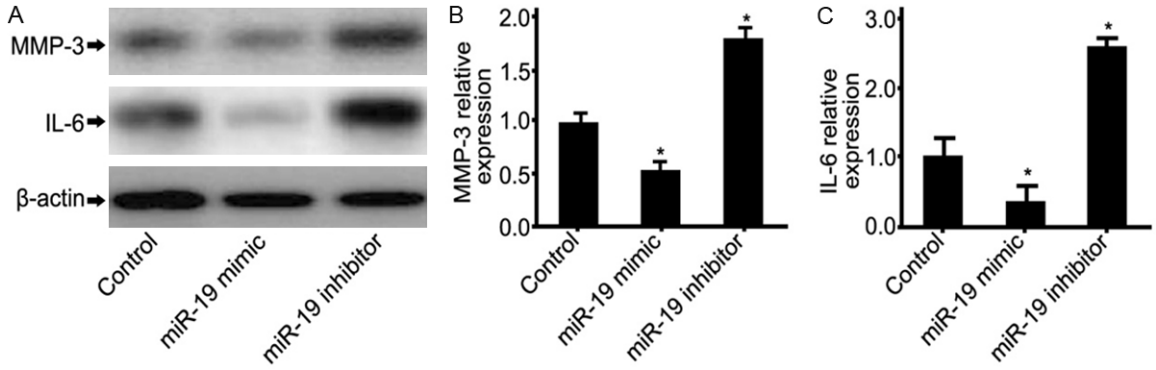

Figure 6. Western blot detection of cytokines level. miR-19 mimics and inhibitor were designed and transfected to FLS followed by measuring the expression of MMP-3 AND IL-6 by western blot. A: Western-Blot detection. B: MMP-3 relative expression in FLS. C: IL-6 relative expression in FLS. *P < 0.05, compared with control.

kin 1 receptor related protein (IRAK), tumor necrosis factor receptor related factor (TRAF-6), and mitogen-activated protein kinase (MAPK). They can activate these signal transducers to further activate nuclear factor (NF-κB) signaling pathway. NF-κB is then translocated into the nucleus and triggers inflammation related genes expression, including IL-6 and MMP-3 [16, 17]. We investigated the influence of miR-19 expression on FLS by cell transfection and Western blot. It was revealed that miR-19 overexpression declined TLR2 level, and reduced inflammation-related cytokines release, such as IL-6 and MMP-3, indicating that miR-19 may inhibit inflammatory cytokines release through TLR2.

Currently, it is still unclear about miRNA regulation in FLS from RA. It was thought that miRNA expressions were altered through manipulating the methylation level of miRNA transcription promoter [18]. It was also believed that miRNA targeted gene coding proteins can reversely regulate miRNA level [19]. More in-depth experimental studies are required in the future to clarify the mechanism by how miR-19 is regulated in FLS from RA.

The pathological process of RA is often accompanied by bone destruction and severe joint deformities, eventually influencing movement function. There is still lack of effective treatment for RA [20]. This study confirmed the role of miR-19 in FLS from RA through in vitro cell transfection and expression experiments, providing theoretical basis for the treatment of RA and other autoimmune diseases in the future.

Conclusion

MiR-19 was downregulated in FLS from RA, leading to elevated TLR2 level which promotes inflammatory cytokines release.

Acknowledgements

Research supported by The General Financial Grant from the China Postdoctoral Science Foundation (No. 2015M572805).

Disclosure of conflict of interest

None.

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