MiR-451 suppresses inflammatory responses in ankylosing spondylitis by targeting macrophage migration inhibitory factor

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Abstract

Objective

To investigate the associations of miR-451 and macrophage migration inhibitory factor (MIF) with disease activity, radiographic progression, and cytokine levels of ankylosing spondylitis (AS).

Methods

Peripheral blood mononuclear cells (PBMCs) were isolated and cultured from 43 AS patients, 11 peripheral spondyloarthritis (pSpA) patients, and 31 healthy controls. ASDAS-CRP and mSASSS were assessed at the time of blood sampling. Expression levels of miR-451 and MIF were determined using quantitative real-time PCR, and the supernatant concentrations of MIF and cytokines were measured using ELISA. After transfection of miR-451 synthetic mimic or FAM-labelled negative control mimic to AS PBMCs, MIF and cytokine levels were determined using quantitative real-time PCR or ELISA.

Results

Level of miR-451 expression was lower in AS PBMCs than in pSpA and control PBMCs, while MIF expression was significantly increased in AS PBMCs compared with those in pSpA and control PBMCs. MIF, TNF-α, and IL-6 concentrations in cell supernatants of AS PBMCs were significantly higher than those of pSpA and control PBMCs. miR-451 expression level did not show significant correlation with clinical parameters, but MIF expression level was elevated in PBMCs from AS patients with high mSASSS (12 or more). Treatment of AS PBMCs with the miR-451 synthetic miRNA mimic significantly reduced mRNA expression levels and cell supernatant concentrations of MIF, TNF-α, and IL-6.

Conclusion

The MIF level was elevated in AS patients with greater radiographic damage and overexpression of miR-451 suppressed the MIF and inflammatory cytokine levels. These findings suggest miR-451/MIF may be a novel therapeutic target in the treatment of AS.

Key words

ankylosing spondylitis, miR-451, MIF, cytokine

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Introduction

MicroRNAs (miRNAs, miR), a group of small noncoding RNA molecules, can post-transcriptionally regulate mRNA expression (1). They predominantly suppress the expression of target mRNA by repressing translation or through direct cleavage of genes. They are also involved in various physiologic and pathologic processes, including immune response, inflammation, cell growth and differentiation, and tumourigenesis (2-4). Among the miRNAs, miR-451, located within chromosome 17q11.2, has been identified to suppress tumour evolution in several forms of malignancies (5, 6). Recent studies showed that miR-451 was also significantly down-regulated in conditions of immune-mediated inflammatory responses, and that systemic administration or overexpression of miR-451 significantly reduced neutrophil migration and inflammatory cytokine production (7-9).

Ankylosing spondylitis (AS) is characterised by entheseal inflammation and pathologic bone remodeling. The disease usually starts with inflammation in the sacroiliac joints and leads to erosion and sclerosis of subchondral bones, after which tissues are gradually replaced by fibrocartilage and ossified with deposition of matrix proteins. Fibrous replacement and abnormal new bone formation result in ankylosis of the spine in advanced stages of the disease (10). Various inflammatory mediators, such as tumour necrosis factor (TNF)-α, interleukin (IL)-6, IL-17, and IL-23, play a dominant role in these inflammatory and proliferative cascades of AS (11). A recent report by Ranganathan *et al.* documented that macrophage migration inhibitory factor (MIF) level is elevated in serum and synovial fluid from AS patients, and that MIF induced production of TNF-α and β-catenin, indicating an essential role of MIF in AS through crosstalk between inflammation and pathologic bone formation (12).

MIF is an evolutionarily highly conserved pleiotropic cytokine that is secreted by various cell types, including macrophages and lymphocytes (13-15). MIF has been demonstrated to be involved in cell proliferation, differentiation, angiogenesis, and tumourigenesis (16-18). Additionally, MIF displays a dominant role in diseases that are characterised by pro-inflammatory pathways such as severe chronic inflammatory diseases and many types of cancers (19-21). Recent advances have indicated that MIF is one of the most important targets of miR-451 and the dominant roles of miR-451 in inflammatory response are thought to be mediated by targeting MIF (6, 18, 22).

These data, taken together, suggest that MIF up-regulation represents one of the molecular mechanisms for which miR-451 down-regulation could promote inflammatory responses. The present study was performed to investigate the role of miR-451 in AS by determining its expression level in conjunction with MIF expression and production status and to find associations of their levels with clinical parameters and inflammatory cytokine concentrations in patients with AS.

Subjects and methods

Study subjects

Blood samples were collected from 43 patients with AS (36 men and 7 women; mean age 33.0±9.2 years) who fulfilled the modified New York criteria (23). At the time of blood collection, Ankylosing Spondylitis Disease Activity Score-C reactive protein (ASDAS-CRP) was determined to assess disease activity of AS (24), and the modified Stoke AS Spinal Score (mSASSS) was scored to assess radiographic severity (25). Two experienced investigators scored the mSASSS using cervical and lumbar spine radiographs. Control blood samples were obtained from 11 peripheral spondyloarthritis (SpA) patients (8 men and 3 women; mean age 38.210.1 years) and 31 healthy blood donors (25 men and 6 women; mean age 34.8±10.3 years) without any evidence of medical illness. This study was approved by our Ethics Committee, and all study subjects provided signed informed consent prior to study enrolment (IRB no. 3-2018-0023).

Isolation and culture of PBMCs

Blood samples were collected into EDTA tubes from all study subjects, and peripheral blood mononuclear cells (PBMCs) were isolated from the buffy

coat by density gradient centrifugation using the Ficoll-Hypaque method (GE Healthcare Bioscience AB, Uppsala, Sweden). PBMCs were cultured in RPMI 1640 medium at 37°C in 5% CO_2 with 10% fetal bovine serum, 100 U/mL penicillin, and 100 U/mL streptomycin.

RNA extraction and quantitative real-time PCR

Total RNA was extracted using RNeasy (Qiagen, Valencia, CA, USA) and DNase I (Qiagen), and RNA was homogenised using Qiashredder columns (Qiagen). The obtained RNA pellet was dissolved in 30 μl of RNase-free water and stored at -80°C. RNA was reverse transcribed into cDNA using a SuperScript III synthesis kit (Invitrogen, Carlsbad, CA, USA). Gene expression was analysed by TaqMan Master Mix (Applied Biosystems, Foster City, CA, USA) on an ABI 7300 Real Time PCR System. The conditions for amplification were as follows: 50°C for 2 min and 95°C for 10 min, followed by 30 cycles of denaturation at 95°C for 15 sec and primer annealing/extension at 60°C for 1 min. PCR was performed in 50 μl volumes, and each PCR sample underwent a 30-cycle amplification to ensure that the reactions had not reached the plateau phase of amplification. All reactions were performed in triplicate. The relative mRNA expression values were calculated using the $2^{-\Delta\Delta Ct}$ method and estimated by normalisation with GAPDH expression.

Transfection of miRNA mimic

PBMCs were seeded at 5×10^4 cells per well in a 6-well plate. Then, cells were transfected with miR-451 synthetic miRNA mimic (Thermo Fisher Scientific, Waltham, MA, USA) at 100 nM by siIMPORTER Transfection Reagent (Millipore, Billerica, MA, USA). FAM-labeled control mimic (Thermo Fisher Scientific) was transfected as the negative control. Cells were incubated for 72 h at 37°C in a 5% CO_2 incubator.

Assessment of cell viability

After transfection, cells were plated at a density of 2×10^4 cells per well in 96-well plate and the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Sigma, St. Louis, MO, USA) was performed; after 24 or 48 h of incubation, cells were incubated with MTT solution (0.5 mg/ml) at 37°C for 4 h, then washed with PBS three times and dimethyl sulfoxide was used to dissolve the insoluble formazan product. Cell viability was determined by the absorbance read at 570/690 nm by SpectraMax 340 (Molecular Devices Co., Sunnyvale, CA, USA).

Enzyme-linked immunosorbent assay (ELISA)

Concentrations of MIF, TNF-α, and IL-6 in cell culture supernatants were determined using commercially available ELISA kits (R&D Systems Inc., Minneapolis, MN, USA) according to the manufacturer's protocols. Each experiment was repeated 3 times with duplicate samples. Optical density (OD) was measured at 450 nm, and the concentrations were calculated from a standard curve according to the manufacturer's protocol. Intra- and inter-assay coefficients of variation were lower than 8% for all cytokine assays.

Statistical analysis

All statistical analyses were performed using SPSS v. 20, and results were expressed as mean ± standard deviation (SD). Comparisons of variables between groups were performed with independent *t*-test, χ-square test, or Mann-Whitney U-test as appropriate. Correlations were calculated using Pearson's correlation test. A *p*-value less than 0.05 was considered to be statistically significant.

Table I. Characteristics of AS patients, pSpA patients and healthy controls.

Characteristics	AS patients	pSpA patients	Controls	*p*-value
Number of subjects	43	11	31	-
Age, mean±SD (years)	33.0 ± 9.2	38.2 ± 10.1	34.8 ± 10.3	NS
Sex, Males/Females	36/7	8/3	25/6	NS
Disease duration, mean±SD (months)	28.1 ± 18.7 (8-53)	16.4 ± 12.1 (3-38)	NA	-
Positive HLA-B27 (%)	43 (100)	1 (9.1)	NA	-
ASDAS-CRP	2.1 (0.8-5.3)	NA	NA	-
mSASSS	18.6 ± 11.7 (1-51)	NA	NA	-
NSAIDs (%)	43 (100)	10 (91.0)	NA	-
DMARDs (%)	14 (32.5)	7 (63.6)	NA	-

AS: ankylosing spondylitis; pSpA: peripheral spondyloarthritis; ASDAS-CRP: Ankylosing Spondylitis Disease Activity Score-C-reactive protein; mSASSS: modified Stoke Ankylosing Spondylitis Spinal Score; NSAIDs: non-steroidal anti-inflammatory drugs; DMARDs: disease-modifying anti-rheumatic drugs; NS: not significant; NA: not applicable.
p-values of <0.05 were considered statistically significant.

Results

Subject characteristics

The demographic and clinical characteristics of the study subjects are shown in Table I. Age and sex distributions were similar among AS patients, pSpA patients, and controls. The mean disease duration of AS patients from symptom onset to study enrolment was 28.1±18.7 months (range: 8–53 months), and all AS patients had positive HLA-B27. At the time of study enrolment, all patients were taking non-steroidal anti-inflammatory drugs, and 14 of them were also taking sulfasalazine. None of the AS patients had been treated with anti-TNF or anti-IL-17A inhibitor. Peripheral SpA group included 6 patients with psoriatic arthritis, 3 patients with reactive arthritis, 2 patients with inflammatory bowel disease-associated arthritis. Mean disease duration of pSpA patients was 16.4±12.1 months (range: 3–38 months). One patient with inflammatory bowel disease-associated arthritis had positive HLA-B27. Ten patients with pSpA were taking NSADs and 7 with pSpA were taking sulfasalazine or methotrexate. No pSpA patients had history of biologic treatment prior to study enrolment.

miR-451, MIF, and cytokine levels in AS PBMCs

The relative mRNA expression levels

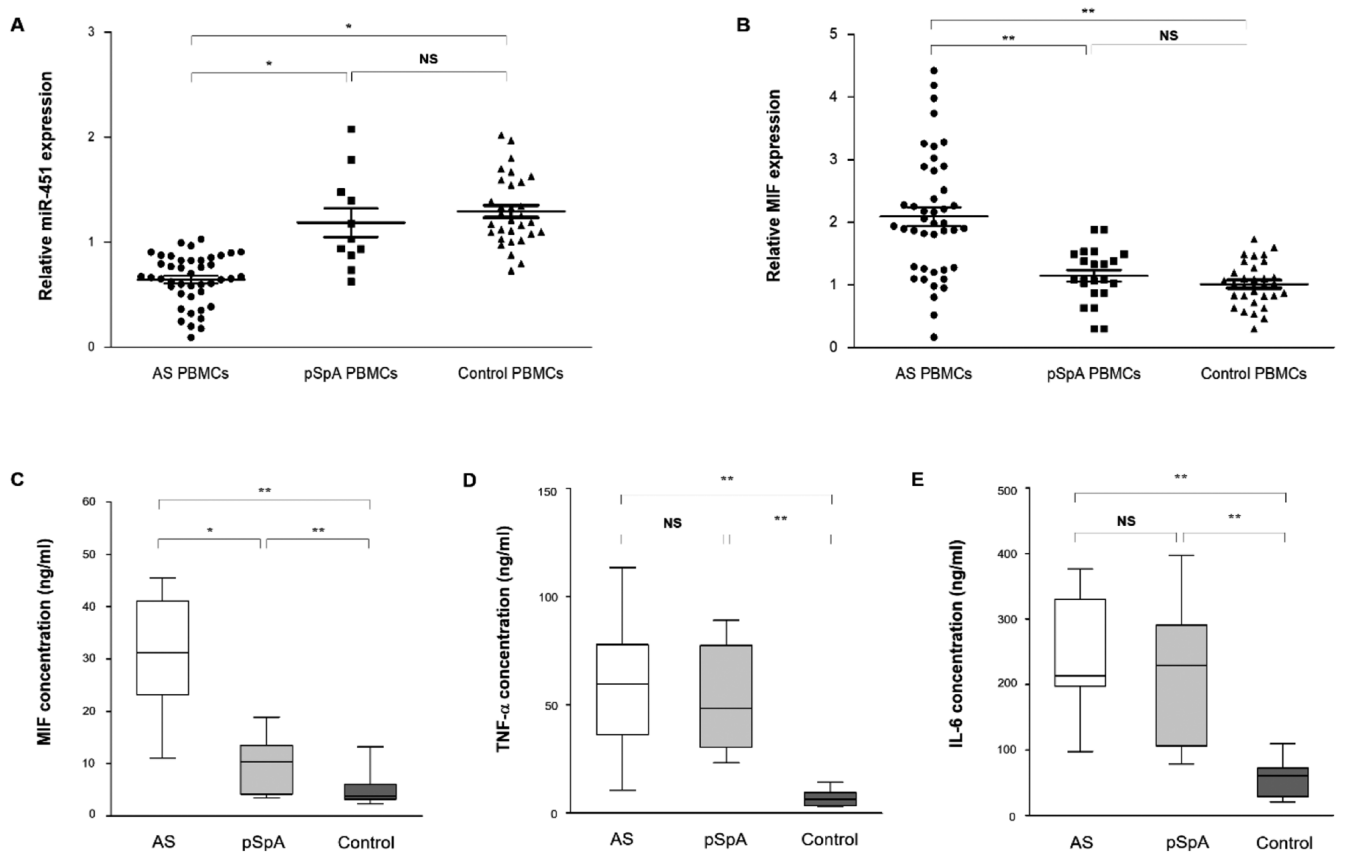

Fig. 1. Comparison of miR-451, MIF, and cytokine levels of AS, pSpA and control PBMCs.
A-B: Expression levels of miR-451 and MIF in PBMCs from AS patients, pSpA patients and healthy controls were determined using quantitative real-time PCR.
C-D-E: Concentrations of MIF and inflammatory cytokines in culture supernatants were measured using ELISA.
Data are shown as mean ± SEM. * $p<0.05$ and ** $p<0.01$ *versus* controls.

of miR-451 and MIF in PBMCs from AS patients, pSpA patients and healthy controls were determined by qRT-PCR. The data showed that miR-451 expression level was significantly decreased (0.64±0.24 for AS PBMCs, 1.19±0.45 for pSpA PBMCs, and 1.29±0.33 for control PBMCs, $p<0.05$, Fig. 1A) and MIF expression level was significantly increased (2.09±0.98 for AS PBMCs, 1.14±0.45 for pSpA PBMCs, and 1.01±0.35 for control PBMCs, $p<0.01$) in AS PBMCs compared with PBMCs from the pSpA and control group (Fig. 1B).

Concentrations of MIF measured by ELISA in cell supernatants of AS PBMCs were significantly higher than those of pSpA and the healthy controls (31.43±9.35 ng/ml for AS PBMCs, 10.0±5.2 ng/ml for pSpA PBMCs, and 4.72±2.43 ng/ml for control PBMCs, $p<0.01$, Fig. 1C). Concentrations of TNF-α and IL-6 in cell culture supernatants of AS PBMCs were also significantly increased compared with those of pSpA and controls (TNF-α; 58.11±28.05 ng/ml for AS PBMCs, 51.623.8 ng/ml for pSpA PBMCs, and 7.063.68 ng/ml for control PBMCs, IL-6; 243.81±96.48 ng/ml for AS PBMCs, 208.9±103.4 ng/ml for pSpA PBMCs, and 55.24±25.77 ng/ml for control PBMCs. all $p<0.01$, Fig. 1D-E).

Associations of miR-451 and MIF levels with clinical variables

To explore the associations of miR-451 and MIF levels with disease activity and radiographic severity of AS patients, we performed correlation tests and found that the mRNA expression level of miR-451 did not show significant linear correlation with ASDAS-CRP or mSASSS. When AS patients were divided into two groups according to ASDAS-CRP, the higher ASDAS group (ASDAS-CRP 2.1 or more, n=16) and the lower ASDAS group (ASDAS-CRP less than 2.1, n=27), the expression levels of miR-451 and MIF did not show significant differences between groups (Fig. 2A-B). Similarly, when AS patients were divided into the higher mSASSS group (score 12 or more, n=20) and the lower mSASSS group (score less than 12, n=23), expression levels of miR-451 were not different between groups (Fig. 2C); however, the MIF level of the higher mSASSS group was significantly elevated compared with that of lower mSASSS group (Fig. 2D).

Effects of miR-451 upregulation on MIF and cytokine levels

To investigate the effect of miR-451 upregulation on MIF and cytokine levels, synthetic miR-451 mimic or negative control miRNA mimic were transfected to PBMCs from AS patients. The MTT assay showed no significant difference in cell viability between miR-451 mimic-treated and control mimic-treated cell lines (Fig. 3A). Quantitative real-time PCR showed that the expression of miR-451 was significantly elevated

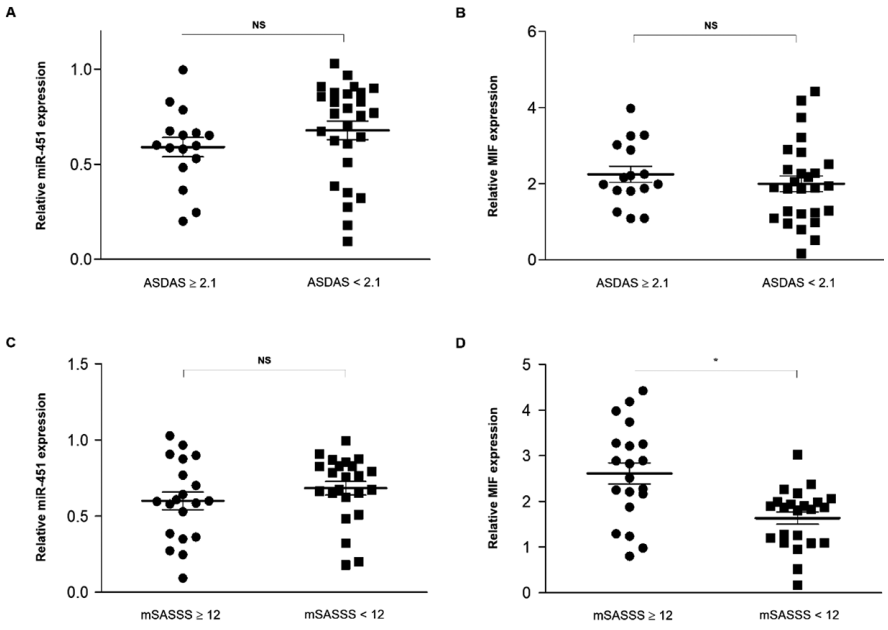

Fig. 2. Associations of miR-451 and MIF expression levels with clinical parameters.
A-B: Comparison of miR-451 and MIF expression levels according to ASDAS-CRP.
C-D: Comparison of miR-451 and MIF expression levels according to mSASSS.
Data are shown as mean ± SEM. *p<0.05 between groups.
ASDAS: Ankylosing Spondylitis Disease Activity Score; mSASSS: modified Stoke Ankylosing Spondylitis Spinal Score; NS: not significant.

in synthetic miR-451 mimic-treated cell lines but not in negative control-treated cell lines, indicating successful transfection of miRNA mimic into PBMCs (Fig. 3B). Up-regulation of miR-451 expression significantly reduced the mRNA expression of MIF from PBMCs (Fig. 3C). Furthermore, miR-451 overexpression induced significant reductions of MIF, TNF-α, and IL-6 concentrations in cell supernatants (Fig. 3D-F).

Discussion

In this study, we report a novel role for miR-451 in regulating inflammatory responses and its possible targeting against MIF in AS. Our results demonstrated that miR-451 exhibited a lowered expression level in PBMCs from AS patients, and that miR-451 overexpression suppressed the levels of several key inflammatory cytokines involved in pathogenesis of AS. Moreover, we found that MIF level was increased in AS PBMCs, and its gene and protein levels were downregulated by miR-451 overexpression.

Growing evidence has shown that miRNAs exert regulatory functions in many pathophysiological processes such as cell proliferation, metabolism, apoptosis, and inflammatory responses depending on the role of their targets (2-4). Accordingly, miRNAs have been shown to regulate polarisation of inflammatory cells and subsequent effects on inflammation (26). A certain

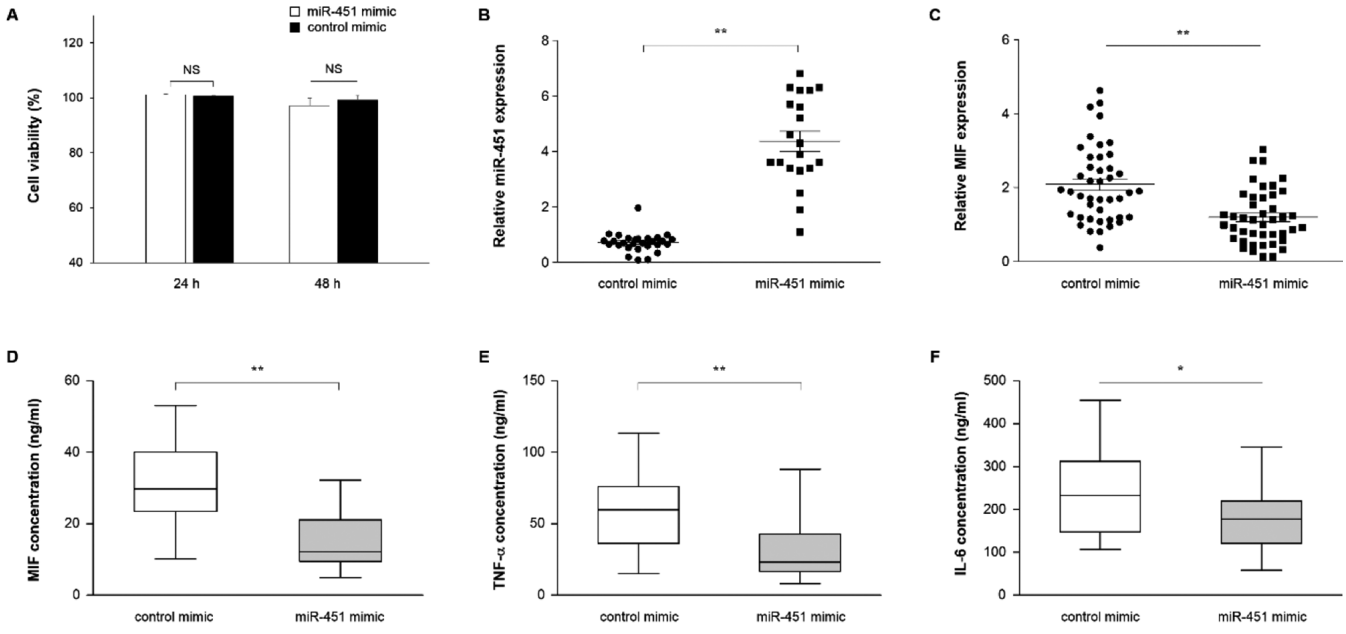

Fig. 3. Effect of miR-451 mimic transfection to AS PBMCs.
A: Cell viability of AS PBMCs transfected with synthetic miR-451 mimic or negative control mimic was assessed using MTT assay.
B-C: Expression levels of miR-451 and MIF in AS PBMCs transfected with synthetic miR-451 mimic or negative control mimic were determined using quantitative real-time PCR.
D-E-F: MIF and inflammatory cytokine levels in cell culture supernatant were measured using ELISA after transfection of miR-451 mimic or negative control mimic. Data are shown as mean ± SEM. *p<0.05 and **p<0.01 between groups.

role of miR-451 has been suggested in inflammatory conditions. Wang *et al*. reported that miR-451 treatment inhibited proliferation of rheumatoid synovial fibroblasts and secretion of inflammatory cytokines (7). Murata *et al*. demonstrated that lowered miR-451 expression in rheumatoid arthritis may be relevant for pathogenesis of the disease as a result of dysregulated neutrophil migration into inflammation sites, and miR-451 treatment suppressed the severity of arthritis in an animal model (9). Until now, there has been a lack of evidence regarding an association of miR-451 with inflammation of AS. Our results showed that miR-451 expression is decreased in AS PBMCs compared to PBMCs from healthy controls and that enhanced miR-451 expression by synthetic miR-451 mimic transfection resulted in significant suppression of inflammatory cytokine levels in the supernatants of AS PBMCs. These findings indicate the anti-inflammatory effects of miR-451 by down-regulating inflammatory cytokine production in AS patients. To our knowledge, the present study is the first to show the role of miR-451 in AS pathogenesis.

Adenosine monophosphate-activated protein kinase (AMKT)/AKT, p38 mitogen-activated protein kinases (MAPK), and prostaglandin E2 have been reported to be target genes of miR-451 in inflammatory diseases (7-9, 27). However, one miRNA can have multiple target genes in the same disease and can have different target genes depending on disease type. Thus, identifying a dominant target gene in specific disease subset will help develop more effective treatments for the disease. Among the candidate target genes of miR-451, MIF was the focus of the present study due to its role as an inducer of inflammatory cytokine production, including TNF-α and IL-17 (28, 29), thereby acting as a key mediator of inflammation in AS. MIF is a cytokine produced by virtually all types of human body cells in response to stress caused by different factors leading to pathological conditions such as chronic inflammation (14, 15). A recent study that investigated the association between MIF and AS showed that higher MIF level predicted radiographic progression in AS patients, suggesting a certain role of MIF as a link between inflammation and new bone formation in AS (12). Similar to this previous observation, we found that MIF gene expression and protein production were increased in AS patients compared to healthy controls. Although MIF level did not show a linear correlation with a clinical parameter reflecting radiographic severity, AS patients with greater radiographic damage had higher MIF level compared to that of patients with lesser radiographic damage. These findings indicate that MIF contributes to the pathologic bone formation of AS, and targeting MIF may be a therapeutic option for reducing progression of spinal ankylosis in AS patients.

As a target gene of miR-451, MIF expression can be regulated by miR-451 (18, 22). To explore the association of miR-451 with MIF in AS patients, we enhanced the expression of miR-451 in AS PBMCs by miR-451 mimic transfection and found inhibitory effects of miR-451 on gene expression and protein production of MIF. Overexpression of miR-451 also induced down-regulation of TNF-α and IL-6, which are known to play crucial roles in AS pathogenesis. Although close correlations of miR-451 and MIF levels with disease activity scores of our patients were not found in the present study, these findings suggest that miR-451 inhibited inflammatory response by targeting expression of MIF and indicate therapeutic potential for miR-451 as a suppressor of MIF in AS. Further investigations including larger numbers of patients are needed to clarify the association of miR-451 and the inflammatory burdens of AS.

In summary, we report that miR-451 expression is decreased and MIF expression is increased in AS PBMCs. AS patients who had more severe radiographic damage had higher MIF level and overexpression of miR-451 inhibited inflammatory cytokine production by downregulating the expression of MIF. These findings may provide new insight into the mechanisms of AS progression and suggest miR-451/MIF as a novel therapeutic target for treatment of AS.

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