Knockdown of circular RNA *hsa_circ_0003307* inhibits synovial inflammation in ankylosing spondylitis by regulating the PI3K/AKT pathway

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Abstract

Background. Ankylosing spondylitis (AS) has a high disability rate, and an early diagnosis is difficult.

Objectives. To explore the possible functions and underlying mechanism of circular RNAs *Homo sapiens (hsa)_circ_0003307* in ankylosing spondylitis.

Materials and methods. The *hsa_circ_0003307* expression levels were investigated in the peripheral blood mononuclear cells (PBMCs) of 30 AS patients and 30 healthy controls (HC) using quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis. Primary fibroblast-like synoviocytes (FLS) were separated from synovial tissues, established as cell lines and cultured for subsequent cell experiments involving transfection with different vectors. The qRT-PCR analysis was used for evaluating the levels of *hsa_circ_0003307* in AS-FLS. Phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT) pathway-related protein levels were measured using western blotting and immunofluorescence. Enzyme-linked immunosorbent assay (ELISA) was used to detect the levels of inflammatory cytokines. Spearman's correlation analysis was used to assess the correlation between *hsa_circ_0003307* and clinical characteristics.

Results. The expression level of *hsa_circ_0003307* was significantly high in AS patients and was positively associated with erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), Bath Ankylosing Spondylitis Disease Activity Index (BASDAI), and Bath Ankylosing Spondylitis Functional Index (BASFI). We found that *hsa_circ_0003307* overexpression could promote the activation of the PI3K/AKT pathway and expression of inflammatory cytokines – tumor necrosis factor alpha (TNF-α) and TNF-α-induced protein 2 (TNFAIP2). However, *hsa_circ_0003307* knockdown reduced the expression of TNF-α and TNFAIP2.

Conclusions. The expression level of *hsa_circ_0003307* was associated with inflammatory response, and it was revealed that *hsa_circ_0003307* knockdown could reduce the inflammatory response of AS by regulating the PI3K/AKT pathway.

Key words: ankylosing spondylitis, PI3K/AKT, *hsa_circ_0003307*

Background

Ankylosing spondylitis (AS) is a chronic refractory inflammatory arthritis, characterized by chronic nonspecific inflammation.[1] The sacroiliac joints and spine are the body parts most commonly affected by AS.[2] If not treated in time, it affects the movement of the spine joints, and even spinal joint stiffness and deformity will appear.[3] This seriously worsen the quality of life of patients. This disease is mainly painful in the early stage, when the symptoms of unfavorable spinal joint movement are not obvious, and it is not easy to be diagnosed. Often, when AS is clearly diagnosed, irreversible joint damage has already occurred, and other system diseases may also be observed. The treatment of AS brings a huge economic burden to the families of the patients and the whole community. Therefore, an early diagnosis is very important for AS patients and their families. There is an urgent need to identify new biomarkers that could be used as indicators for the diagnosis or prognosis of AS. The discovery of such biomarkers may have inestimable value for the early diagnosis and treatment of AS.

Circular RNA (circRNA) is a unique RNA composed of exons, introns, or the products of reverse splicing of both.[4] Because circRNA has no 5′ or 3′ ends, it can withstand RNase digestion and is more stable than most linear RNAs.[5] In addition to its characteristics of a relatively high stability, circRNA often exhibits tissue/developmental stage-specific expression,[6,7] and is therefore more suitable as a biomarker than linear RNA.[8] Previous studies have confirmed that circRNA may control gene transcription by isolating target microRNAs (miRNAs) and regulating RNA-binding proteins, thereby acting as an "miRNA sponge".[9] There is increasing evidence that certain circRNAs may be related to the risk of neurological, atherosclerotic vascular, prion, cancer, and autoimmune diseases.[10–13] This supports the hypothesis that circRNAs may become new diagnostic and prognostic biomarkers, and new disease treatment targets.[14,15] However, the current understanding of circRNA in AS patients is limited.

Phosphatidylinositol-3 kinase (PI3K)/protein kinase B (AKT) is an important inflammatory pathway that participates in a variety of physiological and pathological processes in the body. After activation, it participates in cell signal transduction, growth, angiogenesis, and carcinogenic transformation.[16] The PI3K is affected by cytokines to change the protein structure of AKT and activate it, thereby regulating the release of pro-inflammatory mediators.[17] Studies have shown that the PI3K/AKT//mammalian target of rapamycin (mTOR) signaling pathway plays a role in cartilage degeneration, subchondral bone dysfunction and synovial inflammation.[18,19] Therefore, this study verified the diagnostic effect of *circ_0003307* by observing the influence of *circ_0003307* on the PI3K/AKT pathway.

Objectives

The objective of this study was to explore the possible role of a certain circRNA, *Homo sapiens (hsa)_circ_0003307*, in AS. To achieve this, we detected the expression level of *circRNA_0003307* in peripheral blood mononuclear cells (PBMCs) of AS patients. Thereafter, we verified the possible role of the edited *circRNA_0003307* in the inflammatory response of AS-fibroblast-like synoviocytes (AS-FLS).

Materials and methods

Patients and healthy controls

Thirty AS patients were recruited from the Department of Rheumatology, Anhui Provincial Hospital of Traditional Chinese Medicine, Hefei, China. These patients were diagnosed by interviewers in accordance with the New York criteria revised by the American College of Rheumatology.[20,21] At the same time, we also recruited 30 healthy subjects whose age and gender matched those of AS patients as healthy controls (HC). Both the patient and the HC group ruled out the history of other diseases. The research protocol was in line with the Declaration of Helsinki. This study was approved by the Medical Ethics Committee of Anhui Provincial Hospital of Traditional Chinese Medicine (approval No. 2020AH-08).

Isolation of PBMCs and extraction of total RNA

Peripheral blood (5 mL) of the subjects was collected using an ethylenediaminetetraacetic acid (EDTA) anticoagulation tube. A discontinuous density gradient (Histopaque-1077; Sigma-Aldrich, St. Louis, USA) was used to separate PBMCs. Total RNA was extracted using TRI-Reagent (Invitrogen, Carlsbad, USA) and stored at –80°C. The RNA concentration was determined with a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, USA), and RNA integrity was assessed with agarose gel electrophoresis.

Cell culture

Synovial tissue specimens were obtained from AS patients undergoing hip replacement surgery. The specimens were cut into small pieces and mixed with 4 mg/mL collagenase (type I) (Sigma-Aldrich) for 1 h. Then, the cells were digested with 0.25% trypsin. Thereafter, AS-FLS cells were collected and added to Dulbecco's modified Eagle's culture medium (DMEM; HyClone Laboratories, Inc., Logan, USA) containing 10% fetal bovine serum (FBS; Sigma-Aldrich) and 1% streptomycin and penicillin (Beyotime, Shanghai, China). Cells were maintained at 37°C and 5% CO_2. The isolated AS-FLS were cultivated from the 3rd to the 6th generation for further study.

Cell transfection

The pcDNA3.1-*hsa_circ_0003307* was generated by amplifying the coding sequence of *circRNA_0003307* and inserting it into pcDNA3.1(+). Small interfering RNAs (siRNAs) were targeted to *circRNA_0003307* (siRNA1: 5′-AGGCUGGAAACCAUCGACGTT-3′, siRNA2: 5′-C'UGGAAACCAUC GACGAGUTT-3′, siRNA3: 5′-GAAACCAUCGACGAGUACATT-3′). Nonsense control siRNA (si-NC) was purchased from GenePharma (Shanghai, China). Lipofectamine 2000 (Invitrogen) was used for the transient transfection of vectors at room temperature. After that, the cells were incubated for 24 h before their use in subsequent experiments.

qRT-PCR

Extracted total RNA was reverse transcribed to cDNA using the Prime Script™ RT Reagent Kit (TaKaRa, Dalian, China) with gDNA Eraser. After that, according to the manufacturer's instructions, TB Green™ Premix Ex Taq™ (Tli RNase H Plus; TaKaRa, Kusatsu, Japan) was used for the quantitative reverse transcription polymerase chain reaction (qRT-PCR). The internal control β-actin primer sequence is shown in Table 1. Primers were synthesized by Sangon Biotech (Shanghai, China). The $2^{-\Delta\Delta ct}$ method was used to calculate the relative expression level of *circRNA_0003307.*

Western blotting

Table 1. Primer sequences for *hsa_circ_0003307*

Name	Size (bp)	Sequence (5′→3′)
β-actin	96	F: CCCTGGAGAAGAGCTACGAG R: GGAAGGAAGGCTGGAAGAGT
circRNA0003307	77	F: CTGTCATCAACCTGGGAAGG R: ACGGGTTGGTGGTAGCAT

Commercial kits (Pierce, Rockford, USA) were used to extract cytoplasmic and nuclear proteins from AS-FLS. A sample was prepared for electrophoresis on a Novex 10% sodium lauryl sulphate/polyacrylamide gel (Thermo Fisher Scientific). Then, the membrane was blocked with 5% skimmed milk powder in Tris-buffered saline and combined with rabbit anti-human anti-phosphorylated (p)-PI3K (dilution 1:1000; cat. No. ab182651; Abcam, Cambridge, USA) or rabbit anti-human anti-p-AKT (dilution 1:2000; cat. No. 4060s; Abcam) and incubated overnight. After washing 3 times, the membrane was incubated with anti-rabbit immunoglobulin G secondary antibody (dilution 1:20,000; cat. No. ab6721; Abcam) at room temperature for 1.5 h. Proteins were detected using enhanced chemiluminescence (ECL; Merck Millipore, Burlington, USA) and quantified using ImageQuant™ LAS 4000 (GE Healthcare Life Science, Pittsburgh, USA).

Immunofluorescence assay

After 48 h of transfection, AS-FLS were permeabilized with 0.5% Triton X100 in phosphate-buffered saline (PBS), and blocked with 2% bovine serum albumin for 15 min at room temperature. The cells were then incubated with primary antibodies and diluted in blocking buffer at 4°C overnight. This was followed by the incubation with the fluorophore-conjugated secondary antibody (1:1000; Invitrogen) in blocking buffer for 1 h at room temperature. The nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI; Invitrogen). Images were obtained using a Zeiss LSM710 confocal microscope (Carl Zeiss AG, Jena, Germany).

ELISA

Enzyme-linked immunosorbent assay (ELISA) was performed according to the instructions of the tumor necrosis factor alpha (TNF-α; product No. JYM0110Hu) and TNF-α-induced protein 2 (TNFAIP2; product No. JYM2468Hu) kits (Wuhan Genemei Technology, Wuhan, China) to determine the expression levels of TNF-α and TNFAIP2 in the AS-FLS supernatant. Absorbance was determined at the optical density (OD) of 450 nm. The contents were calculated according to standard curves.

Statistical analyses

Statistical analyses were performed and graphs were created using GraphPad Prism v. 8 software (GraphPad Software, San Diego, USA). Age was analyzed using Student's t-test (Supplementary Table 1A,B). The expression of *circRNA_0003307* in PBMCs was analyzed using Welch's t-test (Supplementary Table 2A,B). Categorical variables were compared using the χ^2 test. Correlations were assessed using the Spearman's analysis because clinical characteristics were not normally distributed (Supplementary Table 3). The *circRNA_0003307* expression in AS-FLS was analyzed using a one-way analysis of variance (ANOVA) followed by the Games–Howell test (Supplementary Table 4A–C). The p-PI3K protein expression in AS-FLS was analyzed using a one-way ANOVA followed by the Games–Howell test (Supplementary Table 5A–C). The p-AKT protein expression in AS-FLS was analyzed using a one-way ANOVA followed by the Games–Howell test (Supplementary Table 6A–C). The TNF-α levels in AS-FLS were analyzed using a one-way ANOVA followed by the Games–Howell test (Supplementary Table 7A–C). The TNFAIP2 levels in AS-FLS were analyzed using a one-way ANOVA followed by Games–Howell test (Supplementary Table 8A–C). A receiver operating characteristic (ROC) curve analysis was performed to judge whether *circRNA_0003307* can be used as a diagnostic indicator for AS. A value of $p < 0.05$ indicates that the difference was statistically significant. All Supplementary Tables with the description of statistical methods and the results of statistical tests are available at https://doi.org/10.5281/zenodo.6244943.

Results

General situation of the research subjects

Thirty AS patients and 30 HC were examined in this study. The general situation of the 2 groups of subjects is exhibited in Table 2. There was no significant difference between AS patients and HC in terms of age (t = 0.1370, p = 0.8915, degrees of freedom (df) = 58) or gender (χ^2 = 0.000, p > 0.999, df = 1) (Table 2).

Expression of *circ_0003307* in PBMCs

To detect the level of *circRNA_0003307* in PBMCs of AS patients, qRT-PCR was performed. The results showed that the level of *circRNA_0003307* in PBMCs of AS patients was significantly higher than that of the HC group (t = 15.32, p < 0.0001, df = 47.30; Fig. 1A). To evaluate the diagnostic value of *circRNA_0003307*, a ROC curve analysis was performed. The area under the curve (AUC) of *circRNA_0003307* was 0.8533 (95% confidence interval (95% CI): [0.7564; 0.9503]). The results indicate that *circRNA_0003307* has potential value in diagnosing AS (Fig. 1B).

Correlation analysis of *circRNA_0003307* and clinical characteristics of AS patients

The results of the Spearman's analysis (the distribution of the correlated variables was non-normal – cf. Supplementary Table 3) showed that the expression level of *circRNA_0003307* in PBMCs of AS patients was positively correlated with clinical characteristics, including erythrocyte sedimentation rate (ESR; r = 0.7188, p < 0.0001; Fig. 2A), C-reactive protein (CRP) level (r = 0.6309, p = 0.0002; Fig. 2B), Bath Ankylosing Spondylitis Functional Index (BASFI) (r = 0.4126, p = 0.0235; Fig. 2C), and Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) (r = 0.6295, p = 0.0002; Fig. 2D). The overall evidence indicate that the abnormal expression of *circRNA_0003307* may be related to the pathogenesis of AS.

CircRNA_0003307 expression in AS-FLS

The expression of *circRNA_0003307* in AS-FLS was detected using the qRT-PCR. In addition, qRT-PCR was used to detect the expression following overexpression and knockdown. These results showed that, through transfection of the *circRNA_0003307* overexpression vector, *circRNA_0003307* was significantly increased (t = 13.68, p < 0.0001, df = 7.171), while *circRNA_0003307* knockdown with siRNA resulted in a significant decrease (t = 18.15, p < 0.0001, df = 8.776, Fig. 3).

Effect of abnormal expression of *circRNA_0003307* on the PI3K/AKT pathway

To determine the effect of abnormal expression of *circRNA_0003307* on AS-related pathways, we tested the expression of PI3K/AKT pathway-related proteins through

Table 2. General situation of the research subjects

Variables	AS (n = 30)	HC (n = 30)	$\chi^{2/t}$	p-value	df
Sex (M/F)	24/6	24/6	χ^2 = 0.0000	>0.9999	1
Age [years], mean ±SD	36.4300 ±10.3300	36.1000 ±8.4170	t = 0.1370	0.8915	58
ESR [mm/h], median (Q1, Q3)	30.00 (17.00, 45.25)	N/A	N/A	N/A	N/A
CRP [mg/L], median (Q1, Q3)	37.13 (23.03, 71.11)	N/A	N/A	N/A	N/A
BASDAI score, median (Q1, Q3)	5.60 (5.40, 6.60)	N/A	N/A	N/A	N/A
BASFI score, median (Q1, Q3)	6.45 (6.20, 6.80)	N/A	N/A	N/A	N/A

ESR – erythrocyte sedimentation rate; CRP – C-reactive protein; BASDAI – Bath Ankylosing Spondylitis Disease Activity Index; BASFI – Bath Ankylosing Spondylitis Functional Index; N/A – not applicable; SD – standard deviation; Q1 – 1st quartile; Q3 – 3rd quartile; AS – ankylosing spondylitis; HC – healthy controls; df – degrees of freedom.

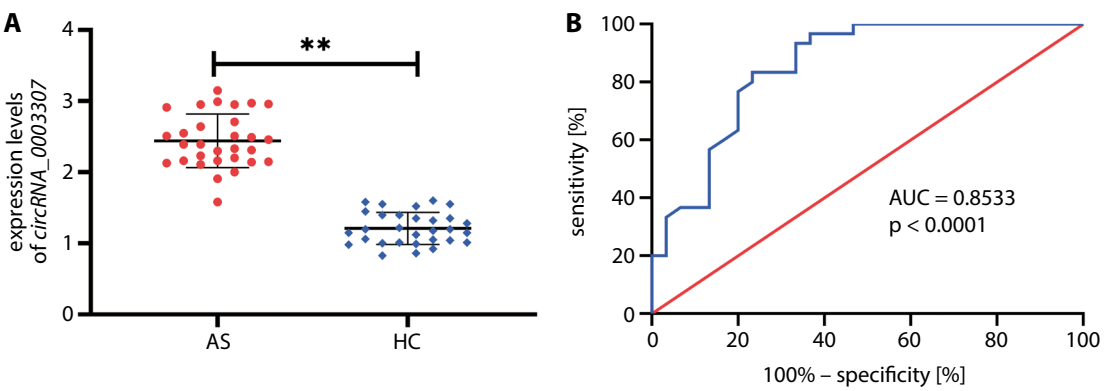

Fig. 1. Validation of abnormal expression of *circRNA_0003307* and receiver operating characteristic (ROC) curve analysis

AUC – area under the curve; AS – ankylosing spondylitis; HC – healthy controls.

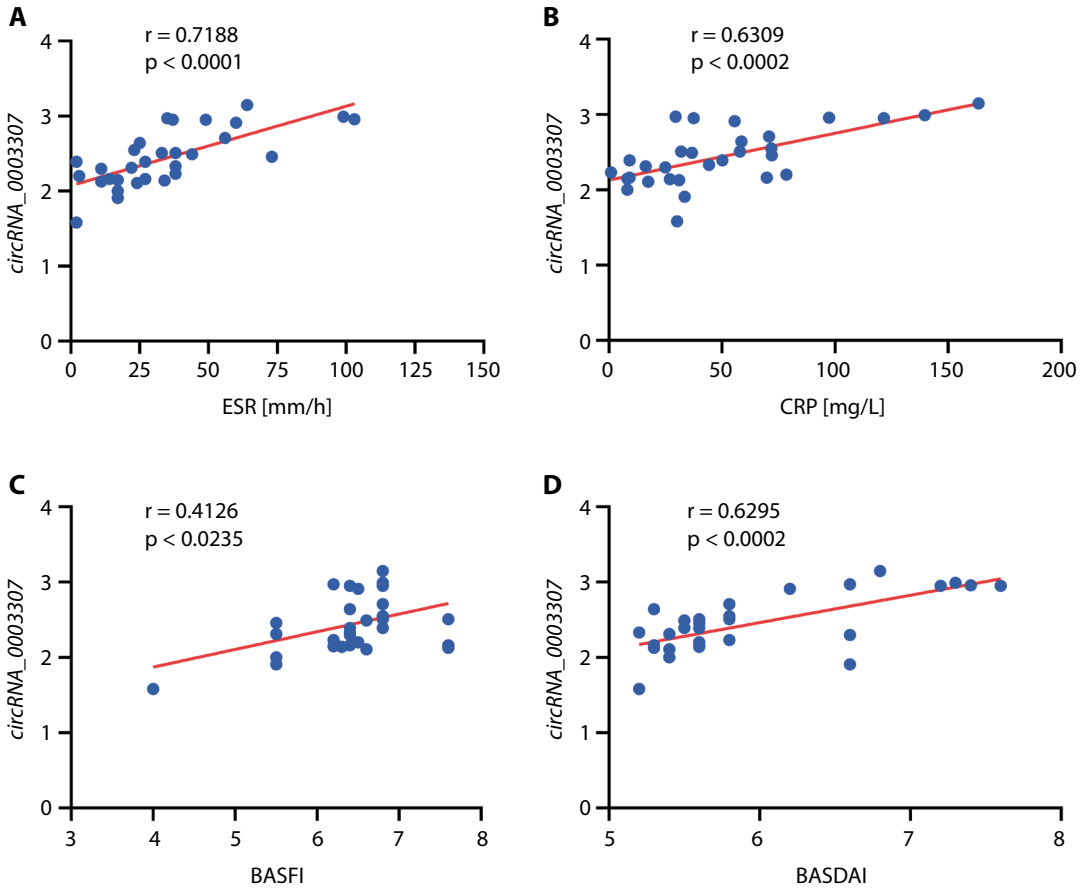

Fig. 2. Spearman's correlation analysis of *circRNA_0003307* and clinical characteristics of ankylosing spondylitis (AS) patients

ESR – erythrocyte sedimentation rate; CRP – C-reactive protein; BASFI – Bath Ankylosing Spondylitis Functional Index; BASDAI – Bath Ankylosing Spondylitis Disease Activity Index.

western blotting and immunofluorescence (IF) assay. Compared with the AS-FLS group, AS-FLS transfected with pcDNA3.1-*hsa_circ_0003307* showed a significant increase in the protein expression of p-PI3K ($t = 21.81$, $p = 0.0015$, $df = 2.806$) and p-AKT ($t = 4.744$, $p = 0.0451$, $df = 3.768$, Fig. 4A–C), whereas these protein levels were significantly decreased in AS-FLS transfected with si-*hsa_circ_0003307* compared with those in the AS-FLS group ($t_{p\text{-}PI3K} = 11.13$, $p_{p\text{-}PI3K} = 0.0050$, $df_{p\text{-}PI3K} = 3.167$; $t_{p\text{-}AKT} = 4.851$, $p_{p\text{-}AKT} = 0.0396$, $df_{p\text{-}AKT} = 3.884$, Fig. 4A–C). The IF assay used to analyze protein expression in these AS-FLS yielded similar results (Fig. 4D,E). These results indicate that the PI3K/AKT signaling pathway can be activated by *circRNA_0003307*, which is highly expressed in AS.

Effects of aberrant *circRNA_0003307* expression on inflammatory cytokines

To study the effect of *circRNA_0003307* on the inflammatory response of AS following the activation of the PI3K/AKT pathway, ELISA was used to detect the expression of the inflammatory factors – TNF-α and TNFAIP2. The results showed that TNF-α and TNFAIP2 levels in AS-FLS significantly decreased when AS-FLS was transfected with si-*hsa_circ_0003307* ($t_{TNF\text{-}\alpha} = 4.686$, $p_{TNF\text{-}\alpha} = 0.0110$, $df_{TNF\text{-}\alpha} = 7.662$; $t_{TNFAIP2} = 5.066$, $p_{TNFAIP2} = 0.0157$, $df_{TNFAIP2} = 5.536$, Fig. 5A,B) compared with si-NC. In addition, AS-FLS induced with pcDNA3.1-*hsa_circ_0003307*

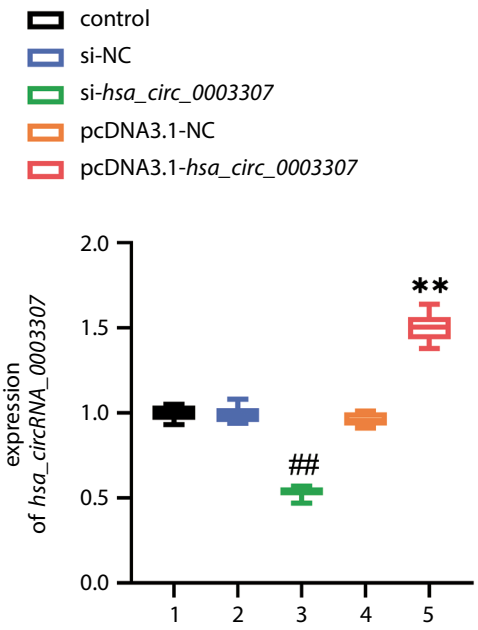

Fig. 3. Differential expression of *circRNA_0003307* in ankylosing spondylitis fibroblast-like synovial cells

si-NC – nonsense control siRNA.

showed higher levels of TNF-α and TNFAIP2 than pcDNA3.1-NC ($t_{TNF\text{-}\alpha} = 11.51$, $p_{TNF\text{-}\alpha} < 0.0001$, $df_{TNF\text{-}\alpha} = 6.077$; $t_{TNFAIP2} = 28.84$, $p_{TNFAIP2} < 0.0001$, $df_{TNFAIP2} = 8.741$, Fig. 5A,B).

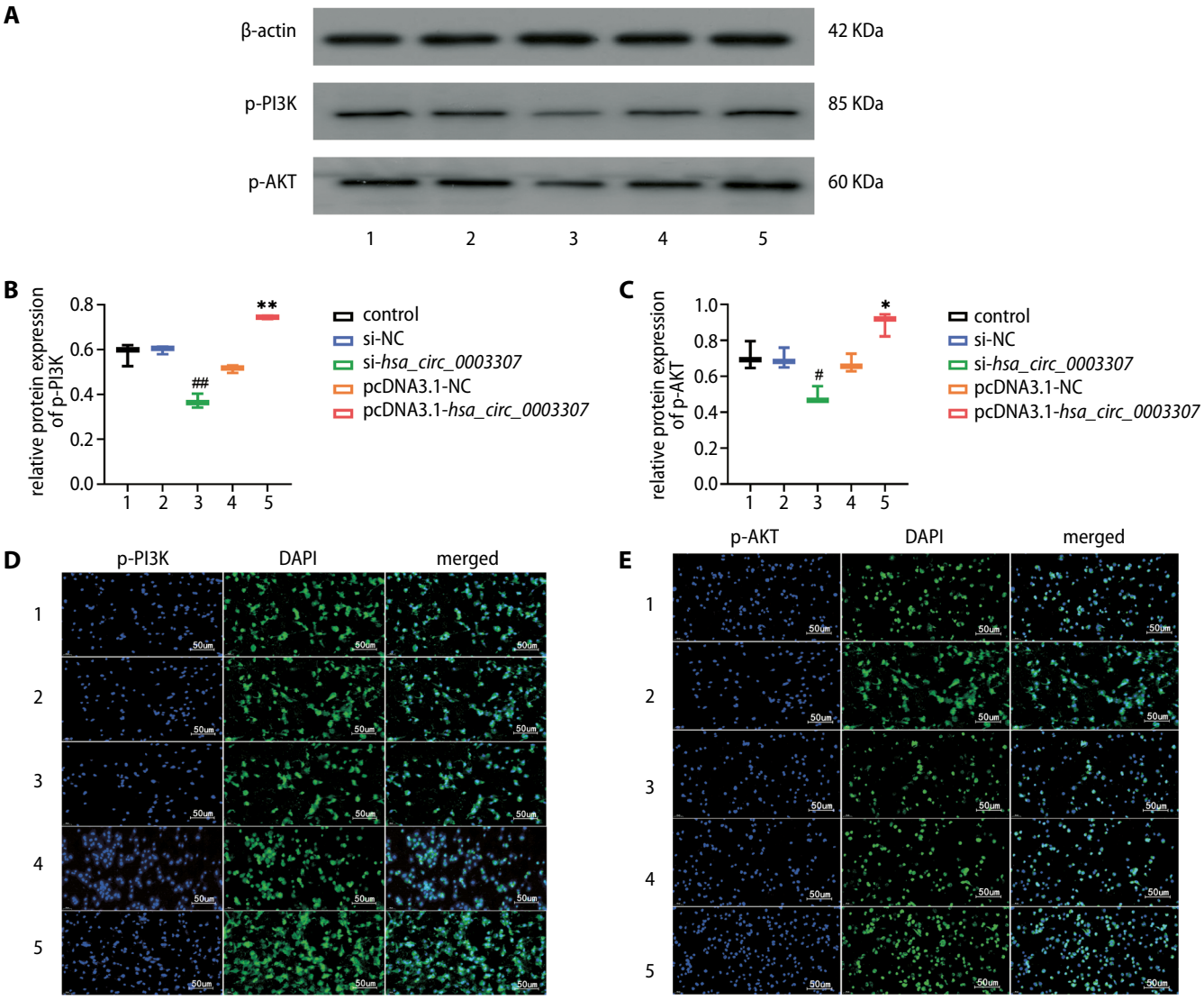

Fig. 4. Boxplots of the effect of *circRNA_0003307* on the phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT) pathway

si-NC – nonsense control siRNA.

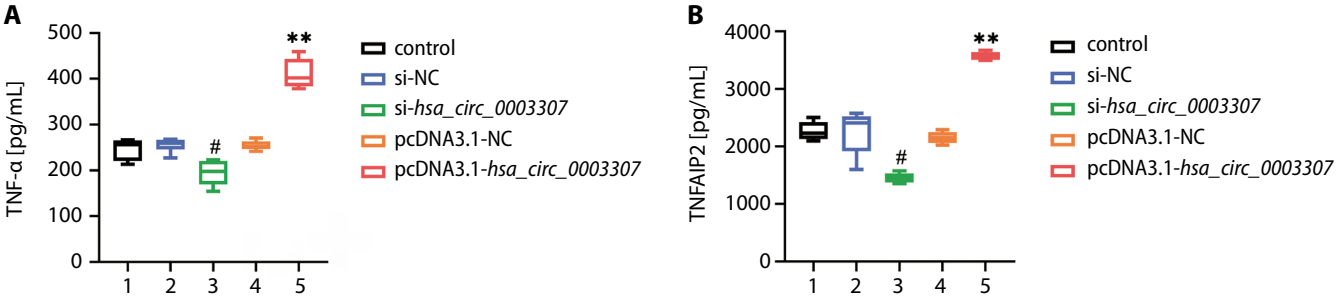

Fig. 5. Boxplots of the effect of *circRNA_0003307* on inflammatory cytokines

si-NC – nonsense control siRNA; TNF-α – tumor necrosis factor alpha; TNFAIP2 – TNF- α-induced protein 2.

Discussion

The discovery of circRNAs has provided novel insights into AS treatment, and it is of vital significance for identifying new diagnostic and therapeutic targets for AS. A growing number of studies in recent years have greatly broadened our horizons regarding circRNA functions and increased its value in disease diagnosis. Many circRNAs are dysregulated in rheumatic disorders, and the clinical significance of dysregulated circRNAs in such disorders

has been investigated previously. For example, Ouyang et al. confirmed that *circRNA_002453* may serve as a diagnostic marker for AS.[22] They have proven that the upregulated circRNA in the plasma of AS patients can aggravate the degree of renal involvement. Wang et al. indicated that circIBTK may also act as a therapeutic target for systemic lupus erythematosus (SLE), and its therapeutic mechanism might regulate DNA demethylation and downstream signaling pathways by targeting miR-29b in SLE.[23]

These findings confirm the existence of useful information about mRNA profiles in the PBMCs of AS patients.[15] In addition, researchers have used bioinformatics methods to predict the genes that were differentially expressed in AS, and research the role of these differential genes in the pathogenesis of AS. This information allows us to better understand the pathogenesis of AS, and it is possible to discover new diagnosis and treatment methods from it. For this study, we selected *circRNA_0003307*. To determine the pathway of *circRNA_0003307* in AS-FLS involved in the pathogenesis, we chose PI3K/AKT as the research pathway. The reason is that PI3K/AKT plays a key role in the pathogenesis of AS and provide a breakthrough in AS treatment. Terlemez et al., Yan et al. and Liu et al. proved that miRNAs affects the phenotype of FLS in rheumatism by regulating the expression of PI3K/AKT, thereby affecting the pathogenesis of rheumatism.[24–26] Therefore, we speculate that there may be a regulatory relationship between *circRNA_0003307* and the PI3K/AKT pathway.

The results of this study showed that high expression level of *circRNA_0003307* was positively correlated with the severity of AS. Li et al. reported that the *circ_0056558* level was highly expressed in AS tissue, and was achieved through the PI3K/AKT pathway.[27] In our study, *circRNA_0003307* was overexpressed in PBMCs of AS patients compared with healthy participants. There was a positive correlation between *circRNA_0003307* levels and ESR, CRP level, BASDAI, and BASFI. Luo et al. demonstrated that low expression of *hsa_circ_0079787* in peripheral blood of AS patients was negatively correlated with BASDAI, which was in concert with our data.[28] Collectively, the expression level of *circRNA_0003307* was associated with disease activity.[29]

CircRNA_0003307 activated the PI3K/AKT signaling pathway and affected the expression of the downstream inflammatory factors – TNF-α and TNFAIP2. The results of the study confirmed our hypothesis stating that the protein expression of p-PI3K and p-AKT was altered by both overexpression and knockdown of *circRNA_0003307*. Li et al. demonstrated that *hsa_circ_0056558* inhibited the PI3K/AKT pathway by targeting miR-1290 in AS, and reduced the protein expression of p-AKT.[27] A further analysis showed that the overexpression of *circRNA_0003307* markedly increased the protein expression level of p-PI3K and p-AKT. However, for *circRNA_0003307* knockdown, the protein expression level was significantly reduced. Thus, PI3K/AKT is involved in immune-mediated inflammatory responses. Simultaneously, the findings showed that the overexpression of *circRNA_0003307* increased the expression of the downstream inflammatory factors – TNF-α and TNFAIP2, while the knockdown of *circRNA_0003307* showed the opposite results, reducing the expression of TNF-α and TNFAIP2.

Kou et al. demonstrated that circRNAs may be involved in the PI3K/AKT signaling pathways associated with inflammation-induced apoptosis in chondrocytes.[30] The activation of p-PI3K could induce downstream p-AKT. Inflammatory cytokines, such as TNF-α and interleukin 17 (IL-17), are involved in the pathogenesis of AS.[31] Taken together, the expression level of *circRNA_0003307* is closely related to the AS inflammatory response.

Limitations

This study has certain limitations. In our subjects, we found that the overexpression of *circRNA_0003307* increased the levels of p-PI3K, p-AKT, TNF-α, and TNFAIP2 in AS-FLS. The knockdown of *circRNA_0003307* showed the opposite results, reducing the expression of p-PI3K, p-AKT, TNF-α, and TNFAIP2. However, the detailed mechanism of *circRNA_0003307*-targeted activation of the PI3K/AKT pathway remains unclear. We also lacked patients with other autoimmune diseases as a control group to clarify that *circRNA_0003307* is specific to AS.

Conclusions

The present study revealed that the knockdown of *circRNA_0003307* inhibits synovial inflammation in AS by regulating the PI3K/AKT pathway. In addition, the expression level of *circRNA_0003307* was associated with disease activity. The *circRNA_0003307* was highly expressed in AS-PBMCs and AS-FLS, and *circRNA_0003307* knockdown reduced the inflammatory response of AS-FLS by regulating the PI3K/AKT pathway. These findings suggest that targeting *circRNA_0003307* offers a promising therapeutic strategy for AS patients and may serve as a potential target for AS treatment. Based on these findings, our research team will explore the possible competing endogenous RNA molecular mechanism of *circRNA_0003307* in future studies of AS.

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