# Endothelial mesenchymal transition promotes pannus formation in ankylosing spondylitis by activation of TGF-β/SMAD signalling pathway

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## Abstract

### Objective

*To explore the role of endothelial-mesenchymal transition (EndMT) mediated by the TGF-β/SMAD signalling pathway in the pathogenesis of ankylosing spondylitis (AS).*

### Methods

*Serum levels of TGF-β1 were measured by enzyme-linked immunosorbent assay (ELISA) in 48 patients with AS and 15 healthy subjects. The expression levels of TGF-β1, SMAD7, CTGF, CD34 and EndMT-related markers (α-SMA, vimentin, FSP-1, VE-cadherin) in the sacroiliac joint (SIJ) of three AS patients were detected by immunohistochemistry, and three non-spondyloarthritis (SpA) autopsy samples were used as controls.*

### Results

*Serum TGF-β1 level of AS patients was significantly higher than that of healthy controls (22971 ± 7667 pg/ml vs. 14837±4653 pg/ml, $p<0.01$). Compared with the non-SpA control group, the microvascular density (MVD) at the pannus formation site of SIJ in AS patients was significantly increased, accompanied by respectively increased expressions of TGF-β1, CTGF, α-SMA, vimentin, and FSP-1 (all $p<0.05$), whereas respectively decreased expressions of VE-cadherin and SMAD7 ($p<0.01$). The expression level of FSP-1 was positively correlated with levels of TGF-β1 and MVD, and negatively correlated with SMAD7.*

### Conclusion

*Our findings show that EndMT is involved in the promotion of pannus formation by TGF-β/SMAD signalling pathway activation in AS.*

**Key words**

ankylosing spondylitis, TGF-β1, endothelial-mesenchymal transition, pannus formation

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*Significance and innovations*
*In this study, we aimed to explore the pathogenesis of sacroiliitis.*
*We found that serum TGF-β1 levels of AS patients were significantly higher than those of healthy controls, and the TGF-β signalling pathway is activated in AS patients with active sacroiliitis.*
*Endothelial-mesenchymal transition (EndMT) is involved in the promotion of pannus formation by TGF-β/SMAD signalling pathway activation.*
*The results of this study are helpful to further elucidate the immunopathological mechanism of sacroiliitis in AS.*

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## Introduction

Ankylosing spondylitis (AS) is a chronic inflammatory disease that mainly invades the axial joints and is recognised as the most typical form of spondyloarthritis (SpA). The prevalence of AS ranges from 0.2% to 0.54% among Han ethnic Chinese (1), mostly in young men. With progression of the disease, AS eventually leads to spinal rigidity, which seriously affects the physical function and quality of life of patients, and also causes a heavy burden to families and society. The sacroiliac joint (SIJ) is a key site involved in AS, and sacroiliitis is the essential indicator for the diagnosis of AS. However, pathological mechanism responsible for sacroiliitis has not been fully elucidated, and the relationship between inflammation and structural damage and the related molecular mechanisms need to be further explored.

In recent years, evidence has increasingly shown that neovascularisation plays an important role in the immunopathological injury in SpA (2-5). In 2000, Bollow *et al*. (2) performed CT-guided SIJ biopsies on 32 cases of SpA (18 cases of AS, 12 cases of undifferentiated spinal arthritis, and 2 cases of psoriatic arthritis) and showed that, in the patients with active sacroiliitis on MRI, there is an abundance of neovascularisation and fibrous connective tissue formation in the bone marrow of the SIJ. Subsequently, Francois *et al*. (3) performed open SIJ biopsy on 5 AS patients, followed by immunohistochemical detection, and showed that a large number of inflammatory cells ($CD3^+$ T cells and $CD68^+$ macrophages) and high levels of anti-tumour necrosis factor antibodies were found in the area of pannus invasion in early sacroiliitis. We previously performed CT-guided SIJ biopsy and showed that the formation of the subchondral pannus was the most valuable pathological sign of early SIJ inflammation (4, 5), and the invasion of pannus into cartilage was an independent risk factor for the radiological progress of sacroiliitis (5).

Endothelial-mesenchymal transition (EndMT) has been proven to promote neovascularisation (6) and fibrosis (7). The TGF-β signalling pathway is one of the main regulatory pathways of EndMT (8). Previous studies have shown that the TGF-β levels in peripheral blood (9) and SIJ tissues (3) of AS patients are elevated, suggesting that the TGF-β signalling pathway may be activated in AS. The purpose of this study is to explore whether EndMT promotes pannus formation in ankylosing spondylitis by activation of the TGF-β/SMAD signalling pathway.

## Methods

*Subjects*

A total of 48 Chinese patients with AS in The First Affiliated Hospital, Shantou University Medical College, China were enrolled for the serological study from July 2020 to September 2021. All of them were met the modified New York criteria for AS (10). Patients with other chronic systemic diseases, other rheumatic diseases, malignancy, or pregnancy were excluded in this study. Fifteen sex-matched and age-matched healthy subjects were randomly selected as controls.

Source of SIJ specimens: with the same screening criteria as patients in the above serological study, three AS SIJ specimens were obtained previously by CT-guided fine needle aspiration biopsy in the SIJ. The SIJ control specimens were obtained from the autopsy specimens of three non-SpA fresh deaths, and the causes of death were myocarditis, coronary heart disease and car accident respectively.

This study was approved by the Ethics Committee of The First Affiliated Hospital of Shantou University Medical College. Informed consent was obtained from each enrolled patient. Postmortem SIJ tissues were collected with family consent.

*Research methods*

Clinical parameters collected including gender, age, initial symptoms, symptom (ever), human leucocyte antigen (HLA)-B27 status, C-reactive protein (CRP) level (>8 mg/L was defined as elevated), erythrocyte sedimentation rate (ESR) (>15 and >20 mm/hour for males and females were defined as elevated). Bath AS Disease Activity Index (BASDAI, ≥4 was defined as active disease),

Bath AS Functional Index (BASFI), SIJ imaging assessment (on pelvic plain radiograph or CT) and the treatments, including non-steroidal anti-inflammatory drug (NSAID) use, conventional synthetic disease-modifying anti-rheumatic drug (csDMARD) use, tumour necrosis factor (TNF) Inhibitors or interleukin-17 (IL-17) Inhibitors use, etc. Sera from 48 patients with AS were collected and stored at -80°C for measurement. Serum TGF-β1 was measured by enzyme-linked immunosorbent assay (ELISA) using a TGF-β1 kit according to the manufacturer's instructions (Shenzhen Xinbosheng Biotechnology Co., Ltd).

The fine needle aspiration biopsy procedure of SIJ was reported in our previous studies (4, 5). The SIJ biopsy was performed using a Franseen lung biopsy needle (Cook Company, DFBN-18-15) under the guidance of CT with the patient in the prone position. The SIJ tissues were extracted by negative pressure. Immunohistochemistry was performed using the streptomyces avidin-peroxidase (SP) method. Primary antibodies were monoclonal antibodies against TGF-β1, connective tissue growth factor (CTGF), fibroblast specific protein-1 (FSP-1), VE-Cadherin (Abcam, UK), SMAD7 (Novus Biologicals Inc), vimentin, alpha-smooth muscle actin (α-SMA) and CD34 (Cell Signaling Technology, Inc), respectively. An Ultrasensitive SP kit and DAB kit were purchased from Fuzhou Maixin Biotechnology Development Co., Ltd.

All sections were stained for the same time and imaged at 400x under the same exposure conditions. Five visual fields at the junction of the cartilage and bone plate of AS SIJ and five visual fields at the corresponding location of non-SpA SIJ were observed. Image-Pro Plus was used to analyse the average optical density and proportion of positive expression area, which was multiplied as a quantitative measure of antigen expression.

The scoring of microvessel density (MVD, marked with CD34) at the cartilage-bone plate interface was manually counted from five high-power visual fields by two experienced pathologists, and the average value was taken.

*Statistical methods*

SPSS for Windows, v. 26.0 (SPSS Inc IMB Company) was used for data analysis. Kolmogorov-Smirnov test of normality was performed for continuous data. Variables were presented as means with standard deviation (SD) and medians with interquartile range (IQR) for normally and non-normally distributed variables, respectively. Frequency (%) was given for counts. Mann-Whitney test was used to compare continuous values between groups. Pearson (normally distributed variables) or Spearman (non-normally distributed variables) correlation analysis was used to analysed the correlation among the expression levels of TGF-β signalling pathway-related factors, MVD and End-MT markers. *p*-values of <0.05 were considered statistically significant.

## Results

*General clinical data*

Table I shows the baseline demographic and clinical characteristics of the patients. Among the 48 patients, 36 were male and 12 were female (male: female ratio at 3:1), the median age was 25.0 (7.0) years; the median disease duration was 8.0 (5.0) years. The positive rate of HLB-27 was 86.0%.

*Serum level of TGF-β1 is elevated in AS patients*

We detected the serum TGF-β1 levels by ELISA in 48 AS patients and 15 healthy subjects. Serum levels of TGF-β1 in AS patients were significantly higher than those in healthy subjects (22971±7667 pg/ml *vs*. 14837±4653 pg/ml, $p<0.01$) (Fig. 1).

*Pathological findings of radiographic sacroiliitis*

Figure 2 shows the representative pathological findings of radiographic sacroiliitis in three cases of SIJ biopsy specimens (HE staining). Specimen 1 (a) was from a 21-year-old female patient (Case 1) with a history of low back pain for 2 years, grade II radiographic sacroiliitis. Pathological findings showed that synovial pannus formed and invaded the surface of cartilage, resulting in cartilage degeneration, abnormal proliferation of chondrocytes and matrix loss.

**Table I.** Baseline demographics of 48 cases of AS.

| Characteristics | Total (n=48) |
|---|---|
| Age, (years)* | 25.0 (7.0) |
| Sex, M/F | 36/12 |
| Disease duration (years)* | 8.0 (5.0) |
| Initial symptoms, n (%) * | |
| Axial | 41 (85.4) |
| Peripheral | 7 (14.6) |
| Symptoms (ever), n (%)* | |
| Axial only | 24 (45.8) |
| Axial and peripheral | 26 (54.2) |
| Extra-articular | 1 (2.1) |
| HLA-B27 positivity, n (%) | 37/43 (86.0) |
| ESR (mm/h) * | 12.0 (17.2) |
| CRP (mg/L) * | 7.0 (11.8) |
| BASDAI** | 3.15 ± 1.87 |
| BASFI** | 2.06 ± 2.26 |
| Treatments | |
| NSAIDs n (%) | 23 (47.9) |
| csDMARDs n (%) | 18 (37.6) |
| TNF-α or IL-17 inhibitors | 5 (10.4) |

AS: ankylosing spondylitis; HLA-B27: human leucocyte antigen B27; ESR: erythrocyte sedimentation rate; CRP: C reactive protein; BASDAI: Bath AS Disease Activity Index; BASFI: Bath AS Functional Index; NSAIDs: non-steroidal anti-inflammatory drugs; csDMARDs: conventional synthetic disease-modifying anti-rheumatic drugs; TNF-α: tumour necrosis factor; IL-17: interleukin-17.
*Values are median (IQR),
** Values are means ± SD.

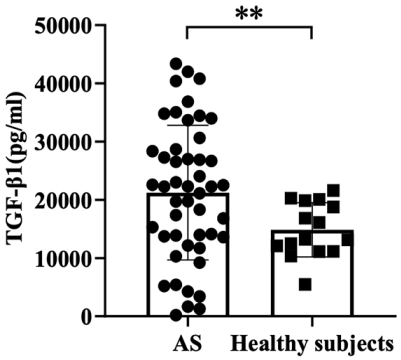


**Fig. 1.** Serum TGF-β1 levels in patients with AS and healthy subjects.
Serum TGF-β1 levels in patients with AS (n=48) and healthy subjects (n=15) were determined by ELISA. **$p<0.01$.

Specimen 2 (b) was from a 35-year-old female patient (Case 2) with a history of low back pain for 2 years and grade II radiographic sacroiliitis. Pathological findings showed that a large number of fibrous granulation tissues rich in blood vessels formed in the bone marrow, invaded and destroyed the subchondral bone plate. Specimen 3 (c) was from a 23-year-old male patient (Case 3) with

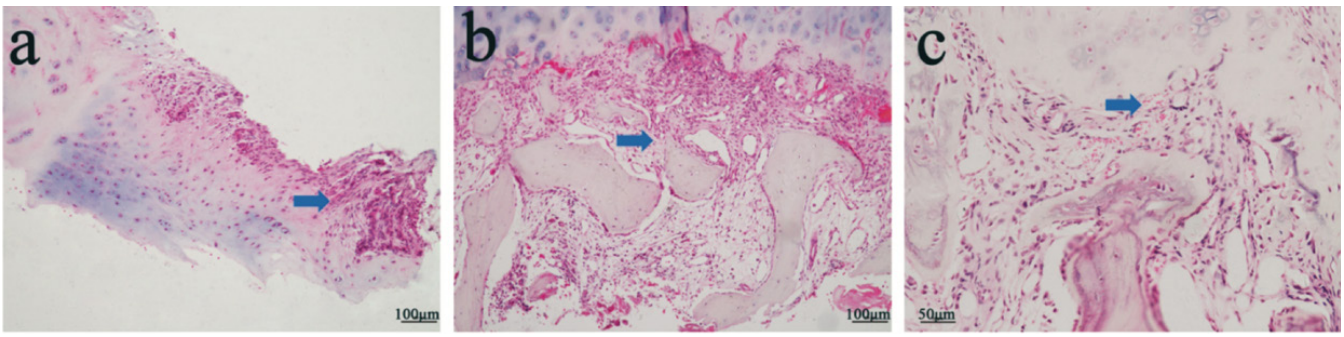


**Fig. 2.** Pathological findings of radiographic sacroiliitis.
The representative pathological findings (blue arrow) of radiographic sacroiliitis in three cases of SIJ biopsy specimens was showed as below (HE staining).
**a**. The representative pathological findings of Case 1 showed that synovial pannus formed and invaded the surface of cartilage, resulting in cartilage degeneration, abnormal proliferation of chondrocytes and matrix loss (100x, Scale bar, 100 μm).
**b**. The representative pathological findings of Case 2 showed that a large number of fibrous granulation tissues rich in blood vessels formed in the bone marrow, invaded and destroyed the subchondral bone plate (100x, Scale bar, 100 μm).
**c**. The representative pathological findings of Case 3 showed that a large number of fibrous granulation tissues formed and destroyed the subchondral bone plate, invaded the cartilage, resulting in cartilage fibrosis (200x, Scale bar, 50 μm).

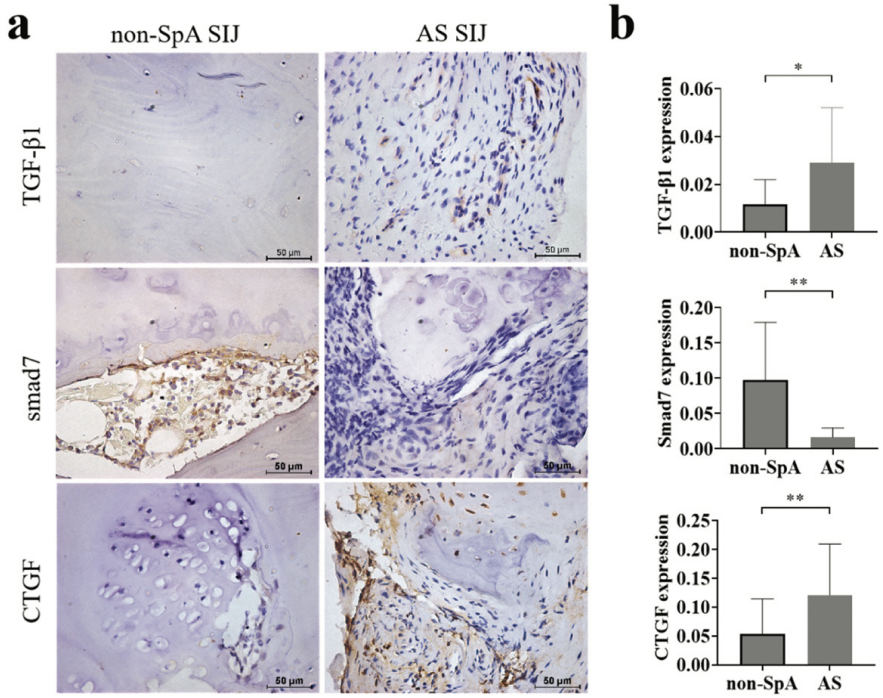


**Fig. 3.** Expression levels of TGF-β1, SMAD7 and CTGF in sacroiliac joint of AS and non-SpA.
Expression levels of TGF-β1, SMAD7 and CTGF were detected by IHC in sacroiliac joint of AS (n=3) and non-SpA (n=3).
**a**. A large number of fibrous vascular tissues formed and invaded into the cartilage in SIJ tissue with AS, where high expression levels of TGF-β1 and CTGF were present. Moreover, the expression levels of SMAD7 were low. While no pannus invasion was found in the cartilage of SIJ tissue with non-SpA, where low levels of TGF-β1 and CTGF were observed, with high expression levels of SMAD7.
**b**. Expression levels of TGF-β1 and CTGF in SIJ tissue with AS were higher than those in non-SpA, while expression levels of SMAD7 were lower in AS.
*$p$<0.05 and **$p$<0.01 *vs*. non-SpA group. All data are representative of three independent experiments. Means ± SD are shown.

a 3-year history of low back pain and grade II radiographic sacroiliitis. Pathological findings showed that a large number of fibrous granulation tissues formed and destroyed the subchondral bone plate, invaded the cartilage, resulting in cartilage fibrosis.

*The TGF-β signalling pathway is activated in AS patients with sacroiliitis*

(a) A large number of fibrous vascular tissues formed and invaded the cartilage in SIJ tissue with AS, where high expression levels of TGF-β1 and CTGF were present. Moreover, the expression levels of SMAD7 were low. While, no pannus invasion was found in the cartilage of SIJ tissue with non-SpA, where low levels of TGF-β1 and CTGF were observed, with high expression levels of SMAD7. (b) Expression levels of TGF-β1 and CTGF in SIJ tissue with AS were higher than those in non-SpA, while expression levels of SMAD7 were lower in AS. Compared with the SIJ specimens with non-SpA, a large number of fibrous vascular tissues formed was found and invaded into the cartilage in SIJ tissue with AS, where high expression levels of TGF-β1 and its downstream factor CTGF was present, whereas the expression levels of negative regulator SMAD7 were decreased in the SIJ specimens with AS (Fig. 3).

*EndMT promotes pannus formation in AS patients*

As shown in Figure 4, compared with non-SpA SIJ tissues, a large amount of fibrous granulation rich in $CD34^+$ blood vessels were formed in the AS patient SIJ, result in penetrating the subchondral bone plate and eroding the cartilage. The expression of endothelial markers VE-cadherin was decreased at the site of pannus invasion, while the expression levels of mesenchymal markers α-SMA, vimentin and FSP-1 were significantly increased (all $p$<0.01).

*Correlation between TGF-β signalling pathway-related factors and EndMT markers*

We analysed the correlation between

the expression levels of TGF-β signalling pathway-related factors and EndMT markers. As shown in Figure 5, the expression of TGF-β1 was positively correlated with the EndMT marker FSP-1, and the expression of FSP-1 was positively correlated with microvessel density. However, the expression of SMAD7 was negatively correlated with FSP-1. Except for the correlation of the above markers, none of the other markers showed any correlation.

## Discussion

EndMT is a type of epithelial-mesenchymal transition, which participates in various physiological and pathological processes *in vivo*. In the process of EndMT, endothelial cells lose contact with other cells, endothelial markers such as VE-cadherin decrease, as well as mesenchymal cell markers including α-SMA, vimentin and FSP-1 become expressed, and the original cobblestone-like morphology is transformed into a slender spindle structure. At the same time, cell migration and invasion are enhanced. In the process of angiogenesis, EndMT may play an important role in apical cell generation of a vascular plexus and migration to surrounding tissues. Vascular supporting cells, such as pericytes or smooth muscle cells, may come from the endothelium itself, and EndMT may be an important means of recruiting these pericytes during angiogenesis. In addition, these vascular support cells are also an important part of mature blood vessels. In angiogenesis, EndMT plays an important role in stabilising new blood vessels (11, 12). Therefore, inhibition of EndMT may inhibit the process of angiogenesis, thus delaying the progress of pannus formation.

At present, EndMT in inflammatory arthritis has yet to be reported. Our results show that the level of the endothelial marker VE-cadherin is decreased, while the levels of mesenchymal markers α-SMA, vimentin and FSP-1 are increased in the site of pannus formation in SIJ inflammation. The expression level of FSP-1 is positively correlated with the local microvessel density, suggesting that EndMT occurs in sacroiliitis and may be involved in pannus formation.

TGF-β1 is the principal member of TGF-β growth factor superfamily (13). Previous studies have shown that the TGF-β signalling pathway is one of the main pathways regulating EndMT (14). In the SMAD-dependent signalling pathway (13), TGF-β1 first binds to the transforming growth factor type II receptor (TβR II) on the endothelial cell membrane. Then the TβR II kinase phosphorylates and binds to TβRI, forming a heterodimer or tetramer structure that then phosphorylates receptor-activated SMAD2 and SMAD3 of the SMAD protein family. Then, p-SMAD2/3 forms a complex with universal SMAD4, which translocates to the nucleus to regulate gene expression. SMAD7, as a negative regulator, can inhibit the activation of TGF-β/SMAD7 signalling pathway.

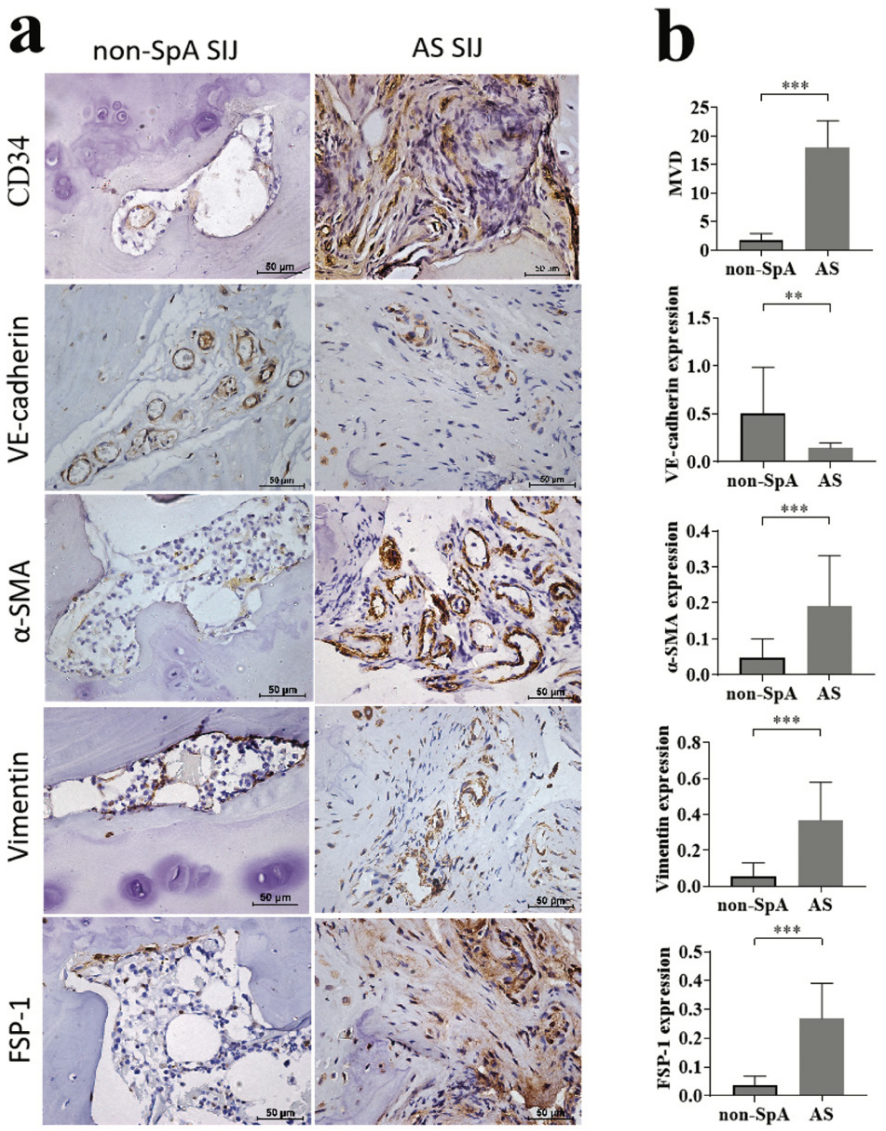


**Fig. 4.** Expression levels of CD34 and EndMT markers in sacroiliac joint of AS and non-SpA. Expression levels of CD34 (calculated as MVD) and EndMT markers were detected by IHC in sacroiliac joint of AS (n=3) and non-SpA (n=3).

**a**. Compared with non-SpA SIJ tissues, in AS SIJ tissue, a large amount of fibrous granulation rich in CD34+ blood vessels were formed and penetrated the subchondral bone plate in areas of eroded cartilage. In addition, lower expression levels of VE-cadherin and higher expression levels of α-SMA, Vimentin and FSP-1 were also observed in AS SIJ tissue.

**b**. Expression levels of CD34 was manually counted as MVD, Evaluation and quantitation of EndMT markers were finished by Image-Pro Plus. Expression levels of VE-cadherin in AS SIJ tissue was lower than those in non-SpA, whereas MVD and the expression levels of α-SMA, Vimentin and FSP-1 in AS SIJ tissue were higher than those in non-SpA tissue.

*$p$<0.01 and **$p$<0.001 *vs*. non-SpA group. All data are representative of three independent experiments. Means ± SD are shown.

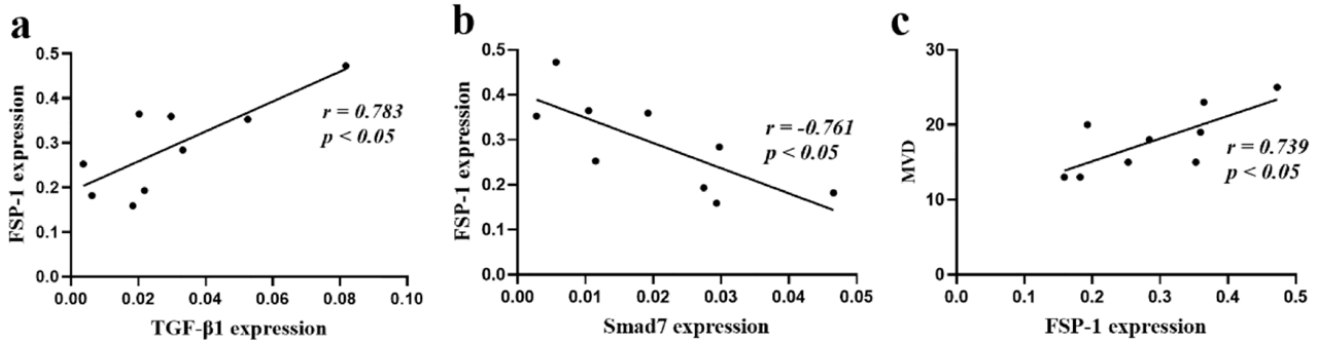


**Fig. 5.** Correlation between TGF-β signalling pathway-related factors, MVD and EndMT markers. Pearson correlation analysis was performed between TGF-β1 and FSP-1, SMAD7 and FSP-1, FSP-1 and MVD in AS SIJ tissues.
**a**. The expression level of TGF-β1 in AS SIJ tissues was positively correlated with FSP-1 ($p$<0.05, $r$=0.783).
**b**. The expression level of SMAD7 was negatively with FSP-1 ($p$<0.05, $r$= -0.761).
**c**. The expression level of FSP-1 was positively with MVD ($p$<0.05, $r$=0.739).

Our results show that the serum TGF-β1 level in AS patients is higher than that in healthy controls. Compared with non-SpA SIJ specimens, the expression levels of TGF-β1 and its downstream factor, CTGF, were increased in AS patients with SIJ inflammation, as well as, the expression level of negative regulatory factor, SMAD7, was decreased in AS SIJ tissue. Correlation analysis showed that the expression level of TGFβ1 was positively correlated with that of FSP-1, which is a mesenchymal marker. Additionally, the expression level of SMAD7 was negatively correlated with that of FSP-1. Previous studies have shown that both TGF-β1 levels are increased in SIJ tissues or peripheral blood of patients with AS (3, 9, 15). It has also been reported that, compared with non-SpA subjects, the expression levels of TGF-β and p-SMAD3 in the SIJ tissue of AS patients were increased, while the expression level of SMAD7 is decreased. These results suggest that TGF-β/Smad signalling pathway is activated and may promote local pannus formation by inducing EndMT in AS sacroiliitis (15).
The limitation of this study is that only three SIJ biopsy specimens were included, and the conclusions need to be further expanded for verification.

## Conclusions

In conclusion, pannus formation plays an important role in inflammation and structural destruction of sacroiliitis in AS. The TGF-β/SMAD signalling pathway may be activated and promote local pannus formation by inducing EndMT in AS sacroiliitis.

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