Increased expression of alpha 1-anti-trypsin in the synovial tissues of patients with ankylosing spondylitis

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Abstract

Objectives

Evidence indicates that the hyperplasia and inflammation of synovial tissues are significantly related to the pathogenic process of ankylosing spondylitis (AS). Using a proteomics approach, we detected a significantly increased expression of alpha 1-anti-trypsin (ATA1) in synovial membranes of patients with AS.

Methods

We continued to investigate the expression level and location of ATA1 in synovial tissue of AS. We also investigated the genetic effect of the gene encoding ATA1 on AS. Western blot analysis was applied to determine the expression of ATA1 in synovial tissues by comparing the expression profiles of AS (n=8), rheumatoid arthritis (RA, n=9) and osteoarthritis (OA, n=9) samples. Immunohistochemistry was used to localise the expression of ATA1 in the synovial membrane. Taqman method was used to genotype tag SNPs (rs2753934, rs2749531 and rs6575424) with 56 AS cases, 260 RA cases and 160 healthy controls.

Results

We detected an increased expression of ATA1 in synovial membranes of AS as compared with samples from RA and OA. We immuno-localised the significant expression of ATA1 in AS tissues. No significant association was found between the ATA1 polymorphism and AS or RA. Haplotype analysis did not reveal a haplotype to be associated with AS or RA.

Conclusions

It has been reported that ATA1 is related with inflammation and new bone formation, two important features of AS. The current findings suggest that ATA1 contributes to the pathogenesis of AS by up-regulating the gene expression in the synovial tissues.

Key words

ankylosing spondylitis, alpha 1-anti-trypsin, genotype, haplotype, susceptibility, rheumatoid arthritis

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Introduction

Evidence has indicated that the hyperplasia and inflammation of synovial tissues are significantly related to the pathogenic process of ankylosing spondylitis (AS) (1, 2). Using a proteomics approach, we previously screened AS-specific proteins within synovial tissues among patients with AS. By comparing these samples with those obtained from patients with rheumatoid arthritis (RA) and osteoarthritis (OA), we detected a significantly increased expression of alpha 1-anti-trypsin (ATA1) in synovial membranes of AS (3). ATA1 is a glycoprotein and is generally known to inhibit serum trypsin. ATA1 is also referred to as a serine protease inhibitor (serpin) due to its inhibition of a wide variety of proteases. It protects tissues from enzymes, especially elastase, released by inflammatory cells (4-6). Thus, changes in the expression of ATA1 may affect lymphocyte responses, complement activation and leukocyte migration, ultimately exacerbating immunologic and inflammatory responses (7). Additionally, ATA1 can influence the autolytic effects of leukocytic enzymes on tissues and may inhibit some aspects of coagulation and fibrinolysis (4-6). Many studies have linked some mildly deficient phenotypes of ATA1 with a variety of chronic immunologic and inflammatory disorders, such as RA, juvenile chronic arthritis and systemic lupus erythematosus (7). It has been reported that proteolytic enzymes are involved in both the formation of new bone and the remodeling of mature bone (8, 9). ATA1, the inhibitor to proteases, may contribute to bone formation. AS is characterised by two key pathologic features: sacroiliac joint and spinal inflammation and new bone formation with possible bone fusion, usually in the axial skeleton (10, 11). Therefore, the increased expression of ATA1 in AS synovial tissues may play a role in the pathogenic process of AS. In the present study, we continued to investigate the expression of ATA1 in synovial tissue of patients with AS. We applied western blot analysis to confirm the increased expression of ATA1 in synovial tissues by comparing the expression profiles of AS, RA and OA samples. We used immunohistochemistry to localise the expression of ATA1 in AS synovial membranes. In addition, we genotyped tag SNPs to determine the possible association of this gene with AS risk.

Materials and methods

Patients and sample collection

Synovial tissues were collected during joint replacement surgery from patients with AS (n=8; all male, 24–54 years old, mean 34), RA (n=6,4 female; 30–63 years old, mean 50) or OA (n=9,2 female; 40–71 years old, mean 62). The AS tissues were collected from the hip joints of patients, and the RA and OA tissues were collected from the knee joints of patients. The samples were dissected from the connective tissues and were immediately stored at -80°C until use. AS patients had an average disease duration of seven years and were positive for the HLA-B27 antigen. Their symptoms fulfilled the modified New York criteria for AS (12). The diagnosis of RA fulfilled the American College of Rheumatology criteria. The patients with RA had disease durations of 3 to 10 years and were classified as having erosive RA (Larsen class IV-V). They had high levels of C-reactive protein (6-192 mg/litre, mean 70 mg/litre), anti-CCP (92-394 U/ml) and RF (78-1280 U/ml). Patients with AS and RA took disease-modifying anti-rheumatic drugs (DMARDs) before surgery. Patients with AS, RA and OA were also medicated with non-steroidal anti-inflammatory drugs (NSAIDs), which help reduce pain and swelling of the joints and decrease stiffness. Thus, the medical pretreatment does not influence the results and the experimental results are comparable.

Blood was collected from patients with AS (n=51, 10 female) and RA (n=267, 183 female). The patients with AS had a mean age of 35.9 years. The patients with RA had a mean age of 51.7 years. The diagnosis of RA and AS was described as above. The patients were selected from the same population living in the Shandong area of northern China. A total of 160 (female 58) healthy people having a mean age of 48.0 years, with no evidence of any personal or family history of other serious illness,

served as blood donors. Control individuals were from the same geographical area. The blood samples were put into Monovette tubes containing 3.8% sodium citrate.

Both patients and controls gave their written consent to participate in the study and to allow their biological samples to be analysed genetically. Approval for the study was provided by the ethics committee of Shandong Academy of Medicinal Sciences.

Western blot analysis

Two hundred micrograms of each sample tissue from synovial tissues were homogenised in Cell Lysis Solution (Sigma) and centrifuged at 16,000 x g for 5 min at 4°C. The supernatant was collected after centrifugation, and the protein concentration was determined using the BCA protein assay kit (Pierce). Five micrograms of total protein was loaded and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto nylon membranes and probed with mouse monoclonal anti-alpha anti-trypsin (abcam, USA). The antibody was raised with full-length alpha 1 anti-trypsin protein and had been confirmed to exhibit no cross-reactivity. Immunoreactive signals were detected with alkaline phosphatase-conjugated secondary antibodies and visualised using a Western Blotting Luminol Reagent (Amersham). Images of western blots were acquired on Typhoon Trio (GE Healthcare). Quantification was conducted using ImageQuant5.2 software. Another membrane prepared by the same protocol was probed with anti-GADPH antibody (Santa Cruz) to normalise for sample loading.

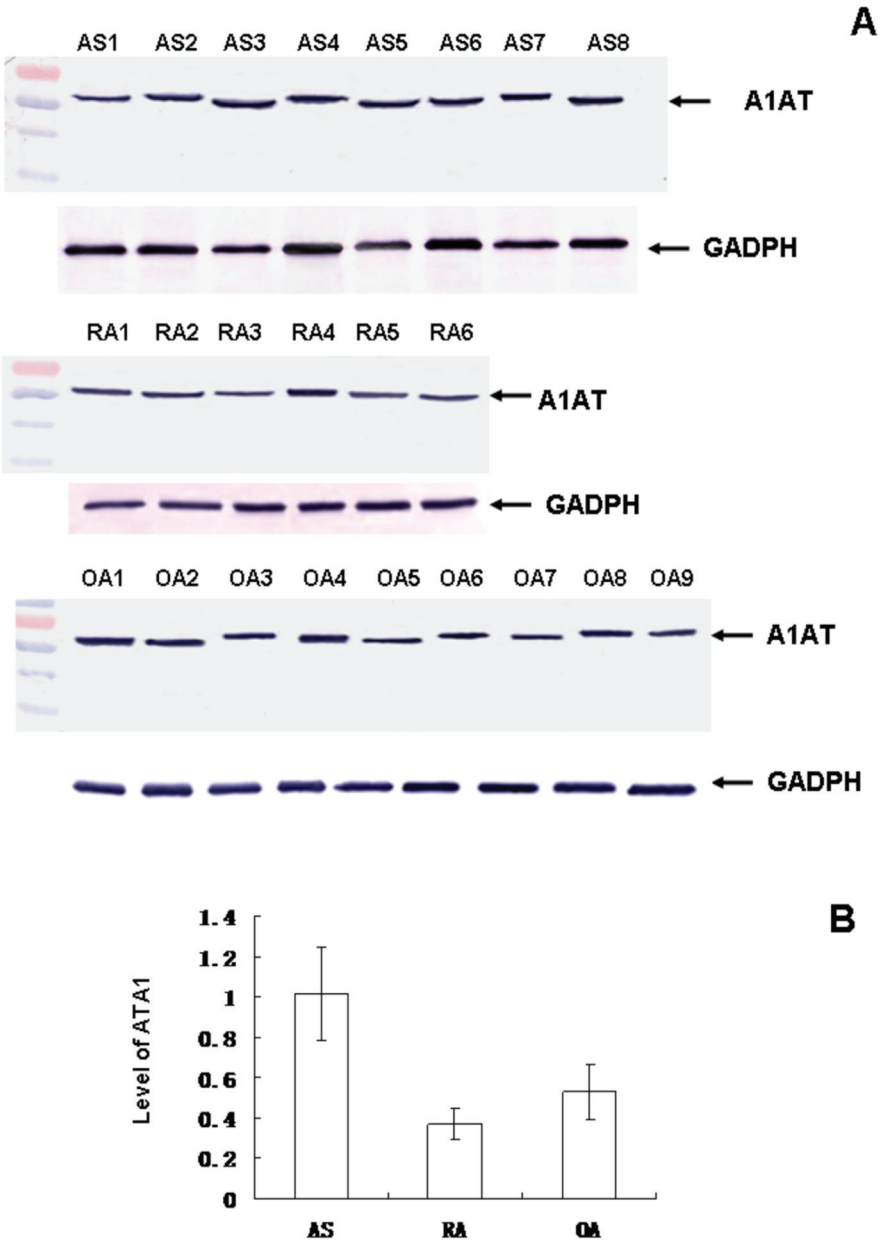

Fig. 1. Western blot analysis of ATA1 in synovial membranes from AS, RA and OA patients. (**A**) ATA1 expression was detected in the synovial samples. The molecular weight of ATA1 is 52 kDa. (**B**) The loading of samples was normalised using GADPH (37 kDa). ATA1 was expressed at a significantly higher level in AS synovial tissue than in RA and OA synovial tissues.

Immunohistochemistry

Synovial tissues were fixed in 10% neutral buffered formalin for 12 hours at room temperature, embedded in paraffin and sectioned by standard procedures. Tissue sections were de-paraffinised and re-hydrated using standard procedures. To increase immunostaining intensity, the sections were heated at 95°C for 10 min in citrate buffer (0.01 M, pH 6.0). Sections were incubated with the monoclonal anti-alpha 1 anti-trypsin antibody overnight at 4°C. Following the incubation, tissue sections were washed three times for three minutes each in PBS and were then processed with the UltraSensitive TM S-P kit (Maixin-Bio, China) according to the manufacturer's instructions. Immunoreactive signals were visualised using DAB substrate, which stained the target protein a brown color. Tissue structure of the section was defined by counterstaining with haematoxylin. In order to determine antibody specificity and optimise antibody dilution, the tissue samples were incubated (1) with pre-immune serum (Maixin-Bio, China) or (2) treated by the modification buffer without addition of antibody.

Genomic DNA extraction

Genomic DNA was extracted from peripheral blood leukocytes with a routine salting-out method using the DNA blood mini-kit from Qiagen (Germany), according to the manufacturer's guidelines. Briefly, 5 ml of blood was mixed

with Triton lysis buffer (0.32 M sucrose, 1% Triton X-100, 5 mM $MgCl_2$, H_2O, 10 mM Tris-HCl, pH 7.5). Leukocytes were spun down and washed with H_2O. The pellet was incubated with proteinase K at 56°C and was subsequently salted out at 4°C using a substrate NaCl solution. Precipitated proteins were removed by centrifugation. The DNA in supernatant fluid was precipitated with ethanol. The DNA pellet was dissolved in 400 µl H_2O.

SNP selection and genotyping

Tag SNPs (rs2753934, rs2749531 and rs6575424) were selected from the HapMap CEU data (www.hapmap.org) with a pair-wise r^2 ≥0.8. Minor allele frequencies (MAF) of the SNPs are over 0.05. These three tag SNPs are located in introns of ATA1.

Genotyping was performed using the TaqMan SNP genotyping assays. The assays were run on a LightCyclerH 480 Instrument (Roche) and were evaluated according to the manufacturer's instructions. Allele-specific probes were labeled with VIC and FAM fluorescent dyes, respectively. The PCR reaction was carried out in a total reaction volume of 10 ul using the following amplification protocol: denaturation at 95°C for 10 minutes, followed by 40 cycles of denaturation at 92°C for 15 seconds and finally annealing and extension at 60°C for 1 minute. The genotype of each sample was determined by measuring allele-specific fluorescence using SDS 2.3 software for allelic discrimination (Roche). Duplicate samples and negative controls were included to check the accuracy of genotyping.

Statistical analysis

SNPs were analysed for association by comparison of the minor allele frequency in cases and controls. Associations of particular SNPs with ATA1 were evaluated using odds ratios (OR) with 95% confidence intervals (CI). Fisher's exact test was used for comparison between categorical variables. *p*-values less than 0.05 were considered statistically significant. After genotyping, SNP markers were evaluated for significant deviation from Hardy-Weinberg equilibrium. The calculation was performed using SHEsis (http://analysis.bio-x.cn/myAnalysis.php), a powerful web-based platform for analyses of linkage disequilibrium, haplotype construction and genetic association at polymorphism loci (13).

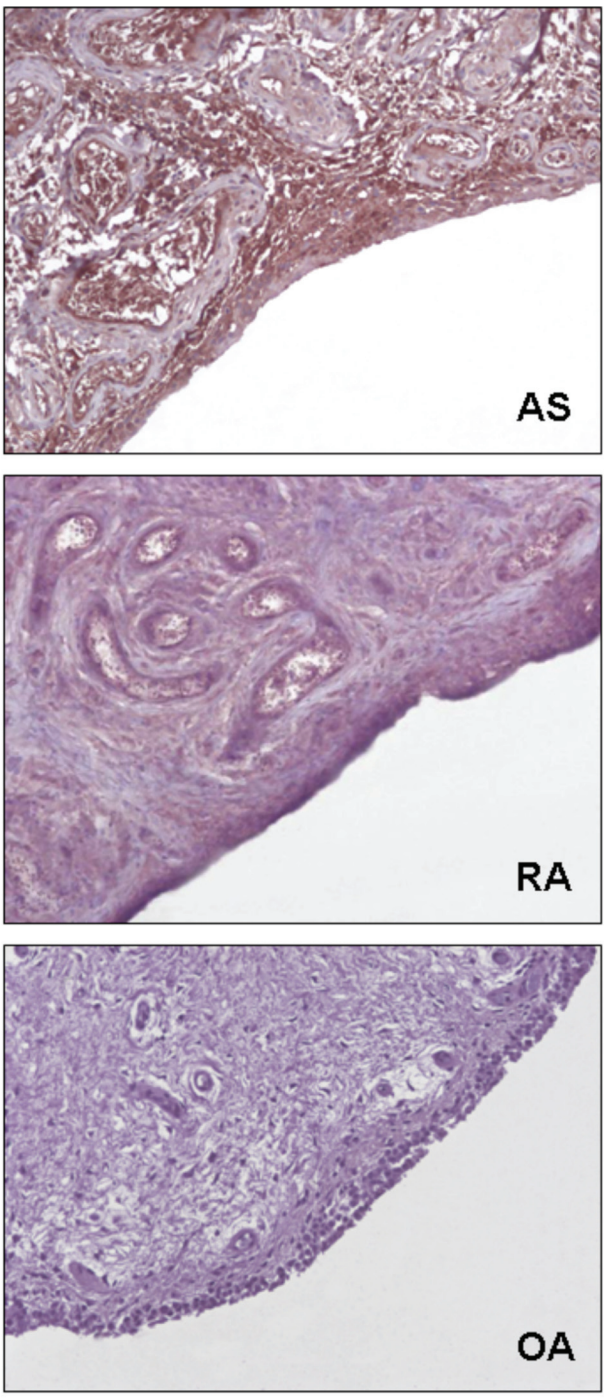

Fig. 2. Immunodetection of ATA1 in the synovial membranes of AS, RA and OA patients. ATA1 has strong immunosignal in AS synovial tissue. Original magnification: 200X.

Results

Western blot analysis was performed with the monoclonal antibody against ATA1. ATA1 was detected in synovial tissues of patients with AS, RA and OA. Using GADPH as a reference, ATA1 (molecular weight 52 kDa) displayed significantly higher levels of expression in synovial membranes of AS patients than in samples from RA and OA patients. The result was observed in all synovial membranes tested. The results are presented in Figure 1. A similar result was also obtained using β-actin as a reference (results not shown).

Immunohistochemistry detected extensive expression of ATA1 in all AS synovial tissues with strong intensity signals.

ATA1 was significantly expressed in the lining layer, the endothelial cells surrounding small blood vessels and fibroblast-like cells under the upper layer in synovial membranes of AS patients. In synovial membranes of RA patients, ATA1 was detected in some fibroblast-like cells under the thick lining layer, and the intensity of the signals was relatively low. ATA1 was also detected in synovial membranes of OA patients, in which the protein was detected in fibroblast-like cells under the thin layer and the intensity of the signals was relatively low. The tissue distribution of ATA1 is shown in Figure 2. This result corresponded to those obtained with western blot analysis.

A case-control study was conducted using TaqMan method to genotype the three tag SNPs of ATA1. Allelic frequency and genotype frequencies were in Hardy-Weinberg equilibrium in all cases and controls. Genotype frequencies and allelic frequency of the tag SNPs were compared between AS patients and controls and between RA patients and controls. Each allelic frequency of the SNP in the AS group and the health groups was nearly equal. No association was seen when samples were compared independently. These genotype frequencies of the ATA1 polymorphisms in AS patients and controls did not show significant differences. Similarly, the assessment of ATA1 gene polymorphisms did not disclose significant differences in genotypic and allelic frequencies between the RA patients and controls. The results are presented in Table I and Table II.

To evaluate the extent of linkage disequilibrium (LD), D' between all possible pairs of polymorphisms were calculated. LD analysis defined one block consisting of rs2753934 and rs2749531 in the ATA1 gene among the AS population, with a mean pair-wise D' of 0.926. LD analysis also identified this block in ATA1 among the RA population, with a mean pair-wise D' of 0.99. The results are presented in Figure 3. Three haplotypes, GA, GG and AG, were defined following haplotype analysis within the AS cohort and RA cohort, respectively. No significant haplotype associations were seen with AS or RA susceptibility.

Table I. Allele and genotype frequencies of ATA1 in AS patients and controls and association results.

dbSNP identity	Allele/ genotype	n. of patients with AS (%)	n. of controls (%)	Fisher's *p*-value	Odds ratio (%95 CI)
rs2753934	A	6 (0.059)	31 (0.097)	0.23677	0.984372 (0.615500–1.574309)
	G	96 (0.941)	289 (0.903)		
	A/A	0 (0.000)	1 (0.006)	0.47493	
	A/G	6 (0.118)	29 (0.181)		
	G/G	45 (0.882)	130 (0.812)		
rs2749531	A	66 (0.647)	210 (0.656)	0.86507	1.090255 (0.812753–1.462504)
	G	36 (0.353)	110 (0.344)		
	A/A	23 (0.451)	69 (0.431)	0.68037	
	A/G	20 (0.392)	72 (0.450)		
	G/G	8 (0.157)	19 (0.119)		
rs6575424	A	34 (0.333)	90 (0.281)	0.31467	1.045148 (0.768790–1.420848)
	G	68 (0.667)	230 (0.719)		
	A/A	9 (0.176)	12 (0.075)	0.08333	
	A/G	16 (0.314)	66 (0.412)		
	G/G	26 (0.510)	82 (0.512)		

160 controls and 51 AS cases observed.

Table II. Allele and genotype frequencies of ATA1 in RA patients and controls and association results.

dbSNP identity	Allele/ genotype	n. of patients with AS (%)	n. of controls (%)	Fisher's *p*-value	Odds ratio (%95 CI)
rs2753934	A	51 (0.096)	31 (0.097)	0.9476	0.582661 (0.235910–1.439084)
	G	483 (0.904)	289 (0.903)		
	A/A	3 (0.011)	1 (0.006)	0.8327	
	A/G	45 (0.169)	29 (0.181)		
	G/G	219 (0.820)	130 (0.812)		
rs2749531	A	358 (0.675)	210 (0.656)	0.5642	0.960317 (0.601975–1.531974)
	G	172 (0.325)	110 (0.344)		
	A/A	116 (0.438)	69 (0.431)	0.5545	
	A/G	126 (0.475)	72 (0.450)		
	G/G	23 (0.087)	19 (0.119)		
rs6575424	A	155 (0.290)	90 (0.281)	0.7781	1.277778 (0.791931–2.061689)
	G	379 (0.710)	230 (0.719)		
	A/A	21 (0.079)	12 (0.075)	0.958	
	A/G	113 (0.423)	133 (0.498)		
	G/G	66 (0.412)	82 (0.512)		

160 controls and 267 cases observed.

Discussion

ATA1 is the most important plasma inhibitor of serine proteases present in numerous polymorphic varieties. The liver usually synthesises ATA1 and releases the protein into the bloodstream. Breit *et al*. suggested that ATA1 phenotypes may be found more frequently in the context of connective tissue disease (7). In the present study, western blot analysis detected an increase in the expression of ATA1 in synovial tissues of AS patients. Immunohistochemistry also localised extensive expression of ATA1 in synovial membranes of the diseased joints. This result suggests the up-regulation of ATA1 in synovial tissues during the pathogenic process of AS.

ATA1 forms a non-immune complex with immunoglobulin A (IgA-ATA1) through disulphide bonding between an active thiol group available on the cysteine residue of alpha heavy chains of IgA and a cysteine in position 232 of

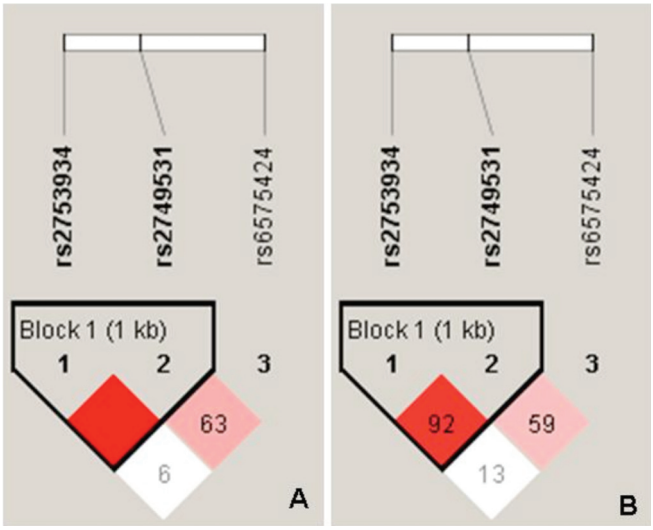

Fig. 3. Linkage disequilibrium of the genotyped SNPs in the ATA1 region. Linkage disequilibrium between the three ATA1 SNPs in the AS group (**A**) and RA group (**B**). Red areas represent higher levels of LD. Dark triangles represent haplotype blocks.

single-polypeptide chain ATA1. Elevated levels of the complex were found in a number of rheumatic diseases (14). Struthers *et al*. reported that 34% patients with AS displayed elevated levels of IgA-ATA1 in their sera (15). Sheehan *et al*. also found positive correlations between ESR, acute phase proteins (APP), CRP and ATA1 levels in AS (16). Significant reductions in levels of both IgA and IgA-ATA1 were seen in patients treated with sulphasalazine, in association with significant improvement in clinical and laboratory measures of disease (17). ATA1 level was significantly decreased in the remission/partial remission group of AS patients, indicating that ATA1 may be a good indicator of disease activity in AS (18, 19). It has been reported that ATA1 is related with inflammation and new bone formation, two important features of AS. In the current study, ATA1 had a significantly higher expression in synovial tissues of patients with AS than in synovial tissues of RA and OA patients, which supports the above findings and suggests that ATA1 is involved in the pathogenesis of AS by up-regulating the gene expression in the synovial tissues.

Linssen *et al*. investigated pathogenic roles of genes on chromosome 14q in HLA-B27-associated diseases. The authors reported that phenotype and allotype frequencies of ATA1 were not statistically different from those in B27+ AS patients who developed anterior uveitis (AAU), B27+ AAU patients without AS, B27+ AS patients without AAU, B27+ patients with Reiter's syndrome, B27+ patients with low back pain, B27- AAU patients or normal controls (20). They suggested that it is unlikely that ATA1 gene variation plays a role in the pathogenesis of B27-associated diseases. In the present study, we found that tag SNPs located on the ATA1 gene were not associated with AS or RA, which is in line with other studies. This evidence suggests that ATA1 contributes to AS pathogenesis by up-regulation rather than genetic variation. In conclusion, we detected the increased expression of ATA1 in synovial membranes of AS joints. ATA1 has no genetic effect on AS or RA. The results suggest that ATA1 contributes to the pathogenesis of AS by up-regulating the gene expression in the synovial tissues.

Key messages

- ATA1 has significantly increased expression in synovial tissues of AS.
- The gene encoding ATA1 is not associated with developing risk of AS.

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