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ORIGINAL ARTICLE

Ankylosing spondylitis is associated with aberrant DNA methylation of IFN regulatory factor 8 gene promoter region

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Abstract

Objectives To investigate the role of methylation levels of the IFN regulatory factor 8 (*IRF8*) gene promoter in the development of ankylosing spondylitis (AS).

Methods In this study, we compared the methylation levels of the *IRF8* gene promoter between 99 AS patients and 99 healthy controls using MethylTarget approach. Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) was performed to compare the mRNA levels of the *IRF8* gene in the other 19 AS patients and 19 healthy controls.

Results Differential methylation was found in 91 CpG sites of the *IRF8* gene promoter, and 4 CpG regions were highly methylated in AS patients compared to healthy controls ($p < 0.05$). In the verification stage, we found that the mRNA levels of the *IRF8* gene in AS patients were significantly lower than that in controls (AS 0.77 (0.39–1.74), $P = 0.038$). Positive correlations between methylation of the *IRF8* gene and the duration of disease, BASFI, and ESR were observed in AS patients.

Conclusions We found a significant hypermethylation of the *IRF8* gene promoter and a downregulation of the mRNA levels of the *IRF8* gene in AS patients. This suggests that aberrant methylation of the *IRF8* gene promoter may probably contribute to the development and pathogenesis of AS through regulating the expression of mRNA.

Keywords Ankylosing spondylitis · Epigenetics · IRF8 · Methylation

Introduction

Ankylosing spondylitis (AS) is the most common form of spondyloarthropathy (SpA). AS is characterized by long-term chronic inflammation in the spine and the sacroiliac joints and predominantly affects males [1]. The prevalence of AS varies from 0.1% to 1.6% worldwide, and the number is 0.22% in the Chinese population [2, 3]. It is well accepted that genetic factors are critical for the development and pathogenesis of AS [4, 5]. The strongest genetic risk factor for AS is human leucocyte antigen (*HLA*)-*B27*, and approximately 95% of AS patients are *HLA-B27*-positive [6]. However, *HLA-B27* and other susceptible genes can only explain around 30% of the total genetic effect of AS [7]. Thus, other factors such as epigenetics may be related to AS susceptibility. Recent evidence suggests that environmental components, including drinking [8], diet [9, 10], and infections [11], may play a role in AS by combining with susceptible genes.

Epigenetic modification is an intermediary between environmental factors and gene expression, which includes DNA methylation, histone modification, and RNA interference [12]. DNA methylation adds a methyl group to the fifth carbon position of cytosines with Sadenosyl-methionine (SAM) as the methyl donor [13]. DNA methylation of gene promoter regions often results in transcriptional gene repression, which was mediated by DNA methyltransferase (DNMT) enzymes.

Mengya Chen and Meng Wu contributed equally to this work and should be considered co-first.

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Substantial evidence has indicated the presence of aberrant methylation in autoimmune diseases, including rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), and AS [14–18]. Previous study has demonstrated that methylation status of the interleukin(*IL*)-*6* gene promoter was correlated with *IL6* messenger RNA levels and RA [19]. Moreover, DNA methylation alterations were associated with SLE in various immune cell populations [20]. In the genome-wide DNA methylation profile analysis, 1915 differentially methylated CpG sites in AS have been detected [21], suggesting that aberrant methylation may act as a pivotal part in the development and pathogenesis of AS.

IFN regulatory factor 8 (*IRF8*), a member of the *IRF* family, plays a role in the differentiation and function of B cell [22], *Th1*/*Th17* [23, 24], dendritic cell (DC) [25], and osteoclast [26]. T helper (Th) type 1 and Th17 cell are important effector T cell subsets. Th1 mainly secretes *IL-2*, *IL-12*, and interferon-γ (*IFN-γ*) and mediates cytotoxic T cells and immune responses [27]. Th17 predominantly produces *IL-17* that can drive inflammation response [28, 29]. DCs belong to the professional antigen-presenting cells (APCs) that regulate the activation of different effector T cell subsets. DCs were related to a number of autoimmune diseases, including AS [30]. In addition, *IRF8* can activate or suppress the expression of some critical immune response genes through interacting with other factors [10]. Thus, we hypothesized that *IRF8* is related to the pathogenesis of AS.

Recent genetic association studies showed that genetic variation of *IRF8* was associated with multiple autoimmune diseases, such as SLE [31], multiple sclerosis [32], autoimmune thyroid disease [33], and Behçet's disease (BD) [34]. Moreover, abnormal methylation of the *IRF8* gene has also involved in the development of autoimmune diseases, including BD [35] and Vogt-Koyanagi-Harada (VKH) disease [36]. As an autoimmune disease, AS may have a common pathogenesis with BD and VKH, so we hypothesized that methylation levels of the *IRF8* gene promoter are associated with AS.

Therefore, the aims of this study were to evaluate the methylation levels of the *IRF8* gene promoter and their effect on the transcript levels of the *IRF8* gene.

Materials and methods

Subjects

AS patients enrolled were diagnosed according to the 1984 Modified New York criteria. The exclusion criteria for the cases were as follows: (1) had other autoimmune diseases; (2) had malignant tumors, neurodegenerative diseases, or mental disease; and (3) had chronic diseases or systemic infections. Age- and sex-matched healthy controls were also recruited for this study. The exclusion criteria for the controls were as follows: (1) had a family history of rheumatism, (2) used drugs (e.g., hormone, immunosuppressive drugs) in the last month, (3) a history of surgery within the last 6 months. A total of 99 AS patients and 99 healthy controls were enrolled from the First Affiliated Hospital of Anhui Medical University for DNA methylation testing, and the other 19 AS patients and 19 healthy controls were enrolled to test mRNA expression level. Five milliliters of peripheral blood was obtained from each participant, and a questionnaire was collected only in AS patients. All participants provided written informed consent. The research was approved by the Local Ethics Research Committee of Anhui Medical University, and all procedures have complied with the 1964 Declaration of Helsinki. Systemic inflammation indexes such as erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) were measured. Bath Ankylosing Spondylitis Disease Activity Index (BASDAI), Bath Ankylosing Spondylitis Functional Index (BASFI), and Ankylosing Spondylitis Disease Activity Score (ASDAS) were used to assess disease severity and functional disabilities.

DNA extraction and CpG sites selection

Genomic DNA was extracted from 5 ml of whole blood using a QIAGEN kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions and was stored at − 80 °C before the detection of DNA methylation. Quality control was applied for DNA samples. Methylation analysis was performed with a working concentration of 20 ng/μL. CpG islands located in the promoter of the *IRF8* gene were selected from 2 k upstream of transcriptional start site(TSS) to 1 k downstream of first exon according to the following criteria: (1) the ratio of observed/expected dinucleotides CpG should be 0.60 or higher, (2) 200 bp minimum length, and (3) the content of GC should be no less than 50%. Finally, we selected 4 CpG regions of the *IRF8* gene promoter including 91 CpG sites (Fig. 1). The details of the CpG regions are listed in Table 1.

Bisulfite conversion and multiplex amplification

The methylation levels of the *IRF8* gene promoter were analyzed by MethylTarget™ (Genesky Biotechnologies Inc., Shanghai, China), an NGS-based multiple methylation-specific PCR analysis method. Specifically, the selected genomic regions were analyzed and converted into bisulfite-converted DNA sequences by geneCpG software. We designed the PCR primers using primer3 software (http://primer3.ut.ee/) from the bisulfite-converted DNA.

Bisulfite conversion of genomic DNA (400 ng) was conducted using the EZ DNA Methylation™-GOLD Kit (Zymo Research), which converts unmethylated cytosine in genomic DNA to uracil. The samples with a rate of bisulfite conversion of DNA less than 98% were firstly filtered out. The multiple-

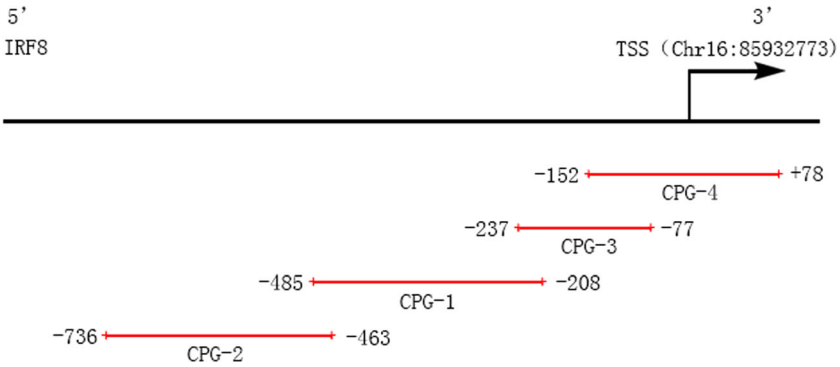

Fig. 1 CpG regions sequenced around promoter of IRF8. Red lines indicate four CpG regions analyzed in this study. Range of each region is indicated by its relative distance (in bp) to TSS

PCR was performed to amplify the bisulfite-modified DNA sequence using indexed primers. PCR amplicons (170 bp–270 bp) were separated by agarose electrophoresis and purified using QIAquick Gel Extraction kit (QIAGEN).

Methylation detection of IRF8

The detection of *IRF8* methylation was performed on Illumina Hiseq/Miseq 2000 using bidirectional sequencing verification with 2x150bp sequencing mode according to the manufacturer's protocol. The methylation levels of the 91 CpG sites from + 78 to − 736 with respect to TSS were measured (additional file: Table S1). The methylation levels of each CpG were equal to the ratio of methylated cytosine to total cytosine.

Quantitative real-time PCR (qRT-PCR)

Peripheral blood mononuclear cells (PBMCs) from peripheral blood were isolated using Ficoll-Hypaque density gradient centrifugation method. Total cellular RNA was extracted using miRNeasy Mini Kit (Qiagen, Germany). The quality of RNA was examined by NanoDropTM 2000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA), and then RNA was reverse-transcribed to complementary DNA (cDNA) using a PrimeScript™ RT reagent kit (Takara Bio Inc., Japan). The mRNA expression levels of *IRF8* were measured using the quantitative Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) and SYBR Green (Takara Bio Inc., Japan) master mix. Data were normalized to the internal control β-actin and each reaction was run in duplicate. The forward and reverse primers of IRF8 and β-actin were as follows: IRF8, forward: 5′-GAAGACGAGGGTTACGCTGTG-3′, reverse: 5′-TCCTCAGGAACAATTCGGTAA-3′; β-actin, forward: 5′-TACTCATACTCCTTGTTGTCCC-3′, reverse: 5′-AGTTGAAGGTAGTTTCGTGGAT-3′. Relative quantification was achieved using $2^{-\Delta\Delta Ct}$ method as described previously.

Statistical analysis

Data analysis was carried out using SPSS software version 23.0 (SPSS, Chicago, IL, USA). The data did not meet the normality assumption; therefore, the results were described as median (max-min). Mann–Whitney *U* test was used to compare methylation and mRNA levels of the *IRF8* gene between AS patients and healthy controls. Spearman correlations were adopted for bivariate correlation analyses. To draw the graphs, we used the GraphPad Prism version 7 for windows (GraphPad Software, La Jolla, CA USA, www.graphpad.com). *P* value ≤ 0.05 was considered statistically significant.

Table 1 The position of the four CpG regions and primer sequences

Target	Chr	Gene	TSS	Start	End	Length	Distance	Primer sequences	
								Fforward	Rreverse
CpG_1	16	IRF8	85932773	85932565	85932288	278	− 485	GTTTYGTTTTTTATATTTGGGGTTT	AACATCACTACCCAATATCCAAC
CpG_2	16	IRF8	85932773	85932310	85932037	274	− 736	GTTGGATATTGGGTAGTGATGTT	ACTAATCCCACAAAAAACCTCTT
CpG_3	16	IRF8	85932773	85932536	85932696	161	− 237	TTTTTGGATTTTAGGTGTGAGGAG	ACCRACCAATAACRTCAACTTAAC
CpG_4	16	IRF8	85932773	85932621	85932851	231	− 152	GGAAAGTGATTTTTYGGAAAGTAGAG	CCTACCCRCCCACTATACCTACCTAC

IRF8 IFN regulatory factor 8, *Chr* Chromosome, *TSS* transcription start site, *Distance* CpG site relative distance (in bp) to TSS, *F* forward primer, *R* reverse

Table 2 Demographic features of 99 AS patients and 99 healthy controls

Property	AS patients ($n = 99$)	Healthy controls ($n = 99$)	P value
Age (year)	31.14 ± 9.93	31.77 ± 8.69	0.635
Gender			
Male (%)	77(77.8%)	83(83.8%)	0.353
Female (%)	22(22.2%)	16(16.2%)	
HLA-B27 status			
Positive (%)	83(83.8%)	–	
BMI (Kg/m^2)	22.58 ± 4.36	–	
ESR (mm/L)	20.71 ± 19.38	–	
CRP (m/L)	18.56 ± 27.83	–	
BASDAI (cm)	2.37 ± 1.52	–	
BASFI (cm)	1.50 ± 1.70	–	
ASDAS (cm)	3.63 ± 4.46	–	
Disease duration (year)	7.26 ± 6.36	–	

AS ankylosing spondylitis, *ESR* erythrocyte sedimentation rate, *CRP* C-reactive protein, *BASDAI* Bath Ankylosing Spondylitis Disease Activity Index, *BASFI* Bath Ankylosing Spondylitis Functional Index, *ASDAS* Ankylosing Spondylitis Disease Activity Score

Results

Study population characteristics

Demographic and clinical features of the 99 AS patients and 99 healthy controls at the first stage are listed in Table 2. There were no statistically significant differences in sex and age between AS patients and healthy controls (age 31.14 ± 9.93 vs. 31.77 ± 8.69 years, $P = 0.635$; male/female 77/22 vs. 83/16, $P = 0.353$, respectively.). Eighty-three *HLA-B27*-positive individuals were observed in the AS group, and the ESR and CRP levels were elevated (ESR 20.71 ± 19.38 mm/h; CRP 18.56 ± 27.83 mg/L, respectively.). The mean disease duration of AS cases was 7.26 ± 6.36 year. In AS patients, BASDAI, BASFI, and ASDAS scores were 2.37 ± 1.52, 1.50 ± 1.70, and 3.63 ± 4.46, respectively.

Methylation levels of IRF8 promoter

Differential methylation analyses were performed for the 91 CpG sites, and the results showed a significant change of DNA methylation in the *IRF8* gene promoter in AS patients (additional file: Table S2). Four CpG regions of the *IRF8* gene promoter were highly methylated in AS patients compared to healthy controls (AS vs. HC: CpG-1 1.53% (1.03–2.17%) vs. 1.40% (0.30–8.57%), $P < 0.001$; CpG-2 3.43% (2.66–4.54%) vs. 3.20% (2.12–10.37%), $P < 0.001$; CpG-3:1.23% (0.80–1.87%) vs. 1.16% (0.81–11.75%), $P < 0.001$; CpG-4 1.40% (0.92–2.15%) vs. 1.34% (0.79–11.85%), $P = 0.02$, respectively) (Fig. 2).

Subgroup analysis of IRF8 methylation

Previous reports indicated that drugs could reverse the methylation levels of DNA. Furthermore, the most important heritability of AS comes from the *HLA-B27* antigen [37, 38]. Therefore, subgroup analysis by drug (received or not) and *HLA-B27* (positive or negative) were performed. The results showed that AS patients who received treatments had lower methylation levels compared to those who did not (*IRF8*: 2.42% (1.67–12.60%) vs. 2.23% (1.58–3.08%), $P = 0.012$) (Fig. 3a). The methylation levels of *IRF8* in *HLA-B27*-positive groups and *HLA-B27*-negative patients were higher than that in healthy controls, but there were no significant differences between the two groups.(*IRF8*: *HLA-B27*(−) vs. *HLA-B27*(+) 2.44% (1.90–4.26%) vs. 2.28% (1.58–12.60%), $P = 0.091$; HC vs. *HLA-B27*(−) 1.44% (1.16–7.67%) vs. 2.44% (1.90–4.26%), $P < 0.001$; HC vs. *HLA-B27*(+) 1.44% (1.16–7.67%) vs. 2.28% (1.58–12.60%), $P < 0.001$, respectively) (Fig. 3b–d).

Correlation of methylation with clinical manifestations

We analyzed the correlations of the methylation levels of the *IRF8* gene promoter with ESR, CRP, duration, BASDAI, BASFI, and ASDAS. Positive correlations between methylation of the *IRF8* gene promoter and disease duration and BASFI in CpG-3 (duration: $r = 0.222$, $P = 0.032$; BASFI: $r = 0.260$, $P = 0.010$) and CpG-4 (duration: $r = 0.322$, $P = 0.001$; BASFI: $r = 0.261$, $P = 0.009$) were observed (Table 3). Moreover, a significant correlation was identified

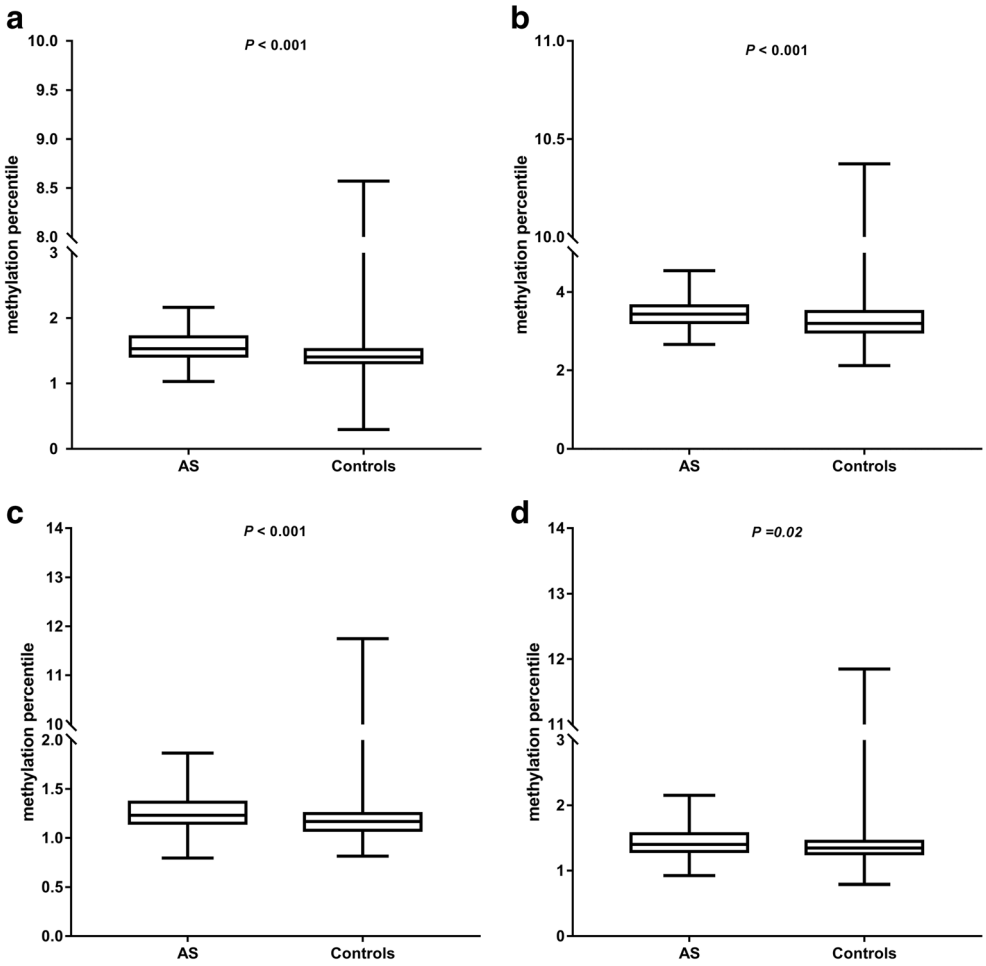

Fig. 2 The methylation levels of IRF8 promoter region in AS: The methylation levels of CpG_1 (**a**), CpG_2 (**b**), CpG_3 (**c**) and CpG_4 (**d**) in AS were significantly higher than that observed in healthy controls

between ESR and methylation of CpG-2. These results suggested that the methylation levels of the *IRF8* gene were associated with the degree of inflammation and functional disabilities in AS.

qRT-PCR validation

To further validate the functional relevance of the *IRF8* gene for AS, we compared the mRNA levels of the *IRF8* gene in PBMCs between 19 AS patients and 19 healthy controls. Gender and age between the two groups showed no significant differences (age: 32.21 ± 7.58 vs. 32.05 ± 7.75 years, $P = 0.950$; male/female: 13/6 vs. 14/5, $P = 0.721$, respectively.). The mRNA levels of the *IRF8* gene in AS patients were significantly lower than that in controls. The average mRNA level of *IRF8* in AS is 0.77 (0.39–1.74), $P = 0.038$ (Fig. 4).

Discussion

Accumulating evidence shows that aberrant DNA methylation plays an important role in the development and pathogenesis of AS. Aberrant methylation of *SOCS-1*, *BCL11B*, and *DNMT1* could be observed in AS patients [39–41]. In addition, a genome-wide DNA methylation profile analysis has detected 1915 differentially methylated CpG sites in AS [21]. Previous reports demonstrated that methylation of the *IRF8* gene promoter was related to autoimmune disease, so we hypothesized that methylation of the *IRF8* gene promoter is associated with the pathogenesis of AS.

Consistent with our hypothesis, the differential methylation was found in 91 CpG sites of the *IRF8* gene promoter and 4 CpG regions were highly methylated in AS patients compared with healthy controls. This was the first report that aberrant DNA methylation of the *IRF8* gene was found in AS, and thus it can further support that aberrant DNA methylation plays an important role in the development and pathogenesis of AS. In the verification stage, we found that the mRNA level of the *IRF8* gene in AS was significantly lower than that in controls. On one hand, *IRF8* can stimulate the expression of many important immune response genes including *IL-12* [42]. Increased expression of these genes could contribute to the breakdown of immune tolerance and lead to chronic inflammation. On the other hand, *IRF8* mutation resulted in a complex

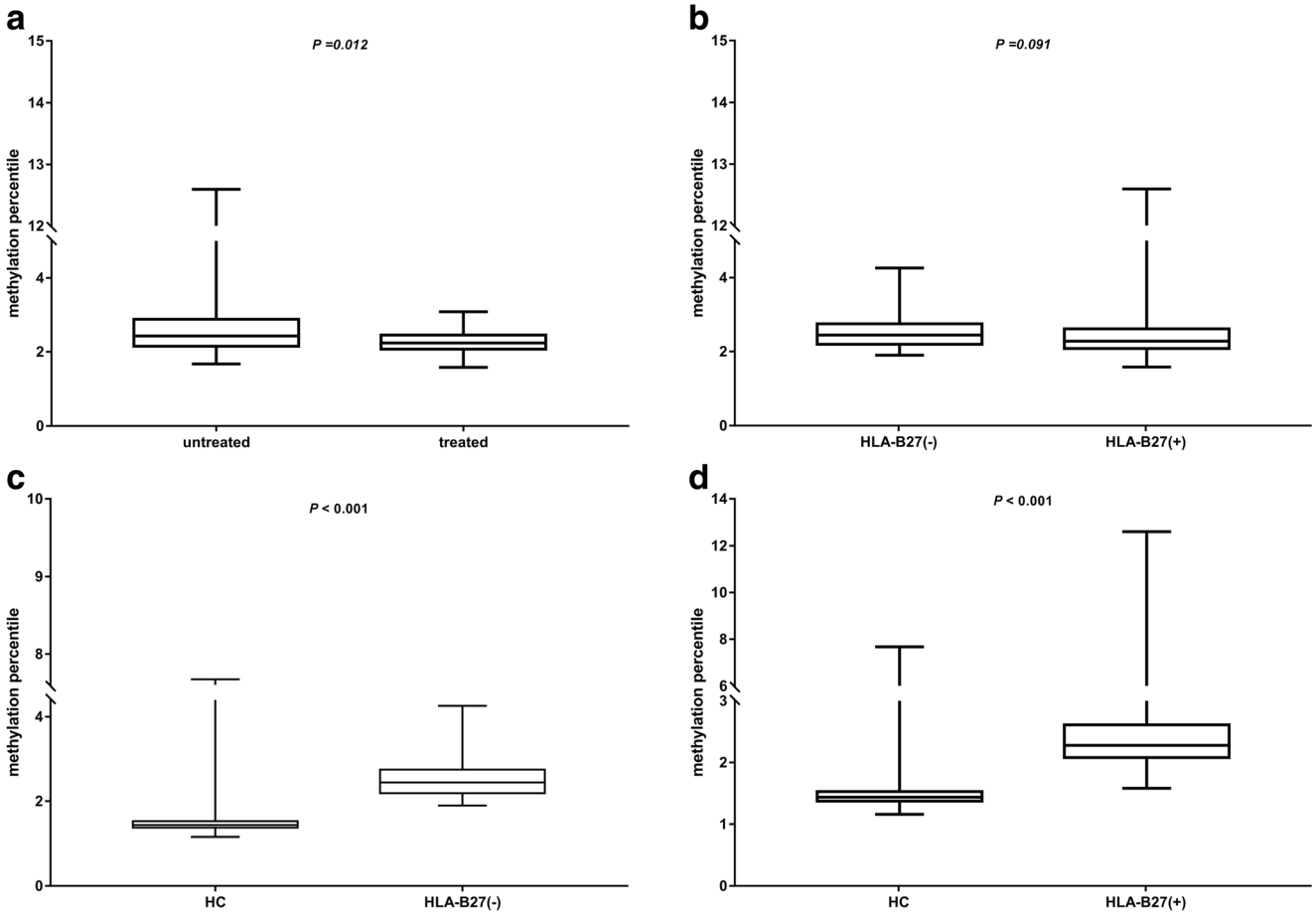

Fig. 3 Subgroup analysis of *IRF8* methylation based on therapy and HLA-B27 antigen. **a** Untreated AS patients vs. treated AS patients. **b** HLA(−) AS patients vs. HLA(+) AS patients. **c** HLA(−) AS patients vs. HC. **d** HLA(+) AS patients vs. HC

immunodeficiency syndrome with DC deficiency, monocytopenia, and immune dysregulation [43]. This has been confirmed by animal studies showing that *IRF8*$^{-/-}$ mice impaired T cell function and resulted in Th1 polarization defects of early immune response [44]. Therefore, hypermethylation of the *IRF8* gene promoter, alongside with gene silencing [41], may contribute to the pathogenesis of AS.

Previous studies have shown an association between DNA methylation and clinical manifestation [39, 45], so we analyzed the correlations of methylation of the *IRF8*

Table 3 Association between methylation levels of *IRF8* and clinical manifestations

	CpG-1		CpG-2		CpG-3		CpG-4	
	r[a]	*p* value	r[a]	*p* value	r[a]	*p* value	r[a]	*p* value
ESR	− 0.136	0.182	− 0.220*	0.030	0.021	0.840	− 0.076	0.457
CRP	− 0.071	0.485	− 0.176	0.081	0.029	0.776	0.036	0.720
Duration	0.220*	0.032	0.189	0.066	0.222*	0.032	0.322**	0.001
BASFI	− 0.040	0.694	− 0.012	0.907	0.260**	0.010	0.261**	0.009
BASDAI	0.018	0.863	− 0.017	0.869	0.147	0.149	0.169	0.095
ASDAS	− 0.029	0.776	− 0.128	0.206	0.118	0.246	0.036	0.723

IRF8 IFN regulatory factor 8, *ESR* erythrocyte sedimentation rate, *CRP* C-reactive protein, BASDAI Bath Ankylosing Spondylitis Disease Activity Index, *BASFI* Bath Ankylosing Spondylitis Functional Index, *ASDAS* Ankylosing Spondylitis Disease Activity Score

*Correlation is significant at the 0.05 level (two-tailed); **correlation is significant at the 0.01 level (two-tailed)

[a] Spearman correlation coefficient

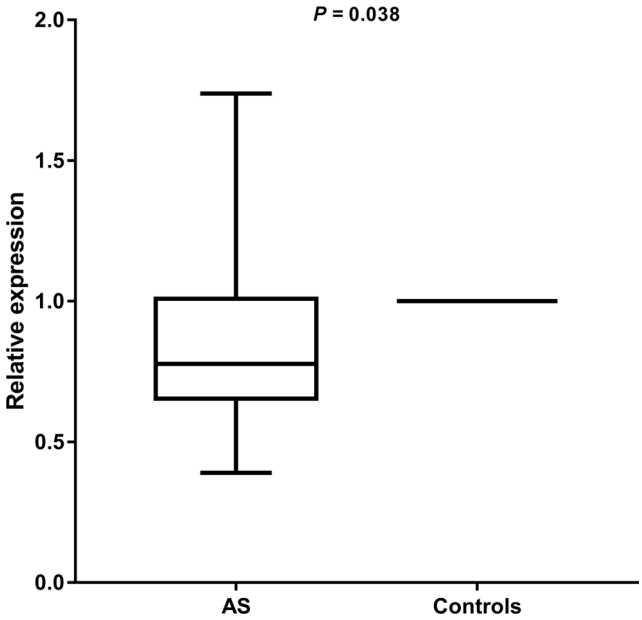

Fig. 4 The results of *IRF8* mRNA expression levels. $P < 0.05$ was considered significant

gene promoter with ESR, CRP, duration, BASDAI, BASFI, and ASDAS. The positive correlations of methylation of *IRF8* with duration, ESR, and BASFI further verified that methylation of the *IRF8* gene promoter was related to inflammation and disease severity of AS, indicating a role of *IRF8* in AS pathogenesis.

Subgroup analysis showed that treated groups had a lower methylation compared with untreated groups in AS. This suggests that therapeutics may affect the methylation status of gene promoters. Consistent with our findings, Kim et al. identified that genomic DNA hypomethylation was associated with inflammatory arthritis, and it could be reversed by methotrexate [38]. In cancer, celecoxib was found to be associated with a reduced DNA methylation and an increased mRNA expression [46]. In this study, most of the AS patients had been treated with celecoxib; this further demonstrated that DNA methylation levels can be reduced by celecoxib. The methylation levels of *IRF8* in *HLA-B27*-positive groups and *HLA-B27*-negative patients were higher than that in healthy controls, but there were no significant differences between the two groups. Although it is well known that the most important heritability of AS comes from the *HLA-B27* antigen, the results suggested that *HLA-B27* status may not be related to the expression of methylation.

This study has some potential limitations. First, this work did not analyze methylation levels of the *IRF8* gene promoter among different cell subtypes in PBMCs. It has been reported that methylation profiles were highly specific for individual cell types [47]. Second, we only measured *IRF8* methylation levels in AS patients but not in RA and osteoarthritis (OA). However, our next study will address this issue by adding two more arms involving RA and OA as examples of autoimmune disease and non-inflammatory disease, respectively.

In conclusion, we found a hypermethylation of the *IRF8* gene promoter and a downregulation of the mRNA levels of *IRF8* in AS patients compared with healthy controls. This suggests that aberrant methylation of the *IRF8* gene promoter may probably contribute to the development and pathogenesis of AS through regulating the expression of mRNA.

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Compliance with ethical standards

The study was approved by the Local Ethics Research Committee of Anhui Medical University, and all participants provided written informed consent. The procedures were in accordance with the 1964 Helsinki Declaration and ethical standards of the institutional.

Disclosures None.

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