

ARTICLE



DNA methylation and transcriptome signatures of the *PDCD1* gene in ankylosing spondylitis

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Ankylosing spondylitis (AS) is an autoimmune-related inflammatory arthritis. The association between the DNA methylation and mRNA expression of *PDCD1* gene with the susceptibility to AS remains unclear. In this case-control study, the methylation level of *PDCD1* promoter was detected in 80 AS patients and 80 healthy controls by MethylTarget method. The transcriptional level of *PDCD1* gene was measured in 47 AS patients and 47 healthy controls by real-time quantitative PCR. Finally, 17 methylation sites mapped to one CpG island were detected. Compared to healthy controls, the promoter of *PDCD1* was hypermethylated ($p < 0.001$) and the mRNA expression was downregulated ($p < 0.001$) in AS patients. Significantly negative correlation was identified between the DNA methylation and mRNA expression of *PDCD1* gene ($r_s = -0.470$, $p < 0.001$). The receiver operating characteristic (ROC) results showed that *PDCD1* island had a sensitivity of 61.3% and a specificity of 82.5%, and *PDCD1* mRNA had a sensitivity of 87.2% and a specificity of 89.0%. The methylation level of *PDCD1* was positively correlated with the ESR, CRP and ASDAS of AS, and was not affected by HLA-B27 status, gender or medicine intake.

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INTRODUCTION

Ankylosing spondylitis (AS) is an autoimmune disease characterized by axial joint inflammation and new bone formation that can eventually lead to spinal deformities, joint stiffness, and even lifelong disability [1]. Extra-articular symptoms also frequently occur in AS, such as psoriasis, uveitis, and intestinal inflammation [2]. AS is estimated to influence 0.1–0.5% of the global population [3]. It usually initially appears during the third decade of life, and causes severe impairment of spinal movement and bodily function [4].

Despite the pathogenesis of AS keeping unclear, susceptibility to AS and the severity of manifestations are remarkably considered heritable [5, 6]. The gene known to be associated with AS is human leukocyte antigen (HLA)-B27, which is positive in almost 95% of patients with AS [7]. However, only about 1–2% of HLA-B27 carriers suffer from AS [8]. Twin and familial studies of AS have illustrated that HLA-B27 accounts for merely 20% of the overall risk of AS, suggesting that other genetic factors are compactly related to AS [9].

The role of epigenetics in the occurrence and development of autoimmune disorders has attracted broad attention [10]. Epigenetics is the study of heritable changes in gene activity or function that are independent of any changes in the DNA sequence itself [11]. Considerable evidence suggests that epigenetic modifications exert a central role in the cellular programming of gene expression [12]. It is sensitive to external stimuli and can mediate the interaction between genes and environment. DNA methylation is the most common epigenetic modification and can be steadily inherited through multiple cell divisions. DNA

methylation is a process by which a methyl group was added onto the 5' carbon site of cytosine in cytosine-phosphate-guanosine (CpG) dinucleotides, resulting in the modification of the chromatin structure and ultimately gene silencing [13]. Aberrant DNA methylation has been found to be involved in the pathogenesis of AS and other autoimmune disorders [14–16]. For example, the significant hypermethylation of the IFN regulatory factor 8 (IRF8) gene promoter and the downregulation of the mRNA level of the IRF8 gene have been reported in AS patients [17]. In addition, hypomethylation of IL-6 and IL-10 promoters is associated with their elevated expression in peripheral blood mononuclear cells (PBMCs) from patients with rheumatoid arthritis (RA) [18, 19]. These findings indicated that DNA methylation may participate in the occurrence and development of autoimmune diseases by negatively regulating gene transcription.

Programmed Cell Death Protein 1 (*PDCD1*), also known as CD279, is a member of the CD28/B7 receptor family. *PDCD1* is a 55-kDa transmembrane protein with an extracellular IgV-like domain and a 97 amino acid cytoplasmic tail containing an immunotyrosine inhibitory motif and an immunotyrosine switching motif [20]. *PDCD1* is primarily expressed on T lymphocytes, B lymphocytes, myeloid cells, natural killer (NK) cells, and thymus cells [21, 22]. Numerous studies have demonstrated that *PDCD1* exerts an important role in restraining T cell signal transduction, mediating tolerance mechanisms, and holding immune homeostasis [23]. *PDCD1* knockout mice exhibited overactive immune phenotypes [24]. Targeting *PDCD1* pathway to treat cancer showed benefits in many patients and has already been applied to clinical cancer therapy [25]. Given that the breakdown of self-

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tolerance and the self-attack of immune system are the prominent features of autoimmune diseases, *PDCD1* may also play a critical role in regulating autoimmune and inflammatory disorders [26, 27]. Published epigenome-wide association studies (EWAS) have identified the association between DNA methylation levels of *PDCD1* and various traits including Sjogren syndrome and RA [28, 29]. However, the relationship between *PDCD1* promoter methylation, transcript level, and AS susceptibility remains further investigation.

In this case-control study, we explored the association of *PDCD1* gene with AS susceptibility in eastern Chinese Han population based on the methylation and transcriptome signatures. Moreover, the correlation between the methylation and mRNA expression of *PDCD1* gene as well as the clinical index of AS patients was also assessed.

MATERIALS AND METHODS

Human subjects

During July 2021 to August 2021, confirmed AS cases and healthy subjects were consecutively recruited from the Department of Rheumatology and Immunology, the First Affiliated Hospital of Anhui Medical University (Hefei, Anhui, China), and the medical examination center of the same hospital, respectively. AS cases were clinical diagnosed according to the New York criteria amended by the American College of Rheumatology in 1984. AS cases and healthy controls (HCs) were matched according to age and gender. Five milliliters peripheral venous blood was extracted from all the participants. Demographic and clinical information, such as age, sex, height, weight, HLA-B27 status, disease course, serum erythrocyte sedimentation rate (ESR), and C-reactive protein (CRP), were obtained from structured questionnaires and patients' medical records. Additionally, Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) and Ankylosing Spondylitis Disease Activity Score (ASDAS) were used to evaluate disease activity. Bath Ankylosing Spondylitis Functional Index (BASFI) was applied to assess functional disabilities. The current study was in line with the Declaration of Helsinki and supported by the ethics committee of Anhui Medical University. Signed informed consents were acquired from the whole participants prior to registration.

DNA methylation detection

Genomic DNA was isolated from 2 ml peripheral blood of AS cases and HCs using the Qiagen® FlexiGene® DNA kit under the manufacturer's instruction, and then stored at -80°C before detecting DNA methylation. After quality control, DNA samples were diluted to 20 ng/ul for quantitative methylation analysis. The CpG islands located in the promoter region of *PDCD1* gene were selected from 2 k upstream of the transcription start site (TSS) to 1 k downstream of the first exon based on the criteria as follows: (1) excessive than 200 bp length; (2) 0.6 or higher ratio of observed or expected dinucleotides CpG; (3) 50% or higher cytosine-guanine content. Finally, 17 CpG methylation sites from one island of the *PDCD1* promoter were selected and sequenced (Supplementary Table S1).

Genomic DNA (400 ng) was treated with sodium bisulfite using EZ DNA Methylation™-GOLD Kit (ZYMO RESEARCH, CA, USA) based on the manufacturer's instructions, which will transform unmethylated cytosine to uracil. Samples with DNA bisulfite conversion rate below 98% were filtered out and all samples were eligible in this study. The optimized primer combinations were used for multiple PCR amplification of bisulfite modified DNA sequences. PCR amplicons (170–270 bp) were separated by agarose electrophoresis and purified using the QIAquick Gel Extraction kit (QIAGEN, Hilden, Germany), and *PDCD1* methylation test was carried out by using Illumina HiSeq/Miseq 2000 according to the manufacturer's protocol. The sequences of sense and antisense primers for *PDCD1* were as follows: forward: 5'-TGAAATTGTTGATATTAGTGATGAGATG-3', reverse: 5'-CCACCCACACAACCTCAC-3'. The methylation level of the *PDCD1* promoter was analyzed using MethylTarget™ (Genesky Biotechnologies Inc., Shanghai, China).

Real-time quantitative PCR (RT-qPCR)

Peripheral blood mononuclear cells (PBMCs) were freshly separated from 3 ml peripheral blood of AS patients and HCs by Ficol-Hypaque density gradient centrifugation method within 4 h. Total RNA was extracted from cells using the miRNeasy Mini Kit (Qiagen, Germany). RNA concentration

and purity were detected using a NanoDrop™2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). Eligible RNA samples were then reverse-transcribed into complementary DNA (cDNA) using a PrimeScript™ RT reagent kit (Takara Bio Inc., Japan). The relative expression level of *PDCD1* mRNA was measured in the Real-Time quantitative PCR System (Applied Biosystems, Foster City, CA, USA) using SYBR Premix Ex Taq II (Takara Bio, Japan). $2^{-\Delta\Delta\text{Ct}}$ method normalized to endogenous control was used for calculating the relative expression level of *PDCD1*. The sequences of sense and antisense primers for *PDCD1* and β -actin are as follows: *PDCD1*, forward: 5'-CCAGGATGGTCTTAGACTCCC-3', reverse: 5'-TTTAGCACGAAGCTCTCCGAT-3'; β -actin, forward: 5'-TGACGTGGACATCCGCAAAG-3', reverse: 5'-CTGGAAGGTGGACAGCGAGG-3' [30–32].

Statistical analysis

SPSS 23.0 software (SPSS Inc, Chicago, IL, USA) was chosen for statistical analysis of the data, and GraphPad Prism 5.01 software (GraphPad Inc, CA, USA) was applied for plotting. Continuous data conforming to normal distribution were represented by mean \pm standard deviation (SD), and independent t-test was used for comparison between the two groups; skewness distribution data were described by median and interquartile (IQR), and comparison between the two groups was conducted by Mann–Whitney *U* test. Categorical data were presented in absolute numbers and percentages, and compared with Pearson chi-square test. Spearman's rank correlation coefficient test was conducted to investigate the association between *PDCD1* gene and clinical index. The predictive performance of *PDCD1* as a biomarker for AS was evaluated by receiver operating characteristic (ROC) curve and area under curve (AUC). A two-side *p* value <0.05 was defined as statistically significant.

RESULTS

Characteristics of study subjects

In the first stage, the DNA methylation levels of *PDCD1* gene were detected in 80 AS patients and 80 HCs. In both groups, there were 60 (75.00%) males and 20 (25.00%) females. The mean age of AS and HCs were 35.14 ± 9.93 and 34.83 ± 9.52 , respectively, and no significant differences were found in either age ($p = 0.839$) or gender ($p = 1.000$). In the second stage, 47 AS patients and 47 HCs were randomly selected from the first stage to detect the mRNA expression of *PDCD1* gene. There were 37 (78.72%) males and 10 (21.28%) females in both groups. The mean age of AS and HCs were 34.66 ± 8.62 and 36.00 ± 8.99 , respectively. The age ($p = 0.462$) and gender ($p = 1.000$) of AS patients were also comparable to those of HCs. The other specific demographic and clinical features of all participants are summarized in Table 1.

Methylation level of *PDCD1*

The methylation level of each CpG site was calculated by the ratio of methylated cytosine to total detected cytosine. A total of 17 CpG sites at one CpG island in the promoter region of *PDCD1* gene were detected to be methylated. The result showed that all 17 CpG sites were significantly hypermethylated in AS patients compared with HCs (Supplementary Table S1). To further understand the overall methylation status of *PDCD1* gene in AS patients, the methylation level of CpG island was determined by calculating the average methylation level of all CpG sites. Compared with HCs, the CpG island in the promoter region of *PDCD1* gene was also highly methylated in AS patients (AS vs. HCs: 73.53% (67.41–79.19%) vs. 65.67% (56.50–70.70%), $p < 0.001$, Fig. 1). To assess the potential of *PDCD1* gene methylation as a biomarker for AS, ROC curve analysis was conducted on the CpG island. The AUC of ROC curve was 0.764 (95% CI = 0.690–0.837, $p < 0.001$) with a sensitivity of 61.3% and a specificity of 82.5% (Fig. 2A).

Subgroup analysis of *PDCD1* methylation

It is well known that the most significant heritability of AS comes from the B27 antigen and the male population. In addition, drug use may affect DNA methylation levels. Thus, subgroup analysis was performed by HLA-B27 antigen, gender and medicine intake to further explore the role of *PDCD1* methylation in AS. The results

Table 1. Demographic and clinical characteristics of participants.

	Methylation stage		RT-qPCR stage	
	AS (n = 80)	HC (n = 80)	AS (n = 47)	HC (n = 47)
Age (year)	35.14 ± 9.93	34.83 ± 9.52	34.66 ± 8.62	36.00 ± 8.99
Gender (male, %)	60 (75.00%)	60 (75.00%)	37 (78.72%)	37 (78.72%)
BMI (kg/m ²)	26.35 ± 25.47		23.59 ± 2.90	
HLA-B27 (+, %)	52 (65.00%)		28 (59.57%)	
ESR (mm/l)	10.00 (4.25–19.50)		10.00 (5.00–20.00)	
CRP (m/l)	4.30 (1.60–10.30)		4.50 (2.60–11.30)	
Disease duration (month)	51.50 (11.75–124.75)		72.00 (33.00–135.00)	
BASFI	0.40 (0.00–1.95)		0.30 (0.00–1.96)	
BASDAI	2.07 (1.01–3.36)		2.40 (1.40–3.40)	
ASDAS	2.18 (1.67–2.72)		2.24 (1.83–2.64)	

Normal variables were given as means ± standard deviation (SD), skewed data were expressed as median (interquartile range).

AS ankylosing spondylitis, ASDAS Ankylosing Spondylitis Disease Activity Score, BASFI Bath Ankylosing Spondylitis Functional Index, BASDAI Bath Ankylosing Spondylitis Disease Activity Index, BMI body mass index, CRP C-reactive protein, ESR erythrocyte sedimentation rate, HC healthy controls, RT-qPCR real-time quantitative PCR.

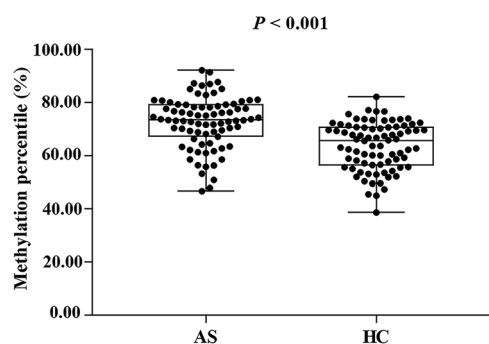


Fig. 1 The methylation level of *PDCD1* promoter in AS patients and healthy controls. Mann–Whitney *U* test was performed, data were expressed as median and interquartile range, $p < 0.05$ was considered significant.

showed that the methylation levels in the promoter region of *PDCD1* gene in HLA-B27-positive patients and HLA-B27-negative patients were all significantly higher than that in healthy subjects (HLA-B27(+) vs. HC: 73.15% (65.26%–78.55%) vs. 65.67% (56.50%–70.70%), $p < 0.001$; HLA-B27(–) vs. HC: 75.83% (68.92%–79.45%) vs. 65.67% (56.50%–70.70%), $p < 0.001$, respectively) (Fig. 3A, B). Moreover, in the subgroup analysis of gender, the hypermethylation of *PDCD1* promoter was also detected in both male and female AS patients (AS-male vs. HC-male: 73.34% (66.52%–79.29%) vs. 62.88% (55.59%–70.70%), $p < 0.001$; AS-female vs. HC-female: 75.28% (68.69%–78.93%) vs. 67.79% (60.94%–70.81%), $p = 0.003$, respectively) (Fig. 3C, D). However, no significant differences were found between treatment groups in the current study (Supplementary Table S2).

mRNA expression level of *PDCD1*

We further measured the mRNA expression level of *PDCD1* gene in the PBMCs of 47 AS patients and 47 HCs using RT-qPCR technology. The relative expression level of *PDCD1* gene was significantly decreased in AS patients than that in HCs (AS vs. HCs: 0.586 (0.330–0.888) vs. 1.991 (1.243–3.442), $p < 0.001$) (Fig. 4A). ROC results showed that the AUC was 0.909 (95% CI = 0.852–0.967, $p < 0.001$) for the *PDCD1* mRNA level with a sensitivity of 87.2% and a specificity of 80.9% (Fig. 2B). There was a significantly negative correlation between the DNA methylation level of *PDCD1* promoter and the expression level of *PDCD1* gene ($r_s = -0.470$, $p < 0.001$, Fig. 4B).

Methylation level and clinical characteristics

We analyzed the associations between the DNA methylation and mRNA levels of *PDCD1* gene and clinical manifestations, and the results were provided in Table 2. The DNA methylation level of *PDCD1* promoter was positively correlated with the ESR ($r_s = 0.306$, $p = 0.006$), CRP ($r_s = 0.306$, $p = 0.006$) and ASDAS ($r_s = 0.287$, $p = 0.012$) of AS patients. Moreover, there was no significant correlation between the *PDCD1* mRNA and clinical characteristics of AS.

DISCUSSION

Epigenetics, which refers to the functional modification of DNA without changing the sequence, can be inherited from one cell cycle to the next and is largely responsible for the expression of cell-specific genes [33]. DNA methylation is one of the best studied and understood epigenetic modifications, which plays a key role in regulating gene transcription and nuclear tissue, and ultimately affecting cellular function [29]. Emerging evidence has linked the aberrant DNA methylation with the pathogenesis and development of AS [34]. A genome-wide DNA methylation profile analysis of 5 AS patients and 5 healthy individuals identified 1915 differentially methylated CpG sites mapped to 1214 genes in the peripheral blood of AS, including *PDCD1*. However, the adjusted *P* value was not significant and data on the transcription level of *PDCD1* gene were lacking [35].

In the current study, we investigated the relationship between the DNA methylation and mRNA expression of *PDCD1* gene and the susceptibility to AS. In the first stage, a total of 80 AS patients and 80 HCs were included to detect the methylation level of *PDCD1* promoter, and the result showed that all 17 CpG sites and the CpG island in the promoter region of *PDCD1* gene were highly methylated in AS. In the second stage, 47 AS patients and 47 HCs were randomly selected from the first stage to measure the mRNA expression of *PDCD1*. We found that the mRNA level of *PDCD1* was significantly lower in AS patients compared with HCs, and there was a significantly negative correlation between the DNA methylation and mRNA expression level of *PDCD1* gene, further supporting of the functional relevance of *PDCD1* in AS. Moreover, subsequent analyses indicated that the methylation level of *PDCD1* gene was positively correlated with the ESR, CRP and ASDAS of AS, and not influenced by HLA-B27, gender and medicine use.

PDCD1 is an inhibitory receptor that plays an important role in suppressing immune response and promoting self-tolerance by

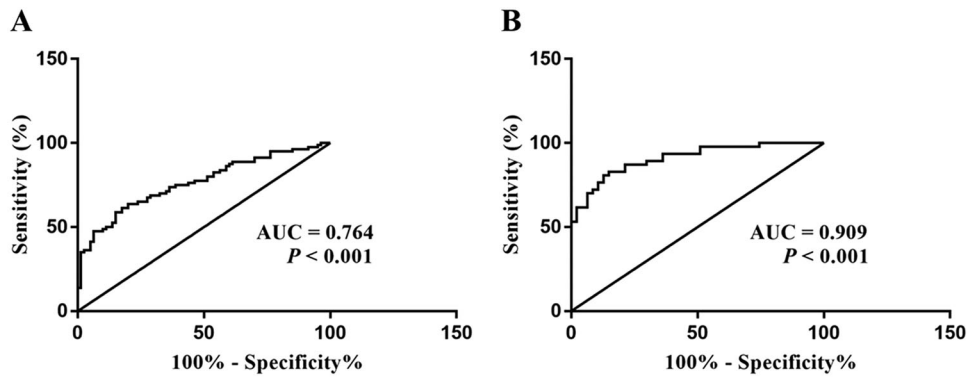


Fig. 2 ROC analysis of *PDCD1* gene in AS. **A** ROC analysis of *PDCD1* methylation in AS; **B** ROC analysis of *PDCD1* expression in AS. Sensitivity, specificity and AUC were provided, $p < 0.05$ was considered statistically significant.

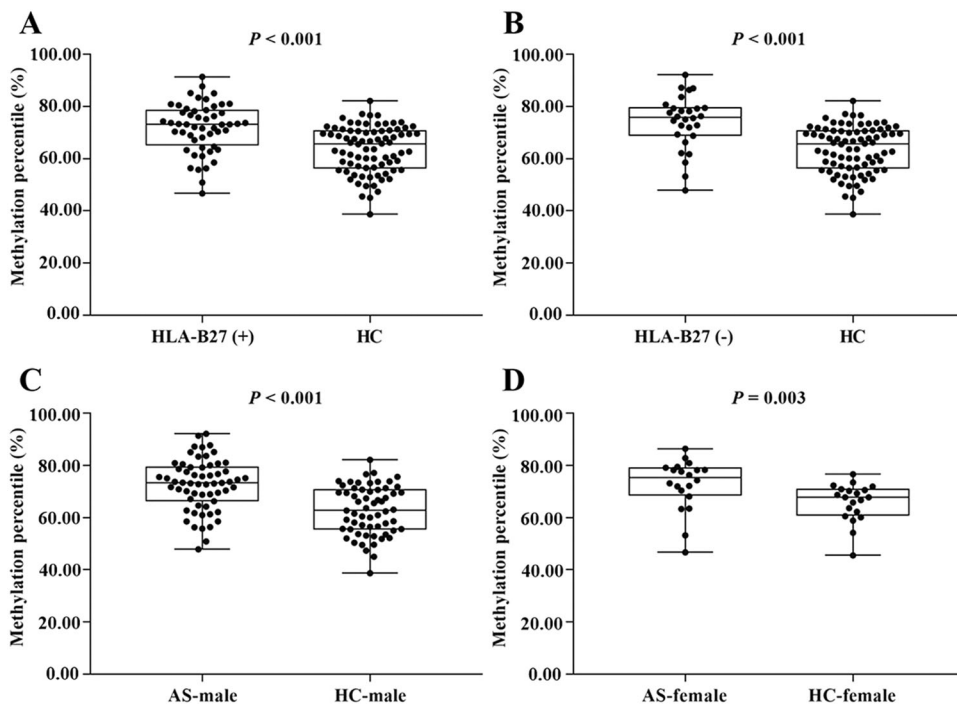


Fig. 3 Subgroup analysis of *PDCD1* methylation based on HLA-B27 antigen and gender. **A** HLA-B27 (+) AS patients vs. healthy controls; **B** HLA-B27 (-) AS patients vs. healthy controls; **C** male AS patients vs. male healthy controls; **D** female AS patients vs. female healthy controls. Mann–Whitney U test was performed, data were expressed as median and interquartile range, $p < 0.05$ was considered significant.

regulating T cell activity, activating antigen-specific T cell apoptosis and inhibiting regulatory T cell apoptosis [36]. Increasing evidence supports the role of *PDCD1* pathway in the prevention of autoimmune diseases [37]. For example, *PDCD1* knockout mice on the C57BL/6 background developed lupus like glomerulonephritis, while pre-morbid BWF1 mice treated with anti-*PDCD1* were protected from lupus nephritis [38, 39]. The serum levels of soluble *PDCD1* and the mRNA expression of *PDCD1* were downregulated in the peripheral blood of RA, and knockout of the *PDCD1* gene in mice increased the incidence and severity of collagen II-induced arthritis [40, 41]. The majority of the studies on the role of *PDCD1* in AS focus on gene polymorphisms, and several single nucleotide polymorphisms have been reported to be correlated with the risk of AS [42, 43]. In this study, we performed gene-specific methylation and RT-qPCR assays, and the hypermethylation of *PDCD1* promoter as well as its downregulated mRNA expression were detected. The main finding is that the aberrant DNA methylation and expression level of *PDCD1* gene may participate in the pathogenesis of AS, and *PDCD1* may be

a promising biomarker for AS. The lack of same observation in previous published EWAS may be due to differences in race and sample size [15, 35].

Several limitations of this study are worth considering. Firstly, this was a single-center case-control study based on one hospital, and the positive rate of HLA-B27 was relatively low in the included AS patients, so selection bias may be inevitable. Secondly, we only detected the mRNA expression level of *PDCD1* gene, but not its protein level. Thirdly, functional studies on the role of *PDCD1* in AS are lacking in this study. Fourthly, β -actin was used as an endogenous control without validation. Thus, multi-center studies with larger sample sizes are required to deeply investigate the role of *PDCD1* in AS to provide more comprehensive evidence.

CONCLUSION

DNA methylation and mRNA expression of *PDCD1* gene were significantly associated with the susceptibility to AS. The

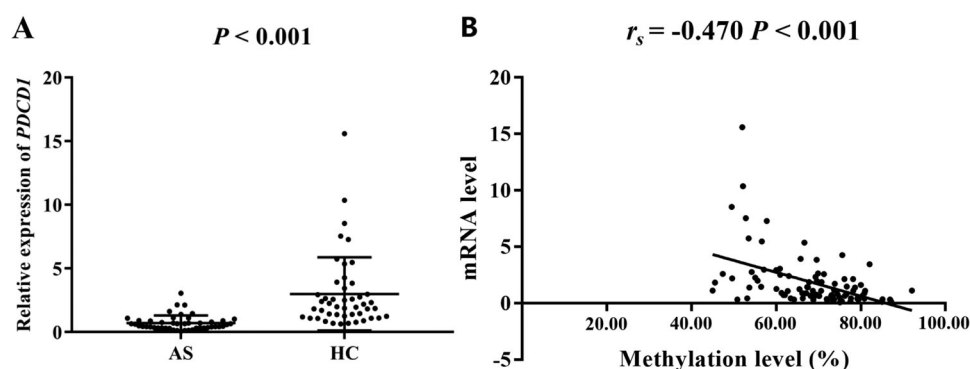


Fig. 4 Results of *PDCD1* mRNA expression level and correlation analysis. **A** The expression level of *PDCD1* mRNA in AS patients and healthy controls, Mann–Whitney *U* test was performed, data were expressed as median and interquartile range; **B** The correlation analysis between methylation level and mRNA level of *PDCD1*, Spearman's rank correlation coefficient test was conducted. $p < 0.05$ was considered statistically significant.

Table 2. Correlation between methylation and transcription of *PDCD1* and clinical characteristics.

Indicators	<i>PDCD1</i> methylation		<i>PDCD1</i> mRNA	
	r_s	p	r_s	p
ESR	0.306	0.006	-0.135	0.366
CRP	0.306	0.006	-0.245	0.100
Disease duration	0.133	0.252	0.154	0.313
BASFI	-0.020	0.862	-0.128	0.407
BASDAI	0.122	0.281	0.043	0.775
ASDAS	0.287	0.012	-0.208	0.175

ASDAS Ankylosing Spondylitis Disease Activity Score, BASDAI Bath Ankylosing Spondylitis Disease Activity Index, BASFI Bath Ankylosing Spondylitis Functional Index, CRP C-reactive protein, ESR erythrocyte sedimentation rate; r_s Spearman's rank correlation coefficient. $p < 0.05$ was considered statistically significant.

hypermethylation of the *PDCD1* gene promoter and the down-regulation of its transcription may be involved in the pathogenesis of AS. *PDCD1* may be a promising biomarker for the diagnosis and evaluation of AS.

DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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AUTHOR CONTRIBUTIONS

YW: conceptualization, methodology, software, investigation, formal analysis, writing—original draft; YC: data curation, writing—original draft; XS: visualization, investigation; YD: resources, supervision; MN: software, validation; FP: conceptualization, funding acquisition, resources, supervision, writing—review and editing.

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COMPETING INTERESTS

The authors declare no competing interests.

ETHICAL APPROVAL

The study was approved by the Ethical Committee of Anhui Medical University (Hefei, China) and all procedures have complied with the Declaration of Helsinki.

INFORMED CONSENT

All the subjects were given an informed consent and were well told of the study protocol.

ADDITIONAL INFORMATION

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