Original Article

Iranian Journal of Immunology

June 2024; 21(2):166-175.

# Evaluation of PD-1 Gene Expression Profile and Methylation of the Regulatory Regions in Patients with Ankylosing Spondylitis

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## ABSTRACT

**Background:** Ankylosing spondylitis (AS) is a chronic autoimmune disorder characterized by the fusion of vertebral joints and axial arthritis. The programmed death-1 (PD-1) inhibitory receptor has a pivotal role in controlling T cell function and may have a significant impact on the pathogenesis of autoimmune diseases such as AS pathogenesis.

**Objective:** To investigate PD-1 gene expression and its epigenetic regulation by detecting methylated CpG islands in the regulatory sites of the gene. This will provide insight into the mechanisms involved in the disease.

**Methods:** 30 AS patients and 30 healthy individuals were examined to detect the 16 CpG islands in intron 1 using bisulfite conversion and methylation-specific PCR technique. In addition, RNA samples were isolated from fresh peripheral blood mononuclear cells (PBMCs), and after complementary DNA (cDNA) synthesis, the expression level of the PD-1 gene was evaluated using Real-Time PCR.

**Results:** The CpG islands located in the intronic zone of the PD-1 gene were hyper-methylated in both the patients with AS and the healthy controls. The gene expression of PD-1 was significantly downregulated in AS patients compared with the controls ($p$=0.017). A negative correlation between the Bath Ankylosing Spondylitis Disease Activity Index and PD-1 gene expression was also revealed.

**Conclusion:** The low level of PD-1 gene expression is implicated in the pathogenesis of AS. However, in both groups, the methylation level of the intron 1 CpG islands of the PD-1 gene suggests that other regulatory mechanisms are more relevant to PD-1 gene expression than methylation in the intron.

**Keywords:** Ankylosing Spondylitis, DNA Methylation, Epigenetics, Gene Expression, Programmed Cell Death 1

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*Cite this article as:*
Soleimanifar N, Assadiasl S, Al-Shammari MS, Rostamian A, Sadr M, Shahkarami S, Mojtahedi H, Nicknam MH. Evaluation of PD-1 Gene Expression Profile and Methylation of the Regulatory Regions in Patients with Ankylosing Spondylitis. *Iran J Immunol.* 2024; 21(2):166-175, doi: 10.22034/iji.2024.101565.2757.

Received: 2024-02-03
Revised: 2024-05-11
Accepted: 2024-05-13

## INTRODUCTION

Ankylosing spondylitis (AS) is the most common type of Spondyloarthropathies (SpA) that mainly affects the lower spine and sacroiliac joints. However, AS patients often experience peripheral arthritis, enthesitis, and certain extra-articular manifestations, suggesting an extended autoimmune condition (1, 2). Disease progression can lead to the fusion of the axial skeleton, and limited spinal mobility, ultimately resulting in significant severe deformities and physical disability in the long term. Furthermore, specific extra-articular complications, in particular cardiovascular and respiratory injuries, have the potential to be life-threatening (3). Although the precise etiology of ankylosing spondylitis is unknown, a combination of genetic and environmental factors, including immune regulatory genes, dietary patterns, air pollution, and physical trauma, contribute to the initiation and progression of chronic inflammation (1, 4, 5).

Ankylosing spondylitis typically impacts young males, with different prevalence based on geographical and ethnic variations. The estimated prevalence of AS is 0.24% in Europeans, 0.17% in Asians, 0.32% in North Americans, and 0.10% among Latin Americans (6). However, it is less common in African and Japanese people since these groups have a lower prevalence of the main genetic factors associated with AS, such as human leukocyte antigen (HLA)-B27 (7). Although HLA-B27 accounts for approximately 20% of the genetic risk in AS (8), genome-wide association studies have implied some non-HLA genes also involved in triggering the AS pathogenesis (9, 10). Moreover, the analysis of transcriptional profiles utilizing microarray technology on the entire blood samples of individuals with AS has shown changes in genes related to inflammation, as well as genes implicated in the regulation of cartilage and bone metabolism (11). Aside from HLA-27, the main genes linked to AS pathogenesis are endoplasmic reticulum aminopeptidase 1 (ERAP1), IL-23R (12, 13), and co-inhibitory receptors like programmed cell death protein 1 (PD-1) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) (14). The PD-1 gene, also known as PDCD1, encodes the PD-1 protein, which acts as a receptor located on the surface of immune cells. PD-1 plays a crucial role in regulating immune responses and preventing autoimmunity. Most immune cells, including activated T cells, dendritic cells (DCs), natural killer (NK) cells, B lymphocytes, and monocytes contain PD-1 on the cell surface (15). PD-1 expression is stimulated by transcription factors nuclear factor of activated T cells (NFAT), NOTCH, and Forkhead box protein (FOX) O1 (16). It acts as a checkpoint to prevent T cell activation and maintain immune tolerance. Studies indicate that peripheral tolerance induction and maintenance depend on the PD-1/PD-L pathway; therefore, any dysfunction in this pathway could potentially lead to autoimmunity. The dysregulation of PD-1 gene expression in ankylosing spondylitis may contribute to immune system dysfunction and the chronic inflammation observed in affected individuals (17).

A crucial alteration known as DNA methylation entails the methyl group being attached to a DNA molecule, typically at cytosine residues in CpG dinucleotides. This modification can alter the accessibility of the DNA to transcription factors and other regulatory proteins, thereby impacting gene expression. In mammals, more than 98% of DNA methylation takes place in CpG islands found in somatic cells (18). These regions are distinguished by a high density of CpG dinucleotides, often located in promoter regions, enhancer elements, and other cis-regulatory elements that control gene expression (19). In the context of the PD-1 gene, DNA methylation in the regulatory regions can directly affect the interaction between transcription factors and other regulatory proteins, leading to changes in PD-1 gene expression. Multiple studies have shown that distinct DNA

methylation patterns in CpG islands located in the promoter regions are associated with modified PD1 gene expression (20, 21). For instance, hypermethylation of the promoter region has been linked to a decrease in PD-1 expression (22).

However, there are still limited studies investigating the methylation status of other cis-regulatory elements located within CpG islands and their effects on PD-1 gene expression. This study aims to evaluate the expression level of the PD-1 gene and investigate the methylation pattern of a cis-regulatory elements of the PDCD1 gene located in intron one. Further investigation of the involvement of PD-1 in AS may offer insightful information about the mechanisms underlying the disease and potential therapeutic targets.

## MATERIALS AND METHODS

*Patients*

Thirty patients with AS were recruited from a single center (Rheumatology Department, Imam Khomeini Hospital, Tehran, Iran). Modified New York classification criteria were used to confirm the diagnosis of AS (23). Additionally, a control group consisting of 30 normal individuals who were age and gender-matched, participated in the study. Disease severity and functional disabilities were assessed using the BASDAI (24) and BASFI (25, 26) indices, and BASMI (26), BAS-G (27) and ASQoL (28) indices to evaluate different aspects of AS. Allergic or autoimmune diseases were used as exclusion criteria for both groups in the study. Additionally, healthy individuals with close family members affected by spondyloarthropathies were excluded. Peripheral blood samples were collected from individual participants after obtaining informed consent. All the techniques carried out in the present study involving human participants followed the guidelines set by the institutional research committee and were in compliance with the Helsinki Declaration and its later amendments, or comparable ethical standards. The study has obtained the ethical approval of the Tehran University of medical sciences (Code: IR.TUMS.CHMC.REC.1398.055). Patients signed informed consent regarding publishing their data. The demographic characteristics of the studied population are presented in Table 1.

*Preparation of PBMC for RNA and DNA Extraction*

The method of density gradient was used to extracte peripheral blood mononuclear cells (PBMCs) by Ficoll-Hypaque (29).

*DNA Extraction Procedure*

A phenol-chloroform technique was applied for DNA extraction. Briefly, the cell and nuclear membranes are dissolved by sodium dodecyl sulfate (SDS) and proteinase K. Organic solvents like phenol and chloroform are used to collect the cell debris, and then DNA is precipitated, washed, dried, and redissolved in solution buffer (30).

*Bisulfite Conversion for DNA Treatment*

Bisulfite conversion: According to the manufacturer's instruction (Promega. inc USA), 20 µl of each DNA sample was deposited into a 200 µl microcentrifuge tube. After adding 130 µl of bisulfite ME conversion, a brief centrifugation was performed. The reaction was incubated using a thermocycler in the following manner: The sample was heated for 8 min at a temperature of 98 °C, followed by a further 60 min at a temperature of 54 °C. Subsequently, the samples were stored at 4 °C and protected from light, for a maximum duration of 20 h.

DNA desulfonation and maximum duration of 20 h: To process each sample, the ME spin column was inserted into one of the provided collection tubes, and following an addition of 600 µl of ME to the column. The bisulfite-treated samples were transferred to the column. Then, the samples were centrifuged at maximum speed (≥10000 ×g)

**Table 1. Demographic data of ankylosing spondylitis patients and the healthy controls**

| | | AS Patients (n=30) | Healthy Controls (n=30) | *p*-value |
|---|---|---|---|---|
| Gender | Male | 26(86.7%) | 26(86.6%) | ns |
| | Female | 4(13.3%) | 4(13.3%) | ns |
| Age | | 8.94±34.7 | 8.77±35.02 | ns |
| Smoking | Yes | 9(30%) | 2(6.7%) | ns |
| | No | 21(70%) | 28(93.4%) | ns |
| Disease Duration (Y) | | 0.9±4.59 | - | - |
| HLA-B27 | Positive | 22(73.3%) | - | - |
| | Negative | 8(26.7%) | - | - |
| BASDAI | | 2.36±4.15 | - | - |
| BASMI | | 2.33±2.77 | - | - |
| BASFI | | 3.17±2.71 | - | - |
| BAS-G | | 3.16±4.76 | - | - |
| ASQoL | | 5.42±6.43 | - | - |
| Family History | Yes | 14(46.7%) | - | - |
| | No | 16(53.3%) | - | - |
| Drugs | | | | |
| NSAIDs | | 5(16.6%) | - | - |
| DMARDs | | 2(6.6%) | - | - |
| Biologic | | 3(10%) | - | - |
| NSAIDs+DMARDs | | 6(20%) | - | - |
| NSAIDs+DMARDs+ Biologic | | 3(10%) | - | - |
| No drug (new patient) | | 11(36.6%) | - | - |

HLA: Human leukocyte antigen; BASDAI: Bath Ankylosing Spondylitis Disease Activity Index; BASFI: Bath Ankylosing Spondylitis Functional Index; BASMI: Bath Ankylosing Spondylitis Metrology Index; BAS-G: Bath Ankylosing Spondylitis Global Score; ASQoL: Ankylosing Spondylitis Quality of Life; DMARD: Disease-modifying anti-rheumatic drugs; NSAID: Non-steroidal anti-inflammatory drugs; TNF: Tumor necrosis factor; ns: Not significant

for 30 seconds. A volume of 100 µl of 1X ME wash buffer (with ethanol added) was added and centrifuged at maximum speed for 30 seconds. Next, 200 µl of ME desulfonation buffer was added to each ME spin column and incubated at room temperature for a duration of 15 min. Subsequently, it was rotated at maximum speed for 30 seconds. Then we added, 200 µl of ME wash solution and spun at the highest speed for 30 seconds. This step was repeated. Then the DNA was eluted in 10µl of ME elution after spun at maximum speed. The ME spin column was then removed and discarded. The eluted DNA was stored at a temperature of 4 °C, protected from light, and used for PCR amplification.

*DNA Targeting Amplification*

The desired amplicon is a CpG island within intron one with 388 base pair (bp) length located at the 2q37.3 band. The primer encompassed a total of 16 CpG sites. The Methprimer 2.0 website (http://www.urogene.org/methprimer2) was applied for primer designe. The forward sequence of 5′-TAGAATTGGAGATTTTGGGTAAAAG-3′ and reverse of 5′-AAATAACCACATCTAAATCAAAACCC-3′ with the amplicon size 388 bp. The targeted segments were amplified through the standard protocols. The gel electrophoresis was used for the validation of the amplification process (Fig. 1). Afterward, the samples were sequenced by Macrogen in Seoul, Korea. The CodonCode Aligner version 2 software was applied for analyzing the results.

*RNA Extraction and Complementary DNA Synthesis*

RNA was extracted using the High Pure

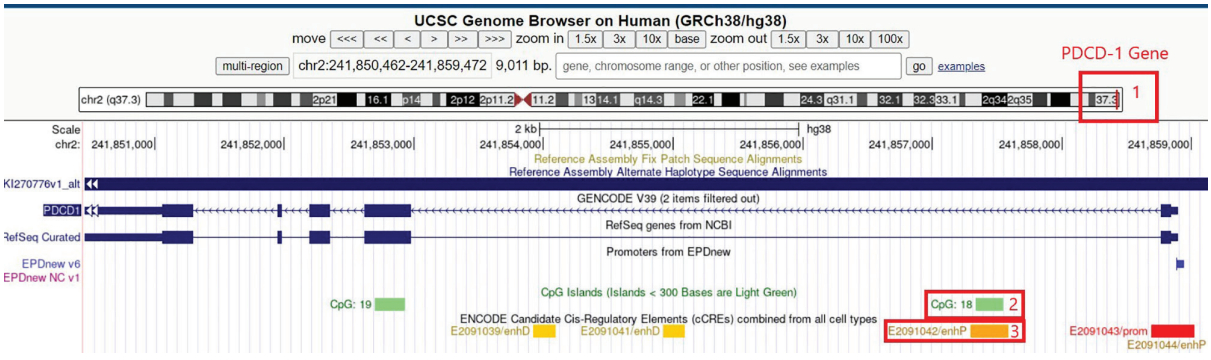


**Fig. 1.** Graphical information about the PDCD1 gene from the UCSC genome browser. Red boxes indicate 1: the location of the PD-1 gene on chromosome 2, 2: the target CpG island, and 3: the candidate Cis-Regulatory Elements (cCREs) enhancer.

RNA Isolation kit (ROJE Technologies, Iran) through the standard protocol. The quality of RNA was analyzed with a NanoDrop ND1000, Thermo Scientific. The absorbance ratios of A260/A280 within the range of 1.8 to 2.2 and the A260/A230 ratios of 2 to 2.2 were acceptable. The complementary DNA (cDNA) was synthesized using a Transcriptor First Strand cDNA Synthesis kit (ROJE Technologies, Iran) (31).

*Expression of PDCD1 Gene*

The gene expression experiment utilized the following primers: PDCD1-forward: CAGCCTGGTGCTGCTAGTCTG; reverse: GTCCACAGAGAACACAGGCAC, GPDH-forward:GTCTCCTCTGACTTCAACAGCG; reverse: ACCACCCTGTTGCTGTAGCCAA, manufactured by Methabion (Germany) and then compared with the reference gene of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (32). All the tests were conducted in a duplicate form and contained two non-template controls and then analyzed by relative quantification mRNA expression=$(2^{-\Delta Ct}) \times 10^3$ (31, 33).

*Statistical Analysis*

Data were presented as mean±standard deviation (SD) or mean±standard error of the mean (SEM). Kolmogorov–Smirnov test examined the normality of the sample population. The independent sample T-test, Pearson chi-square, and odds ratio tests were also performed . P-values less than 0.05 were considered significant (SPSS 26.0; SPSS Inc., Chicago, USA).

## RESULTS

*Demographic and Clinical Characteristics of the Patients and the Control Group*

The AS patients and the healthy individuals were matched for sex, age, and smoking. 73.3% of the patients were HLA-B27 positive, and 46.7% had a family history of spondylitis. Non-steroidal anti-inflammatory drugs (NSAID) used by the patient group were naproxen, indomethacin, or piroxicam; disease-modifying anti-rheumatic drugs (DMARDs) included methotrexate, Hydroxychloroquine, or sulfasalazine, and biologic agents (anti-tumor necrosis factor) were Remicade (infliximab), Enbrel (etanercept), Humira (adalimumab), administrated alone or in combination. Clinical and demographic characteristics of the patients and the controls are summarized in Table 1.

*PD-1 Gene Expression in the AS and the Healthy Controls*

PD-1 gene expression in the healthy individuals was significantly higher than in the AS patients (95.91±22.25 vs. 32.15±12.55 (mean±SEM), $p$=0.017). Moreover, in the patients group, the expression of the PD-1 gene showed a negative correlation with the BASDI disease index, with a Pearson correlation of 0.34 and $p$-value of 0.041 (Fig. 2).

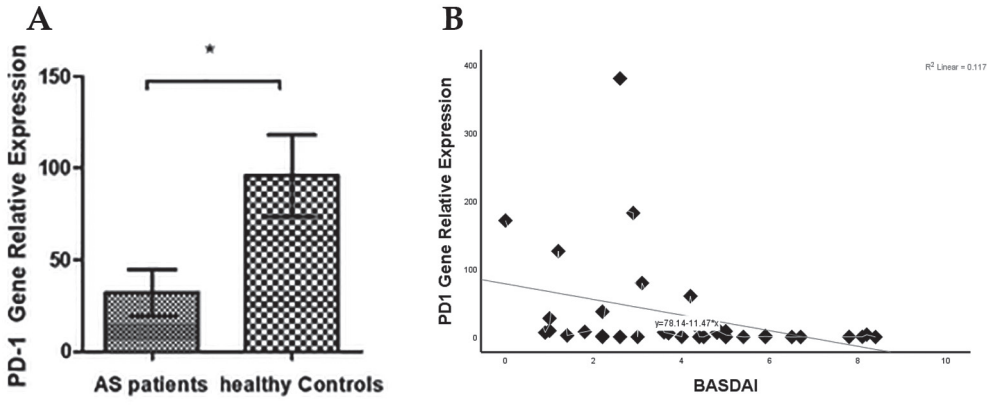


**Fig. 2.** (A) PD-1 gene expression in the healthy group was significantly higher than in the AS patients (*$p$<0.05); (B) negative correlation between PD-1 expression and BASDAI score (r: 0.34, $p$=0.041).

*Sequencing Results of Intron-1 CpG Methylation*

The sequenced target DNA, with 388 bp length, covered a CpG island within intron one with 230 bp of length and 18 GCs inclusion. This position is a regulatory region susceptible to candidate Cis-Regulatory Elements (cCREs) enhancer attachment. Upon examining all the CpG sites together, methylation analysis revealed hypermethylation in intron-1 of the PD-1 gene in both the AS patients and the healthy controls. Therefore, there was no significant difference between the two groups (Figs. 1, 3-5).

## DISCUSSION

In this study, it was found that the level of PDCD1 gene expression in patients is significantly lower than that in those of the healthy ones, and this decrease had a significant negative relationship with the disease activity index, BASDAI.

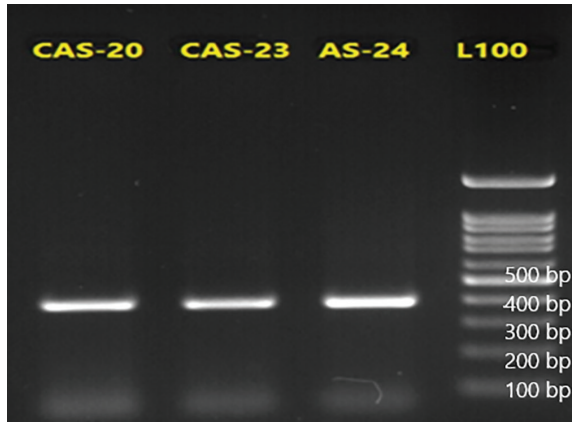


**Fig. 3.** A gel electrophoresis image of PCR products from people with AS and the healthy control group (CAS) after bisulfite treatment.

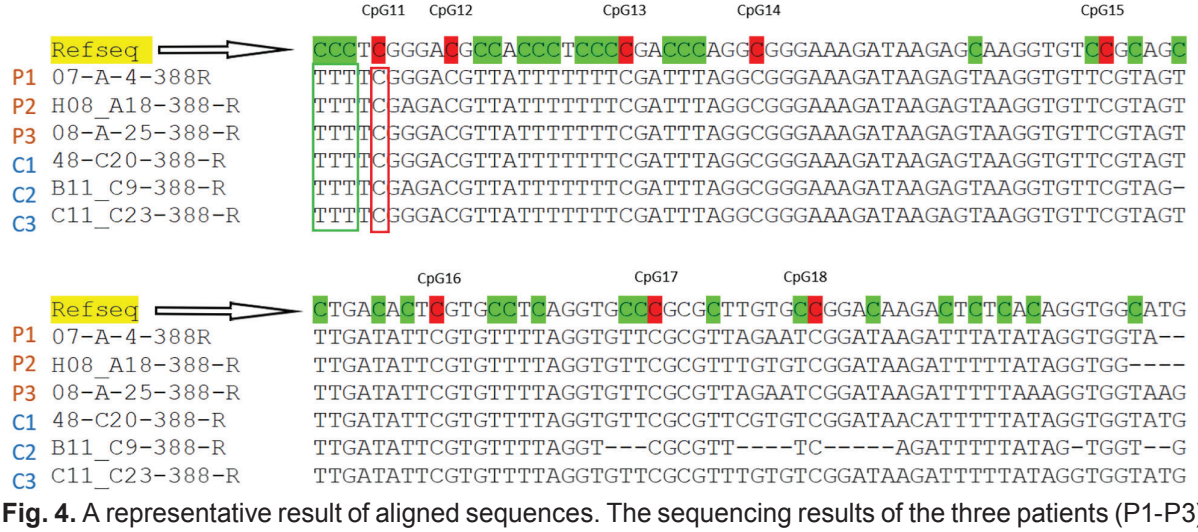


**Fig. 4.** A representative result of aligned sequences. The sequencing results of the three patients (P1-P3) the 3 controls (C1-C3), aligned to the reference sequence, are shown here. The green Cs are cytosines converted to thymidine through bisulfite treatment, and the red Cs are the ones methylated in CpGs.

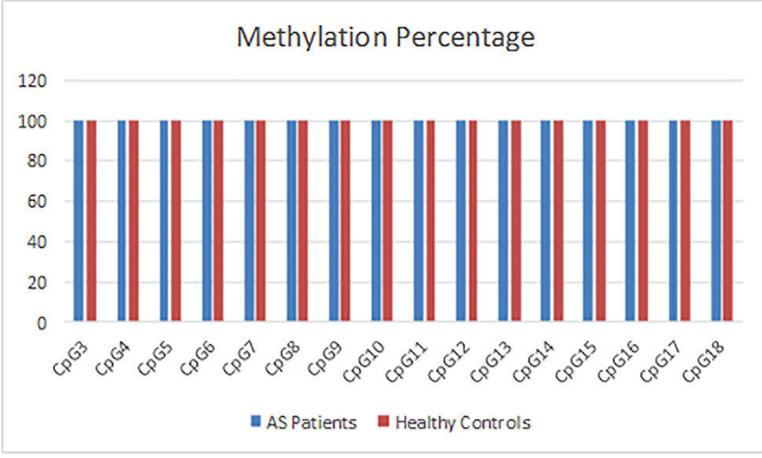


**Fig. 5.** Comparison of methylated CGs percentage in AS patients and the healthy controls in 16 covered CpGs (from CpG3-CpG18).

The methylation pattern of the investigated intronic region was not significantly different from normal subjects in terms of methylation percentage, and all CpG islands of this region were completely hypermethylated, similar to what was seen in the control group.

Autoimmune diseases are multifactorial disorders that have unknown triggers and produce unpredictable outcomes. Studies have shown that both environmental elements and genetic ones can contribute to the pathogenesis of autoimmune disorders. In recent years, the field of epigenetics has seen significant growth and has become a prominent area of research for understanding disease development. Studies on autoimmune diseases (AIDs), such as AS, suggest that both genetics and environmental factors play a major role in triggering these diseases. It is now believed that environmental factors can alter self-antigens through post-translational modifications or molecular mimicry, leading to immunological responses. Therefore, comprehending the development of AS necessitates a thorough understanding of epigenetics.

The PD-1 molecule is an altering regulator of T cells. Previous studies have demonstrated a higher percentage of $CD4^+$ T cells in the AS patients compared with the healthy ones. However, those with AS who have more severe disease activity exhibit notably lower percentages of $PD\text{-}1^+CD3^+$ T cells and $PD\text{-}1^+CD4^+$ T cells. These findings have indicated that the higher number of T helper cells without PD-1 may be associated with joint destruction and the consequent spinal radiologic manifestations in AS patients (10, 34). Additionally, two PD-1 gene polymorphisms have shown a correlation with increased susceptibility to AS. The frequency of the T allele of the PD-1.9 T/C polymorphism was higher in the Korean male population with AS than in the control group. The frequency of the CT haplotype (PD-1.5 C/T and PD-1.9 T/C) was also higher in the AS patients (35). Furthermore, the development of AS appears to be associated with the combined genotypes of PD-1 G-536A, PD-L1 A8923C, and PD-L2 C47103T (36).

Studies have shown that methylation of the promoter region regulated the PD-1 gene expression. This is supported by the observation of an inverse correlation between promoter methylation and PD-1 mRNA levels (21). A study has demonstrated that the dysfunction of methylation mechanisms and altered microRNA patterns play a role in the development of rheumatoid arthritis (RA) (37); likewise, it has been found that DNA methylation at the regulatory region of PDCD1 was lower in RA patients compared with the healthy controls (38). A study by Varun Sasidharan Nair et al. revealed significant hypo-methylation of multiple immunological checkpoints, including PD-1 the CpG islands in the promoter regions, in

primary breast tumor cells compared with normal tissues (39).

There is evidence to show that DNA methylation has a role in AS pathogenesis. For example, a study found that individuals with AS had higher levels of promoter methylation in the BCL11B gene compared with the healthy controls. The study also demonstrated an association of hyper-methylation of CpG3 and CpG5 with the risk of developing AS (40). The elevated hyper-methylation of the IRF8 gene promoter in AS patients, compared with the healthy controls, provides evidence for the involvement of epigenetic dysregulation in patients (41). In addition, studies have shown that two CpG islands (IL-12B-1, IL-12B-2) were hyper-methylated in individuals with AS in comparison with the healthy controls (42). It was also discovered that the methylation level of the DNMT1 promoter was significantly higher in AS patients compared with the control group; however, this increase in methylation level was not significantly related to the gene expression (43).

As described here, the majority of the studies are focused on the promoter region of PD-1 gene; however, other regulatory sequences also play a pivotal role in regulating the gene expression. PD-1 gene has two CpG islands the first covered in intron and is suggested to be a cis-regulatory element, potentially serving as an attachment site for an enhancer protein. In the present study, our main focus was on the evaluation of the methylation level of the PDCD1 gene at the regulatory region inside the intron 1. We discovered that this gene is hyper-methylated in both the AS patients and the healthy controls; however, the gene expression of PD-1 was significantly lower in AS patients. This discovery reveals that methylation of this location does not have a significant effect on PD-1 expression, but it does highlight the important involvement of regulatory genes in AS pathogenesis. This study is limited to the one CpG island, that covered 16 out of 18 CGs in this region. However, it is recommended to examine the methylation status of both CpG islands. Although the study population is sufficient according to the statistical analysis, the greater sample size would enhance the precision of the results and enable the identification of significant associations.

Consequently, these findings indicate that PD1-relative gene expression decreased in the AS patients. However, the regulatory mechanisms responsible for this reduction are not clear. The methylation of other cis-elements in the PD-1 gene or other epigenetic modifications such as non-coding RNAs, could potentially influence the gene expression of PD-1. Obviously, further research is required to clarify the dysregulations of DNA modification in ankylosing spondylitis.

## CONCLUSION

According to recent studies, the PD-1 gene is significantly involved in the development of AS. The decreased expression of the PD-1 gene in the AS patients suggests a potential involvement of epigenetic regulation. More precisely, the methylation level of CpG islands in regulatory sites, mainly in intron 1 of the PDCD1 gene, was observed to be hyper-methylated in both the AS patients and the healthy individuals. Despite similar methylation levels, the expression of the PD-1 gene was disrupted in the AS patients. Further investigation is required to understand the underlying mechanisms and consequences of these findings in the development of AS.

## ACKNOWLEDGMENT

This research was funded by the Tehran University of Medical Sciences (Grant number: 97-03-93-40497).

## AUTHORS' CONTRIBUTION

NS and MHN conceived the research and designed the experiments. NS, MSA, and

SS interpreted the data. NS, SA and MSA prepared the manuscript. MSA, MS and HM collected samples and performed the tests. AR evaluated the patient's criteria and clinical manifestations.

## CONFLICTS OF INTEREST

The authors declare no conflict of interest.

## REFERENCES


1. Assadiasl S, Soleimanifar N. An Overview to Ankylosing Spondylitis and Spondyloarthropathies. In: Nicknam MH, editor. Ankylosing Spondylitis - Axial Spondyloarthritis: Cellular, Molecular and Environmental Factors. Singapore: Springer Singapore; 2022. p. 3-21.
2. El Maghraoui A. Extra-articular manifestations of ankylosing spondylitis: prevalence, characteristics and therapeutic implications. European Journal of Internal Medicine. 2011;22(6):554-60.
3. Sieper J, Poddubnyy D. Axial spondyloarthritis. The Lancet. 2017;390(10089):73-84.
4. Soleimanifar N, Nicknam MH, Bidad K, Jamshidi AR, Mahmoudi M, Mostafaei S, et al. Effect of food intake and ambient air pollution exposure on ankylosing spondylitis disease activity. Advances in Rheumatology. 2019;59.
5. Mohebbi B, Soleimanifar N. Spondyloarthropathies and Food Intake. Ankylosing Spondylitis-Axial Spondyloarthritis: Springer; 2022. p. 157-69.
6. Dean LE, Jones GT, MacDonald AG, Downham C, Sturrock RD, Macfarlane GJ. Global prevalence of ankylosing spondylitis. Rheumatology. 2014;53(4):650-7.
7. Stolwijk C, van Onna M, Boonen A, van Tubergen A. Global prevalence of spondyloarthritis: a systematic review and meta-regression analysis. Arthritis care & research. 2016;68(9):1320-31.
8. Chatzikyriakidou A, Voulgari PV, Drosos AA. What is the role of HLA-B27 in spondyloarthropathies? Autoimmunity Reviews. 2011;10(8):464-8.
9. Reveille JD, Sims A-M, Danoy P, Evans DM, Leo P, Pointon JJ, et al. Genome-wide association study of ankylosing spondylitis identifies non-MHC susceptibility loci. Nature genetics. 2010;42(2):123.
10. Soleimanifar N, Amirzargar AA, Mahmoudi M, Pourfathollah AA, Azizi E, Jamshidi AR, et al. Study of programmed cell death 1 (PDCD1) gene polymorphims in Iranian patients with ankylosing spondylitis. Inflammation. 2011;34:707-12.
11. Fan X, Qi B, Ma L, Ma F. Screening of underlying genetic biomarkers for ankylosing spondylitis. Molecular Medicine Reports. 2019;19(6):5263-74.
12. Mahmoudi M, Jamshidi AR, Amirzargar AA, Farhadi E, Nourijelyani K, Fallahi S, et al. Association between endoplasmic reticulum aminopeptidase-1 (ERAP-1) and susceptibility to ankylosing spondylitis in Iran. Iranian Journal of Allergy, Asthma and Immunology. 2012:294-300.
13. Zhong L, Wang W, Song H. Complex role of IL-23R polymorphisms on ankylosing spondylitis: a meta-analysis. Expert Review of Clinical Immunology. 2018;14(7):635-43.
14. Chen S, Li Y, Deng C, Li J, Wen X, Wu Z, et al. The associations between PD-1, CTLA-4 gene polymorphisms and susceptibility to ankylosing spondylitis: a meta-analysis and systemic review. Rheumatology international. 2016;36(1):33-44.
15. Kim KH, Kim CG, Shin E-C. Peripheral blood immune cell-based biomarkers in anti-PD-1/PD-L1 therapy. Immune Network. 2020;20(1).
16. Staron MM, Gray SM, Marshall HD, Parish IA, Chen JH, Perry CJ, et al. The transcription factor FoxO1 sustains expression of the inhibitory receptor PD-1 and survival of antiviral CD8+ T cells during chronic infection. Immunity. 2014;41(5):802-14.
17. Dai S, Jia R, Zhang X, Fang Q, Huang L. The PD-1/PD-Ls pathway and autoimmune diseases. Cellular Immunology. 2014;290(1):72-9.
18. Moore LD, Le T, Fan G. DNA methylation and its basic function. Neuropsychopharmacology. 2013;38(1):23-38.
19. Chen F, Zhang Q, Deng X, Zhang X, Chen C, Lv D, et al. Conflicts of CpG density and DNA methylation are proximally and distally involved in gene regulation in human and mouse tissues. Epigenetics. 2018;13(7):721-41.
20. Illingworth RS, Gruenewald-Schneider U, Webb S, Kerr AR, James KD, Turner DJ, et al. Orphan CpG islands identify numerous conserved promoters in the mammalian genome. PLoS genetics. 2010;6(9):e1001134.
21. Goltz D, Gevensleben H, Dietrich J, Ellinger J, Landsberg J, Kristiansen G, et al. Promoter methylation of the immune checkpoint receptor PD-1 (PDCD1) is an independent prognostic biomarker for biochemical recurrence-free survival in prostate cancer patients following radical prostatectomy. Oncoimmunology. 2016;5(10):e1221555.
22. Sasidharan Nair V, Toor SM, Taha RZ, Shaath H, Elkord E. DNA methylation and repressive histones in the promoters of PD-1, CTLA-4, TIM-3, LAG-3, TIGIT, PD-L1, and galectin-9 genes in human colorectal cancer. Clinical

epigenetics. 2018;10(1):1-9.
23. Braun J, Sieper J. Ankylosing spondylitis. The Lancet. 2007;369(9570):1379-90.
24. Garrett S, Jenkinson T, Kennedy LG, Whitelock H, Gaisford P, Calin A. A new approach to defining disease status in ankylosing spondylitis: the Bath Ankylosing Spondylitis Disease Activity Index. The Journal of rheumatology. 1994;21(12):2286-91.
25. Calin A, Garrett S, Whitelock H, Kennedy LG, O'hea J, Mallorie P, et al. A new approach to defining functional ability in ankylosing spondylitis: the development of the Bath Ankylosing Spondylitis Functional Index. J rheumatol. 1994;21(12):2281-5.
26. Jenkinson TR, Mallorie PA, Whitelock H, Kennedy LG, Garrett S, Calin A. Defining spinal mobility in ankylosing spondylitis (AS). The Bath AS Metrology Index. The Journal of rheumatology. 1994;21(9):1694-8.
27. Jones S, Steiner A, Garrett S, Calin A. The bath ankylosing spondylitis patient global score (BAS-G). Rheumatology. 1996;35(1):66-71.
28. Doward L, Spoorenberg A, Cook S, Whalley D, Helliwell P, Kay L, et al. Development of the ASQoL: a quality of life instrument specific to ankylosing spondylitis. Annals of the rheumatic diseases. 2003;62(1):20-6.
29. Panda SK, Ravindran B. Isolation of human PBMCs. Bio-protocol. 2013;3(3):e323-e.
30. Demeke T, Jenkins GR. Influence of DNA extraction methods, PCR inhibitors and quantification methods on real-time PCR assay of biotechnology-derived traits. Analytical and bioanalytical chemistry. 2010;396:1977-90.
31. Alamdari A, Minoo FS, Assadiasl S, Freidoon M, Pour-Reza-Gholi F, Soleimanifar N, et al. Expression of Programmed Cell Death 1 and Helios Genes Correlates With rs872071A> G and rs12203592C> T Single-Nucleotide Polymorphisms of InterferonRegulatory Factor 4 in Patients with T-Cell-Mediated Rejection of Renal Allograft. Experimental and clinical transplantation: official journal of the Middle East Society for Organ Transplantation. 2021.
32. Zhao F, Maren NA, Kosentka PZ, Liao Y-Y, Lu H, Duduit JR, et al. An optimized protocol for stepwise optimization of real-time RT-PCR analysis. Horticulture Research. 2021;8(1):179.
33. Assadiasl S, Ahmadpoor P, Nafar M, Lessan Pezeshki M, Pourrezagholi F, Parvin M, et al. Regulatory T cell subtypes and TGF-β1 gene expression in chronic allograft dysfunction. Iranian Journal of Immunology. 2014;11(3):139-52.
34. Chen MH, Chen WS, Lee HT, Tsai CY, Chou CT. Inverse correlation of programmed death 1 (PD-1) expression in T cells to the spinal radiologic changes in Taiwanese patients with ankylosing spondylitis. Clin Rheumatol. 2011;30(9):1181-7.
35. Lee SH, Lee YA, Woo DH, Song R, Park EK, Ryu MH, et al. Association of the programmed cell death 1 (PDCD1) gene polymorphism with ankylosing spondylitis in the Korean population. Arthritis Res Ther. 2006;8(6):R163.
36. Huang C-H, Wong R-H, Wei JC-C, Tsay M-D, Chen W-C, Chen H-Y, et al. Effects of genetic polymorphisms of programmed cell death 1 and its ligands on the development of ankylosing spondylitis. Rheumatology. 2011;50(10):1809-13.
37. de la Rica L, Urquiza JM, Gómez-Cabrero D, Islam AB, López-Bigas N, Tegnér J, et al. Identification of novel markers in rheumatoid arthritis through integrated analysis of DNA methylation and microRNA expression. J Autoimmun. 2013;41:6-16.
38. Tseng CC, Lin YZ, Lin CH, Li RN, Tsai WC, Ou TT, et al. Genetic and epigenetic alteration of the programmed cell death 1 in rheumatoid arthritis. Eur J Clin Invest. 2019;49(10):e13094.
39. Sasidharan Nair V, El Salhat H, Taha RZ, John A, Ali BR, Elkord E. DNA methylation and repressive H3K9 and H3K27 trimethylation in the promoter regions of PD-1, CTLA-4, TIM-3, LAG-3, TIGIT, and PD-L1 genes in human primary breast cancer. Clinical epigenetics. 2018;10:1-12.
40. Karami J, Mahmoudi M, Amirzargar A, Gharshasbi M, Jamshidi A, Aslani S, et al. Promoter hypermethylation of BCL11B gene correlates with downregulation of gene transcription in ankylosing spondylitis patients. Genes & Immunity. 2017;18(3):170-5.
41. Chen M, Wu M, Hu X, Yang J, Han R, Ma Y, et al. Ankylosing spondylitis is associated with aberrant DNA methylation of IFN regulatory factor 8 gene promoter region. Clinical Rheumatology. 2019;38(8):2161-9.
42. Zhang X, Lu J, Pan Z, Ma Y, Liu R, Yang S, et al. DNA methylation and transcriptome signature of the IL12B gene in ankylosing spondylitis. International Immunopharmacology. 2019;71:109-14.
43. Aslani S, Mahmoudi M, Garshasbi M, Jamshidi AR, Karami J, Nicknam MH. Evaluation of DNMT1 gene expression profile and methylation of its promoter region in patients with ankylosing spondylitis. Clinical rheumatology. 2016;35(11):2723-31.