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CD28 and CTLA4 polymorphisms associated with ankylosing spondylitis: a study in the context of HLA-B27

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Abstract

Background The human leukocyte antigen (HLA)-B27 gene is highly associated with ankylosing spondylitis (AS). However, not everyone who carries the HLA-B27 antigen develops AS, indicating that factors beyond the HLA-B27 gene contribute to the disease's onset. AS is an autoimmune disease in which co-stimulatory systems have been widely explored. Therefore, we aimed to analyze the association between single-nucleotide polymorphisms (SNPs) in co-stimulatory/inhibitory molecules and AS to identify other key factors involved in developing the disease.

Results This study recruited 32 patients with AS and 32 controls. DNA was extracted from whole blood, and PCR amplification was performed to target the promoter regions of the CTLA4, CD28, and PDCD1 genes. Chi-square and Fisher's exact tests were used under various genetic models to assess differences in genotype and allele distribution between cases and controls. The results showed that rs201801072 of the CD28 gene (TT + CT vs. CC, $p = 0.001$) and rs11571319 of the CTLA4 gene were associated with AS (GG vs. AG + AA, $p = 0.001$). Logistic regression analysis showed that rs201801072 (CD28) and rs11571319 (CTLA4) were independently associated with AS. A significant positive interaction was observed between these SNPs and HLA-B27 positivity, further increasing the risk of AS (T-allele: OR = 6.15; G-allele: OR = 13.30, both $p < 0.001$). HLA-B27 carriers exhibited an extremely high risk of AS (OR = 65.0, $p = 1.19E-06$).

Conclusions The elevated frequencies of specific alleles in AS patients compared to controls highlight the potential involvement of these SNPs as key factors in the pathogenesis of AS, offering new insights into the genetic mechanisms underlying the disease.

Keywords Ankylosing spondylitis, HLA-B27, CD28, CTLA4, Single nucleotide polymorphism (SNP)

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Background

Ankylosing spondylitis (AS) is a chronic autoimmune disease that primarily affects the axial joints of the spine, with a prevalence of approximately 2–3 in 1,000 [1]. Most patients develop the condition between the ages of 16 and 40, with the peak incidence occurring between 20 and 30 years [2, 3]. Typical symptoms include chronic low back pain, morning spinal stiffness, and limited range of motion, often centered around the sacroiliac joints. Classification criteria developed in 2009 by the Assessment of Spondyloarthritis International Society (ASAS) relied much on human leukocyte antigen (HLA)-B27 positivity to establish a diagnosis for axial spondyloarthritis [4]. HLA-B27 is strongly associated with triggering inflammatory responses through T-cell immune reactions and the unfolded protein response, leading to damage to fibrocartilage or cartilage in AS [5].

Studies on HLA-B27 subgroups have identified that *HLA-B27:02*, *HLA-B27:04*, *HLA-B27:05*, and *HLA-B27:07* are related to AS, while *HLA-B27:06* and *HLA-B27:09* appear to be unrelated to the disease [6]. *HLA-B27:05* is commonly found in Koreans [7], whereas *HLA-B27:04* is prevalent among the Chinese population [8, 9] and shows a strong association with AS in Hunan Province [10]. In the East Asian population, 5–10% of HLA-B27-positive individuals carry the *HLA-B27:04* subtype. Over 95% of AS patients are HLA-B27 positive, while 5% of AS patients are HLA-B27 negative. HLA-B27 positivity does not guarantee AS, and factors beyond *HLA-B27* also influence AS pathogenesis [11, 12].

Because T cells are activated by HLA-mediated antigen-specific signals, followed by immune checkpoint signals (ICS), we focused on genes involved in this process. Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4, encoded by *CTLA4* gene) interacts with B7, while programmed death-1 (PD-1, encoded by *PDCD1* gene) interacts with programmed cell death-ligand 1 (PD-L1) to suppress T cell responses and maintain self-tolerance [13]. CD28, a receptor responsible for stimulatory ICS, was found to be associated with AS-related pulmonary involvements with certain variants [14]. Data on expression level and possible CD28 polymorphism in AS are minimal [15]. This study aims to identify the single-nucleotide polymorphisms (SNPs) of the T-cell regulation genes (*CD28*, *CTLA4*, and *PDCD1*) associated with AS beyond *HLA-B27*. Although HLA-B27 is strongly associated with AS, only 1–2% of HLA–B27–positive individuals eventually develop the disease [16], suggesting that additional genetic factors may contribute to AS susceptibility. Identifying such variants may provide further insights into the pathogenesis of AS.

Table 1 Basic information of the AS patients and controls

	Patients (*N* = 32)	Controls (*N* = 32)
Gender (male: female)	22:10	5:27
Age (average ± SD)	49.9 ± 15.8	37.2 ± 6.9
Onset age	30.2 ± 10.3	-
Grade of AS		
I	1	
II	15	
III	3	
IV	1	
Unknown	12	
(Peripheral) arthritis	12 (37.5%)	-
Extra-axial manifestations	18 (56.3%)	-
HLA-B27 genotype		
Positive (%)	30 (94%)	4 (12.5%)
Negative (%)	2 (6%)	28 (87.5%)

(Peripheral) arthritis: patients with RA or affecting the hip, knee, and ankle joints. In addition, enthesopathy is also commonly observed

Extra-axial manifestations: including enthesitis, dactylitis, uveitis, inflammatory bowel disease (IBD), psoriasis, cardiopulmonary involvement, etc

Methods

Subjects

A total of 32 AS patients from the Taiwanese population were recruited based on a confirmed clinical diagnosis made by board-certified rheumatologists according to the modified New York criteria. Although the sacroiliitis grade was not consistently documented for all patients, the diagnosis was based on clinical features including chronic inflammatory back pain, reduced spinal mobility, and peripheral arthritis, supplemented by radiographic findings when available. Among them, 22 patients were male and 10 were female. The HLA-B27 status of the patients was obtained from medical records, where the hospital's laboratory tested the antigen at the attending physician's request. Blood samples were collected using acid-citric dextrose blood collection tubes, and the presence of HLA-B27 antigens in serum was detected through flow cytometry. However, 8 AS patients in this study did not have their HLA-B27 status (positive or negative) indicated in their medical records. Thus, we identified their HLA-B27 status using polymerase chain reaction (PCR). The healthy control group was also analyzed using the same PCR method for consistency. Additionally, 32 control cases without immune-related diseases were collected, with an average age of 37.2 years, including 5 males and 27 females (Table 1). The samples were residual specimens from HLA-B27 patients' routine follow-up examinations or health check-ups of the patients. These healthy controls were individuals who underwent health check-ups at our hospital or hospital employees who were invited to participate in institution-wide health examinations. Participants were informed about the study's objectives and research materials before providing consent. They reviewed the consent form, which outlined the study details, and voluntarily agreed to participate by signing the document.

HLA-B27 genotyping

For *HLA-B27* genotyping via PCR, specific primers were used to amplify target regions indicative of the antigen's presence. Two sets of primers, SC1 and SC2, were utilized for this purpose. The primer sequences are as follows: SC1-F (5'-GCTACGTGGACGACACGCT-3'), SC1-R (5'-GTCTGTGCCTTGGCCTTGC-3'), SC2-F (5'-GACGCCGCGAGTCCGAGA-3'), and SC2-R (5'-CACGTCGCAGCCATACATAT-3'). For controls, two additional primers were employed: control-C5 (5'-TGCCAAGTGGAGCACCCA-3') and control-C3 (5'-GCATCTTGCTCTGTGCAGAT-3'). The PCR conditions were optimized to ensure specificity and efficiency in amplifying the respective regions, facilitating accurate genotyping of *HLA-B27*. This methodology was applied to both patient and control samples to ascertain the antigen status reliably.

Candidate SNPs

Promoter regions play a critical role in gene expression regulation, as they contain binding sites for transcription factors that initiate or modulate the transcription process [17]. Variations in these regions, such as single nucleotide polymorphisms (SNPs), can alter transcription factors' binding efficiency, leading to gene expression changes [18]. Given the immune-regulatory function of CD28, CTLA-4, and PD-1 in T-cell activation and immune tolerance, any disruption in their expression could significantly impact the immune response and contribute to autoimmune conditions, such as AS [19]. Therefore, analyzing promoter SNPs in these genes allows us to identify potential regulatory variants that may influence susceptibility to AS by altering gene expression patterns, making these SNPs important candidates for understanding the genetic basis of the disease. Additionally, this study included key hotspot loci of the gene, such as the CT60 site (rs3087243) located in the 3'-UTR of the *CTLA4* gene and rs3116496 (INT3 + 17) in intron 3 of the *CD28* gene. These SNPs (Table S1) have been shown to be associated with disease susceptibility in various autoimmune diseases, including systemic lupus erythematosus (SLE) [20] and rheumatoid arthritis (RA) [21].

PCR and SNP sequencing

The DNA samples used for PCR were extracted from whole blood collected in ethylenediaminetetraacetic acid-coated vacuum tubes. DNA extraction was performed using the QIAamp DNA Blood Mini Kit (Qiagen, Valencia, California, USA). This study focused on investigating the promoter regions and hotspots of the *CTLA4* gene on chromosome 2, as well as the *CD28* and *PDCD1* genes. Based on the SNP database from the National Center for Biotechnology Information (NCBI), six pairs of primers were designed (Table S2) to amplify genomic DNA fragments covering 30 SNPs (Table S1). The PCR reaction mixture included 50 ng of DNA, 1 μL each of forward and reverse primers (10 μM), and Hotstar Taq DNA Polymerase (Qiagen GmbH, Hilden, Germany), with ddH_2O added to bring the total volume to 25 μL. The PCR conditions used for the amplification of each fragment are shown in Table S1.

Statistical analysis

In the genotype groups (AA, Aa, and aa), the more frequent allele was referred to as the major allele "A," while the other was termed the minor allele "a." All data were analyzed through the homozygous model (AA vs. aa), heterozygote model (AA vs. Aa), dominant model (AA vs. Aa + aa), recessive model (AA + Aa vs. aa), and additive model (AA vs. Aa vs. aa). All allele frequencies in the control group complied with Hardy-Weinberg equilibrium (HWE). The SNPs with call rates < 95%, monomorphic SNPs, and those with a minor allele frequency (MAF) < 0.005 have been filtered out. Allele and genotype frequencies between AS patients and healthy controls were compared using chi-square and Fisher's exact tests, with odds ratios (OR) and 95% confidence intervals (CI) reported. These statistical calculations were performed using SPSS 17.0. In SNP analysis, due to multiple comparisons, we applied the Bonferroni correction by setting α at p = 0.05/6 (one SNP analyzed under allelic, additive, dominant, recessive, homozygous, and heterozygous models). Therefore, the significance threshold was set at 0.0083. D-prime (D')was used to estimate linkage disequilibrium (LD) by comparing observed and expected frequencies of alleles at different loci involved in a haplotype. The block was defined by showing little evidence of historical recombination in the region [22]. LD data were generated using Haploview 4.2 (https://www.broadinstitute.org/haploview/haploview).

We performed a logistic regression analysis to assess whether SNPs (rs201801072 and rs11571319) and HLA-B27 positivity are independently associated with AS. The variables were coded as follows: rs201801072 (TT = 2, CT = 1, CC = 0), rs11571319 (GG = 2, AG = 1, AA = 0), AS status (test = 1, control = 0), and HLA-B27 positivity (positive = 1, negative = 0). Additionally, we examined the interaction effects between SNPs and HLA-B27 positivity (SNP × HLA-B27 status) on AS risk. A significance threshold of 0.05 was applied for statistical testing.

Results

This study analyzed 32 patients with AS and 32 healthy controls, in which AS patients 94% were HLA-B27 positive, and controls 12.5% were HLA-B27 positive (Table 1). Among the AS patients, 68.8% were male, which is consistent with the known male predominance in AS.

We used the genotype with the largest case numbers as a reference and calculated the OR for developing AS in individuals carrying the minor allele. This approach allowed us to assess the risk associated with the presence of the minor allele in comparison to the reference genotype. The genotype analysis results (Table 2) showed that the one SNP located in the promoter region of the *CD28* gene was associated with AS. Rs201801072 (-880 C > T) was associated with AS according to the additive model (TT vs. TC vs. CC, $p = 0.0001$), the recessive model (TT + TC vs. CC, OR = 0.054, 95% CI = 0.006–0.446, $p = 0.001$), the homozygous model (TT vs. CC, OR = 0.079, 95% CI = 0.009–0.668, $p = 0.005$), and the heterozygous model (TT vs. TC, $p = 0.004$). Additionally, one SNP in the 3 prime untranslated region (3'-UTR) of the *CTLA4* gene, rs11571319 (G > A), was associated with AS according to the additive model (GG vs. GA vs. AA, $p = 0.001$), the dominant model (GG vs. GA + AA, OR = 0.111, 95% CI = 0.034–0.366, $p = 0.0001$), and the heterozygous model (GG vs. GA, OR = 0.117, 95% CI = 0.035–0.387, $p = 0.0002$). In allele analysis, a significant difference in allele frequencies of the *CD28* gene's rs201801072 was observed between AS patients and control subjects, with the T-allele being significantly more frequent in AS patients than in controls. Additionally, in the *CTLA4* gene, the G-allele of rs11571319 was significantly more frequent in AS patients ($p = 0.0004$, OR = 5.763, 95% CI = 2.014–16.49) (Table 2). The complete analysis data are shown in Table S3.

In the LD analysis chart (Fig. 1), the color of the squares represents the degree of LD between two SNPs. The LD analysis of immune regulatory genes shows that there is one haplotype block in the *CTLA4* gene, consisting of 7 SNPs. Among these, 5 are located in the promoter region, 1 in exon 1, and 1 in 3'-UTR, including rs11571315, rs733618, rs11571316, rs62182595, rs5742909, rs231775, and rs3087243. The AS-associated SNP, rs3181096, rs3181097, rs201801072, and rs11571319, were not included in this block.

Logistic regression analysis (Table 3) was performed to investigate whether SNPs are associated with AS independently of HLA-B27 and whether SNPs associated with AS exhibit an interaction effect with HLA-B27 positivity. The results showed a significant positive interaction between the T-allele of rs201801072 and HLA-B27 positivity, indicating that in HLA-B27 positive individuals, each additional T-allele further increases the risk of AS by 515% (OR = 6.15, 95% CI.=2.85–13.28, $p < 0.001$). Similarly, a significant positive interaction was observed between the G-allele of rs11571319 and HLA-B27 positivity, where each additional G-allele further increases the risk of AS by 1230% (OR = 13.30, 95% CI.=4.80-36.86, $p < 0.001$). Moreover, individuals carrying HLA-B27 had an exceptionally high risk of AS, with an OR of 65.0 (OR = 65.0, 95% CI: 12.1-350.3, $p = 1.19E-06$).

Discussion

We found that the rs201801072 of the *CD28* gene and the rs11571319 of the *CTLA4* gene were associated with AS. Then, we analyzed whether these SNPs were independently associated with AS regardless of HLA-B27 status and gender and whether there was an interaction influencing the risk of AS.

As for rs201801072 (-880 C > T), the CC genotype appeared to have a protective effect against AS, and T-allele carriers had increased susceptibility under several genetic models. Notably, each additional T-allele was associated with a 5.15-fold increase in AS risk. Currently, no studies are showing significant associations between this SNP and AS or other diseases. Given rs201801072 in the promoter region, it should be prioritized for functional studies, such as clustered regularly interspaced short palindromic repeats (CRISPR)-based editing [23], to determine its regulatory impact on *CD28* expression.

Table 2 SNPs significantly associated with ankylosing spondylitis

Gene	SNP		Allele		genotype			Model	*p*-value	OR (95% CI)
			major	minor (MAF)	*n* (%)					
CD28	rs201801072		T	C	T/T	C/T	C/C	Additive	0.0001*	NA
		AS	52	12	21 (51.2)	10 (100)	1 (7.7)	Recessive	0.001*	0.054 (0.006–0.446)
		Control	40	24	20 (48.8)	0 (0)	12 (92.3)	Homozygous	0.005*	0.079 (0.009–0.668)
			92 (71.9)	36 (28.1)				Heterozygous	0.004*	NA
								T-allele	0.018	2.600 (1.161–5.823)
CTLA4	rs11571319		G	A	G/G	G/A	A/A	Additive	0.001*	NA
		AS	59	5	27 (69.2)	5 (20.8)	0 (0)	Dominant	0.0001*	0.111 (0.034–0.366)
		Control	43	21	12 (30.8)	19 (79.2)	1 (100)	Heterozygous	0.0002*	0.117 (0.035–0.387)
			102 (79.7)	26 (20.3)				G-allele	0.0004*	5.763 (2.014–16.49)

Additive: AA vs. Aa vs. aa; Dominant: AA vs. Aa + aa; Recessive: AA + Aa vs. aa; Homozygous: AA vs. aa; Heterozygous: AA vs. Aa

AS ankylosing spondylitis, *SNP* single nucleotide polymorphism, *OR* odds ratio, *CI* confidence interval, *NA* not applicable, *MAF* Minor allele frequency

*$p < 0.0083$. Bonferroni correction was applied, with the significance threshold set at $\alpha = 0.0083$

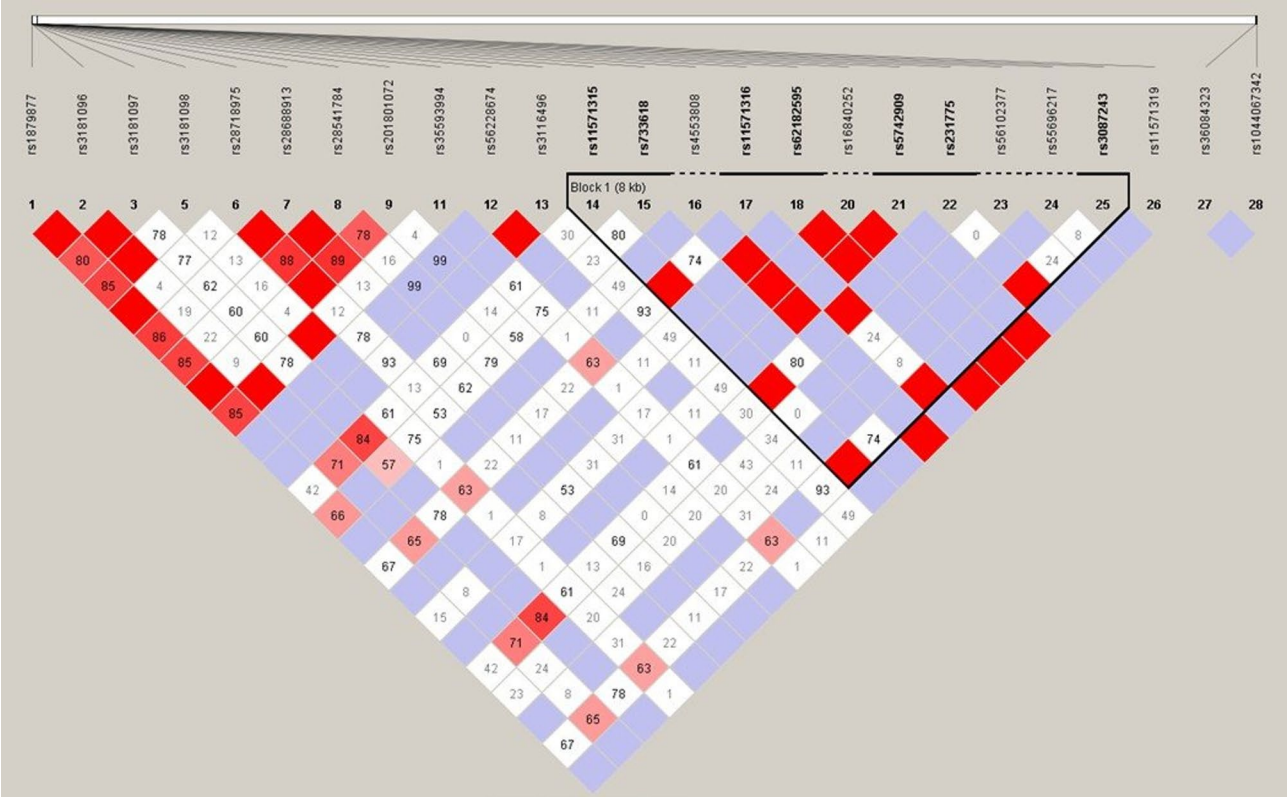

Fig. 1 Linkage disequilibrium (LD) analysis of receptor genes in the CD28, CTLA4, and PDCD1 gene. The color gradient within the boxes transitions from white to red, indicating increasing strength of LD. Purple indicates the absence of LD

Table 3 Logistic regression analysis of the independent associations and interactions of SNPs, gender, and HLA-B27 positivity with AS

variant	OR (95% CI.)	*p*-value
rs201801072_T-allele	3.55 (1.22–10.30)	0.020*
rs11571319_G-allele	8.71 (2.67–28.43)	0.004*
B27 positive	65.0 (12.1-350.3)	1.19E-06*
rs201801072_T-allele * B27 positive	6.15 (2.85–13.28)	3.83E-06*
rs11571319_G-allele * B27 positive	13.30 (4.80-36.86)	6.43E-07*
gender	11.88 (3.54–39.93)	6.29E-05*
rs201801072_T-allele * male	4.05 (1.77–9.23)	0.001*
rs11571319_G-allele * male	4.72 (2.24–9.99)	4.77E-05*

OR odds ratio, *CI* confidence interval

* $p < 0.05$

The rs11571319 (G > A) in the 3'-UTR region of the *CTLA4* gene showed a significant association with AS when comparing the GA + AA genotypes to the GG genotype, suggesting that carrying at least one A-allele was protective against AS. This finding aligns with prior studies implicating the G-allele in increased risk for autoimmune diseases such as SLE, RA, and Graves' ophthalmopathy [20, 21, 24]. Additionally, previous studies have also reported a higher frequency of the GG genotype in rs11571319 among patients with axial spondyloarthropathies in Morocco [25]. These results suggest that rs11571319 may influence disease susceptibility through mechanisms of immune regulation.

To further evaluate these associations, we also analyzed the SNPs rs201801072 in *CD28* and rs11571319 in *CTLA4*, and the results showed that these genetic variants were significantly associated with the risk of AS, with their effects being independent of HLA-B27. Furthermore, we observed a significant positive interaction between these SNPs and HLA-B27 positivity, which further increased the risk of AS. Each additional T-allele of rs201801072 was associated with a 515% increase in the risk of AS, while the effect was even more pronounced for rs11571319, where each additional G-allele increased the risk by 1230%. These findings suggest that these SNPs are independently associated with AS and highlight an

additive effect between these SNPs and HLA-B27 status in contributing to genetic susceptibility to the disease. This pattern is consistent with findings in the Taiwanese population, where specific *ERAP1* variants, such as *ERAP1-001*, significantly promote the development of AS in the presence of HLA-B27 [26]. Additionally, HLA-B27 itself exhibited an exceptionally strong association with AS, with HLA-B27 positive individuals having a 65-fold risk (OR = 65.0, 95% CI: 12.1-350.3, $p = 1.19E-06$), further reinforcing its role as the major genetic risk factor for AS.

Although there is a mismatch in age between the experimental and control groups, it is important to note that most AS patients present between the ages of 16 and 40, with a peak incidence between 20 and 30 years [1, 2]. Therefore, the age difference between the groups is within the typical age range for AS onset and is unlikely to significantly affect the relevance of the genetic associations observed in this study. Due to the gender imbalance between cases and controls, particularly the limited number of male participants in the control group, we did not further analyze gender-SNP interaction in this study. Future studies with gender-matched controls are warranted to evaluate potential sex-specific genetic associations. These findings underscore the impact of these SNPs on the risk of AS. Furthermore, these were preliminary data due to this study's sample size is relatively small, and susceptibility genes for AS may vary across different populations.

Despite the exploratory nature of this study, the identification of significant associations provides valuable insights into potential genetic contributions to AS, paving the way for future large-scale investigations. Further investigation with larger sample sizes and in diverse populations is necessary to better understand the role of these SNPs in AS susceptibility and to identify population-specific genetic markers.

Conclusion

SNP analysis revealed that three SNPs in the *CD28* gene and one SNP in the *CTLA4* gene are associated with the development of AS. Notably, the SNP rs201801072 in the *CD28* promoter region and rs11571319 in the 3' untranslated region of the *CTLA4* gene showed independently significant associations with AS, and these SNPs had a positive interaction effect on AS with HLA-B27 and gender. This suggested that analyzing SNPs in *CTLA4* and *CD28* genes may predict AS susceptibility. The study highlights that these findings could pave the way for genotype-based precision medicine in AS, enabling personalized treatment options, especially for HLA-B27-negative patients.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12865-025-00720-9.

Supplementary Material 1.

Supplementary Material 2.

Supplementary Material 3.

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Not applicable.

Authors' contributions

D.P.C. studied conception, wrote and reviewed the final draft; G.H.C.W. wrote and reviewed the final draft; C.J.Y. funding acquisition, studied conception, wrote and reviewed the final draft; W.T.L. analyzed, interpreted data, and wrote the manuscript draft; F.P.H. performed the experiments, analyzed, interpreted data, and wrote the manuscript draft; K.H.Y. provided samples and reviewed the final draft. All authors read and approved the final manuscript.

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Data availability

The genetic variation data supporting this study's findings have been submitted to the dbSNP database under submission numbers 2137544612 and 10182363154-10182363181. Upon release, these data can be accessed via the NCBI dbSNP database (https://www.ncbi.nlm.nih.gov/snp/).

Declarations

Ethics approval and consent to participate

This study was conducted in accordance with the principles of the Declaration of Helsinki. Ethical approval was obtained from the Institutional Review Board (IRB) of Chang Gung Memorial Hospital (CGMH), with approval ID 202300764B0. All participants provided written informed consent prior to their inclusion in the study.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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