Immunology Letters 217 (2020) 31–38

Contents lists available at ScienceDirect

Immunology Letters

journal homepage: www.elsevier.com/locate/immlet

Evaluation of *ERAP1* gene single nucleotide polymorphisms in immunomodulation of pro-inflammatory and anti-inflammatory cytokines profile in ankylosing spondylitis

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ARTICLE INFO

Keywords:
Ankylosing spondylitis
Endoplasmic reticulum aminopeptidase 1
Single nucleotide polymorphism
IL-17/IL-23 axis

ABSTRACT

Background: Ankylosing spondylitis (AS) is a prototype of chronic inflammatory arthritis termed seronegative spondyloarthropathies that typically affects the joints. Among the non-Human leukocyte antigen (HLA) loci, the strongest association has been observed with *Endoplasmic reticulum aminopeptidase 1* (*ERAP1*) gene single nucleotide polymorphisms (SNPs). Moreover, the effect of *ERAP1* gene SNPs on the pro-inflammatory and anti-inflammatory cytokines in AS disease has still been poorly elucidated. In this study, we aimed to determine the association of *ERAP1* gene SNPs (rs30187 and rs2287987) with AS risk as well as their effect on the mRNA expression of pro-inflammatory and anti-inflammatory cytokines, with emphasis on the immunoregulation of the IL-17/IL-23 pathway, in an Iranian population.

Methods: We performed Single specific primer (SSP)-PCR for genotyping of 160 AS patients and 160 healthy controls. After isolation of peripheral blood mononuclear cells (PBMCs), total RNA of PBMCs was isolated, complementary DNA (cDNA) was synthesized, and quantitative analyses of mRNA expression of cytokines were performed by Real-time PCR for 40 HLA-B27 positive AS patients and 40 healthy individuals as controls.

Results: It was seen that T allele of rs30187 (OR = 1.54, 95% CI = 1.07–2.22, P = 0.017) and C allele of rs2287987 (OR 1.50, 95% CI 1.05–2.14, P = 0.024) were associated with the risk of AS. Both of these alleles were associated more strongly in the HLA-B27 positive AS patients. There was a significant overexpression of mRNAs of pro-inflammatory (IL-17A, IL-17F, IL-23, TNF-α and IFN-γ), while downregulation of anti-inflammatory cytokines (IL-10 and TGF-β) in PBMCs from 40 HLA-B27 positive AS patients in comparison to controls. AS patients with rs30187 SNP TT genotype expressed mRNA of IL-17A, IL-17F, and IL-23 significantly higher than patents with CT and CC genotypes for this SNP.

Conclusions: This study represented the association of *ERAP1* gene rs30187 and rs2287987 polymorphism with the risk of AS. Additionally, it appears that rs30187 polymorphism may be involved in the immunomodulation of the IL-17/IL-23 pathway in the AS disease.

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https://doi.org/10.1016/j.imlet.2019.10.016
Received 2 March 2019; Received in revised form 21 October 2019; Accepted 24 October 2019
Available online 09 November 2019

1. Introduction

Ankylosing spondylitis (AS) is a prototype of chronic inflammatory arthritis termed seronegative spondyloarthropathies that mainly affects the spine and pelvis in young men. Chronic inflammation in joints causes change of the joint architecture with bone neoformations [1,2]. The unique structural alterations in AS are syndesmophyte formation and ankylosis of the vertebrae [3,4]. However, the link between bone neoformations and inflammation in AS has not yet been fully understood. The prevalence of AS is different in the various populations, and it has been established that geographical and environmental factors impress the prevalence rate. Although genetic and epigenetic contributing factors along with environmental stimuli have been blamed in the disease etiopathogenesis, however, the exact underlying mechanisms have been elusive [5–7,48].

Genetic susceptibility to AS disease became obvious in 1973 with the recognition of its relation with HLA-B27. However, it is currently known that genes outside the HLA region also contribute to AS susceptibility. Recently, genome-wide association studies (GWAS) have provided novel insights into the AS immunopathogenesis, such as identification of new variants responsible for AS predisposition other than HLA-B27, including *Endoplasmic reticulum aminopeptidase* (*ERAP*) *1 and ERAP2*, *interleukin* (*IL*) *1* gene cluster, *IL1 receptor 1* (*IL1R1*), *IL6R*, *IL1R2*, *IL23R*, *IL12B*, and *IL7R* [8,9]. Among the non-HLA loci, the second most important gene has been established to be *ERAP1* gene, contributing to 30% of AS risk [10–12].

ERAP1 is encoded by *ERAP1* gene, located on chromosome 5q15, and belongs to the M1 family of aminopeptidases in the Endoplasmic reticulum (ER). ERAP1 plays a role in a variety of innate and adaptive immune response [12]. The difference in the activity of ERAP1 enzyme is probably due to its different intracellular localizations. ERAP1 is mainly localized in the ER in human cells [13]. However, ERAP1 also might be released into the extracellular region or be expressed on the external layer of the cell membrane [14,15]. One of the roles of *ERAP1* is a “molecular ruler” that is effective in the N-terminal proteolysis of peptides to trim them for a length optimum for loading onto major histocampatiblity complex (MHC) class I molecules [16,17]. Additionally, *ERAP1* might be involved in the formation of the soluble forms of pro-inflammatory cytokine receptors, such as Tumor necrosis factor receptor 1 (TNFR1), IL6R, and type II IL-1 decoy receptor (IL-1RII), hence preventing cytokine signalling [18,19]. This function of ERAP1 is known as the “receptor sheddase”. On the other side, the involvement of IL-23/T helper (Th) 17 axis has been hypothesized in the pathogenesis of AS. Defective function of ERAP1 may culminate in an aggregation of aberrant structures of HLA-B27 in ER, resulting in activation of an unfolded protein response (UPR) pathway in immune cells from AS patients. UPR pathway activation in macrophages from AS patients causes upregulation of IL-23 [20,21], which can bind to IL-23R on $CD4^+$ T cells, leading to expansion of pathogenic Th17 cells [22].

A bulk of studies has disclosed that polymorphisms in the *ERAP1* gene have potential roles in the inflammatory pathways in AS disease. Substitution of amino acids in ERAP1 may lead to alterations in the aminopeptidase activity [23]. These substitutions may also lead to the dysregulation of peptide processing and modulation of immune cells through TNF, IL-1, and IL-6. The supposed potential of *ERAP1* variant substitutions to generate unusual peptides is considered as the aminopeptidase influence on AS progression, which demonstrates remarkable impression of genetic variations in *ERAP1* gene on the risk of AS [24]. The effects of genetics, like polymorphisms in *ERAP1* gene, in AS development may vary among the various ethnic groups worldwide [8,25]. Hence, identification of genetic variations in different populations may be essential in precisely determination of AS pathogenesis [26]. However, the role of *ERAP1* variants in impressing the important inflammatory immune pathway of IL-17/IL-23 has not been explored. Therefore, the current study aimed first to determine the association of alleles and genotypes in the *ERAP1* gene SNPs (rs30187 and rs2287987) with the risk of AS in an Iranian population; second, we attempted to observe if such variants act as immunomodulatory factors in the regulation of the IL-17/IL-23 pathway in AS.

2. Materials and methods

2.1. Patients and healthy controls

This case-control study was conducted between November 2015 and April 2017. All subjects submitted their written informed agreement forms for this study and the Ethical Review Board in Tabriz University of Medical Sciences, Tabriz, Iran (Permission Number: TBZMED.REC.1395.1360) approved the study protocol. The study group consisted of 160 Iranian patients with AS, recruited from Rheumatology clinic of Imam Reza Medical Research and Training Hospital of Tabriz, and 160 normal healthy controls. The examination of AS was based on the 1984 modified New York Criteria [27] and diagnosis was conducted by a qualified rheumatologist (Mehrzad Hajaliloo: Connective Tissue Research Center, Tabriz University of Medical Science, Iran). The control group was composed of 160 age-, gender-, and ethnically-matched healthy individuals without a personal or family history of AS and other autoimmune diseases. The prevalence of HLA-B27 was determined in the collected normal healthy controls and AS patients. Bath AS Disease Activity Index (BASDAI) and Bath AS Functional Index (BASFI) were used to assess the disease activity and physical function of the AS patients. Patients’ baseline characteristics, laboratory parameters, and medications are presented in detail in Table 1.

2.2. PBMC isolation and DNA extraction

About 10 ml of fresh whole blood from each participant was collected into tubes containing EDTA. After diluting the whole blood samples with PBS, PBMCs were isolated using Ficoll/Hypaque 1.077 g/ml (Lymphodex, inno-Train, Kornberg, Germany) density-gradient centrifugation and immediately were stored at -80 °C. Genomic DNA of whole blood samples from AS subjects and healthy controls were extracted using the QIAamp DNA Mini Kit (Qiagen, Hilden Germany),

Table 1
Clinical characteristics and Demographic of patients with AS and controls.

Characteristics	AS Patients (N = 160)	Healthy controls (N = 160)	*P* value
Age, mean (range) years	38.5 (23-62)	36.1 (23-59)	> 0.05
Female/male, N (%)	20/140 (87.5)	20/140 (87.5)	> 0.05
HLA-B27 Positive, N (%)	124 (77.5)	10 (6.2)	< 0.001
Disease duration (range) years	10.5 (1-40)	–	–
Family history, N (%)	47 (29.3)	–	–
CRP, (mg/L) Mean ± SD	3.7 ± 4.6	1.5 ± 1.1	< 0.001
BASDAI score, mean (range)	4.41 (0.3-8.6)	–	–
BASFI score, mean (range)	3.17 (0.2-9)	–	–
BASMI score, mean (range)	3.89 (2.1-7.8)	–	–
BASG score, mean (range)	5.03 (0.1-10)	–	–
ASQoL score, mean (range)	7.1 (1-18)	–	–
Arthritis medications			–
NSAIDs	111	–	
Immunosuppressants	–	–	
Biological agents	8	–	

AS: ankylosing spondylitis; CRP: C- reactive protein; BASDAI: Bath Ankylosing Spondylitis Disease Activity Index; BASFI: Bath Ankylosing Spondylitis Functional Index; BASMI: Bath Ankylosing Spondylitis Metrology Index; BASG: Bath Ankylosing Spondylitis global; ASQoL: Ankylosing Spondylitis quality of life; NA: not applicable; NSAIDs: non-steroidal anti-inflammatory drugs. The *t*-test and *Chi square* used to calculate any statistical differences in age, gender, HLA-B27 status, disease duration and CRP level.

according to the manufacturer's protocols and wsd stored at -20 ℃. The DNA concentration was evaluated with Nanodrop (Thermo Scientific, Wilmington, USA).

2.3. RNA extraction and cDNA synthesis

Total cellular RNA extraction from PBMCs was performed using Trizol total RNA extraction kit (GeneAll, Korea) according to the company's protocol. The synthesize of the complementary DNA (cDNA) from the RNA of cells was carried out using the BioFact™ RT Series cDNA Synthesis Kit (Daejeon, Korea) according to the manufacturer's guidelines. Reverse transcription for cDNA production was conducted in the final volume of 20 μL pre tube as follows; first, 5 μL of total RNA was mixed with 1 μL of random hexamer primer and 1 μL of oligo dT primer and 3 μL of RNase-free H_2O, and then incubated at 65 ˚C for 5 min. Microtubes were then chilled on ice, followed by adding a reverse transcription (RT) pre-mix10 μL. Samples were then passed an incubation process at 25 ˚C for 5 min followed by 50 ˚C for 50 min. The reaction was terminated by heating at 95 ˚C for 5 min.

2.4. Primer design

The sequences of *ERAP1* and cytokines gene were picked up from the National Center for Biotechnology Information (NCBI; https://www.ncbi.nlm.nih.gov/) and Ensembl (http://asia.ensembl.org/) databases. For two candidate non-synonymous coding SNPs of the *ERAP1* gene, including rs30187 and rs2287987, the primer pairs were designed using OLIGO7 Software (Molecular Biology Insights, Inc., Cascade, CO., USA) and purchased from Metabion company (Germany). The primer sets used for quantification of cytokines genes were designed using online primer design tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). The sequence of the HLA-B27-specific primers was obtained from Faner et al. [28] study. The sequences of the primer sets and specification for the SNPs are listed in the supplementary Table 1.

2.5. Genotyping for ERAP1 gene polymorphisms and HLA-B27 detection

Two candidate SNPs from *ERAP1* gene, including rs30187 and rs2287987 were selected based on the SNP locations (chromosome position, regulatory and coding regions, and functional relevance in the previous studies) and analyzed by SSP-PCR assay. "PCRBIO Taq Mix Red" (Biosystems, United Kingdom) was used for PCR amplification. PCR reactions were carried out in a SimpliAmp Thermal Cycler (Applied Biosystems, New Jersey, USA) with a total volume of 25 μL (containing 3 μL of DNA, 12 μL of PCRBIO Taq Mix Red, 0.5 μL of one sequence-specific forward primer (for allele C or T), 0.5 μL of the common reverse primer, and 9 μL PCR grade dH_2O). The therocycling conditions were as follows; initial denaturation for 1 min at 94 ℃, followed by 40 cycles for 15 s at 94 ℃, annealing at 63 ℃ for 15 s, and extension at 72 ℃ for 30 s.

HLA-B27 typing for case and control groups was done using amplification by conventional PCR. PCR reactions were carried out in a total volume of 25 μL, with an initial denaturation step at 94 ℃ for 1 min, followed by 40 cycles at 94 ℃ for 15 s, annealing at 60 ℃ for 15 s, and extension at 72 ℃ for 30 s. Primers of β-actin (ACTB) were used as the internal control. Electrophoresis was performed on a 2% agarose gel (Fermentas, USA) for visualizing the PCR products.

2.6. Real-time gene expression analysis

Quantitative analyses of mRNA expression of the cytokines were carried out by Real-time PCR by the SYBR Green PCR Master mix (Ampliqon, Denmark) and Rotor-Gene Q Real-time PCR System machine (Qiagen, USA). Among the 160 AS and healthy subjects, mRNA expression analysis was conducted on 40 HLA-B27 positive AS patients and 40 healthy individuals as controls. The content of each reaction mixture in a total volume of 25 μL was master mix 13 μL, cDNA 4 μL, forward and revers primer 1 μL each, and H_2O 6 μL.. The PCR conditions were as follows: 50 ˚C for 2 min, 95 ˚C for 10 min, then 40 cycles of 95 ˚C for 30 s, 60 ˚C 30 s, and 72 ˚C for 20 s. The comparative C_T method was applied to calculate the relative gene expression. The relative amount of target cytokine mRNAs was normalized to the corresponding 18srRNA mRNA level as the housekeeping gene. The relative expression for each sample was measured using the $2^{-\Delta Ct}$ equation.

2.7. Statistical analysis

Statistical analysis was performed using SPSS software version 22.0 (SPSS, Chicago, IL, USA). The association between *ERAP1* gene rs30187 and rs2287987 SNPs and risk for AS was assessed by calculating the odds ratio (OR) and the corresponding confidence interval (CI 95%). The distribution of the genotypes of *ERAP1* polymorphisms in controls was analyzed using the Hardy-Weinberg equilibrium (HWE). Allelic and genotypic associations of the SNPs were performed by the chi-square (χ^2) test or Fisher's exact test followed by OR and 95% CI calculation with binary logistic regression method. The Kolmogorov-Smirnov test was used to evaluate whether the variables were normally distributed. The pairwise linkage disequilibrium (LD) ($r^2 > 0.8$) was determined using the SHEsis online website (http://shesisplus.bio-x.cn/SHEsis.html). Student *t*-test or analysis of variance (ANOVA) test was used to evaluate the difference of mRNA expression of cytokines profiles. The graphs were designed by Graphpad Prism software version 7.0 (Graph Pad Prism; San Diego, CA, USA). *P* values less than 0.05 were considered as statistically significant.

3. Results

3.1. Subject selection and clinical characteristics

The patient group was consisted of 140 males and 20 females, with a mean age of 38.5 ± 8.9 years (Table 1). The control groups included 140 males and 20 females and had a mean age of 36.1 ± 9.6 years. Therefore, patients and controls were age- and sex- (and ethnically) matched. From 160 AS patients and 160 controls, the HLA-B27 positivity rate was 124 (77.5%) and 10 (6.2%), respectively. HLA-B27 positivity and C-reactive protein level were significantly higher in AS patients than controls ($P < 0.001$).

3.2. Allele and genotype frequencies

The distribution of the genotypes for rs30187 and rs2287987 in healthy individuals did not show significant deviation from the HWE ($P = 0.99$ and 0.62, respectively). The results of the genotypic and the allelic frequency analysis are shown in Tables 2 and 3.

The C allele was the reference for rs30187 SNP, and T allele was reported as the minor global allele according to the SNP database of NCBI (https://www.ncbi.nlm.nih.gov/snp). The T allele of rs30187 SNP was observed more frequently in AS patients relative to the controls (29.1% *vs*. 20.9%). There was a significant association between rs30187 T allele in all AS subjects (OR = 1.54; 95% CI = 1.07–2.22; $P = 0.017$). However, the association was stronger when only the HLA-B27 positive AS subjects were compared with healthy controls (31.1% *vs*. 20.9%; OR = 1.70, 95% CI = 1.16–2.48; $P = 0.006$; Table 3). The CC genotype was the reference for the rs30187 SNP. The TT genotype in rs30187 SNP was prevalent in AS group than controls (8.1% *vs*. 4.4%). As the dominant genotype, the CT + TT model indicated significantly different frequency between HLA-B27 positive AS subjects and healthy controls (52.4% *vs*. 37.5%; OR = 1.83, 95%CI = 1.14–2.95; $P = 0.012$, Table 3).

The T allele was the reference for rs2287987 SNP, and C allele was reported as a minor global allele. There was a significant difference of rs2287987 C allele frequency between all AS subjects and healthy

Table 2
The distribution of allele and genotype frequencies of ERAP1 gene polymorphism in all AS patients and healthy controls.

dbSNP	Allele /Genotype	AS (n = 160) N%	Control (n = 160) N%	OR (95% CI*)	*P*	Adjusted *P***
rs30187 [C/T]	T	93 (29.1)	67 (20.9)	1.54 (1.07-2.22)	0.017*	–
	C (Reference)	227 (70.9)	253 (79.1)	–	–	–
	TT	13 (8.1)	7 (4.4)	1.93 (0.75-4.97)	0.17	0.17
	CT	67 (41.9)	53 (33.1)	1.45 (0.92- 2.29)	0.106	0.159
	CT + TT	80 (50)	60 (37.5)	1.66 (1.06-2.60)	0.024*	0.072
	CC (Reference)	80 (50)	100 (62.5)	–	–	–
HWE			*P* = 0.99			
rs2287987	C	96 (30)	71 (22.2)	1.50 (1.05-2.14)	0.024*	–
[C/T]	T (Reference)	224 (70)	249 (77.8)	–	–	–
	CC	16 (10)	10 (6.2)	1.66 (0.73-3.79)	0.22	0.22
	CT	64 (40)	51 (31.9)	1.42 (0.90-2.25)	0.13	0.195
	CT + CC	80 (50)	61 (38.1)	1.62 (1.04-2.53)	0.032*	0.096
	TT (Reference)	80 (50)	99 (61.9)	–	–	–
HWE			*P* = 0.62			

MAF: minor allele frequency, HWE: Hardy Weinberg Equilibrium, AS: ankylosing spondylitis, OR: odds ratio, CI: confidence interval, dbSNP: database of single nucleotide polymorphisms, **FDR correction for multiple comparisons by Benjamini-Hochberg, *P < 0.05.

controls (OR = 1.50; 95% CI = 1.05–2.14; *P* = 0.024). On the other side, C allele increased the risk of the disease with the strong association in HLA-B27 positive AS patients in compared with healthy controls (OR= 1.60, 95%CI= 1.10–2.34; *P* = 0.013; Table 3). The TT genotype was regarded as the reference genotype for rs2287987 SNP. The CC genotype was more frequent in AS patients compared with healthy controls (10% *vs*. 6.2%). The CT + CC model was the dominant genotype, and it demonstrated more frequency in HLA-B27 positive AS patients compared with the control group (53.2% *vs*. 38.1%); the frequency distribution was significant statistically (OR = 1.84, 95% CI = 1.14–2.97; *P* = 0.011; Table 3).

3.3. Haplotypes frequency

With respect to the haplotypic analysis (rs30187 C/T and rs2287987 C/T), the CT haplotype was less frequently found in AS patients as compared to the healthy individuals (45.6% *vs*. 59.6%) and the difference was statistically significant (OR = 0.566, 95% CI = 0.41-0.77, *P* = 0.001; Table 4).

3.4. LD test

Fig. 1 represents the structure of LD block with the order of rs30187 and rs2287987 polymorphisms and the D′ and r^2 scores are demonstreated. Based on to D′ value, the linkage observed between rs30187 and rs2287987 SNPs was 42%. On the other hand, there was no linkage between rs30187 and rs2287987 SNPs concerning r^2 scores (Fig. 1).

3.5. Gene expression analysis

It was observed that mRNA expressions of IL17A, IL-17 F, IL-23, IFN-γ, and TNF-α (Fig. 2) were significantly upregulated in the PBMCs of AS patients compared with that of healthy subjects (Table 5). Nonetheless, among the evaluated anti-inflammatory cytokines, downregulation of IL-10 mRNA in PBMCs of AS patients in comparison to healthy controls was statistically significant (Fig. 2). However, downregulation of TGF-β mRNA in PBMCs of AS patients in comparison to that of healthy controls was not statistically significant (Fig. 2). AS patients with rs30187 SNP TT genotype expressed mRNA of IL-17A (*P* = 0.015; Fig. 3A), 17 F (*P* = 0.0069; Fig. 3B), and IL-23 (*P* = 0.0002; Fig. 3C) significantly higher than CT and CC genotypes. Nonetheless, the expressions of the mentioned cytokines were not statistically significant for rs2287987 SNP (Supplementary Fig. 1). Moreover, BASDAI, BASMI, and BASFI of HLA-B27 positive AS patients with three genotypes were not statistically significant for rs30187 (Supplementary Fig. 2).

4. Discussion

Previous studies have distinctly established the importance of the *ERAP1* variants in the immunopathophysiology of AS. Despite comprehensive studies, the immunopathogenesis of AS remains obscure [29]. It has been suggested that various genetic and immunologic triggers are involved in immunomodulating the Th17/IL-23 axis in AS disease. It was disclosed that *ERAP1* gene rs30187 SNP had significant

Table 3
Allele and genotype frequencies of ERAP1 gene rs30187 and rs2287987 SNPs HLA-B27 Positive AS patients and healthy controls.

dbSNP	Allele /Genotype	AS (n = 124) N%	Control (n = 160) N%	OR (95% CI*)	*P*	Adjusted *P***
rs30187 [C/T]	T	77 (31.1)	67 (20.9)	1.70 (1.16-2.48)	0.006*	–
	C (Reference)	171 (68.9)	253 (79.1)	–	–	–
	TT	12 (9.7)	7 (4.4)	2.34 (0.893-6.13)	0.083	0.097
	CT	53 (42.2)	53 (33.1)	1.50 (0.928-2.44)	0.097	0.097
	CT + TT	65 (52.4)	60 (37.5)	1.83 (1.14-2.95)	0.012*	0.03
	CC (Reference)	59 (47.6)	100 (62.5)	–	–	–
HWE		*P* = 0.98				
rs2287987 [C/T]	C	78 (31.4)	71 (22.2)	1.60 (1.10-2.34)	0.013*	–
	T (Reference)	170 (68.6)	249 (77.8)	–	–	–
	CC	12 (9.7)	10 (6.2)	1.60 (0.670-3.85)	0.28	–
	CT	54 (43.5)	51 (31.9)	1.64 (1.01-2.68)	0.043*	0.071
	CT + CC	66 (53.2)	61 (38.1)	1.84 (1.14-2.97)	0.011*	0.03
	TT (Reference)	58 (46.8)	99 (61.9)	–	–	–
HWE		*P* = 0.91				

AS; ankylosing spondylitis, SNP; single nucleotide polymorphism, OR; odds ratio, 95% CI; 95% confidence interval, HWE; Hardy-Weinberg equilibrium, **FDR correction for multiple comparisons by Benjamini-Hochberg *P < 0.05.

Table 4
Overall haplotype associations of the SNPs according to Haploview.

Block 1 Haplotypes			Frequencies		OR* (95% CI*)	χ2	*P*	Adjusted *P***
Row	rs30187	rs2287987	Hap Freq. (Case) N (%)	Hap Freq. (Control) N (%)				
1	T	C	15 (4.6)	9 (2.8)	1.69 (0.73-3.94)	1.55	0.211	0.441
2	T	T	78 (24.3)	58 (18.2)	1.45 (0.99-2.13)	3.73	0.159	0.198
3	C	T	146 (45.6)	191 (59.6)	0.566 (0.41-0.77)	12.69	0.001	0.003
4	C	C	81 (25.5)	62 (19.4)	1.41 (0.96-2.04)	3.25	0.159	0.198

*OR; odds ratio, 95% CI; 95% confidence interval, ** adjusted *P* values for the Benjamini & Yekutieli (2001) step-up FDR controlling procedure.

Fig. 1. Pairwise linkage disequilibrium (LD) plot patterns (r^2 and D′) for rs30187 and rs2287987 SNPs of the *ERAP1* gene. Left and right diagrams indicate the D′ and r^2 values, respectively. Each block contains values, which range from 0% to 100%, depicting the score of the related LD measures (D′ or r^2) for alleles of rs30187 and rs2287987 SNPs. The higher the score, the greater the possibility of these alleles of different SNPs to be inherited at the same time. Consequently, a given D′ or r^2 value implies to the possibility, through which these alleles, in combination with each other, might affect the risk of AS.

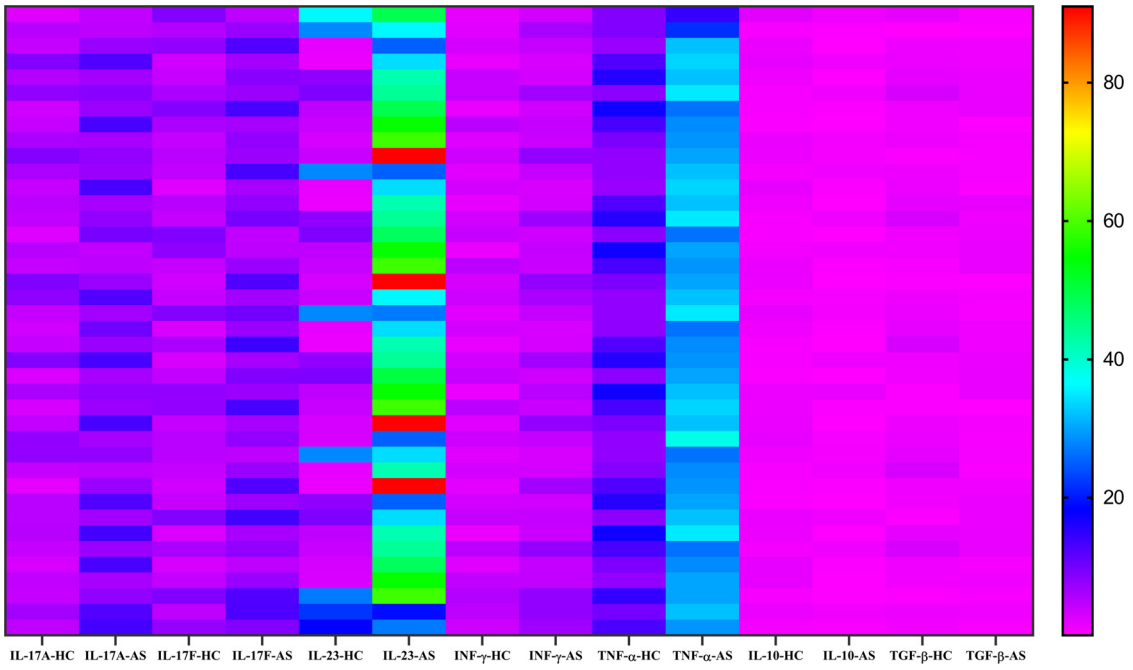

Fig. 2. Relative mRNA expression of pro-inflammatory and anti-inflammatory cytokines genes in HLA-B27 positive AS patients and healthy controls. A heat map of seven cytokines genes (*i.e.*, IL-17A, IL-17 F, IL-23, INF-γ, TNF-α, IL-10, and TGF-β) from HLA-B27 positive AS patients (n = 40) and healthy controls (n = 40). For each gene, an orange signal illustrates underexpression and a red signal illustrates overexpression. The relative amount of target cytokine mRNAs was normalized to the corresponding 18srRNA mRNA level as the housekeeping gene. IL, interleukin; IFN, interferon; TNF, tumor necrosis factor; TGF, transforming growth factor; HC, Healthy control; AS, Ankylosing Spondylitis (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Table 5
Comparison between pro-inflammatory and anti-inflammatory cytokines expression in HLA-B27 positive AS patients and controls.

Cytokines	Fold change (AS patients n = 40 *vs.* Control groups n = 40)	*P* value
Pro-inflammatory		
IL-17A	2.27	0.008*
IL-17 F	2.11	0.001*
IL-23	1.54	0.034*
INF-γ	1.96	0.004*
TNF-α	4.91	0.0001*
Anti-inflammatory		
IL-10	1.14	0.038*
TGF-β	1.35	0.46

AS, Ankylosing Spondylitis; IL, Interleukin; IFN, Interferon; TNF, Tumor Necrosis Factor; TGF, Transforming growth factor (*$P < 0.05$).

allelic associations with AS risk. Importantly, the susceptibility risk genotype of TT for *ERAP1* gene rs30187 SNP was associated with an increased mRNA expression of IL-17A, IL-17 F, and IL-23 in AS patients.

Among non-MHC genetic factors, *ERAP1* is now recognized to be restricted to HLA-B27 positive AS patients. Some of the variants of *ERAP1* have been described to be responsible for 30% of the attributable risk of AS, with *ERAP1* and HLA-B27 together accounting for 70% of the attributable risk for the development of AS [30,31]. The genetic risk of AS has been extensively investigated in the last decades. However, the mechanism by which *ERAP1* predisposes individuals to AS remains unknown. But one theory is that *ERAP1* potentially contributes to the pathogenesis of AS by altering the stability and the antigen presentation pathway mediated by HLA-B27 and, hence, modulating the antigen presentation pathway, leading to inflammatory and pathogenic activation of immune system [32,33]. Another mechanism is shedding and cleavage of cell-surface receptors for proinflammatory cytokines, such as TNFRI, IL-1RII, and IL-6R [34]. Whether cytokine receptor shedding activity of *ERAP1* is involved in AS pathogenesis appears unlikely since studies demonstrated that there were no differences in the serum levels or the soluble receptors of these cytokines between AS patients with the different *ERAP1* polymorphisms and haplotypes [35]. However, polymorphisms in *ERAP1* may induce inflammation by altering both these mechanisms. For example, recently, Chen et al. exhibited that the protective alleles of rs30187 (528R) and rs27044 (730E) variations were associated with reduced HLA-B27 free heavy chain expression in monocytes, while silencing or inhibition of *ERAP1* by siRNA and DG013A were associated with suppressed expansion and responses of Th17 cells [36]. Furthermore, *ERAP1* inhibition may suggest a novel therapeutic option for AS and *ERAP1* inhibitors are recently in the development process [37].

Studies have shown the association of the IL-23/Th17 immune axis with AS pathogenesis. Increased serum concentrations of IL-23 and IL-17 have been reported in patients with AS [38]. It has been theorized that aberrant folding of HLA-B27 and the UPR pathway activation in ER can be responsible for a high level of IL-23 expression in AS patients and the activation and differentiation of IL-17 producing Th17 cells [10]. In current study, we represnted that the patients carrying the susceptibility TT genotype of *ERAP1* gene rs30187 SNP had increased IL-17A, IL-17 F, and IL-23 mRNA expressions in PBMCs.

Recently, studies reported that polymorphisms in *ERAP1* were associated with the risk of autoimmune diseases, such as Crohn's disease [39], Behçet's disease [40] and AS [41]. Lee et al. demonstrated that the rs30187 and rs2287987 polymorphisms of *ERAP1* were associated with the risk of AS in Europeans and East Asian populations [42]. According to this meta-analysis study performed in 2016, the minor C allele of rs2287987 was associated with decreased AS risk in overal as well as sub-group analysis in european population. However, we detected that the minor C allele of this SNP was associated with increased AS risk. This discrepancy might be due to difference in the genetic stratification across various populations. Additionally, Mahmoudi et al. exhibited for the first time in Iranian AS patients that the rs30187, rs27434, and rs13167972 polymorphisms were significantly associated with the risk of AS [43]. Our previous studies also represented the association of *ERAP1* gene rs27044, rs10050860, and rs27038 SNPs with the risk of AS in an Iranian population [44–46]. In the current study, we reported that the minor T allele of rs30187 SNP, as well as the minor C allele of rs2287987 SNP, were associated significantly with increased risk of AS; this association was even stronger in the HLA-B27 positives AS patients. Hence, the role of *ERAP1* polymorphism and HLA-B27 interaction should be further explored.

This study is the first report about the association between *ERAP1* rs2287987 polymorphism and AS risk in an Iranian population. We observed that there was significant association between rs30187 C allele and AS risk. However, the CT haplotype was less frequently found in the AS patients than controls and, therefore, decreased the risk of AS. Besides, it has been shown that these risk alleles cause a significant increase in the aminopeptidase activity of ERAP1 enzyme [47]. In the present study, the presence of the rs30187/rs2287987 haplotype CT for *ERAP1* was associated with a reduced risk in AS patients.

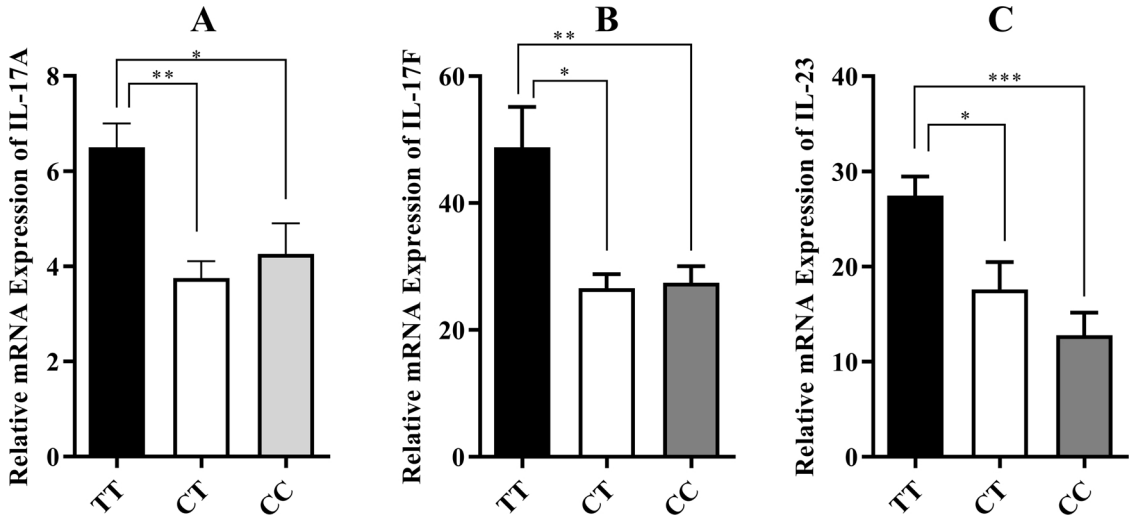

Fig. 3. The mRNA expression levels of cytokines in the peripheral blood mononuclear cells (PBMCs) in HLA-B27 positive AS patients. The mRNA expressions of IL-17A, IL-17 F, and IL-23 in PBMCs of HLA-B27 positive AS patients (n = 40) with three genotypes for rs30187 SNP are indicated in figures A–C, respectively (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

In conclusion, our study represented that *ERAP1* gene rs30187 and rs2287987 polymorphisms conferred a risk to AS susceptibility in an Iranian population. The rs30187 SNP was observed to be involved in the immunomodulation of the inflammatory immune pathway of IL-17/IL-23 in AS patients. However, the gap between *ERAP1* genetic polymorphisms and increased IL-23 and, therefore, an expansion of Th17 cells response requires to be filled. This could be contributing in designing novel therapeutic strategies by immunomodulating the *ERAP1* enzymatic activity and, therefore, improvement of patients *via* relieving the inflammation.

Declaration of Competing Interest

The authors confirm that this article content has no conflict of interest.

Acknowledgments

This study is supported by Immunology Research Center, Tabriz University of Medical Sciences, Tabriz, Iran (Grant No. 58988).

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.imlet.2019.10.016.

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