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Evaluation of *ERAP1* Gene Single Nucleotide Polymorphism in Impressing the Inflammatory Cytokine Profile of Ankylosing Spondylitis Patients

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ABSTRACT

Ankylosing spondylitis (AS), an autoinflammatory disease, has been associated with impaired Endoplasmic reticulum aminopeptidase (ERAP) 1 activity, which is involved in priming antigenic peptides. The purpose of this study was to evaluate if the genetic variant of *ERAP1* gene could impress the inflammation status of the AS patients.

For genotyping, 140 AS cases and 140 healthy controls were enrolled. After isolation of peripheral blood mononuclear cells (PBMCs) and DNA extraction, all the subjects were genotyped for rs27044 polymorphism using SSP-PCR assay. Total RNA of PBMCs was isolated, cDNA was synthesized, and quantitative analyses of mRNA expression of cytokines were performed via Real-time PCR using the SYBR Green Gene Expression MasterMix. To measure the concentration of cytokines in serum of subjects, ELISA was used.

It was observed that the G allele of rs27044 polymorphism was significantly prevalent in AS patients. Moreover, the GG genotype and the GG+GC dominant model had significantly different distribution between study groups. There was a significant overexpression of mRNAs of IL-17A, IL-6, IL-33, TNF-α, and IFN-γ, while IL-10 was significantly downregulated in AS patients. The ELISA results were in line with that of the gene expression analysis. No significant differences in mRNA expression and concentration of cytokine were identified among AS patients with three genotypes for rs27044 SNP.

This study replicated the association of polymorphisms in *ERAP1* gene with the risk of AS in a population from Iranian. However, it did not directly determine the inflammatory profile of the AS patients.

Keywords: Ankylosing spondylitis; Cytokine; Endoplasmic reticulum aminopeptidase; Gene expression; Polymorphisms

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INTRODUCTION

Ankylosing spondylitis (AS), a type of spondyloarthropathies (SpA) group, is an inflammatory disorder, which is defined by major manifestations of sacroiliitis and axial inflammation. The prevalence of AS is approximately 0.1–1.4% in general and affects men more than women.[1] It has been documented that the development of AS stems from a complicated interaction of environmental and genetic contributing factors.[2,3] It has been identified that human leukocyte antigen (HLA)-B27 is one of the cogent genetic factors for AS; nonetheless, it can explain no further than 30 percent of the total genetic risks of AS.[4] As a consequence, a number of genes out of major histocompatibility complex (MHC) region is supposed to be involved in AS pathogenesis. According to previous studies, there have been accumulating results to suggest that non-MHC genes such as *endoplasmic reticulum aminopeptidase* (*ERAP*) *1*, *IL12B*, *interleukin 23 receptor* (*IL-23R*), and *anthrax toxin receptor 2* (*ANTXR2*) are attribute to AS susceptibility.[5,6]

Ankylosing spondylitis is considered by aberrant regulatory pathways of inflammatory cytokines. Although the interleukin (IL)-17/IL-23 axis has repetitively been observed to be the important inflammatory player of AS, several inflammatory cytokines secreted from T helper (Th1) cells, for example interferon (IFN)-γ, IL-17A produced by Th17 cells, and others like IL-6 and tumor necrosis factor (TNF)-α indicate increased levels in blood and joint milieu of AS patients. Along with this increase, a decreased level of immunosuppressive and immunoregulatory cytokines, including IL-10 and transforming growth factor (TGF)-β, has been reported. Other than Th cells, several types of immune cells like natural killer (NK) cells, type 3 innate-like lymphocytes (ILC3), macrophages, and etc., has been blamed as the culprit of inflammatory cytokine source in AS.[7-9]

Arthritogenic peptide hypothesis has been supported by studies demonstrating an association between *ERAP1* gene and AS risk. According to this hypothesis, AS is initiated when a peptide on the surface of antigen presenting cells (APCs) is presented by HLA-B27 molecules. As a result, AS has been underlying the diversity in the HLA-B27 peptide-binding repertoire, which is attributed to variety in antigen-processing components, such as ERAP1 enzyme.[4]

ERAP1 belongs to the M1 family of zinc metallopeptidases, responsible for trimming peptides for HLA class I presentation[10] and also cleave proinflammatory cytokine receptors (TNFR1, IL-1 RII and IL-6 Rα), eventuating in the downmodulation of their signal intensity on the cell surface.[11] Recent findings have indicated association between single-nucleotide polymorphisms (SNPs) in *ERAP1* gene (rs27044) and AS.[12,13] This relation was also confirmed by the studies performed in European and East Asian populations.[14,15] To gain a comprehensive understanding of the susceptibility variants in AS, the results replicated in other ethnic groups could be critical.

ERAP1 is involved in impaired biology of antigen presentation in AS that could result in activation of immune cells to produce inflammatory cytokines.[7] Hence, in this study, we first attempted to evaluate the association between *ERAP1* gene polymorphism (rs27044) and risk of AS in an Iranian population. In the next step, it was the purpose of this study to survey if the inflammatory profile of AS patients could be under impression of *ERAP1* gene polymorphism.

MATERIALS AND METHODS

Patients and Healthy Controls Subjects

This case-control study was done at the Immunology Research Center of Tabriz University of Medical Sciences in Iran between November 2015 and April 2017. All subjects submitted their written informed consent forms, and the Ethical Review Board centrally approved this study protocol in Tabriz University of Medical Sciences, Tabriz, Iran (Permission No. TBZMED.REC.1394.12937). The study group consisted of 140 Iranian patients with AS [120 (85.71%) males and 20 (14.29%) females, age range of range 23-62 years] and 140 healthy normal controls (Table 1). Examination of AS was based on the 1984 modified New York Criteria for AS and diagnosis was carried out by a qualified rheumatologist.[16] The control group encompassed 140 age, gender, and ethnically matched healthy individuals (85.71% males versus 14.29% females) without personal or family history of AS or other autoimmune diseases. The HLA-B27 status was determined in all of the participants.

Table 1. Demographic and clinical manifestations of ankylosing spondylitis patients and healthy individuals used for genotyping of *ERAP1* gene rs27044 SNP and cytokine's gene expression

Characteristic	AS Patients (n=140)	Healthy controls (n=140)	*p* value
Age (years)	37.41±8.21	35.8±6.10	>0.05
Female/Male, n (%)	20 (14.3%)/120 (85.7%)	20 (14.3%)/120 (85.7%)	>0.05
HLA-B27 positive, no (%)	111 (79.28%)	9 (6.4%)	<0.05
CRP (mg/L)	2.81±2.75	1.54±1.08	<0.0001
Disease duration (years)	10.56±7.69	-	-
BASDAI score	4.52±2.34	-	-
BASFI score	3.25±2.33	-	-
BASG score	4.37±2.41	-	-
ASQoL score	7.25±4.74	-	-

AS; ankylosing spondylitis, HLA; human leukocyte antigen, CRP; C-reactive protein, BASDAI; bath ankylosing spondylitis disease activity index, BASFI; bath ankylosing spondylitis functional index, BASG; bath ankylosing spondylitis global score, ASQoL; ankylosing spondylitis quality of life

Table 2. Sequence and amplicon size of the primers used for genotyping of *ERAP1* gene rs27044 SNP and for quantification of cytokine's mRNA expression in ankylosing spondylitis patients and healthy individuals

SNP/Gene name	Sequence of nucleotides (5′–3′)		Amplicon Size (bp)
Genotyping			
rs27044	rs27044-G	CACACAGGCGAGGAGTAGTAGATG	475
	rs27044-C	CACACAGGCGAGGAGTAGTAGATC	
	rs27044- Reverse	GGTACTTGAGGAAGAGGCACAA	
β-Actin	ACTB Forward	GGTCCTCACTGCCTGTCC	140
	ACTB Reverse	CTCGTCATACTCCTGCTTGCT	
HLA-B27	HLA-B27 Forward	TACGTGGACGACACGCT	137
	HLA-B27 Reverse	TGTGCCTTGGCCTTGC	
Growth hormone	GH1 Forward	TGCCTTCCCAACCATTCCCTTA	434
	GH1 Reverse	CCACTCACGGATTTCTGTTGTGTTTC	
mRNA expression			
IL-37	Forward	CCCAGTGCTGCTTAGAAGACC	173
	Reverse	TGCTATGAGATTCCCAGAGTCC	

IL-33	Forward	GAATCAGGTGACGGTGTTGATGG	81
	Reverse	GTTGTTGGCATGCAACCAGAAG	
IL-17A	Forward	CTCTGTGATCTGGGAGGCAAAG	196
	Reverse	GACAATCGGGGTGACACAGG	
IL-10	Forward	AGCTGAGAACCAAGACCCAGAC	100
	Reverse	AAGAAATCGATGACAGCGCC	
IL-6	Forward	AGCCACTCACCTCTTCAGAAC	117
	Reverse	CCTCTTTGCTGCTTTCACAC	
TNF-α	Forward	GCCCCAATCCCTTTATTACCC	72
	Reverse	GCCCCCAATTCTCTTTTTGAGC	
IFN-γ	Forward	GGCAGCCAACCTAAGCAAGA	222
	Reverse	TTGGAAGCACCAGGCATGA	
TGF-β	Forward	GCAACAATTCCTGGCGATACCT	115
	Reverse	TCCCCTCCACGGCTCAAC	
18srRNA	Forward	GATCAGATACCGTCGTAGTTCC	241
	Reverse	CTGTCAATCCTGTCCGTGTC	

In this study, we applied Bath AS Disease Activity Index (BASDAI) and Bath AS Functional Index (BASFI) to assess the disease activity and physical function of the AS patients. Patients' demographics, disease characteristics, including HLA-B27 status, age of symptom onset, BASFI, BASDAI, Bath AS Metrology Index (BASMI), Bath AS Global Score (BASG), and AS quality of life (ASQoL) were described with detail in Table 1. Five mL of peripheral blood was obtained from all the study subjects and the genomic DNA content from whole blood of AS patients and healthy controls was extracted exerting the QIAamp DNA Mini Kit (Qiagen, Germany).

Primer Design

ERAP1 gene sequence and data were obtained from the National Center for Biotechnology Information (NCBI), and Ensembl (http://asia.ensembl.org/) databases. For candidate non-synonymous coding SNP from *ERAP1* gene, namely rs27044, and the related internal control gene (β-Actin) the primer pairs were designed applying the OLIGO7 Software, (Molecular Biology Insights, Inc., Cascade, CO., USA). Moreover, primers for HLA-B27 and the related internal control (Growth hormone 1) were obtained from already published works [17] and are listed in Table 2.

In order to perform quantitative mRNA expression of the cytokines (IL-37, IL-33, IL-17A, IL-10, IL-6, TNF-α, IFN-γ, and TGF-β) as well as the 18srRNA as a housekeeping gene, primers (Table 2) were designed via Primer Express 3.0 (Applied Biosystems, Foster City, CA, USA). To determine the target specificity of primer pairs, they were checked by the Primer BLAST Tool of the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Production of the primers was conducted by the custom oligonucleotide synthesis service (Metabion, Martinsried, Germany).

Genotyping of ERAP1 Gene Polymorphism and HLA-B27 Detection

The candidate non-synonymous coding SNP of *ERAP1* gene, rs27044 (Gln730Glu), was genotyped by

Single Specific Primer-Polymerase Chain Reaction (SSP-PCR) assay (Figure 1.A). "PCRBIO Taq Mix Red" (Biosystems, United Kingdom) was used for PCR amplification; PCR reactions were carried out in a total volume of 25 µL, with the initial denaturation step of 1 min at 94°C, followed by 40 cycles at 94°C for 15s, annealing at 63 °C for 15s and extension at 72 °C for 30s. HLA-B27 typing (Figure 1.B) was done using amplification PCR. PCR reactions were carried out in a total volume of 25 µL, with an initial denaturation step of 1 min at 94°C, followed by 40 cycles at 94°C for 15s, annealing at 60°C for 15s and extension at 72°C for the 30s. Electrophoresis was carried out on a 2% agarose gel. The rate of a genotypes was evaluated by direct counting.

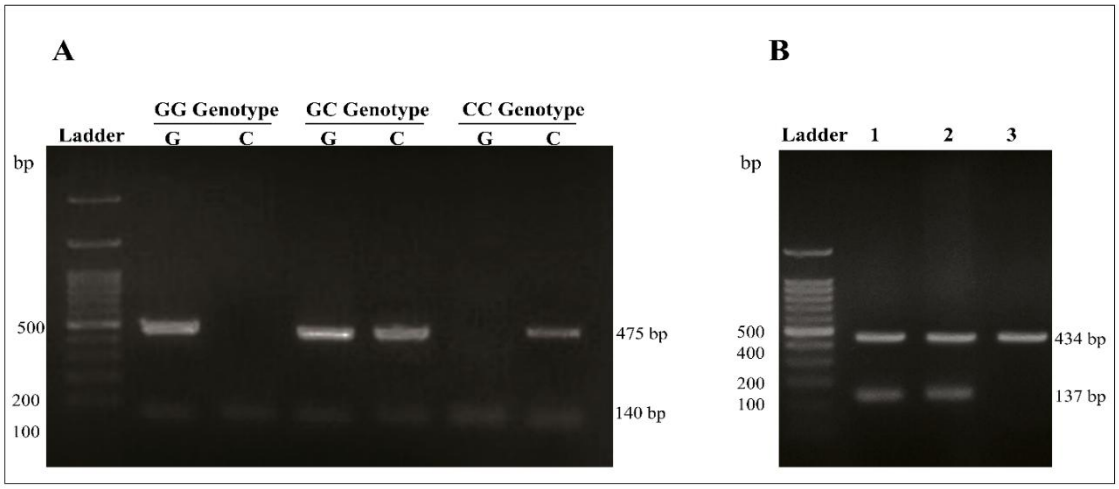

Figure 1. A. Electrophoresis gel imaging for detection of HLA-B27 and *ERAP1* gene rs27044 polymorphism after PCR amplification in DNA samples from ankylosing spondylitis patients and healthy subjects. The detection of genotypes was conducted based on the presence of bands in each lane for G (475 bp) and C (475bp) alleles (the band of internal control, β-Actin, was also demonstrated as 140 bp). B. HLA-B27-related band (137 bp) and the internal control (growth hormone 1 (GH1); 434 bp) are depicted.

PBMC Isolation, RNA Extraction, and cDNA Synthesis

Peripheral blood mononuclear cells (PBMCs) were isolated from fresh whole blood tubes containing EDTA. After diluting with PBS, samples were underlaid using Ficoll/Hypaque 1.077 g/mL (Lymphodex, inno-Train, Kronberg, Germany) density-gradient centrifuged to isolate PBMCs and immediately stored at -80°C until use. RNA extraction from PBMCs was performed using Trizol total RNA extraction kit (GeneAll, Korea) according to company's guidelines. 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 protocol. Reverse transcription for cDNA production was conducted with the final volume of 20 µL pre tube. First, 4 µL of isolated RNA (30 µg) was mixed with 1 µL of random hexamer primer and 7 µL of RNase-free H_2O and then incubated at 65°C for 5 minutes. Then, microtubes were chilled on ice, followed by adding a mixture of reaction buffer 4 µL, RNASe inhibitor 1 µL, dNTP mix 2 µL and reverse transcriptase 1 µL. Samples were then passed an incubation process at 25°C for 5 minutes followed by 42°C for 60 minutes; the reaction was finished by heating at 70°C for 5 minutes.

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 and Rotor-Gene Q Real-time PCR System machine (Qiagen, USA). Among the 140 AS and healthy subjects, mRNA expression analysis was conducted on 75 individuals from each group. The content of each reaction mixture in a total volume of 25 µL was master mix 12.5 µL, cDNA 4.5 µL, forward and revers primer 1 µL each, and H_2O 6 µL. The PCR conditions were as follows: 50 °C for 2 minutes, 95 °C for 10 minutes, then 40 cycles

of 95°C for 30 seconds, 60°C 30 seconds, and 72°C for 30 seconds. The comparative C_T method was applied to calculate the relative gene expression as described by Schmittgen and Livak.[18] 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.

Cytokine Levels in Serum and ELISA

In order to measure the cytokine levels of IL-17A, TNF-α, IFN-γ, IL-6, IL-33, IL-37, IL-10, and TGF-β in the serum samples of AS and healthy control groups, enzyme linked immunosorbent assay (ELISA) was conducted by commercial kit, provided by Invitrogen (Thermo Fisher Scientific, San Diego, CA, USA)

Statistical Analysis

Analysis of data was conducted by SPSS version 24.0 for Windows (IBM Corp., Armonk, N.Y., USA). Data were expressed as either in percentage or mean ± standard deviation (SD). Demographic and clinical manifestations of the study population were assessed by descriptive statistical analysis. For evaluating the normality distribution of data, the Kolmogorov-Smirnov test was used. To compare the clinical data, mRNA expression, and cytokine levels among patients with three different genotypes, the Kruskal-Wallis test was applied. To compare the mRNA expression level of cytokines as well as their level in serum, Mann-Whitney *U*-test was performed. The associations between AS and *ERAP1* gene SNP were analyzed by Logistic Regression and Pearson's chi square ($\chi 2$) to evaluate for significance differences of both allele and genotype frequencies between patients and controls. Odds ratios (OR) and confidence intervals (95% CI) were employed for estimating the risk. *P* values were adjusted by Benjamini-Hochberg Method and regarded statistically significant if they were less than 0.05. Adherence to the Hardy–Weinberg Equilibrium (HWE) was measured using $\chi 2$ test in Package "genetics" of R Software.

RESULTS

Demographic and Laboratory Data

Demographic, laboratory, and clinical specifications of the patients are summarized in Table 1. Most importantly, 111 cases (79.28%) in AS group and 9 cases (6.4%) in healthy control group were HLA-B27 positive. Moreover, CRP level was significantly (p<0.0001) higher in AS cases in comparison to the controls (2.81±2.75 *vs*. 1.54±1.08).

Allele and Genotype Frequencies

The distribution of the genotypes for rs27044 SNP in healthy individuals did not indicate significant deviation from the HWE (p=0.35, Table 3). According to Table 3, the global major C allele was considered as the reference allele for rs27044 SNP according to SNP database of NCBI (https://www.ncbi.nlm.nih.gov/snp). The G allele of this SNP was observed more frequently in AS patients relative to the controls (45.71% *vs*. 34.29%). This allele had significantly different distribution between AS patients and healthy subjects and increased risk of the disease (OR=1.61, 95% bCI= 1.14-2.26; p=0.0059). The CC genotype was the reference and the GG genotype was prevalent in AS group (24.28% *vs.* 13.57%). This genotype increased the risk of AS (OR=2.45, 95% CI=1.24–4.82; p=0.0096). The frequency of GC genotype was higher in the AS patients than in healthy controls (42.86% *vs.* 41.43%) and the frequency difference was not statistically significant (OR=1.41, 95%CI=0.83–2.39; p=0.192). As the dominant genotype, the GG+GC pattern indicated significantly different frequency between the two study groups (67.14% *vs.* 55%; OR=1.67, 95% CI=1.02–2.71; p=0.037).

Association between demographic and clinical data and genotypes

The relation of clinical data, including disease duration, CRP levels, BASDAI, BASFI, BASG, and ASQoL scores with the genotypes of *ERAP1* gene rs27044 SNP are represented in Table 4. There was a statistically significant difference in disease duration (p<0.0001), CRP level (p=0.019), BASFI score (p=0.041), and ASQoL (p<0.0001) among AS patients with three genotypes of GG, GC, and CC.

Gene Expression Analysis

Among the evaluated cytokines, the mRNA expression of IL17A, IL-6, TNF-α, IFN-γ, and IL-10 demonstrated significant difference between cases and controls (Figure 2). It was observed that IL17A (Fold change=2.33, p=0.0009, Figure 2. A), TNF-α (Fold change=2.68, p<0.0001, Figure 2. B), IFN-γ (Fold change=1.55, p=0.033, Figure 2. C), IL-6 (Fold change=1.96, p=0.0023, Figure 2. D), and IL-33 (Fold change=4.83, p=0.0001, Figure 2.E) demonstrated

significant upregulation in the PBMCs of AS patients compared with that of healthy subjects. Nonetheless, upregulation of IL-37 (Fold change=1.13, *p*=0.47, Figure 2.F) mRNAs in PBMCs of AS patients was not statistically significant in comparison to healthy controls. On the other hand, IL-10 mRNA level was downregulated significantly (Fold change=0.5, *p*=0.046, Figure 2.G) in PBMCs of AS patients than healthy subjects.

Moreover, TGF-β demonstrated downregulation in AS patients in comparison to healthy individuals; however, the difference was not statistically significant (Fold change=0.76, *p*=0.42, Figure 2.H).

mRNA expression of cytokines was also evaluated among AS patients with GG, GC, and CC genotypes for rs27044 SNP of *ERAP1* gene. Data demonstrated insignificant differences of mRNA expression of all cytokines in AS cases with three genotypes for rs27044 SNP (Figure 3).

Table 3. Allele and genotype frequencies of *ERAP1* gene rs27044 SNP in ankylosing spondylitis patients and healthy control group and related association analyses

SNP*	Allele /Genotype	AS* (n=140) N%	Control (n=140) N%	OR* (95% CI*)	*p*	Adjusted *P***
rs27044	G	128 (45.71%)	96 (34.29%)	1.61 (1.14 - 2.26)	**0.0059**	**0.019**
	C (Reference)	152 (54.29%)	184 (65.71%)	-	-	
	GG	34 (24.28%)	19 (13.57%)	2.45 (1.24 – 4.82)	**0.0096**	**0.019**
	GC	60 (42.86%)	58 (41.43%)	1.41 (0.83 – 2.39)	0.192	0.192
	GG+GC	94 (67.14%)	77 (55%)	1.67 (1.02 – 2.71)	**0.037**	**0.049**
	CC (Reference)	46 (32.86%)	63 (45%)	-	-	
HWE			*P*=0.35			

*SNP; single nucleotide polymorphism, AS; ankylosing spondylitis, OR; odds ratio, 95% CI; 95% confidence interval, HWE; Hardy-Weinberg equilibrium, ** *P* values for the comparison of both allele and genotype frequencies were adjusted by Benjamini-Hochberg method
The associations between AS and ERAP1 gene SNP were analyzed by Logistic Regression and Pearson's chi square ($\chi 2$)

Table 4. Association of *ERAP1* gene rs27044 SNP with clinical manifestations of ankylosing spondylitis patients

Characteristic	rs27044 (GG)	rs27044 (GC)	rs27044 (CC)	*p*
Disease duration (years)	12.72±3.47	10.24±5.33	9.75±7.36	<0.0001
CRP (mg/L)	3.97 ±2.88	3.31±3.55	1.72±0.97	0.019
BASDAI score	5.11±3.09	4.25±1.71	4.26±1.75	0.428
BASFI score	3.34±2.79	2.51±2.02	3.35±1.48	0.041
BASG score	5.19±3.16	4.36±2.09	4.97±1.32	0.344
ASQoL score	7.26±6.05	5.39±4.61	8.11±3.46	<0.0001

CRP; C-reactive protein, BASDAI; bath ankylosing spondylitis disease activity index, BASFI; bath ankylosing spondylitis functional index, BASG; bath ankylosing spondylitis global score, ASQoL; ankylosing spondylitis quality of life
The Kruskal-Wallis test was used to compare the characteristics among subjects harboring different genotypes

Cytokine Levels in Serum

The results of cytokine concentration in serum of study participants were in accordance with that of mRNA expression (Figure 4). Levels of IL17A (45 vs.23; $p<0.0001$, Figure 4. A), TNF-α (135.2 vs.48.5; $p<0.0001$, Figure 4. B), IFN-γ (153.8 vs. 119.4; $p=0.0098$, Figure 4. C), IL-6 (8.6 vs.2.85; $p=0.0021$, Figure 4. D), and IL-33 (206.1 vs. 133.7; $p=0.0004$, Figure 4. E) were increased significantly in serum samples of AS patients than controls, while IL-10 (2.367 vs. 4.389; $p=0.0035$, Figure 4. G) showed a significantly decreased level. Level of IL-37 was increased (137 vs.124.1; $p=0.39$, Figure 4. F) and TGF-β level was decreased (20.5 vs.24.4; $p=0.15$ Figure 4. H), but both insignificantly.

Evaluation of cytokine levels in serum samples among AS subjects with three genotypes rs27044 SNP of *ERAP1* gene indicated no significand differences (Figure 5).

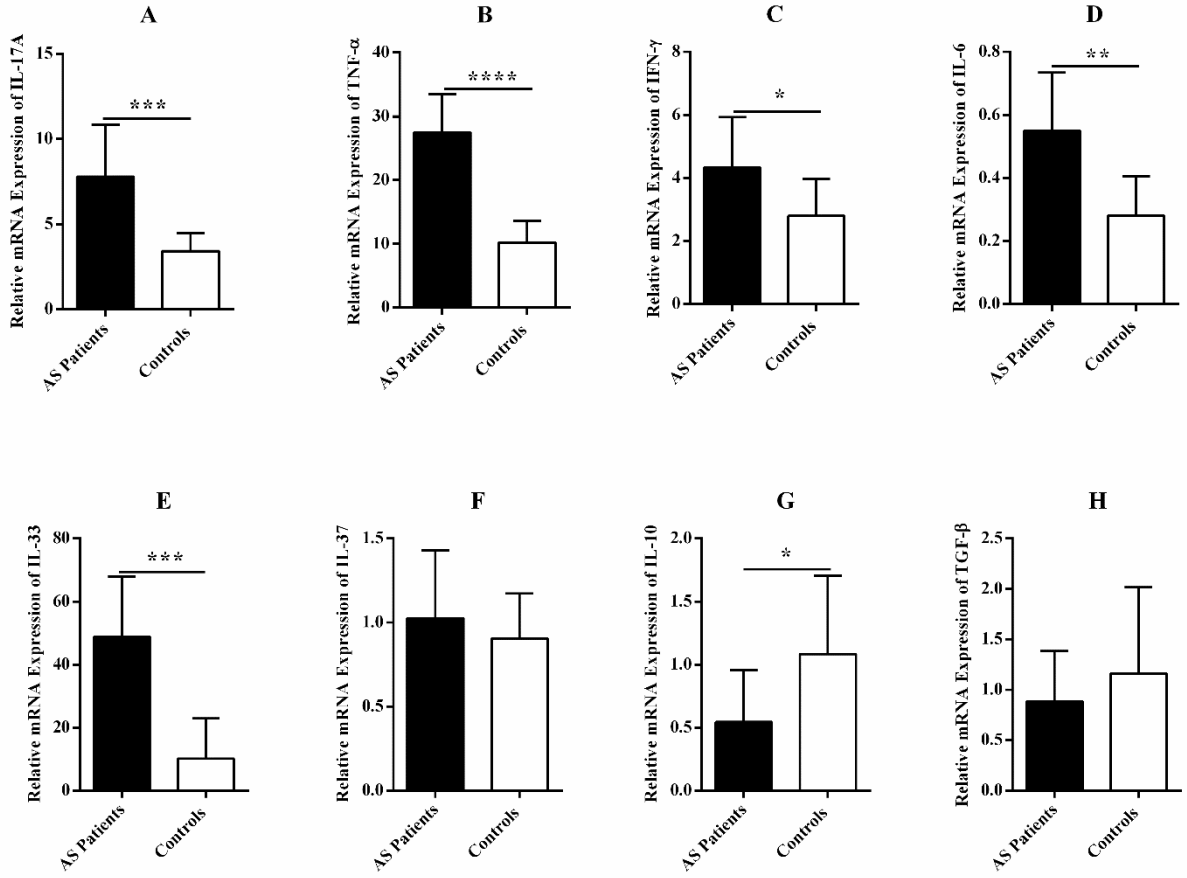

Figure 2. Demonstration of mRNA expression of cytokines in peripheral blood mononuclear cells (PBMCs) between ankylosing spondylitis patients and healthy subjects, as conducted by Real-time PCR gene expression analysis. The comparison was conducted by Mann-Whitney's *U*-test (*demonstrates $p<0.05$, ** demonstrates $p<0.01$, * demonstrates $p<0.001$, **** demonstrates $p<0.0001$).**

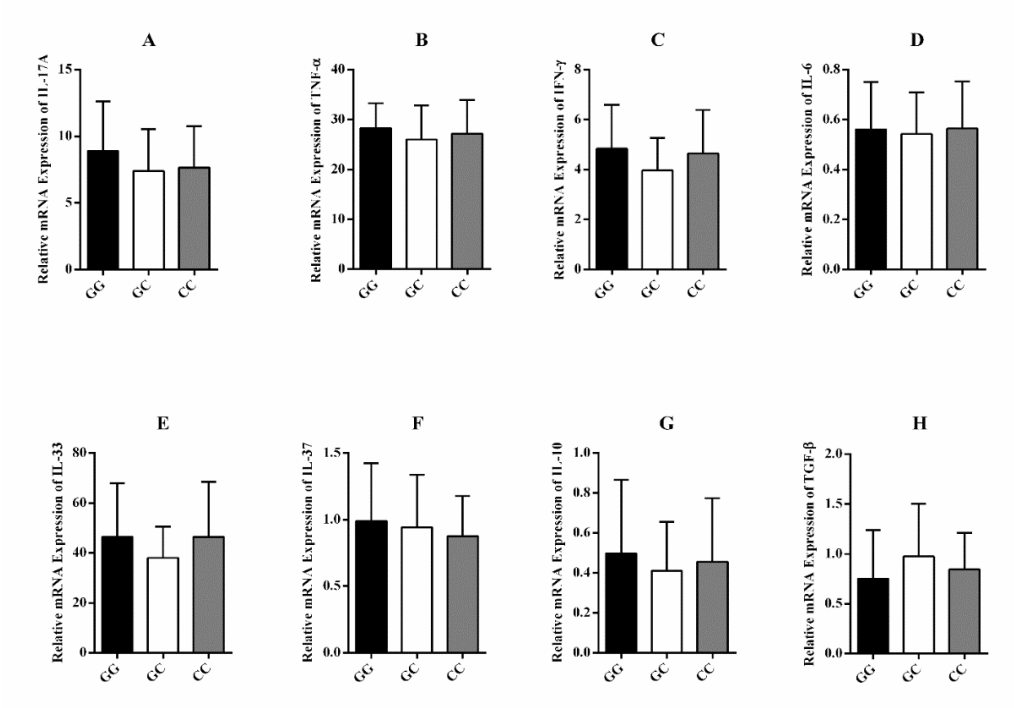

Figure 3. Demonstration of mRNA expression of cytokines in peripheral blood mononuclear cells (PBMCs) between ankylosing spondylitis patients according to three genotypes (GG, GC, and CC) of *ERAP1* gene rs27044 SNP. Real-time PCR was used to evaluate the expression of cytokine's genes. The comparison was conducted by Kruskal-Wallis test.

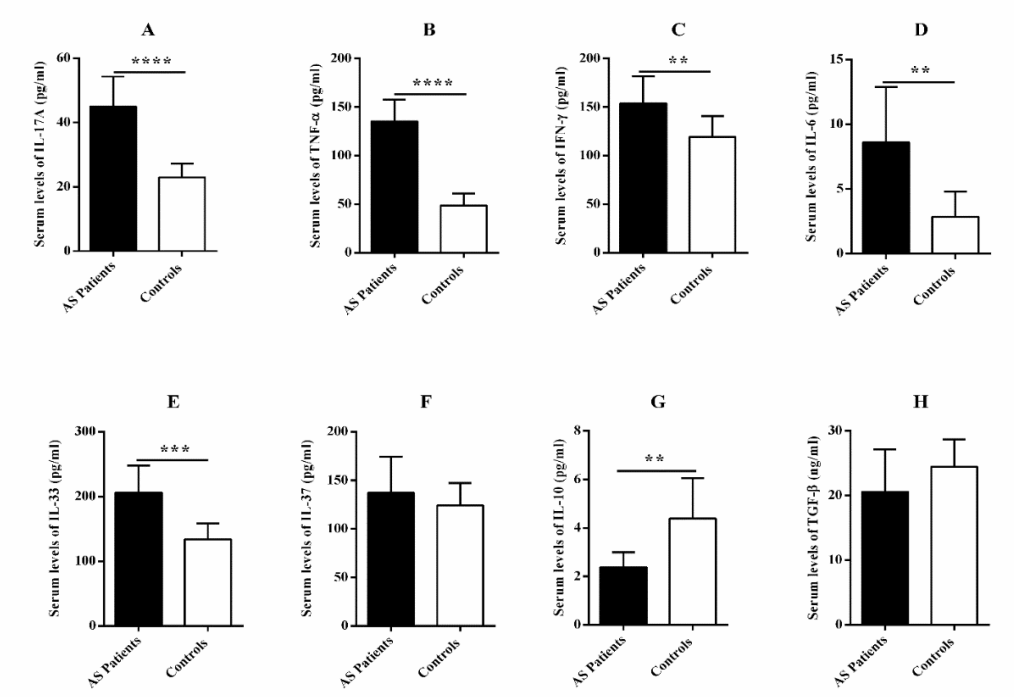

Figure 4. Bar graphs illustration of cytokine concentration in serum of ankylosing spondylitis and healthy control groups. The Enzyme-linked immunosorbent assay (ELISA) was used to evaluate the cytokine's levels. The comparison was conducted by Mann-Whitney's *U*-test (*demonstrates $p<0.05$, ** demonstrates $p<0.01$, * demonstrates $p<0.001$, **** demonstrates $p<0.0001$).**

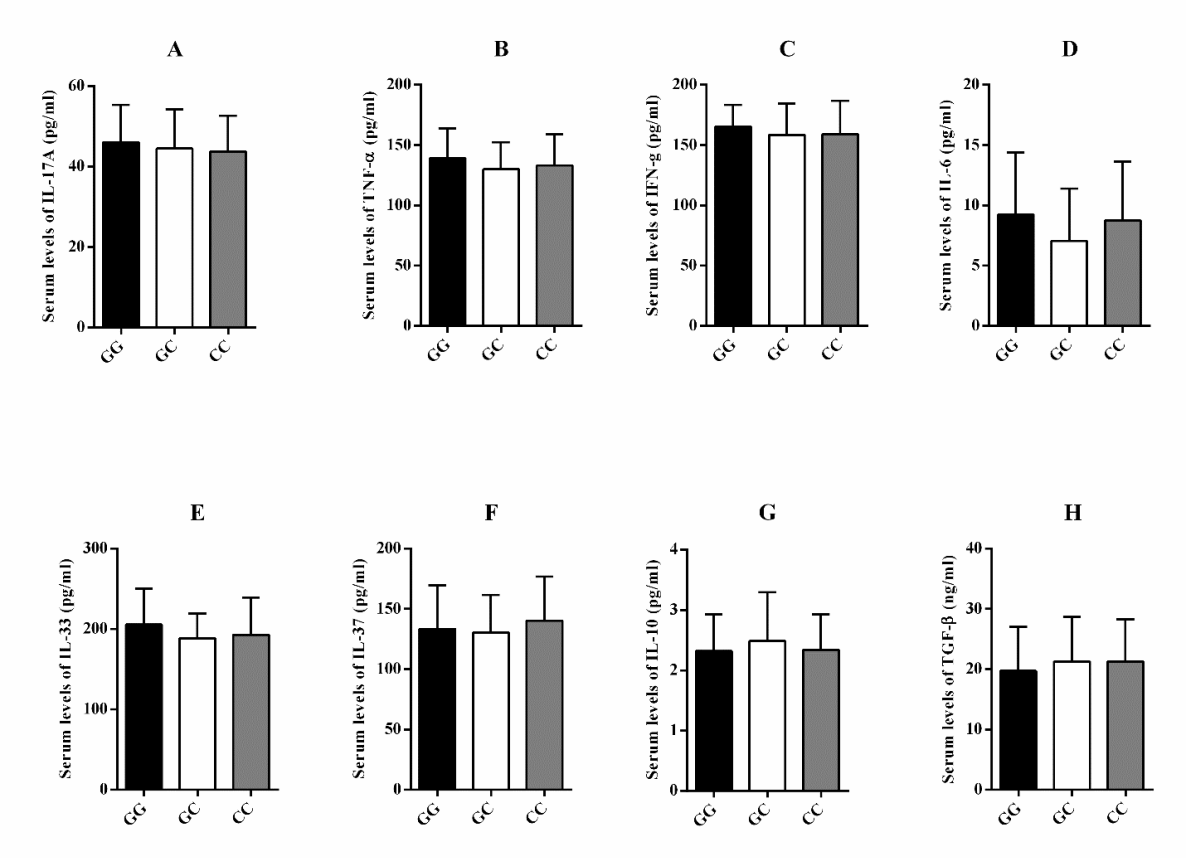

Figure 5. Bar graphs illustration of cytokine concentration in serum of ankylosing spondylitis subjects based on three genotypes (GG, GC, and CC) of *ERAP1* gene rs27044 SNP. The Enzyme-linked immunosorbent assay (ELISA) was used to evaluate the cytokine's levels. The comparison was conducted by Kruskal-Wallis test.

DISCUSSION

Ankylosing spondylitis is defined as an inflammatory rheumatic disease of the axial skeleton that mainly impress young males. Despite greatest genetic risk of AS has been attributed to the MHC class I allele HLA- B*27, GWASs have disclosed more than 60 further risk factors for this disease. Among these genetic risk factors, ERAP1 polymorphisms has been among the initial non-MHC loci identified with susceptibility to AS. In this replication study, we evaluated possible associations of rs27044 polymorphism with the risk of AS in Iranian population in 140 AS cases and 140 healthy subjects and tried to discover if these variants affect the inflammatory profile of AS subjects. Our investigations resulted in identification of association of rs27044 SNP with AS and this variation affected the clinical picture of AS. However, this variation did not impress the inflammatory profile of the AS subjects.

Over the course of past few decades, the genetic risk of AS has been extensively investigated in various populations.[2] Numerous studies have reported that *HLA-B27* is the major gene associated with AS with almost 95% of patients positive for this gene.[4] Nonetheless, only about 5% of subjects carrying the *HLA-B27* gene has AS, suggesting that other genes, in addition to HLA-B27, also participate in disease proneness. Results from twin and familial studies of AS have established that *HLA-B27* gene is responsible for only less than 50% of the total disease risk.[19,20] Up until now, genome-wide and replication studies have revealed new genes associated with AS.[14,21]

It has been reported that four aminopeptidase enzymes, encoded by genes located in the two loci on the chromosomes 5p15 and 17q21, has been associated with AS risk. ERAP1 and ERAP2 are encoded by genes harbored on the chromosome 5p15.[22] In fact,

ERAP1 was the first aminopeptidase that was associated with AS.[14] Until now, the association of ERAP1 has been extensively replicated in many studies, indicating similar allelic, genotypic, and haplotypic associations in various populations like white European and East Asian populations. This observation implies to involvement of common variants in AS pathogenesis across different ethnicities.[4]

ERAP1 participates in processing of peptide antigens for presenting by MHC class I molecules for T lymphocytes by trimming N-terminally extended peptides to the proper final antigenic epitopes.[23] ERAP1 peptidase activity has been investigated *in vitro* to reveal if *ERAP1* gene polymorphisms impress it. These studies have reported that the two protective polymorphisms, namely rs30187 and rs17482078, result in a 40% decreased in peptidase function of ERAP1.[24] Wild type ERAP1 protein carrying rs27044 variant was compared with recombinant ERAP1 and demonstrated that the functional circumstances of ERAP1 due to these variant were under the impression of substrate composition and concentration.[25]

The first replication study of *ERAP1* gene polymorphisms indicated association of rs30187, rs27434, and rs13167972 genotypes and haplotypes with AS susceptibility.[26] This was the second replication study of *ERAP1* gene variants in Iranian AS population. Although, we did not evaluate the association of those polymorphisms, but it was observed in this study that the G allele and GG genotype of rs27044 SNP was associated significantly with increased risk of AS. This may imply that *ERAP1* gene needs to be extensively studied in Iranian AS population that my result in identification of new variants with significant association. On the other hand, a meta-analysis of *ERAP1* genetic polymorphisms indicated that the *ERAP1* gene rs27044, rs17482078, rs10050860, rs30187, and rs2287987 SNPs were associated with the risk of AS in European population. Moreover, the minor allele G of *ERAP1* gene rs27044 were significantly associated with AS risk in European and Asians.[27] It seems that rs27044 SNP is a risk variant for AS in these two populations.

A number of studies have tried to disclose the correlation of genetic polymorphisms with clinical manifestations of AS patients. It has been reported that modified Stoke Ankylosing Spondylitis Spine Score (mSASSS) is under the impression of *ERAP1* gene rs30187 and *LMP2* gene rs17587 polymorphisms. Furthermore, disease duration, sex, and the *LMP2* rs17587 SNP were associated significantly with the baseline mSASSS, which indicates the radiographic severity in AS patients.[28] Here, we noticed that different genotypes of rs27044 could impress the BASFI score and ASQoL in AS patients. To prematurely conclude, the genotypes of this polymorphism may impress the somehow the functional limitation of body movement. This, in turn, may result in decreasing the quality of life in long-time period. This hypothesis requires long-term monitoring of AS cases in large cohorts.

ERAP1 participates in AS pathogenesis by altering the structure of HLA-B27 molecule.[24] In fact, genetic polymorphisms of ERAP1 modulate the length and sequence of peptides that bind HLA-B27.[29,30] Unconventional types of HLA- B27, like free heavy chains (FHCs) can be accumulated in the gut and synovial tissues from spondyloarthritis (SpA) patients.[31] In cellular perspective, FHCs are accumulated within the endoplasmic reticulum (ER), culminating in ER stress. This event, in turn, results in unfolded protein response (UPR). Pathogenesis of AS has been attributed to UPR, since it causes inflammatory responses with promoted production of cytokines, especially those found in IL-17/IL-23 axis, which is a critical pathway in the etiopathogenesis of AS.[32] Dimers of HLA-B27- FHC promote IL-17 production through inducing IL-23 receptor (IL-23R)-positive T cells that express the killer cell immunoglobulin-like receptor (KIR) 3DL2 by FHCs expressed on the surface of antigen-presenting cells (APCs).[33] We tried to evaluate if the genetic variants of *ERAP1* gene could impress the inflammatory setting in AS patients. Although the inflammatory IL-33, IL-17A, IL-6, TNF-α, as well as IFN-γ cytokines demonstrated increased levels in AS patients relative to healthy controls (as well as significant decreased level of immunosuppressive IL-10 cytokine), none of the genotypes of the AS associated SNP (rs27044) had different expression and concentration levels in comparison to other genotypes. This may due to association of *ERAP1* SNPs with the disease risk in AS subjects carrying HLA- B27,[34] as we did not distinguish AS patients in terms of HLA-B27 status when performed cytokine level analysis.

In consideration of all, this was the second replication study of *ERAP1* gene polymorphism with AS susceptibility in Iranian population. We identified

another risk allele for AS in this population, in which the G allele and GG genotype of rs27044 increased the risk of AS. The genotypes of this SNP influenced the clinical manifestation of AS patients. In spite of increased inflammatory cytokines of IL-17A, TNF-α, IL-6, and IL-33 in AS patients in comparison to healthy controls, genotypes of rs27044 SNP did not impress the inflammatory cytokine profile of AS subjects. This could illuminate further aspects of AS etiopathogenesis and, ultimately, we will be armed with more apprehensive knowledge to design much optimal diagnostic and therapeutic tools. Nonetheless, as the limitation of the current study, it is recommended to perform further investigations using large sample sizes as well as evaluation other pathways along with ERAP1 that may affect the inflammatory state in AS patients.

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