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Genetic polymorphism and serum levels of interleukin- 38 in male patients with ankylosing spondylitis

Rehab M. Khadum[1], Reema Mohammed Abed[2]

[1]Department of Biotechnology, College of Science. University of Baghdad, Iraq, Email: rehab@esraa.edu.iq
[2]Department of Biotechnology, College of Science, University of Baghdad, Iraq,
Email: Reema.abed@sc.uobaghdad.edu.iq

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ABSTRACT
Ankylosing spondylitis is a common chronic inflammatory autoimmune disorder affecting 1% of the general population. This study aimed to assess the role of the cytokine IL-38 in AS, examining serum levels, gene expression, and single nucleotide polymorphisms (SNPs). The study enrolled 200 Iraqi males, including 100 AS patients and 100 healthy controls. Baseline characteristics showed the mean age was 41.10 ±13.00 years, AS duration was 8.87 ±6.38 years, and BASFI score was 3.41 ±1.68. There was a higher proportion of married patients (74.0%) compared to non-married (26.0%), and a significant difference in heritable disease between AS and controls ($p<0.001$). IL-38 gene expression was significantly lower in AS (0.34-fold) than controls (1-fold, $p<0.001$). Genotype analysis revealed significant differences in the observed versus expected counts of rs4849146 and rs28992497 SNPs in both groups (all $p<0.05$). The rs4849146 heterozygous AT genotype was higher in AS versus controls (38.0% vs. 20.0%, $p=0.002$), while the homozygous AA genotype was lower (39% vs. 60%, $p=0.003$). Allele A was less frequent and T more frequent in AS ($p=0.012$). In AS, IL-38 expression was higher in TC and CC versus TT genotypes of rs28992497 ($p=0.002$). Haplotype analysis showed significant differences in A-T and T-C frequencies between groups ($p<0.05$). In conclusion, these findings suggest IL-38 gene expression and SNPs may play a role in the pathogenesis of AS.

Keywords: Ankylosing spondylitis, IL-38, SNP, rs4849146, Variants.

INTRODUCTION

Ankylosing spondylitis (AS), also known as radiographic axial spondyloarthritis (axSpA), is a prevalent chronic inflammatory autoimmune rheumatic condition that mostly affects the spinal vertebrae and sacroiliac joints in around 1% of the general population [1]. The disease's cause remains mostly unknown, but there is a strong link between AS incidence and the presence of human leukocyte antigen (HLA)-B27 (an immunogenetic marker) in various ethnic groups. AS etiopathogenesis involves chronic enthesitis associated with aberrant activities of particular immune cells such as T lymphocytes and macrophages[2].

Furthermore, it has been demonstrated that cytokines, which are essential for mediating interactions and communications between these cells, play an important role in the start and regulation of inflammatory processes at enthesitis locations. The inflammatory process in AS has been linked to elevated levels of circulating pro-inflammatory cytokines. Furthermore, it has been revealed that anti-inflammatory cytokines play critical roles in maintaining immunological homeostasis in AS [3]. Various genetic studies have been published the relationships between single nucleotide polymorphisms (SNPs) with such autoimmune diseases, including autoimmune thyroid disease [4], systemic lupus erythematous (SLE) [5], [6].

The interleukin (IL)-1 family consists of both pro-inflammatory (IL-36, IL-33, IL-18, IL-1β and IL-1α) and anti-inflammatory (IL-37 and IL-38) cytokines [3]. Among the newly discovered members of the IL-1 family, IL-36, IL-37, and IL-38 have received little attention in the field of AS studies [7]. One member of the IL-1 family that has anti-inflammatory properties is IL-38, which was found relatively recently [8]. There is mounting evidence that interleukin (IL)-38 plays a critical role in the development of several inflammatory autoimmune disorders, including psoriatic arthritis, systemic lupus erythematosus, and rheumatoid arthritis (RA) [9]. Although IL-38 levels in the blood have not been found, a link between IL38 gene variants and AS risk has been postulated [10].The 152-amino acid (AA) precursor protein, which was derived from a fetal skin library and shows a 12-β-stranded trefoil structure, is the main product of the five exons that make up the IL-38 gene. A number of cell types, including keratinocytes (KCs), B cells, fibroblast-like synoviocytes (FLSs), peripheral blood mononuclear cells (PBMCs), and immune cells, are able to secrete IL-38, even though it lacks a signal peptide, a feature shared by all IL-1F proteins except IL-1Ra. In addition, IL-38 was shown to be strongly expressed in specific

tissues such as skin, tonsils, lungs, spleen, heart, placenta, fetal liver, and thymus, whereas it was found to be less prevalent in inactive immune tissues[11].
Therefore, this study aimed to assess the role of IL-38 serum levels, gene expression and single nucleotide polymorphisms (SNPs) in AS patients.

MATERIALS AND METHODS

Study subject

The present research was a case-control study which was carried out in Department of biotechnology, college of science, university of Baghdad, Baghdad, Iraq. 100male patients with ankylosing spondylitis and 100 healthy controls were enrolled in this study, whereas the participants' ages ranged from 18 to 73 years. All patients had magnetic resonance imaging of the sacroiliac joints and were evaluated for the presence of HLA-B*27. The parameters, including age, disease duration,erythrocyte sedimentation rate (ESR)and Bath Ankylosing Spondylitis Functional Index (BASFI) score which was used to assess the disease activity [12]through questionnaires and reviews of medical records. The control group, were selected according to following the criteria for inclusion: no autoimmune and/or rheumatic diseases, unrelated to the patient group, and belonging to the same ethnic group as the patients, and from the same region

Collection of blood samples

Five mL of peripheral blood from the case and control groups were drawn into two tubes: one without anticoagulant (plain tube) for immunoassay of IL-41, and the other with anticoagulant (EDTA- anticoagulant) for DNA and RNA isolation.

Immunoassay of IL-38

In IL-38 immunoassay, it hasbeen used an ELISA assay to detect serum IL-38. Interleukin (IL-38) concentration was determined in patient and control samples using a sandwich ELISA kit according to the manufacturer's instructions (catalog number In-Hu4228, MyBioSource, Inc., USA). The antibody utilized in this assay does not exhibit any detectable cross-reactivity with other relevant proteins.

Gene expression

RNA extraction

Total RNA was extracted from all samples using the EasyPure® RNA Kit Reagent (TransGen, biotech. ER401) according to the manufacturer's instructions.The 2000c Nanodrop spectrophotometer (Thermo Fisher Scientific, USA) was used to evaluate the concentration and purity of extracted RNA in order to determine the quality of samples for subsequent analysis in RT-qPCR. The samples ranged in RNA concentration from (81-123) ng/µl, while the absorbance of the samples was measured at two distinct wavelengths to determine RNA purity (260 and 280nm). The presence of an A_{260}/A_{280} ratio of around 2.0 suggested that the RNA sample was pure.

Synthesis the cDNA form mRNA

Using the EasyScript® One-Step gDNA Removal and cDNA Synthesis SuperMix Kit, total RNA was reverse-transcribed to complementary DNA (cDNA), according to the manufacturer's instructions.

Quantitative Real Time PCR (qRT–PCR)

Quantitative Real Time PCR (qRT–PCR) was carried out using the QIAGEN Rotor gene Q Real-time PCR System (Germany). The expression levels and fold changes of the IL-38 and GAPDH genes were assessed using the TransStart® Top Green qPCR Super Mix kit and measuring the threshold cycle (Ct). Every reaction was performed twice. The needed volume of each component was determined as follows: 10µl of 2xTransStart® Top Green qPCR Super Mix; 6µl of nuclease free water, 1 µl of forward Primer (10 µM), 1 µl of reverse primer (10 µM) and 2 µl of cDNA. The primer sequences used in qPCR detection of IL-38 gene expression was shown in table 1.

Table 1: Primer sequences used in qPCR detection of IL-38 gene expression.

SNP	Primer	Sequence (5'-3')	Primer size	Replicon size	Ref.
IL-38	F	CCCGAAATATGTCCACATCC	20	161	This study
	R	CTTCCTGCCTCCTTCTTGTG	20		
Housekeeping gene (GAPDH)	F	GAAATCCCATCACCATCTTCCAGG	24	160	[13]
	R	GAGCCCCAGCCTTCTCCATG	20		

*F: forward; R: Reverse.

The cycling protocol was programmed for the following optimized cycles and according to the thermal profile, as represented in table 2 :

Table 2: The thermal profile of gene expression.

Step	Temperature (ºC)	Time (sec.)	Cycles
Denaturation	94	10	1
Annealing	56	15	40
Extension	72	20	

Calculation of gene expression

The relative cycle threshold ($^{2-}\Delta\Delta$Ct) approach, first described by Livak and Schmittgen, was used to quantify fold changes of the quantitative expression of the mature RNAs[14]. The relative gene expression ratio compares the two groups (control and test) and finds the mean.

Genotyping of IL-*38*

The IL-*38* (rs4849146 A/T andrs28992497 T/C) SNPs were identified through Sanger sequencing analysis. Using EasyPure® Blood Genomic DNA Kit (TransGen, biotech. EE121), genomic DNA was isolated from whole blood in accordance with the manufacturer's instructions.NCBI designed primer sequences,as shown in table 3, for two SNPs (rs4849146 A/Tand rs28992497 T/C). After confirming effective PCR amplification with the QIAGEN Rotor gene Q System (Germany), the results were examined under UV light using 1.5 % agarose gel containing 0.5 g/mL ethidium bromide. The 624 bp PCR fragment of the IL-*38*gene that was amplified by PCR was sequenced by Sanger analysis. After alignment with a gene bank reference sequence, the results were analyzed with Geneious software.

Table 3: Primer sequences used in qPCR detection of IL-38 genotyping.

SNP	Primer	Sequence (5'-3')	Primer size	Replicon size	Ref.
rs4849146 A/T	F	ACGCTTGTAATGGAGGGATG	20	624	This study
	R	TGCAGCCAGATAAACAGTGC	20		
rs28992497 T/C	F	CCCGAAATATGTCCACATCC	20	161	This study
	R	CTTCCTGCCTCCTTCTTGTG	20		

Statistical analysis

To summarize the data in this investigation, several descriptive statistical approaches were used. The mean differences in eosinophil counts between patients and controls were examined using an independent two-sample student one-way ANOVA and the T-test. The Mann Whitney test was used, in addition to Median ± Standard Error. A statistically significant p-value of less than 0.05 was utilized. SPSS statistical analysis was used to determine the significant differences in the observed alleles between gene collections. A logistic regression model was used to calculate 95% confidence intervals (CIs) and odds ratios (ORs).

RESULTS

Baseline characteristics of patients and controls

Baseline characteristics were presented in Table 1, which revealed the mean age of males AS patients was 41.10 ±13.00 years with disease duration of 8.87 ±6.38 years. The age of the control 40.82±12.90 years was matched (p = 0.878)with ranged 18-73 years in both cases. In this study, the disease activity indices BASFI was examined and found to be less than 4 (3.41 ±1.68), indicating that the AS patients had an active form of the disease. With respect to marital status, the proportions of married and not married patients were 74 (74.0 %) and 26 (26.0 %); while those of the control group were 62 (62.0 %) and 38 (38.0 %) respectively; with non-significant differences (p = 0.069). Thepositive family history was reported in 24 (24.0 %) cases of patients' group which was significantly higher (p< 0.001) than that reported in control group; 4 (4.0 %), while thecomparison of presence of chronic medical illness and smoking habit between patients' group and control group revealed non-significant difference (p = 0.182; p = 0.767) respectively as shown in table 3.

Table 4: Baseline characteristics of patients' and control subjects

Characteristic	AS (n = 100)	HC (n = 100)	p
Age (years) Mean ±SD	41.10 ±13.00	40.82±12.90	0.878 I NS
Disease duration (years) Mean ±SD	8.87 ±6.38	-	-

BASFI (Mean ±SD)	3.41 ±1.68	-	
Marital status			
Married	74 (74.0 %)	62 (62.0 %)	0.069 C NS
Not married	26 (26.0 %)	38 (38.0 %)	
Heritable disease			
Positive	24 (24.0 %)	4 (4.0 %)	< 0.001 C ***
Negative	76 (76.0 %)	96 (96.0 %)	
Chronic medical illness			
Positive	20 (20.0 %)	13 (13.0 %)	0.182 C NS
Negative	80 (80.0 %)	87 (87.0 %)	
Smoking			
Smoking	36 (36.0 %)	34 (34.0 %)	0.767 C NS
Not smoker	64 (64.0 %)	66 (66.0 %)	

SD: standard deviation; **n**: number of cases; **I**: independent samples t-test; **C**: chi-square test; **NS**: not significant; **: significant at $p \leq 0.01$

Comparison of IL-38 serum levels between patients and control group

The results showed no significant difference in median (interquartile range) of IL-38 serum levels among patients and HCs; 17.70 (8.98) (pg/ml) vs. 17.47 (9.63) (pg/ml), respectively; withp = 0.084, as displayed in figure (1; A). Receiver operating characteristic (ROC) curve analysis was carried out to find the cutoff values of serum levels of IL-38 that can predict positive diagnosis of disease and these results were demonstrated in figures (1; B). The cutoff value of serum IL-38 was≤11.82 pg/ml, but this cutoff was not valid because the area under the curve (AUC) was < 0.700 (0.520) in addition the p-value was not significant (p = 0.623), whereas there was poor sensitivity of 20 % and week accuracy of 52 % despite excellent specificity level of 96 %.

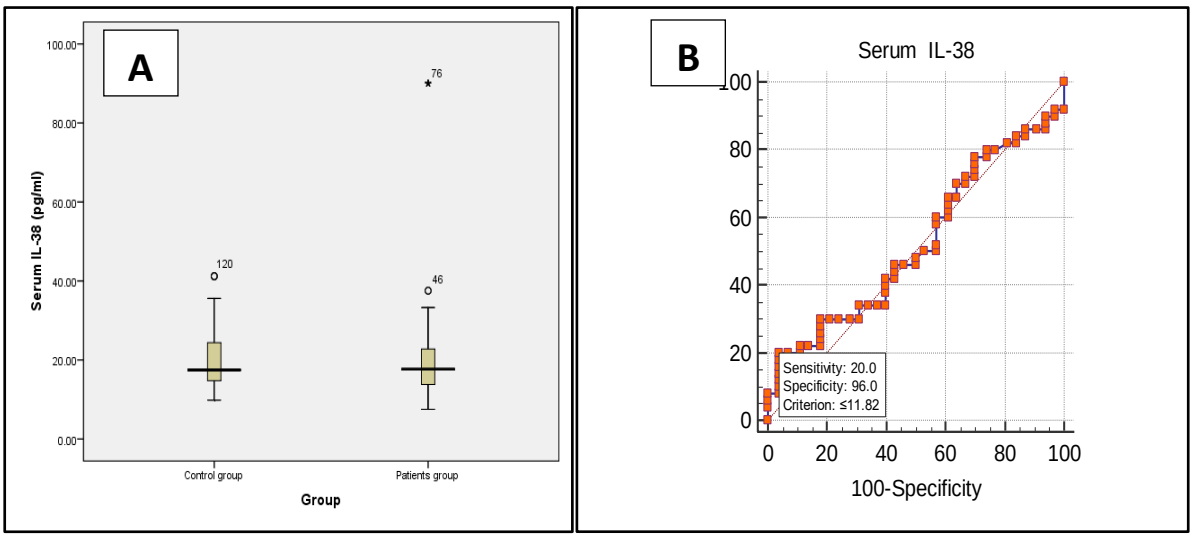

Figure 1: A: Box plot showing comparison of serum IL-38 level between patient group and control group; B: Receiver operating characteristic (ROC) curve analysis to find the cutoff value of serum IL-38 that can predict positive diagnosis of disease.

Comparison of IL-38 gene expression between patients and control group

Comparison of IL-38 gene expression between patients and control group was shown in figure (2; A). Level of IL-38 gene expression was lower significantly (p< 0.001) in patient group in comparison with control group, 0.34 (0.35)-fold change vs. 1-fold change, respectively.The cutoff value of IL-38 gene expression was ≤0.44-fold change, and this cutoff was valid because the area under the curve (AUC) was > 0.700 (0.837) in addition the p-value was significant (p < 0.001), in addition, there was moderate senility of 66 %, excellent specificity of 91 % and very good accuracy of 83.7 %, as displayed in figure (2; B).

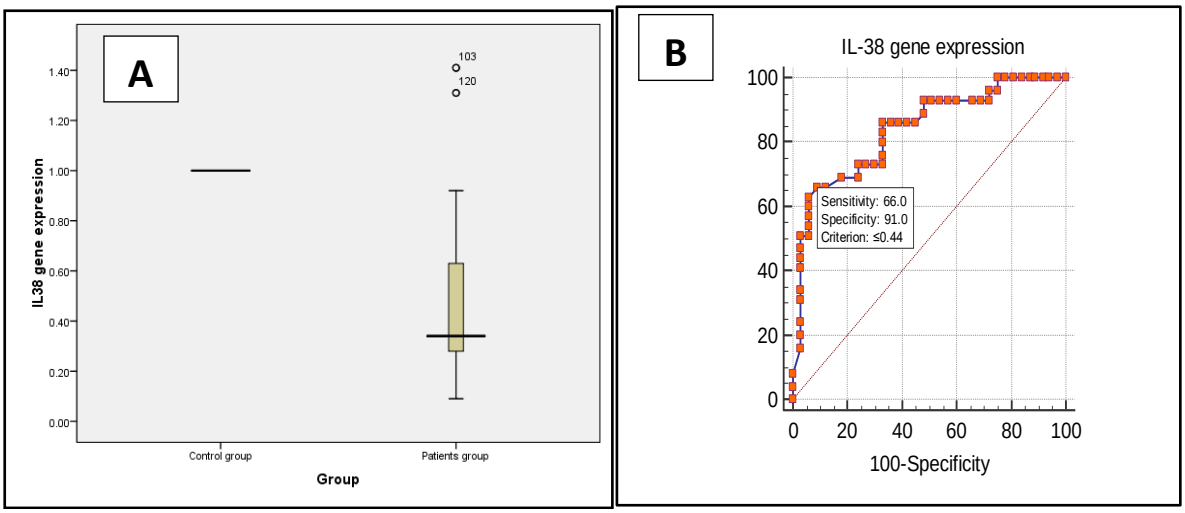

Figure 2: A: Box plot showing comparison of IL-38 gene expression level between patient group and control group; B:Receiver operating characteristic (ROC) curve analysis to find the cutoff value of IL-38 gene expression that can predict positive diagnosis of disease.

Association of IL-38 gene SNPs

All 200 people in this research were genotyped for the SNPs (rs4849146 and rs28992497)of the IL-38 gene. The participants were divided into two groups: 100 AS sufferers and 100 controls.

The frequency of IL-38 (rs4849146) A/T based on Hardy Weinberg equilibrium wasrepresented in table5. The observed counts of genotypes AA, AT and TT were reported in 39, 38 and 23 cases, respectively of patients' group and they showed significant difference in comparison with expected counts (p = 0.028). The observed counts of genotypes AA, AT and TT were reported in 60, 20 and 20 cases, respectively of control group and they showed significant difference in comparison with expected counts (p< 0.001).

Table 5: Frequency of IL-38 (rs4849146) A/T based on Hardy Weinberg equilibrium (HWE)

IL-38 (rs4849146 A/T)	Patients' group (n = 100)	Control group (n = 100)
AA	39	60
AT	38	20
TT	23	20
HWEp-value	0.028*	< 0.001***

*: significant at p ≤ 0.05; ***: significant at p ≤ 0.001

Comparison of genotypes and alleles of IL-38 (rs4849146) A/T between patients' group and control group was showed in table 6. The analysis of the association included codominance mode, dominant mode, recessive mode and alleles was performed. In codominance mode, genotype AA was regarded as the reference genotype. The heterozygous genotype AT was significantly higher in patients'group in comparison with control group, 38.0 % versus 20.0 %, respectively (p = 0.002), for that reason this genotype was associated with the risk of disease with an odds ratio of 2.92. In addition, the homozygous genotype TT was not significantly different in patients' group in comparison with control group, 23 % versus 20 % (p = 0.120), thus it was neither a risk factor nor a protective factor.

Regarding dominant mode, the homozygous AA genotype was contrasted to other 2 genotypes (AT+TT) and the results revealed significantly lower rate in patients' group in comparison with control group, 39 % versus 60 % (p= 0.003), for that reason this genotype was a protective factor with an odds ratio of 0.43 (providing a protection against disease by 57 %).Regarding recessive mode, the homozygous TT genotype was contrasted to other 2 genotypes (AT+AA) and the results revealed no significantdifference in patients' group when compared to control group, 23 % versus 20 % (p = 0.606), making this genotype neither a risk factor nor a protective factor.Allele analysis revealed that allele A was significantly less frequent and that allele T was significantly more frequent in patients' group in comparison with control group, 58.0 % versus 70.0 % % and 42.0 % versus 30.0 %, respectively (p= 0.012), thus, allele A is a protective factor with an odds ratio of 0.59 (41 % protection against the disease) and allele T is a risk factor with an odds ratio of 1.69.

Table 6: Comparison of genotypes and alleles of IL-38 (rs4849146) A/T between patients' group and control group

Mode	Genotype/ allele	AS n = 100 (%)	HC n = 100 (%)	p-value	OR 95% CI
Co-dominance	AA	39 (39.0 %)	60 (60.0 %)	Reference	Reference
	AT	38 (38.0 %)	20 (20.0 %)	0.002 C **	2.92 (1.49 -5.74)
	TT	23 (23.0 %)	20 (20.0 %)	0.120 C NS	1.77 (0.86 -3.64)
Dominant	AA	39 (39.0 %)	60 (60.0 %)	0.003 C **	0.43 (0.24 -0.75)
	TT+AT	61 (61.0 %)	40 (40.0 %)	Reference	Reference
Recessive	TT	23 (23.0 %)	20 (20.0 %)	0.606 C NS	1.19 (0.61 -2.35)
	AA+AT	77 (77.0 %)	80 (80.0 %)	Reference	Reference
Allele	A	116 (58.0 %)	140 (70.0 %)	0.012 C *	0.59 (0.39 -0.89)
	T	84 (42.0 %)	60 (30.0 %)		1.69 (1.12 -2.55)

C: chi-square test; **OR**: odds ratio; **a**: approximate odds ratio**; CI**: 95 % confidence interval; **NS**: not significant; *: significant at $p \leq 0.05$; **: significant at $p \leq 0.01$

The frequency of IL-38 (rs28992497) T/C based on Hardy Weinberg equilibrium was shown in table 7. The observed counts of genotypes TT, TC and CC were reported in 70, 13 and 17 cases, respectively of patients' group and they showed significant difference in comparison with expected counts ($p< 0.001$). The observed counts of genotypes TT, TC and CC were reported in 65, 20 and 15 cases, respectively of control group and they showed significant difference in comparison with expected counts ($p< 0.001$).

Table 7: The frequency of IL-38 (rs28992497) T/C based on Hardy Weinberg equilibrium

IL-38 (rs28992497 T/C)	Patients' group n = 100	Control group n = 100
TT	70	65
TC	13	20
CC	17	15
χ^2	40.760	21.778
HWE p-value	< 0.001 ***	< 0.001 ***

***: significant at $p \leq 0.001$

Comparison of genotypes and alleles of IL-38 (rs28992497) T/C between patients' group and control group was shown in table 8. The analysis of the association included codominance mode, dominant mode, recessive mode and alleles. None of genotypes or allele showed significant variation between patients' group and control group ($p > 0.05$), thus, none of them can be a risk or a protective factor.

Table 8: Comparison of genotypes and alleles of IL-38 (rs28992497) T/C between patients' group and control group

Mode	IL-38 (rs28992497) T/C	Patients' group n = 100	Control group n = 100	p	OR
Co-dominance	TT	70 (70 %)	65 (65 %)	Reference	Reference
	TC	13 (13 %)	20 (20 %)	0.199 C NS	0.60 0.28 -1.31
	CC	17 (17 %)	15 (15 %)	0.897 C NS	1.05 0.49 -2.28
Dominant	TT	70 (70 %)	65 (65 %)	0.450 C NS	1.26 0.69 -2.27
	CC+TC	30 (30 %)	35 (35 %)	Reference	Reference
Recessive	CC	17 (17 %)	15 (15 %)	0.700 C NS	1.16 0.54 -2.48
	TT+TC	83 (83 %)	85 (85 %)	Reference	Reference
Allele	T	153 (76.5 %)	150 (75 %)	0.726 C NS	1.09 0.69 -1.71

	C	47 (23.5 %)	50 (25 %)		0.92 0.58 -1.46

C: chi-square test; **OR**: odds ratio; **a**: approximate odds ratio**; CI**: 95 % confidence interval; **NS**: not significant.

Analysis of correlation

The analysis correlation of mean IL-38 serum level and IL-38 gene expression according to IL-38 (rs4849146 A/T) genotypes in patients" group was shown in table 9. The analysis revealed there was no significant variation in patients' groups, ($p > 0.05$).

Table 9: analysis correlation of mean IL-38 serum level and IL-38 gene expression according to IL-38 (rs4849146 A/T) genotypes in patients' group and control group

No.=100	**Characteristic**	**IL-38 (rs4849146 A/T)**			**p**
		AA	AT	TT	
Patients'	Serum IL38	27.76 (24.74)	22.30 (17.81)	17.77 (16.44)	0.171 K NS
	IL38 gene expression	0.48 (0.31)	0.50 (0.38)	0.40 (0.19)	0.472 K NS

Data were shown as median (inter-quartile range); **K**: Kruskal Wallis test; **NS**: not significant

The analysis correlation of mean IL-38 serum level and IL-38 gene expression according to IL-38 (rs28992497 T/C) genotypes in patients' group was shown in table 10.With respect to patients' group, IL-38 gene expression level was higher significantly ($p = 0.002$) in both TC and CC groups in comparison with TT group, but there was no significant variation in serum level ($p = 0.979$).

Table 10: The analysis correlation of IL-38 serum level and IL-38 gene expression according to IL-38 (rs28992497 T/C) genotypes in patients' group and control group

Group	**Characteristic**	**IL-38 (rs28992497 T/C)**			**p**
		TT	**TC**	**CC**	
Patients' group	serum IL38	23.63 (21.31)	22.35 (21.68)	23.21 (18.49)	0.979 K NS
	IL38 gene expression	0.40 (0.20)	0.64 (0.42)	0.64 (0.49)	0.002 K **

Data were shown as median (inter-quartile range); **K**: Kruskal Wallis test; **NS**: not significant; **: significant at $p \leq 0.01$

The results, as represented in table 11, were indicated that there was highly significant differences in frequency of A-T (83.49 vs. 105.00) and T-C (69.51 vs. 45.00) haplotypes of rs4849AA46 A/T and rsTC899TC497 T/C among patients and controls with p-value (0.031 and 0.007), respectively.The frequency of A-T haplotypes of rs4849AA46 A/T and rsTC899TC497 T/C was significantly increased in HCs than AS patients, while T-C was significantly decreased.However, no significant differences were observed in frequency of other haplotypes among both groups.

Table 11: Numbers and frequencies of haplotypes of IL-38 in patients and controls.

Haplotypes	**Patients group n = 100**	**Control group n = 100**	**OR (95 % CI)**	**p**
rs4849AA46 A/T and rsTC899TC497 T/C)	**AS (Frequency)**	**HC (Frequency)**		
A-C	32.51 (0.163)	35.00 (0.175)	0.915 (0.542~1.545)	0.740
A-T	83.49 (0.417)	105.00 (0.525)	0.648 (0.437~0.962)	0.031*
T-T	14.49 (0.072)	15.00 (0.075)	0.963 (0.455~2.039)	0.922
T-C	69.51 (0.348)	45.00 (0.225)	1.835 (1.180~2.853)	0.007**

OR: odds ratio; CI: confidence interval; *: significant at $p \leq 0.05$; **: significant at $p \leq 0.01$

Correlation of parameters under study

Correlation among IL-38 serum levels and gene expression and other characteristics of patientswas shown in table 12. Serum IL-38 showed significant positive correlation to age and significant negative correlation to BASFI-1. While IL-38 gene expression showed no significant correlations ($p> 0.05$).

Table 12: Correlation among IL-38 serum levels and gene expression and other characteristics of patients'

Characteristic	Serum IL-38		IL38 gene expression	
	r	p	r	p
Age	0.198	0.048*	-0.027	0.787
BASFI	-0.221	0.027*	-0.150	0.137
Marital status	0.172	0.087	-0.032	0.750
Heritable disease	0.169	0.093	-0.101	0.316
Chronic medical illness	0.161	0.110	0.012	0.908
Smoking	-0.170	0.092	-0.117	0.246
Duration of disease	-0.003	0.974	0.126	0.212

BASFI: Bath Ankylosing Spondylit is Functional Index; **IL:** Interleukin.

DISCUSSION

The present study was indicated that the mean age of males AS patients was 41.10 years. The majority of the 2579 individuals with axial spondyloarthritis (axSpA) (92%), regardless of the geographical locations analyzed, had an age at onset of less than 45 years, according to a recent study. Asia has 94%, North America and Europe have 92%, Latin America has 89%, and the Middle East has 91% [15]. In the study of [16], age of AS start in the patients appeared to be a factor influencing family risk. Patients with illness beginning before the age of 25 were at a greater risk than those with disease onset after the age of 25. In agreement with this study, it has been reported that the mean age was 41.96±9.11 in 100 AS patients [17]. In disagreement with study conducted by [18], there was no significant difference in median age of patients and controls (40.7 vs. 40.8 years), respectively. Also, it has been reported that the mean ± SD of age in 132 patients with AS was 37.61 ± 10.0 years, was lower than the finding of this study; but these findings were lower than the findings of study conducted by [19], who found that the mean ± SD of age in 974 patients with AS was 59.3 ± 12.1 years. These results may be attributed to the differences in sample size among various studies. AS often occurs in people under the age of 45 years and is more prevalent in males, although it can occur in female patients too [20]. Regardless of age, the progression and symptoms of AS can vary from individual to another.

In addition, theMean ±SD of disease duration was 8.87 ±6.38 years, which was close to the findings of study reported by [21], whose found the median of disease duration from AS diagnosis was 8.0 years; while it was lower than findings of study reported by [22], whose found that AS duration was 20.5±11.8 years.Different people will experience AS for different duration, and this is due to a combination of causes, including early diagnosis, treatment plans, regular monitoring of the disease, and environmental and genetic factors [23], [24], [25].

Clinical recommendations call for routine monitoring of disease activity and functional impairment in AS patients. The BASFI, which may be scored from 0 to 10, was used to accomplish this. It has been found that a score of 4 or higher indicated active disease, while a value of less than 4.0 shows clinical improvement of AS [26], [27]. The results of this study also found that Mean ±SD of BSAFI was 3.41±1.68, indicated that the disease show clinical development. In corresponding with study represented by [21], whose found that the average of BSAFI was 2.4 for AS patients.

In corresponding with this study, in has been reported, married mothers with AS (67%; n=1322) were not show significant difference in comparison with healthy mother (67%; n=8377) [28].

The results of this study also showed that heritable disease wassignificantly differ among groups of study. Heritability, as shown in study reported by [29], estimated by assuming sibling risks were completely due to genetics, was 77% (95% CI 73, 80). Over 90% of the chance of developing AS, the prototypic seronegative arthropathy, is determined genetically. The condition is known to be highly heritable. The use of family-based or candidate gene techniques to discover the genes implicated in this condition has been progressing at a slower rate than with the majority of common heritable disorders [29]. A phenotypic trait's heritability is the extent to which its variation in genes accounts for the observed variation in that characteristic. In comparison to other phenotypes, such as RA (40%), inflammatory bowel disease (65-75%), and adult height (80-90%), twin studies have shown that AS has a heritability of 90-99% [30]. This would make AS one of the most heritable phenotypes [31]. However, there were relatively few participants in the AS heritability investigations, with just 40 twin pairs, respectively, in the research [30].

Cigarette smoking has been linked to a variety of ailments, prompting concern among healthcare providers worldwide. It affects both innate and adaptive immunity, and can cause dysregulation by either increasing pathogenic immune responses or weakening defensive immunity. However, there is a complex relationship between smoking and spondyloarthropathy. Research indicates that smoking may worsen the condition, but the exact cause remains unknown [32].

The current study assesses the significance of IL-38 as a biomarker and its gene expression and correlation with the risk of AS. The results of the present study was indicated that no significant difference observed among

group of study, suggested that this interleukin not involved in pathogenesis of AS. The widespread consensus is that AS is caused by the chronic inflammatory response. Growing evidence links several cytokine pathways, including those engaged in pro- and anti-inflammatory interactions, to the development of disease [33]. Different studies were assessed the roles of IL-38 in various studies, including AS. One member of the IL-1 family of cytokines, IL-38, was found in the serum at elevated levels in AS patients, and this was linked to a higher chance of developing the condition (OR = 12.94). With the exception of a theory linking an IL-38 genetic variation to an increased risk of the condition, this cytokine has not been studied in AS [34]. However, there is evidence linking IL-38 to the pathophysiology of other inflammatory autoimmune illnesses, including arthritis. According to recent research, IL-38 is expressed more frequently in a variety of inflammatory conditions, such as psoriatic arthritis, rheumatoid arthritis (RA), SLE, primary Sjogren's syndrome, and inflammatory bowel disease (IBD) [35], [36]. From a functional standpoint, IL-38 is often characterized as an anti-inflammatory cytokine. It has been discovered that IL-38 suppresses fungal-induced Th17 responses. Moreover, PBMCs treated in vitro with IL-38 produced fewer pro-inflammatory cytokines, such as IL-17A and IL-22. Furthermore, it has been noted that recombinant IL-38 can prevent pro-inflammatory cytokines including TNF-α, IL-1β, and IL-17A from being produced in vitro. Additionally, pro-inflammatory effects have been noted; these might be influenced by IL-38 concentration. For example, Xie et al. [37] observed that IL-38, IL-22, and IL-17A were reduced at low concentrations but rose at high concentrations. According to these results, IL-38 might be a useful biomarker for anticipating the onset of autoimmune illnesses that cause inflammation, such as AS. It has recently been determined that IL-38 plays a role in controlling etiological pathways, and this role is linked to the pathogenesis of several disorders [38]. In study of [39], results revealed that serum levels of IL-38 were significantly higher in AS patients than in controls. Levels of serum IL-38 did not significantly differ between the total multiple sclerosis (MS) or and systemic sclerosis (SSc) patients compared to controls. However, levels of IL-38 were significantly higher in newly diagnosed patients of MS than in those previously treated [40].The serum levels of IL-38 were measured in patients with Behcet's disease (BD) and controls sera using enzyme-linked immunosorbent assay. The relationship between the serum levels of IL-38 and clinical and laboratory characteristics of the patients were determined. IL-38 serum levels were significantly lower in patients in comparison with healthy controls [41]. Also, according to results of [42], IL-38 levels were significantly differed between healthy individuals and patients with Knee Osteoarthritis.

When comparing the AS group with the control group, in this study, the level of IL-38 gene expression was considerably lower ($p < 0.001$), with a 0.34-fold decrease compared to a 1-fold change, respectively. Expression of IL-38 gene was reported in different studies related to various diseases.IL-38 expression is upregulated in patients with rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE), Furthermore, studies in mice indicated that IL-38 is a negative regulator of inflammatory arthritis [43]. Clinical inflammatory scores were significantly decreased after adeno-associated virus (AAV) IL-38 injection in joints of mice with collagen-induced arthritis (CIA), K/BxN serum transfer-induced arthritis (STIA), but not antigen-induced arthritis (AIA). This decrease was accompanied by reduced macrophage infiltration and a decreased expression of Th17 cytokines (IL-17, IL-23, IL-22) and TNFα. However, IL-38 overexpression had no effect on cartilage or bone destruction. In vitro, the THP-1 monocytic cell line expressed less IL-6, TNFα and IL-23 after IL-38 overexpression. Conditioned media from these cells, containing released IL-38, also exert an anti-inflammatory effect on human primary macrophages and synovial fibroblasts from patients with RA [44]. In patients with Graves' ophthalmopathy, the interleukin IL-38 gene expression was observed to be significantly greater compared to healthy controls ($P<0.01$) [45].

This study was assessed the role of rs4591246 of IL-38 gene in patients with AS. According to this study, all 200 participants were genotyped for the SNPs (rs4849146 and rs28992497**)**of the IL-38 gene. The observed counts of genotypes AA, AT and TT were showed significant difference in comparison with expected counts ($p = 0.028$) and ($p< 0.001$) in AS patients and healthy HCs, respectively. The analysis of the association included codominance mode, dominant mode, recessive mode and alleles was performed. The analysis in this study revealed that heterozygous genotype AT, homozygous genotype AA and allele T and A of IL-38 (rs4849146) increased AS risk, while homozygous genotype TT was not a risk factor. No one of genotypes and alleles of IL-38 (rs28992497) T/C was showed to be risk factor for AS incidence. The SNP rs4591246 variant genotype was correlated with increased abdominal aortic aneurysm. The SNP rs28992497, was not showed significant association in patients with systemic juvenile idiopathic arthritis [46] and COVID-19 [47]. Polymorphisms in the gene encoding IL-38 are associated with AS and RA [48].

There were highly significant differences in the frequency of A-T (83.49 vs. 105.00) and T-C (69.51 vs. 45.00) haplotypes (rs4849AA46 A/T and rsTC899TC497 T/C) between patients and controls, according to the results of this study. The frequency of A-T haplotypes of rs4849AA46 A/T and rsTC899TC497 T/C was significantly increased in HCs than AS patients, while T-C was significantly decreased.In terms of haplotypes (in order: rs7599662, rs28992497 and rs28992498), frequency of CTC haplotype was significantly increased in patients compared to HC, while TTC haplotype showed significantly lower frequency in patients [47].

CONCLUSION

Age is one of the risk factors associated with AS. The lower expression of IL-38 in patients with AS suggests that it may play a role in the disease's incidence. Variations in genotypes and alleles may have a substantial impact on the incidence of AS.Both rs4849146 and rs28992497 SNP of Il-38 gene may effect incidence of AS.

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