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Gene Expression and Serum Levels of Interleukin-40 in Male Patients with Ankylosing Spondylitis

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Abstract: Ankylosing spondylitis (AS) is a kind of arthritis marked by persistent inflammation in the spine's joints, usually at the point where the spine and pelvis meet. Such a disease has different causes, including immunological causes. Therefore, it is necessary to investigate the role of the immune system in this disease to improve a potential treatment strategy. In this context, the present study examined serum levels and gene expression of *IL-40* in a group of Iraqi males with AS to determine their associations in AS pathogenesis. The study included a total of 200 Iraqi male participants (100 with AS and 100 healthy subjects). The serum level of IL-40 was determined using enzyme-linked immunosorbent assay (ELISA), while gene expression was examined by quantitative real-time polymerase chain reaction art- (PCR) technique.Serum level of IL-40 revealed no significant difference between the Median (IQR) of AS and HC groups, 30.63 (18.03) (pg/ml) *versus* 33.05 (24.81) (pg/ml) ($p = 0.557$) with AUC of 0.560. However, the level of *IL-40* gene expression was higher significantly ($p = 0.005$) in the AS group in comparison with the HC group, 1.23 (0.73) fold change versus a 1-fold change.Consequently, it has been suggested that IL-40 gene expression had roles in the susceptibility of AS.

Keywords: Ankylosing spondylitis, Interleukin-40, Genotype, Inflammation, C17orf99 gene

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Introduction

Ankylosing spondylitis (AS) is an immune-mediated inflammatory arthritis that belongs to the wider family of spondyloarthropathies (SpA), which also includes reactive arthritis, enteropathic arthritis, and psoriasis(1,2). Typically, the axial skeleton—specifically, the sacroiliac and spinal joints—is the target of AS, which results in considerable disability and persistent discomfort. Ankylosis, the formation of new bone, characterizes severe AS disease, leading to vertebral fusion, impaired mobility, and long-term disability (3,4). This illness is rather prevalent among inflammatory arthritides, accounting for up to 1.40% (5). The illness often begins in the sacroiliac (SI) joints, although it can affect any portion of the spine, including the peripheral joints and entheses (6). Ankylosing spondylitis is more common in men (2-4:1), with the majority of symptoms appearing between the ages of 20 and 35. On average, it takes up to eight years from the start of back discomfort to a clear diagnosis of AS (7).

The processes underlying the disease are not entirely understood. Understanding this mechanism is essential for treating the disease. A complex network of cytokines is

implicated in the pathogenesis of AS (8). Glycoproteins called cytokines help regulate inflammatory responses by acting as both pro- and anti-inflammatory messengers between cells. They are also involved in immune activity coordination (9).

The IL-40 has been one of the cytokines that have garnered interest in AS. It seems that IL-40 is implicated in a lot of inflammatory and autoimmune diseases, including rheumatoid arthritis, primary Sjogren's syndrome, ankylosing spondylitis, type 2 diabetes, Graves' disease, liver cell carcinoma, and systemic lupus erythematosus (10). In October of 2017, a novel cytokine called IL-40 was discovered. Immunoglobulin A (IgA) levels were significantly lower in IL-40-deficient mice, which was associated with a decreased number of IgA+ B cells. As a result, its role in regulating inflammatory events and the immune response, especially to B cells, has been postulated (11). The *C17orf99* gene has around 65 SNPs, with a minor allele frequency of > 10% (http://www.ensembl.org). In the previons study none of these SNPs have been studied for their relationship with the risk of any human illness. Several cytokine SNPs have been proposed to modulate serum cytokine levels (12). An IL-40-expressing cell type includes activated B cells, BM cells, and fetal hepatocytes. This protein has a low molecular weight of 27 kDa. This gene, which may be found on human chromosome 17 (17q25.3), is responsible for encoding it. Due to its lack of structural homology with other well-known cytokines, IL-40 is considered an orphan cytokine (13).

In this context, serum levels of IL-40 and gene expression were analyzed in a group of Iraqi males with AS to determine their associations in AS susceptibility.

Materials and Methods

Subject

The study included a total of 200 Iraqi male participants. The participants' ages ranged from 18 to 73 years. Individuals were divided into 100 patients with ankylosing spondylitis and 100 healthy individuals. Blood samples were obtained from each participant from January 2021 to October 2023 at Al-Yarmouk Teaching Hospital in Baghdad, Iraq.The questionnaire format included various information about each individual, patient and control, such as age, marital status, smoking, family history, disease duration, was reported. Both disease activity and functional impairment were evaluated using the Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) and the Bath Ankylosing Spondylitis Functional Index (BASFI), respectively. The patients were divided into two groups in both instances: those with a score below 4.0 (low) and those with a score over 4.0 (high) (14). The current study was accomplished in the Department of Biotechnology, College of Science, University of Baghdad.

Blood sample collection

Three ml of blood was obtained from each participant in this study. Each sample was separated into 2 parts: 2 ml was placed in a gel tube and 250 µl of blood was put in 750 µl TRIzol for RNA extraction. The blood in the gel tube was left to coagulate at room temperature and centrifuge (at 4 ◦C; 15 min; 1000 × g) was used to separate serum. Total RNA was extracted from all samples using the *EasyPure*® RNA Kit Reagent (TransGen, biotech. ER401-01) according to the manufacturer's instructions.The 2000c Nanodrop spectrophotometer (Thermo Fisher Scientific, USA) was used to

evaluate the concentration and purity of extractedRNA to determine the quality of samples for subsequent analysis. The RNA concentration ranged from (81-123) ng/µl, 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 2.0 suggested that the RNA sample was pure, in accordance with (15).

Determination of IL-40 serum levels

A sandwich ELISA kit according to the manufacturer's instructions (catalogue number In-Hu4074, MyBioSource, Inc., USA) was used to quantify serum IL-40 levels following the manufacturer's protocol.

Gene expression Synthesis of the cDNA from 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-40* 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) as shown in table 1 and 2 µl of cDNA. The cycling protocol was programmed for the following optimized cycles and according to the thermal profile, as represented in Table 3.

Table (1): Primer sequences used in qPCR detection of *IL- 40* gene expression.

Genename	Primer	Sequence (5'-3')	Primer size	Replicon size	References
IL-40	F	AGCCCACCTATCACCAACAG	20	124	this study
	R	ACCAGAACCAGTCCGATGTC	20		
GAPDH	F	GAAATCCCATCACCATCTTCCAGG	24	160	[12]
	R	GAGCCCCAGCCTTCTCCATG	20		

*F: forward; R: Reverse.

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

To measure changes in the quantitative expression of mature RNAs, the relative cycle threshold ($2^{-\Delta\Delta Ct}$) method, initially described by Livak and Schmittgen, was employed(16).

Statistical analysis

To analyze statistically significant differences among normally distributed variables, analysis of variance (ANOVA) with post hoc least significant difference (LSD) was used. We used the Mann-Whitney U test to determine if there were statistically significant differences, and we presented skewed variables as median and interquartile range (IQR: 25-75%). Using receiver operating characteristic (ROC) curve analysis, we assessed IL-

40's ability to distinguish between individuals with AS and HC. Statistical measures such as sensitivity, specificity, area under the curve (AUC), 95% CI, cut-off point (as modified by the Youden index), and reliability were reported. Statistical significance was granted to results where the p-values were less than 0.05. To reduce the occurrence of type I errors induced by repeated comparisons, the Bonferroni correction was employed. This required taking the p-value and multiplying it by the pc-value, which is the number of comparisons. We used IBM SPSS Statistics 25.0 (Armonk, NY: IBM Corp.) to do the statistical analyses, and GraphPad Prism 9.4.1 (San Diego, CA, USA) to make the figures.

Results and Discussion

Baseline characteristics

The current study included 100 AS patients with a mean duration of disease of 8.87 ±6.38 years and it ranged from 3 months to 26 years.The study also included 100 health control subjects (HC). Comparison of mean age between AS and HC revealed a significant difference ($p< 0.001$); 41.10 ±13.00 years*versus*33.54 ±7.92, respectively. Concerning marital status, the proportions of married and not married patients were 74 (74.0 %) and 26 (26.0 %); while those of HC were 62 (62.0 %) and 38 (38.0 %); the difference was not significant ($p = 0.069$). Positive family history was reported in 24 (24.0 %) cases of AS group which was significantly higher ($p< 0.001$) than that reported in HC; 4 (4.0 %). Smoking was reported in 36 (36.0 %) AS and 34 (34.0 %) HC; therefore, there was no significant variation ($p = 0.767$), as displayed in table (3).

Table (3): Baseline characteristics of men with ankylosing spondylitis and healthy subjects.

Characteristic	AS n = 100	HC n = 100	P-value
Age (years)			
Mean ±SD	41.10 ±13.00	33.54 ±7.92	< 0.001 I ***
Range	18 -73	18 -52	
Marital status			
Married	74 (74.0 %)	62 (62.0 %)	0.069 C NS
Not married	26 (26.0 %)	38 (38.0 %)	
Family history			
Positive	24 (24.0 %)	4 (4.0 %)	< 0.001 C ***
Negative	76 (76.0 %)	96 (96.0 %)	
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; AS: Ankylosing spondylitis; HC: Healthy controls; n: number of cases; I: independent samples t-test; C: chi-square test; NS: not significant; *: significant at p ≤ 0.01; p: probability (significance was determined using one-way analysis of variance (ANOVA) post-hoc least significant difference (LSD) test; significant p-value is indicated in bold).**

A study found that out of 2579 patients with axial spondyloarthritis (axSpA), the vast majority (92%) had an age at onset of less than 45 years, and this was true across all geographic regions evaluated. There are 94% in Asia, 92% in Europe and North America, 89% in Latin America, and 91% in the Middle East (17). In the study of (18), the age at which patients

first experienced AS was a factor in the hereditary risk. The risk was higher for patients whose illnesses started before the age of 25 compared to those whose illnesses started after that age. According to this study, 100 AS patients had an average age of 41.96±9.11 (19). The median age of the patients and controls was 40.7 and 40.8 years old, respectively, which is not significantly different from the results of the study by (20). Additionally, it has been stated that 132 patients with AS had an average age of 37.61 ± 10.0 years, which is lower than the results of this study. However, these results were lower than those of a study by(21), which found an average age of 59.3 ± 12.1 years for 974 patients with AS. One possible explanation for these findings is that different research used different sample sizes. Although female patients can also experience AS it is more common in males and typically affects those less than 45 years old (22). Different people with AS may experience different symptoms and how the disease develops at different ages.

Furthermore, the average ± standard deviation of the time it took for the disease to progress was 8.87 ±6.38 years. This value was similar to that of a study published by (23), which indicated that the median time it took for AS to be diagnosed was 8.0 years; however, it was lower than the value reported by (24), which indicated that the duration of AS was 20.5±11.8 years. Environmental and genetic factors, as well as delays in diagnosis and treatment, as well as the frequency and duration of illness monitoring, all contribute to the fact that AS will last varying amounts of time in various individuals (25,26).

The average age of male AS patients was 41.10 years, according to this study. The Regular assessments of disease activity and functional impairment in AS patients are recommended by clinical guidelines. This was achieved by utilizing the BASFI, which may be evaluated on a scale from 0 to 10. According to previous research, an AS score of 4 or above indicates active disease, while a score below 4.0 indicates clinical improvement of AS (14,27). The condition was determined to be clinically developing, as the Mean ±SD of BSAFI was 3.41±1.68 according to this study's data. Consistent with the results of the study cited in (23), the average BSAFI for AS patients was 2.4.

According to this study, there was no significant difference between married mothers with AS (67%; n=1322) and healthy mothers (67%; n=8377) (28).

Another finding was that there was a statistically significant difference in family history among the research groups. Assuming that risks between siblings were entirely attributable to genetics, a study described by (18) found a heritability of 77% (95% CI 73, 80). Genomic factors account for more than 90% of the risk of AS, the prototypic seronegative arthropathy. It is recognized that the illness has a strong genetic component. Finding the genes involved in this syndrome via candidate gene or family-based approaches has been slower than with most prevalent heritable disorders (18). The degree to which variations in a trait's genes explain the observed variation in that trait is called its heritability. Twin studies have demonstrated that AS has a heritability of 90-99%(29), which is significantly higher than other traits including RA (40%), inflammatory bowel disease (65-75%), and adult height (80-90%). Because of this, AS would be considered a highly heritable trait (30).

On the other hand, only 40 sets of twins were included in the research on AS heredity factors(29).

Medical professionals around the world are worried about the correlation between cigarette smoking and other diseases. It can lead to dysregulation by influencing adaptive and innate immunity, and it can enhance pathogenic immunological responses or decrease defence immunity. Smoking and spondyloarthropathy do, however, have a complicated association. Smoking may exacerbate the illness, according to research, but the exact reason is yet unclear (31).

Serum levels of IL-40

The comparison of serum IL-40 revealed no significant difference between the Median (IQR) of AS and HC groups, 30.63 (18.03) (pg/ml) versus 33.05 (24.81) (pg/ml) ($p = 0.557$), as shown in figure (1. A).Receiver operating characteristic (ROC) curve analysis was carried out to find the cutoff values of serum levels of IL-40 that can predict a positive diagnosis of disease and these results were demonstrated in Figure (1. B). The cutoff value of serum IL-40 was ≤41.6 pg/ml, but this cutoff was not valid because the area under the curve (AUC) was < 0.700 (0.560).

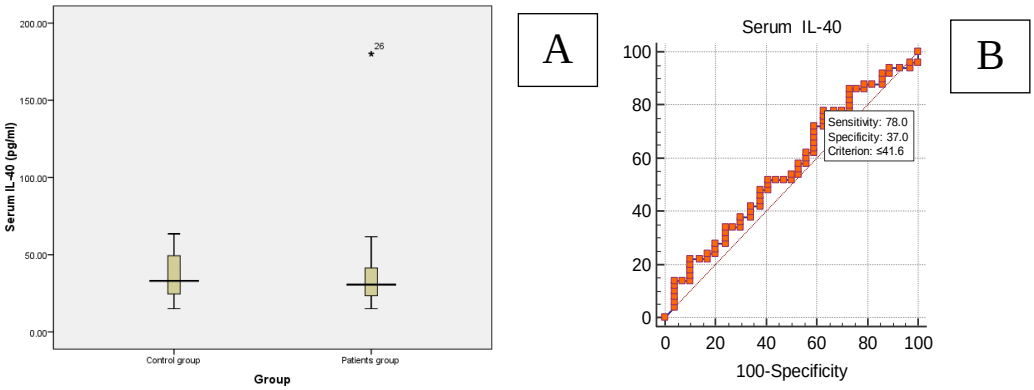

Figure (1): A: Box-whisker plots showing a comparison of serum IL-40 levels between the AS group and HC group. B: Receiver operating characteristic (ROC) curve analysis to find the cutoff value of serum IL-40 that can predict a positive diagnosis of disease.

Gene expression of *IL-40* (*C17orf99*)gene

The level of *IL-40* gene expression was higher significantly ($p = 0.005$) in the AS group in comparison with the HC group, with 1.23 (0.73) fold change versus 1-fold change, as shown in Figure (2. A), respectively. The cutoff value of *IL-40* gene expression was >0.122-fold change, and this cutoff was valid because the area under the curve (AUC) was > 0.700 (0.675) in addition the p-value was significant ($p < 0.001$), in addition, there was poor sensitivity of 53 %, very good specificity of 84 % and moderate accuracy of 67.5 %, as shown in figure (2. B).

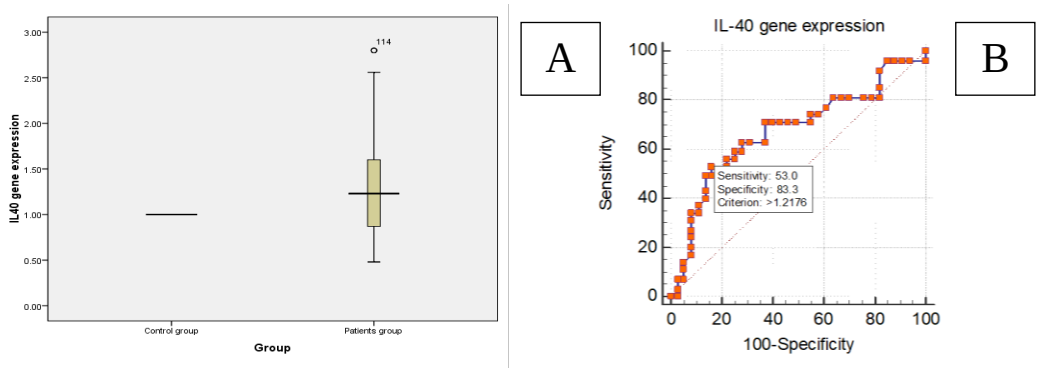

Figure (2): A: Box plot showing a comparison of *C17orf99* gene expression level between the AS group and HC group. B: Receiver operating characteristic (ROC) curve analysis to find the cutoff value of *C17orf99* gene expression that can predict a positive diagnosis of disease.

The chronic inflammatory response is thought to play a causal role in AS. Evidence suggests that several cytokine pathways, including pro-inflammatory and anti-inflammatory interactions, contribute to disease etiology (32,33). The study found that AS patients had a non-significant difference in levels of IL-40 in their serum compared with HC, with an AUC value of 0.560, indicating poor diagnostic performance, in disagreement with Jaber and Ad'hiah(8) who found that the level of IL-40 in sera of AS patients was higher than HC, with its diagnostic performance was excellent as indicated by an AUC value of 0.886. However, the gene expression of *IL-40* was observed as an excellent diagnostic indicator with an AUC value of 0.675 for predicting AS incidence. It was recently shown that serum and synovial IL-40 levels were elevated in RA patients and linked favorably with rheumatoid factor-IgM and anti-cyclic citrullinated peptides. Furthermore, overexpression of IL-40 was detected in IL-40-deficient animals that had a general IgA deficit, and the absence of IL-40 was related to a dysregulated gut flora (11,13).Concerning AS, the latter discovery is significant since it is claimed that the gut microbiota may play a role in the development of AS(34). IL-40 was shown to be overexpressed in rheumatoid arthritis (RA) synovial tissue, notably in the synovial lining and invading immune cells. Rheumatoid arthritis (RA) patients had significantly higher levels of IL-40 in synovial fluid compared to OA patients ($p<0.0001$). RA patients had significantly higher blood levels of IL-40 than those with HC, OA, or SLE ($p<0.0001$) (35).

Correlation analysis

Serum IL-40 showed a significant positive correlation to age, presence of family history, and ESR3 ($p > 0.001$). *IL-40* gene expression showed no significant correlations, as represented in Table 8.

Table (4): Correlation among IL-40 serum levels and gene expression and other characteristics of patients

Characteristic	Serum IL-40		IL40 gene expression	
	r	*p*	*r*	*P*
Age	0.347	<0.001 ***	-0.120	0.234
Marital status	0.139	0.169	-0.159	0.114
Family history	0.274	0.006 **	-0.017	0.870
Smoking	-0.155	0.125	0.068	0.502
Duration of disease	0.124	0.219	0.132	0.191
ESR1	0.158	0.116	-0.120	0.234
ESR2	-0.171	0.089	-0.069	0.498
ESR3	0.231	0.021 *	-0.035	0.726
BASFI-1	-0.146	0.146	-0.126	0.212
BASFI-2	0.050	0.620	-0.037	0.718

Conclusions

Age is considered a risk factor for AS. *IL-40gene* is overexpressed in patients with AS, suggesting that it may have a role in the incidence of the disease.

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