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Gene expression profile of proinflammatory cytokines in Iranian patients with ankylosing spondylitis

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Ankylosing spondylitis (AS) is mostly characterized by inflammation of the sacroiliac joints, enthesis and the spine. This study aims to determine gene expression profile of proinflammatory cytokines and their correlations with disease activity, spinal mobility, functional status, quality of life, and smoking in Iranian patients with ankylosing spondylitis. Peripheral blood mononuclear cells (PBMCs) were isolated from 48 patients with AS and 47 age and gender-matched healthy controls; then total RNA content of leukocytes was extracted, followed by cDNA synthesis from the mRNA of PBMCs. Quantitative polymerase chain reaction was performed to measure mRNA expression of TNF-α, IL-1β and IFN-γ genes. Clinical characteristics were evaluated and their correlations were analyzed with gene expression levels of the prionflammatory cytokines. A significant overexpression of TNF-α was observed in the patient group, but there was no significant difference in expression of other cytokines between groups. A positive correlation (P< 0.01) between TNF-α and Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) and negative correlation (P< 0.05) between IFN-γ and Bath Ankylosing Spondylitis Functional Index (BASFI) were found. Eta-Squared test supported significant results about the smoking effect on Bath Ankylosing Spondylitis Metrology Index (BASMI) (P< 0.05) and IFN-γ (P< 0.01). Our results demonstrated that TNF-α was the most important cytokine responsible for inflammatory-related outcomes of AS in the Iranian population.

Keywords: Ankylosing spondylitis, Proinflammatory cytokines, Gene expression

Introduction

About 0.1 to 1.4% of Caucasian population experiences the debilitating chronic inflammatory disease named ankylosing spondylitis (AS) [1]. This prototypic form of seronegative spondyloarthropathies involves primarily the axial skeleton joints. Enthesis, where ligaments, tendons and joint capsules are attached to the bone, is the main involved site [2]. Although the combination of genetic, environmental and immunological factors can cause the clinical manifestations, it seems that AS is of the strongest genetic background when compared to any other rheumatic diseases [3]. In this regard, the role of human leukocyte antigen (HLA)-B27 in AS pathogenesis has been discovered for more than 40 years [4].

From the immunological point of view, several cytokine pathways have been associated with AS based on both genome-wide association (GWA) studies and experimental investigations [5-7]. Tumor necrosis factor (TNF)-α, interleukin (IL)-1β and interferon (IFN)-γ are the main proinflammatory cytokines, culminating in manifestations of autoimmune disorders [8]. In HLA-B27 mis-folding theory, endoplasmic reticulum (ER) stress leads to increased expression of proinflammatory cytokines [9].

Tumor necrosis factor α is a pleiotropic cytokine, mainly produced by activated macrophages and has different biological functions in different tissues. This proinflammatory cytokine belongs to TNF superfamily, and exerts its function through ligation with two receptors of TNFR1 and TNFR2. Signaling through TNFR1 results in proinflammatory, anti-apoptotic and regulatory processes, but TNFR2 is critical for stabilization of regulatory T cells (Treg) [10-12]. Evidence shows that TNF-α blockade by infliximab is

sufficient to reduce pain and inflammation in AS patients [13].

Single Nucleotide Polymorphisms (SNPs) in TNF-α gene promoter, especially at -238 (G/A) and -308 (G/A) positions, have been demonstrated in a number of population-based studies [14-17]. They rest on the belief that these polymorphisms irrespective of HLA-B27, might have an important role in AS pathogenesis. However, their effect on susceptibility or severity of the AS has remained a subject of debate.

As a proinflammatory cytokine, IL-1β is secreted by all nucleated cells, especially macrophages [18]. Many studies have established the role of IL-1 in autoimmune diseases [19]. In addition, SNPs of IL-1 receptor (IL-1R) have been shown to be associated with AS susceptibility [20]. Moreover, IFN-γ, which is produced and secreted by a variety cells of innate and adaptive immunity, plays an important role in autoimmunity [21]. As mentioned above, ER stress molecules especially unfolded protein response (UPR)-related molecules cause increased IFN-γ expression in HLA-B27 positive patients [22].

Regarding the necessity to address such discrepancies to gain a unique understanding, in this study we evaluated mRNA expression levels of TNF-α, IL-1β and IFN-γ proinflammatory cytokines in peripheral blood mononuclear cells (PBMCs) of AS patients in comparison to the healthy control group. Furthermore, it was attempted to find out if there were plausible correlations between cytokine gene expressions with disease manifestations.

Methods and Materials

Patients
Forty-eight (34 males and 14 females) unrelated AS patients (40.02±11.25 years old) undertaking no immunomodulatory therapy, whose diagnosis were fulfilled according to the modified New York criteria [23], were selected from patients recruited in the rheumatology research center, Shariati hospital, Tehran University of Medical Sciences. Forty-seven (36 males, 11 females) age and gender-matched healthy individuals (37.79±9.43 years old) without any history of autoimmune diseases for themselves and their first-degree relatives were also included. The human research ethics committees of the Tehran University of Medical Sciences approved this study and written informed consent was taken from all participants. In order to record demographic, medical, familial and pharmacological history of the study subjects, a questionnaire was exerted. To determine disease activity, quality of life, spinal mobility and functional disability, indexes including Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) [24], Ankylosing Spondylitis Quality of Life (ASQoL), Bath Ankylosing Spondylitis Metrology Index (BASMI) [25] and Bath Ankylosing Spondylitis Functional Index (BASFI) [26] were measured for each patient. About 5 mL of blood from all participants was collected in Ethylenediaminetetraacetic acid (EDTA)-anticoagulated tubes and Erythrocyte Sedimentation Rate (ESR) blood collection test tubes by venipuncture.

Peripheral blood mononuclear cells separation and RNA extraction
Ficoll-Hypaque density gradient centrifugation approach was used in order to isolate PBMCs from peripheral blood of the subjects [27]. Total cellular RNA was extracted using high pure RNA isolation kit (Roche, Germany), according to the manufacturer's manual. Yield and purity of RNA were measured using a NanoDrop spectrophotometer at 260/280 nm (NanoDrop ND-2000c Spectrophotometer, Thermo Fisher scientific, USA).

Reverse transcription and complementary DNA synthesis
Complementary DNA (cDNA) was synthesized from extracted RNA by the Transcriptor first strand cDNA synthesis kit (Roche, Germany), according to the manufacturer's manual. To go through the procedure briefly, first 1 μg of RNA was mixed with 2 μL of random hexamer primer and corresponding amount of RNase-free H_2O to reach a total volume of 13 mL and then incubated at 65°C for 10 minutes. Afterwards, micro-tubes were chilled on ice and a mixture of reaction buffer (4 μL), RNase inhibitor (0.5 μL), dNTP mix (2 μL) and reverse transcriptase (0.5 μL) was added to each sample. Samples were then incubated at 25°C for 10 minutes followed by 50°C for 60 minutes; the reaction was finally terminated by heating at 85°C for five minutes with a final volume of 20 μL pre tube.

Real-time quantitative polymerase chain reaction
Primers for gene mRNA expressions of TNF-α, IL-1β and IFN-γ, and β2M as a housekeeping gene were designed using primer express 3.0 (Applied Biosystems, Foster city, CA, USA). For more details of primers, refer to Table 1. To determine accuracy and specificity, all primers were blasted using the basic local alignment search tool on the USA national center for biotechnology information website (http://www.ncbi.nlm.nih.gov/tools/primerblast/). Primers were synthesized by the

Table 1. Primers used in real-time polymerase chain reaction

Gene	Primer		Product size (bp)	Tm (°C)	Gene ID
IL-1β	F	5′-ATGGCTTATTACAGTGGCAATGAG-3′	138	59.42	576.2
	R	5′-GTAGTGGTGGTCGGAGATTCG-3′		60.20	
TNF-α	F	5′-CCTGCCCCAATCCCTTTATT-3′	81	57.52	594.3
	R	5′-CCCTAAGCCCCCAATTCTCT-3′		58.77	
IFN-γ	F	5′- CTGACTTGAATGTCCAACGCAAAGC-3′	120	63.47	619.2
	R	5′- CTCTTCGACCTCGAAACAGCATCTG-3′		63.58	
β2M	F	5′- CCTGAATTGCTATGTGTCTGGG-3′	244	59.05	4048.2
	R	5′- TGATGCTGCTTACATGTCTCGA-3′		59.83	

custom oligonucleotide synthesis service, Metabion (Martinsried, Germany). Quantitative analysis was performed by real-time polymerase chain reaction using the SYBR Green PCR master mix and StepOne real-time PCR system (both applied Biosystems). Each reaction mixture contained a total volume of 20 μL (master mix 10 μL, cDNA 2 μL, forward primer 0.2 μL, reverse primer 0.2 μL and H_2O 7.6 μL). The quantitative real- time PCR conditions were: 95°C for 10 minutes, then 40 cycles at 95°C for 15 seconds and 60°C for 60 seconds. Using a melting curve, the purity of each amplified product was confirmed. A widely used approach to represent relative gene expression, the comparative CT method, was exploited to evaluate expressions, as previously described by Schmittgen and Livak [28]. Relative amounts of target mRNAs in test samples were calculated and normalized to the corresponding β2M mRNA transcript level as a housekeeping gene. Next, relative expression for each sample was calculated using the following equation: relative mRNA expression $=(2^{-\Delta Ct}) \times 10^3$.

Statistical analysis

Chi-square and independent t-test were performed for matching gender and age in the groups, respectively. Normality test was done using Kolmogorov-Smirnov for scale variables. If data did not have a normal distribution, Mann-Whitney nonparametric test was performed for comparing means. Correlation analysis was done using Spearman's Rank-Order. All the data were analyzed using IBM SPSS version 21.0 (SPSS, Chicago, IL, USA). The P values of less than 0.05 indicated statistical significance.

Results

Demographic and laboratory characteristics of study subjects

Demographic and clinical characteristics of the study participants are summarized in Table 2. There was no significant difference in age or gender between case and control groups. However, smoking rate and ESR level were significantly higher in patients in comparison to healthy subjects.

Table 2. Demographic and clinical characteristics of the study participants

Variables	Groups		P Value
	AS patients (n = 48)	Healthy control (n = 47)	
Age (Year)	40.02 ± 11.25	37.79 ± 9.43	0.298
Sex			
Male	34	36	0.524
Female	14	11	
Smoking (%)	63.83	32.61	0.003
ESR (mm/h)	35.7±35.03	6.48±8.12	< 0.0001
BASDAI	4.32±2.22	-	-
BASMI	4.93±1.88	-	-
BASFI	3.64±2.45	-	-
ASQoL	7.71±4.72	-	-
Disease duration	12.40±9.48	-	-

Abbreviations: AS, Ankylosing Spondylitis; ASQoL, Ankylosing Spondylitis Quality of Life; BASDAI, Bath Ankylosing Spondylitis Disease Activity Index; BASMI, Bath Ankylosing Spondylitis Metrology Index; BASFI, Bath Ankylosing Spondylitis Functional Index; ESR, Erythrocyte Sedimentation Rate.

Gene expression results by the real-time Polymerase Chain Reaction (PCR) method

Relative to the control, mRNA expression levels of the studied cytokines were increased in PBMCs of AS patients. The expression level of TNF-α mRNA was upregulated significantly in AS patients compared with healthy controls (AS vs. healthy controls; fold change= 3.58; P= 0.024; Table 3 and Fig. 1). However, there was no significant increased expression of IL-1β and IFN-γ mRNAs in AS patients relative to healthy controls with the fold changes of 1.73 and 1.36, respectively.

Correlation between gene expression and clinical features

The correlation coefficients (r) and corresponding P values of correlation analysis between cytokine gene expression levels and clinical data of patients are shown in Table 4. A positive significant correlation was observed between TNF-α mRNA expression and BASDAI score (r= 0.416, P= 0.004). Moreover, there was a negative correlation between IFN-γ mRNA expression with BASFI criteria (r= -0.308, P= 0.035).

Smoking effect as measured by Eta-squared, demonstrated a significant positive effect on IFN-γ mRNA expression levels (η^2= 0.240, P= 0.006). Furthermore, a significant effect was observed between smoking and BASMI scores (η^2= 0.339, P= 0.029). However, no significant correlation was observed between BASMI and IFN-γ.

Table 3. Comparison between IL-1β, TNF-α and IFN-γ expression in ankylosing spondylitis patients and controls

Cytokines	Groups		Fold change (AS vs. healthy controls)	P value
	AS patients (n= 48)	Healthy controls (n= 47)		
IL-1β	29.43±48.81	17.04±28.52	1.73	0.170
TNF-α	30.64±45.06	8.54±8.60	3.58	0.024[*]
IFN-γ	2.12±0.93	1.56±0.49	1.36	0.461

AS: Ankylosing Spondylitis; TNF-α: Tumor Necrosis Factor-α; IL-1β: Interleukin-1β; IFN-γ: interferon-γ
* Significant correlation at 0.05 level
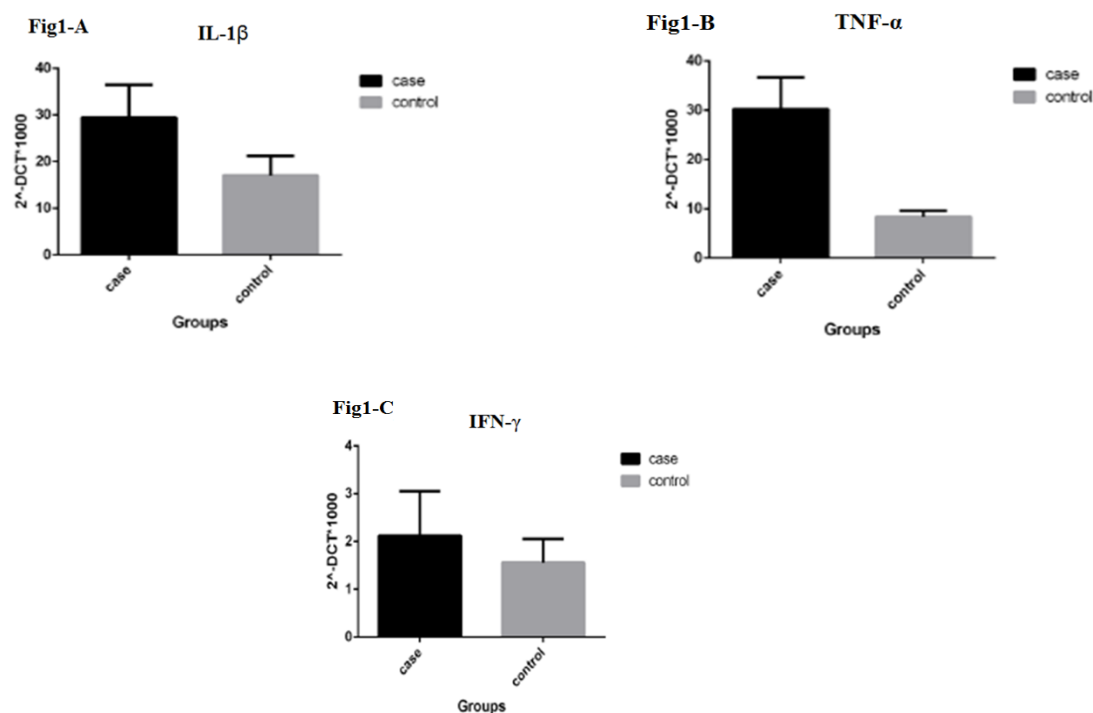
Fig. 1. Relative mRNA expression of IL-1β (A), TNF-α (B) and IFN-γ (C) in PBMCs from AS patients (n= 48) in comparison to healthy controls (n= 47). Data are represented as mean±SEM

Table 4. Correlation between gene expressions and patients' clinical data[a]

Clinical data	TNF-α	IL-1β	IFN-γ
BASDAI	0.416, (0.004)[b]	0.259, (0.078)	0.180, (0.227)
BASFI	0.171, (0.257)	0.201, (0.175)	−0.308, (0.035)[b]
BASMI	−0.136, (0.367)	−0.171, (0.251)	−0.189, (0.203)
Disease duration	0.138, (0.359)	0.077, (0.605)	−0.159, (0.284)
ASQoL	0.135, (0.382)	0.101, (0.510)	−0.122, (0.425)
ESR	0.067, (0.527)	0.104, (0.323)	−0.050, (0.639)

BASDAI: Bath Ankylosing Spondylitis Disease Activity Index; BASFI: Bath Ankylosing Spondylitis Functional Index; BASMI: Bath Ankylosing Spondylitis Metric Index; ESR: Erythrocyte Sedimentation Rate
[a]Data in each cell shown as correlation coefficient, (P value)
[b]Significant correlation at 0.05 level

Discussion

Although the importance of proinflammatory cytokines in inflammatory diseases [29-32] is well established, so far no study has been conducted for their gene expression profiles in AS patients. Studies show incongruent results about cytokine expression pattern in these patients. However, such discrepancies might be explained regarding the different clinical characteristics of various populations and different laboratory methods. So far, several studies about cytokine levels in AS patients were performed based on protein-based techniques. Since the actual levels of cytokines may be underestimated by diagnostic methods at protein levels, a much more sensitive measurement, perhaps through mRNA expression level, is required.

Furthermore, IL-1β and TNF-α are macrophage-derived cytokines that induce autoimmunity setting in many tissues [33]. In rheumatoid arthritis (RA), the subchondral erosion process is mediated primarily by these cytokines [34]. Because such erosions have been also seen in AS [35], these cytokines might also play a role in AS pathogenesis. Another cytokine is IFN-γ, which contributes to organ damage and systemic involvement of inflammatory mechanisms [36].

Earlier evidence of involvement of proinflammatory cytokines in AS pathogenesis came from the study of Gratacos et al. [37]. They evaluated cytokine levels of 69 patients with AS in comparison to 43 RA patients and 36 outpatients with non-inflammatory back pain (NIBP), through an enzyme linked immunosorbent assay (ELISA) approach. By comparing AS and NIBP groups, they observed a higher TNF-α concentration in sera of AS patients; but no detectable IL-1β concentrations were found in all groups. Through examining synovial biopsies of AS patients, increased TNF-α but not IL-1β mRNA expressions were found with the in situ hybridization technique [38]. The study included only three patients for evaluations; therefore, due to the small sample size, experiments could not explain the reliability of the findings. Significantly higher TNF-α and IL-1β concentrations in a Chinese population of AS patients were found. Furthermore, they observed a positive correlation between IL-1β concentration and BASDAI [39]. The correlation result between TNF-α and disease activity index was in accordance with our study. However, contrary to our results, they did not find any correlation between BASFI and cytokine concentrations. Peripheral blood mononuclear cells are important players of immune responses [40]. Rudwaleit et al. [41] analyzed PBMCs of HLA-B27 positive AS patients, and positive and negative healthy controls with flow cytometry. They found lower production of TNF-α and IFN-γ in both HLA-B27 positive patient and control groups with an unknown mechanism. This study divulged an association between HLA-B27 positivity and lower cytokine concentrations. Vazquez-Del Mercado et al. [18] assessed concentrations of TNF-α, IL-1β and some other cytokines in a supernatant soup of cultured PBMCs of 27 AS and 24 control individuals in presence of Phytohemagglutinin (PHA). Higher IL-1β but not TNF-α concentrations were observed in AS patient. However, IL-1β concentration did not correlate with disease activity indices of AS patients. Their results were in accordance with the lower amount of TNF-α in HLA-B27 positive individuals as shown in a previous study, but both studies were in discordance with our results. Toussirot et al. [42] used the Radioimmunoassay (RIA) technique to measure TNF-α and IL-1β levels in spondyloarthritis patients with mixed clinical manifestations. They reported equal IL-1β but raised TNF-α levels in the patient group. Again, they had a low sample size in their study.

To explain these contradictory results first we must consider that in the ELISA method due to cellular consumption of cytokines, it is difficult to estimate their actual levels. Second, this method can detect targets at the protein level. Because of post-transcriptional modifications, RNA measurement through real-time PCR technique does not always reflect the corresponding

protein levels. Other possible factors include ethnic differences in study populations and small sample size in some studies.

In the study underway, we found higher expression of IL-1β, TNF-α and IFN-γ in AS patients; however only TNF-α was significantly overexpressed in PBMCs of AS patients compared with healthy subjects. According to the correlation results shown in this study, by progressing disease due to inflammation effect on axial skeleton, patients dismiss their spinal flexibility. As a result, it is obvious that increasing disease duration enhances BASMI score. In addition, greater functional limitation (higher BASFI score) is associated with poor quality of life (high ASQoL score). Regarding the smoking effect on clinical indexes and mRNA expression levels of cytokines, a significant effect was observed on BASMI and IFN-γ.

The role of smoking as a risk factor of AS development is well-established in a bulk of studies. In this respect, Fallahi et al. [43] revealed significantly higher BASMI scores in current smokers. In a survey by Zhang et al. [44], AS patients with a smoking habit were shown to have a higher BASMI score than non-smokers. Our data was in accordance with their results. However, the limitation of both aforementioned studies was the type of study. In the view that both studies were cross-sectional, unilateral effect of smoking was not concluded on related outcomes. This means that possessing these criteria could result in AS susceptibility. Moreover, in sight of Fallahi et al., other factors might be responsible for restriction in spinal movement.

Many studies have considered smoking as an independent variable for higher functional limitation [45-47]. However in this study, no significant correlation was found between smoking and BASFI scores.

Conclusion

Considering all these facts, this study was conducted to measure mRNA expression profile of proinflammatory cytokines in Iranian patients with ankylosing spondylitis. We found TNF-α as an important factor involved in disease inflammatory mechanism and in determining disease severity and outcomes. Further investigations with emphasis on specific immune cell sorts are mandatory to improve valid apprehension of the AS inflammatory process.

Conflict of interest

The authors declare no conflict of interest.

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