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Upregulated Expression of Runt-related Transcription Factor 3 and Interferon Regulatory Factor 4 Genes in Patients With Ankylosing Spondylitis

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ABSTRACT

Background: Ankylosing spondylitis (AS) is a debilitating autoimmune disease presented by chronic inflammation of large joints and central skeleton. The role of various immune cells, including T cell subsets, has been studied in the pathogenesis of AS. Two critical transcription factors, runt-related transcription factor 3 (*RUNX3*) and interferon regulatory factor 4 (IRF4), are involved in the differentiation and function of T lymphocytes. This study compared the gene expression level of *RUNX3* and *IRF4* between patients with AS and healthy subjects to understand the impact of these factors in the immunopathogenesis of the disease.

Materials and Methods: Thirty patients with AS and 30 age- and gender-matched healthy individuals were recruited to the study, and expression of *RUNX3* and *IRF4* genes was evaluated using the reverse transcription polymerase chain reaction (RT-PCR) technique in their peripheral blood.

Results: The expression of *RUNX3* and *IRF4* genes in AS patients was significantly upregulated compared to the healthy controls (P=0.03 and 0.025, respectively). In addition, there was a direct correlation between *IRF4* gene expression and bath ankylosing spondylitis global score (BAS-G) (correlation coefficient=0.38, P=0.04)

Conclusion: Gene expression of *RUNX3* and *IRF4* transcription factors involved in T cells' differentiation and function was increased in AS. These findings might have prognostic and therapeutic value.

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Introduction

Ankylosing spondylitis (AS), a chronic autoimmune disorder, affects between 0.1% and 1.4% of the population, mainly young men, and causes significant physical, psychological, social, and economic problems [1]. AS presents with chronic inflammation of the spine, hip, and shoulder joints as well as extra-articular complications, including ocular, gastrointestinal, and pulmonary symptoms [2]. According to the inflammatory nature of AS, a thorough understanding of its immunopathogenesis, particularly identifying the key immune elements involved in the development and progress of the disease, might introduce novel diagnostic and therapeutic targets. Indeed, the critical role of T lymphocyte subsets has already been demonstrated in the pathogenesis of AS [3, 4]. For instance, T helper 17 (Th17) cells may be the main T cell subset implicated in initiating and expanding inflammatory responses in joints and the gastrointestinal tract [5, 6]. Moreover, the Th1/Th2 ratio has been reported to be higher in AS patients than healthy individuals [3]. Accordingly, cytokines, chemokines, and transcription factors involved in the differentiation and activation of T cells might be important in describing the immunopathogenesis of AS. Two transcription factors that play a role in the development and function of immune cells, especially T lymphocytes, are runt-related transcription factor 3 (*RUNX3*) and interferon regulatory factor 4 (IRF4) [7, 8]. *RUNX3* and *RUNX1* are expressed in the thymic medulla and cortex, respectively, and are involved in developing CD8 T cells during thymopoiesis [9]. Moreover, it appears that dysregulated expression of *RUNX3* results in Th1/Th2 and Th17/T regulatory (Treg) cell imbalance and enhanced proinflammatory responses [10, 11]. Furthermore, certain *RUNX3* gene polymorphisms have been associated with autoimmune diseases, such as psoriatic arthritis, systemic lupus erythematosus, and AS [12-14]. For instance, rs11249215 and rs4648889 polymorphisms seem to be significantly correlated with developing AS [15, 16].

IRF4 is the other transcription factor differentiating T cell subsets [17]. Its implication has been demonstrated in the pathogenesis of inflammatory disorders such as autoimmune diseases, allergies, and transplant rejection [18-20]. *IRF4* is a major transcription factor in differentiating Th17 lymphocytes [21]. Besides, a significant association has been reported between the risk of single nucleotide polymorphisms (SNP) of *RUNX3* and *IRF4* recruitment to the transcription site in patients with ankylosing spondylitis [22]. However, there is insufficient data about the association of *IRF4* expression or its SNPs with the development and prognosis of spondyloarthropathies.

Therefore, we aimed to evaluate the expression level of *RUNX3* and *IRF4* genes in AS patients and compare it with healthy subjects. It was hypothesized that any upregulated expression of transcription factors involved in T cell differentiation and function might provide a link between T lymphocyte hyperactivation and disease development or activity.

Materials and Methods

Study patients

The present research was conducted at Imam Khomeini Hospital of Tehran University of Medical Sciences between July 2022 and April 2023. Thirty patients diagnosed with ankylosing spondylitis were recruited according to the modified New York criteria [23]. Thirty sex- and age-matched individuals were selected, and their peripheral blood samples were taken. The exclusion criteria were medical history of any autoimmune disease other than AS, malignancy, severe allergy, active infection or fever, receiving tumor necrosis factor (TNF) inhibitors within two months before sampling, and age over 60 years. Disease activity indices, including bath ankylosing spondylitis metrology index (BASMI), bath ankylosing spondylitis disease activity index (basdai), bath ankylosing spondylitis global score (BAS-G), bath ankylosing spondylitis functional index (BASFI), and ankylosing spondylitis quality of life (ASQOL) were used to evaluate disease activity in patients.

RNA extraction and cDNA synthesis

A high pure RNA isolation kit (ROJE Technologies, Tehran, Iran) was used for RNA isolation from peripheral blood. RNA quality was evaluated with a spectrophotometer (NanoDrop ND1000; Thermo Scientific, Waltham, MA, USA). DNase was used in RNA extraction. RNA samples with A260/A280 absorbance ratios of 1.8 to 2.2 and the A260/A230 ratios of 2 to 2.2 were assigned to further process. Complementary DNA (cDNA) synthesis was performed with a transcriptor first-strand cDNA synthesis kit (ROJE Technologies, Tehran, Iran). cDNA samples with A260/A280 ratios of 1.7 to 2 were stored at -70°C until RT-PCR test.

Real-time polymerase chain reaction test

After obtaining acceptable amplification bands of target genes in gel electrophoresis, a relative gene expression assay of *RUNX3* and *IRF4* was performed using SYBR-Green real-time polymerase chain reaction (RT-PCR) test. 18S rRNA was used as the internal control (reverse and forward

primers sequence [metabion GmbH, Germany] have been shown in Table 1). One microliter of assay mix (including forward and reverse primers), 2 μL of diluted sample cDNA (5 ng/μL), 10 μL of master mix (RealQ Plus Green; Ampliqon, Odense, Denmark), and 7 μL of distilled water were added to the wells. The reaction cycles included 50˚C for 2 min, 95˚C for 10 min, 45 cycles of 95˚C for 15 s, and 60˚C for 1 min provided by StepOnePlus™ RT-PCR System (Applied Biosystems, Waltham, MA, USA). Nontemplate controls were used in each run. The primer efficacy ranges from 1.95 to 2.01. All tests were performed in duplicate and quantified relative to the expression of the internal control. Threshold cycle number was used to assess the relative expression of target genes. The relative expression was calculated using the Equation 1:

1. Relative mRNA expression=($2^{-\Delta\Delta C_t}$) (Livak method) [24].

Statistical analysis

SPSS software, version 26 (IBM Corp., Armonk, NY, USA) was used for statistical analysis. The data were presented as Mean±SD or Mean±SD of the mean. The normality of distribution was evaluated with the Kolmogorov-Smirnov test. The independent sample t-test and Mann-Whitney U test were applied to compare the variables with normal and non-normal distributions, respectively. The correlation test was performed with regression analysis presented as correlation coefficient. P<0.05 were considered statistically significant.

Results

Demographic characteristics, clinical, and laboratory data of the study population

Ninety percent of the patients were male, and their mean age was 34.8 years. Seven patients were newly diagnosed with AS, and the mean disease duration was 4.6±2.1 years. Seven patients had a history of anti-TNF therapy, but according to the exclusion criteria, they had not received TNF inhibitors two months before the study. Almost half of the patients had positive HLA-B27 test results. Six patients had a positive family history of spondyloarthropathies in the first- and second-degree relatives, and two of them reported Rheumatoid arthritis in first-degree family members. Regarding disease activity indices, most patients were in the active phase (Table 2).

Higher expression of the *RUNX3* gene in as patients compared to healthy individuals

The expression level of the *RUNX3* gene between patients with ankylosing spondylitis and healthy subjects showed a significant difference. The relative expression of the *RUNX3* gene in the patients group was higher than that of healthy subjects (3.5±1.1 vs 1.45±0.8 [Mean±SEM] P=0.03) (Figure 1).

Upregulated expression of *IRF4* gene in patients with ankylosing spondylitis

Evaluation of *IRF4* gene expression in peripheral blood showed significantly higher expression levels of *IRF4* in the patients group compared to the healthy individuals (8.3±3.1 vs 3.6±1.5 [Mean±SEM] P=0.025) (Figure 2).

Table 1. The sequence, efficacy, and product length of primers used in the RT-PCR test

Gene	Sequence	Product Length	Primer Efficiency	Company
RUNX3	Forward: 5'-GGCGAGGGAAGAGTTTCACC-3'	242 bp	1.97	Metabion
	Reverse: 5'-GAAGTGGCTTGTGGTGCTGA-3'		2	Metabion
IRF4	Forward: 5'-TTGGCGTTCTCAGACTGCCG-3'	102 bp	2.01	Metabion
	Reverse: 5'-AACGCTTGCAGCTCTGACAA-3'		1.95	Metabion
18S rRNA	Forward: 5'-GTAACCCGTTGAACCCCATT-3'	151 bp	2	Metabion
	Reverse: 5'-CCATCCAATCGGTAGTAGCG-3'		1.95	Metabion

Abbreviations: *RUNX3*: Runt-related transcription factor 3; *IRF4*: Interferon regulatory factor 4; 18S rRNA: 18S ribosomal ribonucleic acid.

Table 2. Clinical and laboratory data of AS patients and healthy controls

Variables		No. (%)/Mean±SD		P
		AS Patients (n=30)	Healthy Controls (n=30)	
Gender	Male	27(90)	27(90)	1
	Female	3(10)	3(10)	1
Age (y)		34.9±9.8	34.8±8.8	0.93
Smoking	Yes	11(36.6)	7(23.3)	0.24
	No	19(63.3)	23(76.6)	0.24
Disease duration (y)		4.6±2.1	-	-
HLA-B27	Positive	16(53.3)	-	-
	Negative	10(33.3)	-	-
	Unknown	4(13.3)		
Indices of Disease Activity				
BASDAI-index		5.3±2.8		
BASMI-index		3.9±2.4	-	-
BASFI-index		4.5±2.8	-	-
BAS-G-index		5.9±3.1	-	-
ASQoL-index		10±7.9	-	-
Family history of rheu-matoid diseases	Yes	8(26.6)	-	-
	No	22(73.3)	-	-
Medications				
NSAIDs		8(26.6)	-	-
DMARDs		4(13.3)	-	-
Anti-TNFα		3(10)	-	-
NSAIDs+DMARDs		4(13.3)	-	-
DMARDs+Anti-TNFα		2(6.7)	-	-
NSAIDs+DMARDs+Anti-TNFα		2(6.7)	-	-
No drug (new patient)		7(23.3)	-	-
Anti-TNFα	Yes	7(23.3)	-	-
	No	23(76.7)	-	-

Abbreviations: AS: Ankylosing spondylitis; ASQoL: Ankylosing spondylitis quality of life; BASDAI: Bath ankylosing spondylitis disease activity index; BASFI: Bath ankylosing spondylitis functional index: BAS-G: Bath ankylosing spondylitis global score; BASMI: Bath ankylosing spondylitis metrology index; DMARD: Disease-modifying anti-rheumatic drugs; HLA: Human leukocyte antigen; NSAID: Non-steroidal anti-inflammatory drugs; TNF-a: Tumor necrosis factor-alpha.

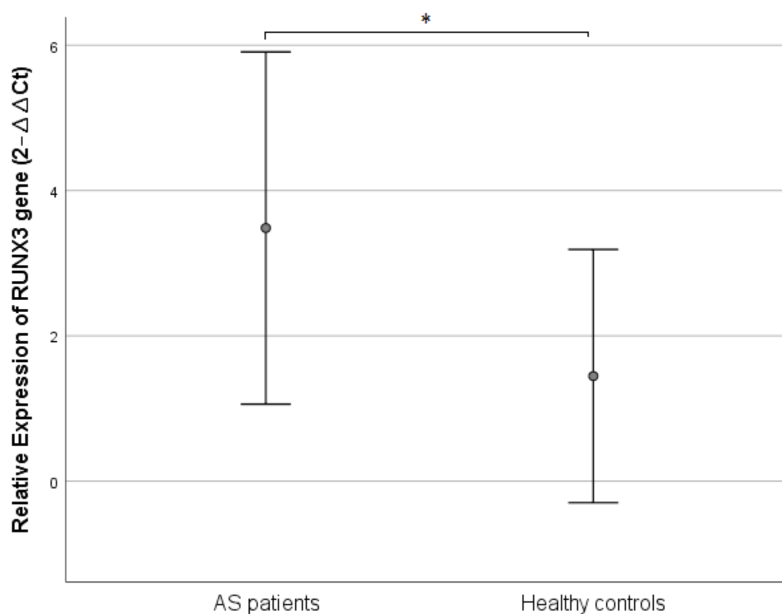

Figure 1. Relative expression of *RUNX3* gene in AS patients and healthy controls (*P=0.03)

Correlation between *IRF4* gene expression and disease activity indices

The regression analysis showed a significant direct correlation between the expression level of the *IRF4* gene and bath ankylosing spondylitis global score (BAS-G) (correlation coefficient=0.38, P=0.04) (Figure 3). However, there was no significant correlation between *IRF4* expression and other disease activity indices. Of note, *RUNX3* expression showed no considerable correlation with studied indices (data not shown).

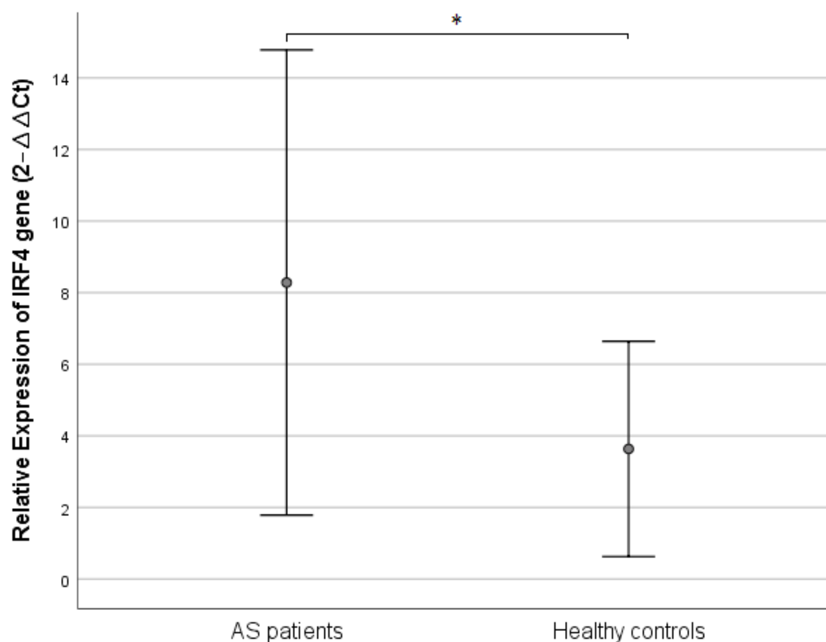

Figure 2. Relative expression of *IRF4* gene in AS patients and healthy individuals (P=0.025)

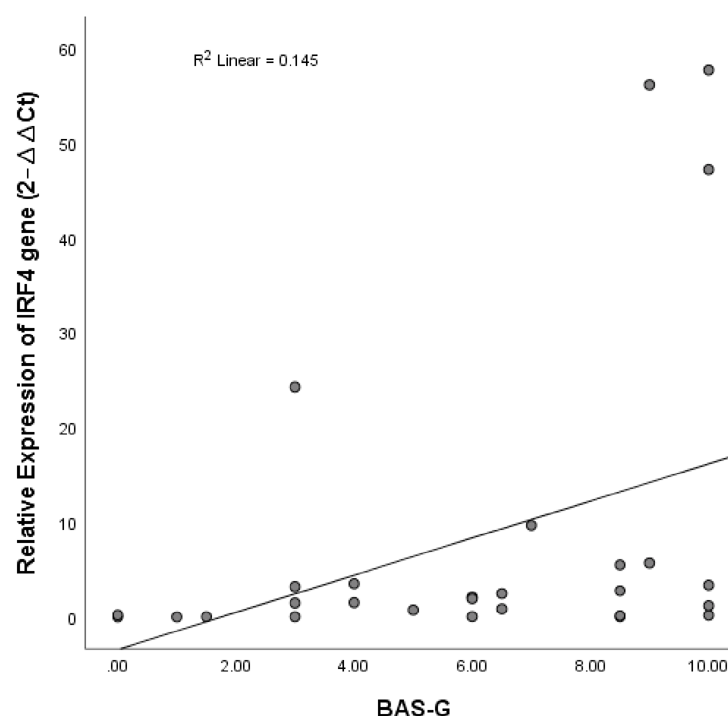

Figure 3. Significant correlation between *IRF4* gene expression andFin ankylosing spondylitis patients (P=0.04)

Discussion

RUNX3 and *IRF4* are critical transcription factors regulating immune cell differentiation and function [25]. According to the chronic inflammation in ankylosing spondylitis that leads to irreversible bone and joint damage, a comprehensive understanding of the molecular mechanisms implicated in initiating and progressing dysregulated immune responses might be helpful. The association of many immune cells, cytokines, and genes has been demonstrated with the development of AS [3, 4, 26]. Nonetheless, the implication of dysregulated transcription factors has barely been studied. Certain *RUNX3* polymorphisms have shown significant correlations with the susceptibility to spondyloarthropathies such as psoriatic arthritis [12] and AS. For instance, an SNP (rs4648889) in the enhancer upstream of the *RUNX3* promoter is associated with an increased risk of developing ankylosing spondylitis [14]. Moreover, *RUNX3* plays a role in the differentiation and function of TCD8+ cells [27]. It also establishes a balance between Th1 and Th2 and Th17 and Treg subsets [10, 11]. Furthermore, it has been demonstrated that increased expression of *RUNX3* was associated with upregulated production of Th17- and Th22-related cytokines in the CD4+ T cells from healthy individuals. Besides, the inhibition of *RUNX3* reduced the levels of these cytokines and decreased the frequency of Th17 and Th22 cells in the CD4+ T lymphocytes from the patients with psoriasis. These findings suggest a significant correlation between *RUNX3* and Th17/Th22 cell function and proposed *RUNX3* as a therapeutic target for treating psoriasis [28]. On the other hand, other reports suggest no association between SNPs of the *RUNX3* gene and susceptibility to AS in different ethnicities such as Chinese Han [29].

Despite growing evidence of *IRF4* involvement in the pathogenesis of autoimmune diseases [18-20], its role in the immunopathogenesis of spondyloarthropathies has barely been understood. One study has reported increased expression of *IRF4* in skin lesions of patients with psoriasis vulgaris [30]. In addition, a significant correlation has been shown between *IRF4* levels and interleukin (IL)-6, IL-17, and IL-22 mRNA expression in experimental colitis, a disease genetically related to spondyloarthropathies [31].

Considering the insufficient evidence linking these transcription factors to the immunopathogenesis of AS, the gene expression of *RUNX3* and *IRF4* was evaluated in the peripheral blood of AS patients compared to healthy controls. Our results showed a significant increase in the expression level of these genes in the AS group, suggesting an uncontrolled inflammatory response in patients.

Previous studies have reported increased percentages of inflammatory T cell subsets, i.e. Th17 and Th22, and elevated proinflammatory cytokine levels in AS patients [32, 33]. The upregulated expression of *RUNX3* and *IRF4* may contribute to the initiation, maintenance, or progression of inflammatory T helper cell subset activity.

Additionally, regarding the high disease activity indices in the patients, correlation analysis was performed between the index and gene expression of *RUNX3* and IRF4, which revealed a significant correlation between *IRF4* expression and disease activity index BAS-G. It appears that higher *IRF4* expression indicates an enhanced activity of T lymphocytes. Similarly, previous research has shown a positive correlation between K-L grades (severity classification scale of osteoarthritis) and serum levels of *IRF4* in patients with osteoarthritis [34]. However, none of the indices showed any remarkable correlation with *RUNX3* expression. These findings may suggest a prognostic role for *IRF4* in ankylosing spondylitis.

In the next step, studying the association of *RUNX3* and *IRF4* expression with the T cell subset frequency and function would be helpful. Inhibition of *RUNX3* with small peptide inhibitors [35] or *IRF4* inhibition using selective antisense oligonucleotide [36] in experimental models might also provide further information about the significance of these transcription factors in the pathogenesis of AS and the feasibility of their inhibition in humans. The low sample size was the other limitation of the present study due to the low incidence of AS in Iran [37] and the high number of patients receiving TNF inhibitors within two months before sampling, which excluded a considerable number of the patients from the study. It is also noteworthy that the effect of non-steroidal anti-inflammatory drugs and disease-modifying antirheumatic drugs, which were administered to studied patients, on the gene expression of *IRF4* and *RUNX3* is to date unknown; however, the influence of these medications on the results of the study cannot be ruled out.

Conclusion

In summary, the expression of *RUNX3* and *IRF4* genes appeared to be increased in the peripheral blood of the patients with ankylosing spondylitis compared to the healthy subjects. Besides, the *IRF4* gene expression level significantly correlated with the BAS-G index in patients. These findings suggest a prognostic role for these transcription factors in AS. They might also be considered potential therapeutic targets to inhibit chronic inflammation in these patients.

Ethical Considerations

Compliance with ethical guidelines

This study has been approved by the Tehran University of Medical Sciences Ethics Committee (Code: IR.TUMS.CHMC.REC.1399.197).

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Authors contribution's

Conceptualisation and patient recruitment: Abdolrahman Rostamian; Study design and literature review: Narjes Soleimanifar; Materials and processing: Maryam Sadran and Abeda Mazari; Test performance: Hanieh Mojtahedi and Maryam Ahmadi; Data collection, data processing and writing: Sara Assadiasl; Supervision, review and editing: Mohammad Hossein Nicknam.

Conflict of interest

The authors declared no conflict of interest.

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