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ORIGINAL ARTICLE

Investigation of methylation status of interleukin-16 and autoimmune regulator gene promoter regions in Behçet's disease

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ABSTRACT

Objectives: The aim of this study was to determine the epigenetic changes in interleukin-16 (IL-16) and autoimmune regulator *(AIRE)* genes in Behçet's disease (BD) and to investigate the relationship between these changes and the disease mechanism.

Patients and methods: Between October 2022 and January 2023, a total of 40 patients (20 males, 20 females; mean age: 37.0±11.4 years; range, 19 to 71 years) who met the 2014 International Criteria for Behçet's Disease with no concomitant diseases and who were either newly diagnosed or under follow-up and 40 age- and sex-matched healthy hospital staff as the control group (20 males, 20 females mean age: 35.1±5.7 years; range, 29 to 45 years) with no chronic diseases or active infections were included. Peripheral blood samples were obtained from all participants, and genomic deoxyribonucleic acid (DNA) was isolated. The DNA samples were modified using a bisulfite modification kit. Subsequently, the promoter methylation profiles of *IL-16* and *AIRE* genes were determined using methylation-specific polymerase chain reaction (MSP) with primers specifically designed for methylation.

Results: In both BD and control groups, methylation was detected in the promoter region of *IL-16*, indicating a weak expression of the *IL-16* gene. In contrast, while the promoter region of the *AIRE* gene was methylated in all control participants, it was unmethylated in all patients with BD.

Conclusion: This is the first study to evaluate the methylation status of both *AIRE* and *IL-16* genes in BD. Our study results suggest that the promoter region of the *AIRE* gene is unmethylated in BD and that *AIRE* gene is activated in BD and produces autoimmune regulatory proteins that eliminate autoreactive T cells, suggesting a tendency toward autoimmunity in BD. These findings also suggest that IL-16, which is involved in the pathogenesis of many rheumatic diseases, does not play a significant role in the pathogenesis of BD.

Keywords: Autoimmune regulator gene, Behçet's disease, interleukin-16.

Behçet's disease (BD) is an inflammatory multisystemic disorder characterized by recurrent episodes of oral aphthae, genital ulcers, arthritis, dermal lesions, and ocular lesions and the involvement of the gastrointestinal and central nervous systems. Unlike other vasculitides, BD can affect vessels of any type and size. The pathogenesis of BD is characterized by neutrophil-mediated endothelial dysfunction as a result of neutrophil hyperactivation (autoinflammation). Although the etiopathogenesis of BD has not been not fully elucidated yet, several epigenetic factors have been shown to be implicated.[1]

The autoimmune regulator *(AIRE)* gene on chromosome 21q22.3 is crucial for the clearance of autoreactive T cells and maintenance of self-tolerance. Experimental evidence indicates that *AIRE* increases the transcription of certain organ-specific self-antigens in medullary, thymic, and epithelial cells, thereby causing negative selection of organ-specific thymocytes.[4]

The *AIRE* is also crucial in shaping the T cell repertoire. Mutations in the *AIRE* gene can affect other aspects of the immune response by affecting deletion of autoreactive T cells in the thymus (directly) and by increasing anti-interferon antibodies (indirectly).[5] Mutations in the *AIRE* gene lead to a clinical condition commonly characterized by chronic candidiasis, autoimmune polyendocrinopathy, hypoparathyroidism, adrenal insufficiency, and skin dystrophy, termed autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED).[6]

Interleukin (IL)-16 was first identified in 1982 as a T cell chemoattractant factor and once its proinflammatory effects were evaluated, it began to be referred to as IL-16.[7] It is synthesized by various immune (T lymphocytes, eosinophils, mast cells, and dendritic cells) and non-immune (fibroblasts, epithelial cells, and neural cells) cells.[8] The IL-16, synthesized as a ligand for CD4+ T lymphocytes, affects the activation and proliferation of T cells.[9] It stimulates the expression of cytokines such as tumor necrosis factor-alpha (TNF-α), IL-1β, and IL-6, which induce inflammation, from mononuclear cells. In addition, IL-16 activates the expression and production of cytokines such as IL-1β, IL-6, IL-15, and TNF-α, which induce inflammation, in human monocytes.[10] Increased IL-16 expression is associated with several immune-mediated disorders, including allergies, asthma, Crohn's disease, multiple sclerosis, rheumatoid arthritis (RA), and systemic lupus erythematosus (SLE).[11,12]

Previous studies have demonstrated the involvement of *AIRE* and *IL-16* in the pathogenesis of various rheumatic diseases. However, no studies have investigated the relationship between *AIRE* and *IL-16* in BD yet. In the present study, we, therefore, aimed to determine the methylation status of *IL-16* and *AIRE* genes in BD and to investigate the possible relationship between these epigenetic changes and the pathogenesis of the disease.

PATIENTS AND METHODS

Study design and study population

This single-center, cross-sectional study was conducted at Ondokuz Mayıs University Faculty of Medicine, Departments of Rheumatology and Internal Medicine between October 1st, 2022 and January 31st, 2023. A total of 40 patients (20 males, 20 females; mean age: 37.0±11.4 years; range, 19 to 71 years) who met the 2014 International Criteria for Behçet's Disease with no concomitant diseases and who were either newly diagnosed or under follow-up were included. Those with a history of malignancy, pregnancy, breastfeeding, allergies, and active infections were excluded from the study. The control group consisted of 40 age- and sex-matched healthy hospital staff (20 males, 20 females; mean age: 35.1±5.7 years; range, 29 to 45 years) with no chronic diseases or active infections. A written informed consent was obtained from each participant. The study protocol was approved by the Ondokuz Mayis University Clinical Research Ethics Committee (date: 31.12.2021, no: OMU-KAEK 2021/634). The study was conducted in accordance with the principles of the Declaration of Helsinki.

Clinical data and sample collection

The patients' age, sex, age at the time of diagnosis, history of oral and genital ulcers, skin manifestations, history of thrombosis, uveitis, arthritis, pathergy test results, medication use, and smoking status were recorded. Detailed medical histories were obtained, and physical examinations were performed for both the patient and control groups. Peripheral blood samples of 5 mL were obtained from patients with BD and healthy participants for laboratory analysis (C-reactive protein [CRP], erythrocyte sedimentation rate [ESR], complete blood count [CBC], blood urea nitrogen [BUN], creatinine, aspartate aminotransferase [AST], and alanine aminotransferase [ALT]).

DNA extraction and bisulfite modification

Genomic deoxyribonucleic acid (DNA) was isolated from all blood samples using a commercial kit (Pure Link® Genomic DNA Mini Kit; Invitrogen, MA, USA) according to the manufacturer's protocol. The concentration and purity of DNA samples were determined using a NanoDrop spectrophotometer (Jenway Genova Nano, Stone, UK). After DNA extraction, using 500 ng/20 mL DNA for each sample, DNA was

modified with bisulfite using EpiJET Bisulfite Conversion Kit (Thermo Fisher Scientific Baltics, Vilnius, Lithuania) according to the manufacturer's protocol.

Methylation-specific polymerase chain reaction

Promoter methylation of *IL-16* and *AIRE* gene promoter regions was analyzed by methylation-specific polymerase chain reaction (MSP) using the methylation-specific primers designed for *IL-16* and *AIRE* gene promoters. For MSP, methylation- and unmethylation-specific primers for *IL-16* and *AIRE* gene promoter regions were used. Of note, MSP primers were specifically designed for *IL-16* and *AIRE* promoter sequences using MethPrimer database (http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi) (Table 1).

The MSP reaction was performed in a total volume of 25 µL, using 3 µL of bisulfite-modified DNA, Hot Start Taq™ DNA polymerase enzyme (Thermo Fisher Scientific Baltics, Vilnius, Lithuania), and MSP-specific primers. The polymerase chain reaction (PCR) analysis was performed using a Thermal Cycler GeneAmp™ PCR System 9700 (Applied Biosystems, MA, USA) according to the following protocol: initial denaturation of 3 min at 95°C, followed by 40 cycles of 40 sec at 95°C, 40 sec at 57°C, and 70 sec at 72°C, followed by a final extension of 7 min at 72°C and storage at 4°C.

The MSP results were analyzed by imaging with an ultraviolet (UV) transilluminator following 2% agarose gel electrophoresis. After the analysis, the gene methylation status was evaluated as methylated versus unmethylated depending on the detection of PCR products from methylation- and unmethylation-specific primers.

Statistical analysis

The study power analysis and sample size calculation were performed using the G*Power version 3.1 software (Heinrich-Heine-Universität Düsseldorf, Düsseldorf, Germany). The sample size for this study was set at 40 participants per group, calculated with an alpha error of 0.05 and a power of 80% and 90%.

Statistical analysis was conducted using the IBM SPSS for Windows version 22.0 software (IBM Corp., Armonk, NY, USA). Continuous variables were presented in mean ± standard deviation (SD) or median (min-max), while categorical variables were presented in number and frequency. The Student t-test was used to compare normally distributed variables between the groups, and Mann-Whitney U test was used to compare non-normally distributed variables. Relationships between the variables were analyzed using the Pearson correlation test for normally distributed variables and Spearman correlation test for non-normally distributed variables. A p value of <0.05 was considered statistically significant.

Table 1. Primer sequences for methylation-specific polymerase chain reaction

AIRE MSP-F	5'-GTCGAGGAGGTAGGATAGGGTTATATTC-3'
AIRE MSP-R	5'-AAAAACACTTCCCTATAATAAAAACG-3'
AIRE USP-F	5'-GTTGAGGTAGGATAGGGTTATTATATTTG-3'
AIRE USP-R	5'-AAACACTTCCCTATAATAAAAACACT-3'
IL-16 MSP-F	5'- TGATTATTTAAGGATTGAGGAGGAGGAGTGTC-3'
IL-16 MSP-R	5'- AAACTAAACATAAAAATTCTCCACGTC-3'
IL-16 USP-F	5'- TTATTTAAGGATTGAGAGTGTTGG-3'
IL-16 USP-R	5'- AAACTAAACATAAAAATTCTCCACATC-3'

AIRE: Autoimmune regulator; MSP-F: Methylation-specific forward primer; MSP-R: Methylation-specific reverse primer; USP-F: Unmethylation-specific forward primer; USP-R: Unmethylation-specific reverse primer; IL: Interleukin.

RESULTS

Demographic characteristics and laboratory data of the BD and control groups are summarized in Table 2. Compared to the control group, the number of smokers (p=0.012), ESR (p=0.002), and creatinine levels (p=0.02) were significantly higher in the BD group (Table 2). The *AIRE* gene was methylated in all participants of the control group, whereas it was unmethylated in all patients with BD. This was not associated with AST (p=0.418) ALT (p=0.723), and BUN (p=0.577), creatinine (p=0.428), sex ,and age. In contrast, the *IL-16* gene was methylated in all patients with BD and controls which was not found to be associated with AST, ALT, BUN, creatinine, sex, and age. Since the *IL-16* gene promoter regions were methylated in all participants, no additional statistical analysis was applied.

In the control group, the promoter regions of the *AIRE* and *IL-16* genes were found to be methylated. Therefore, subgroup analyses regarding sex, hypertension, diabetes mellitus, and smoking status were not performed.

Examination of the clinical findings of the BD group showed that out of 40 patients, 39 had oral ulcers, 27 had genital ulcers, 32 had papulopustular lesions, 26 had erythema nodosum, 18 had positive pathergy test, 16 had arthritis, 15 had uveitis, five had vascular involvement, and two had neurological involvement. In addition, in the BD group, 39 patients received colchicine treatment, while 18 patients received corticosteroid treatment,

Table 2. Demographic characteristics and laboratory data of the study groups

	Healthy control group		Behçet's disease group		
	n	Mean±SD	n	Mean±SD	*p*
Age (year)		35.1±5.7		37.0±11.4	0.3
Sex Female Male	 20 20		 20 20		1
Smoking Yes No	 0 40		 7 33		0.012
Hypertension Yes No	 0 40		 5 35		0.055
Diabetes mellitus Yes No	 0 40		 3 37		0.2
Atherosclerosis Yes No	 0 40		 1 39		1
CRP (mg/dL)		3.5±1.8		7.5±13.1	0.6
ESR (mm/h)		17.9±10.4		29.7±20	0.002
WBC (cells/µL)		7337.5±2807.6		7515±2197.4	0.7
Hemoglobin (g/dL)		13.8±2.4		13.4±1.7	0.4
Platelet (thousand cells/µL)		260.9±55.5		272.8±68.1	0.3
BUN (mg/µL)		11.8±2.6		12.2±3.3	0.5
Creatinine (mg/µL)		0.8±0.1		0.7±0.1	0.02
AST (IU/L)		20.1±7.1		17.7±6.5	0.1
ALT (IU/L)		20.7±14.4		17.6±9.8	0.2

SD: Standard deviation; CRP: C-reactive protein; ESR: Erythrocyte sedimentation rate; WBC: White blood cells; BUN: Blood urea nitrogen; AST: Aspartate transaminase; ALT: Alanine transaminase.

23 azathioprine, 17 anti-TNF therapy, four dapsone treatment, and three methotrexate. In all patients in the BD group, the promoter region of the *AIRE* gene was found to be unmethylated, while the promoter region of the *IL-16* gene was methylated. Therefore, subgroup analyses for the promoter regions of both genes in relation to the existing clinical findings and the treatments were not performed.

DISCUSSION

In the present study, we investigated the potential roles of *AIRE* and *IL-16* in BD patients. The methylation status of *AIRE* and *IL-16* gene promoter regions was investigated in peripheral blood samples of patients with BD and healthy participants. Our study results showed that the promoter region of the *IL-16* gene was methylated in all individuals in both the BD and control groups, and there was no statistically significant difference in *IL-16* gene expression between the two groups. In contrast, the promoter region of the *AIRE* gene was methylated in all healthy participants, whereas it was unmethylated (active) in all participants in the BD group. This is the first study investigating the methylations of *AIRE* and *IL-16* in BD and their possible associations with the clinical features of the disease.

The product of *AIRE* gene is a protein called "autoimmune regulator," and it is responsible for the mechanism of destruction of all autoreactive T cells.[5] In the present study, the *AIRE* gene promoter region was unmethylated (active) in all patients with BD. Under normal conditions, this gene should be inactive in the methylated form, which was the case in the control group. The activation of *AIRE* gene in BD may be due to its attempt to eliminate autoreactive T cell clones.

In diseases such as SLE and RA, single-nucleotide polymorphisms (SNPs) in the *AIRE* gene may increase the risk of the development of these diseases. These SNPs in the *AIRE* gene may lead to changes in gene expression and, consequently, impairment in function, thereby increasing the risk for the development of autoimmune diseases.[13-16] It is not definitively established whether BD is an autoimmune disease. Some studies have indicated that autoimmunity may play a role in the pathogenesis of BD.[17]

In the present study, the *AIRE* gene, which is responsible for the elimination of autoreactive T cells, was active in patients with BD, but not in healthy participants. The activation of the *AIRE* gene in BD may indicate a tendency toward autoimmunity in the pathogenesis of BD. We believe that increased *AIRE* gene expression and the resulting increase in the *AIRE* protein levels in BD facilitate the elimination of autoreactive T cells that emerge during the pathogenesis of the disease. Another interpretation of this could be that there is a tendency toward autoimmunity in BD.

Furthermore, IL-16 is a relatively poorly characterized cytokine. However, it has been investigated in various chronic autoimmune diseases. Murato *et al.*[18] reported that IL-16 might be a useful biomarker associated with clinical response in RA treatment. El Atta *et al.*[19] indicated that IL-16, a proinflammatory cytokine involved in the pathogenesis of RA, might be involved in the development of deformities. In their study with 30 patients diagnosed with SLE, Fava *et al.*[20] showed that IL-16 reflected histological activity in the pathogenesis of lupus nephritis and IL-16 was the leading cytokine expressed in kidneys with lupus nephritis. Consequently, the authors proposed that IL-16 could be used as both a biomarker and therapeutic target in SLE. Lard *et al.*[21] reported that IL-16 levels were higher in patients with severe SLE than in those with mild disease and healthy controls. They also found a significant correlation between SLE disease activity index and IL-16 levels. In a study involving 57 patients diagnosed with systemic sclerosis (SSc), Kawabata *et al.*[22] found that IL-16 was expressed in lymphocytes infiltrating the capillaries. The authors hypothesized that increased serum IL-16 levels in patients with SSc could lead to cutaneous symptoms such as skin sclerosis, erythema, and pigmentation. In contrast to these data, *IL-16* gene was inactive in BD in the present study. These results suggest that IL-16 may not play a significant role in the pathogenesis of BD.

The aforementioned studies indicate that IL-16 is involved in the pathogenesis of chronic autoimmune diseases. Although some features

observed in autoimmune diseases are present in BD, its pathogenesis does not fully conform to the pattern of an autoimmune disease. In all BD patients included in the study, regardless of clinical findings and treatments, the promoter region of the *IL-16* gene was found to be methylated, and no gene expression was detected. The inactivity of *IL-16* in BD may be related to the unique nature of BD, which differs from other autoimmune diseases.

In our study, the promoter regions of the *AIRE* and *IL-16* genes were methylated in all healthy volunteers. Accordingly, no relationship was identified between the parameters (age, sex, smoking, hypertension, diabetes mellitus, liver and kidney functions, ESR, and CRP) evaluated in our study and the activity of the *AIRE* and *IL-16* genes. These data suggest that there is no strong association between the mentioned parameters and the *AIRE* and *IL-16* genes in healthy individuals.

Nonetheless, there are certain limitations to the present study. First, despite examining the methylation status of *AIRE* and *IL-16* gene promoter regions, *IL-16* expression or serum levels were not investigated in the present study. Similarly, evaluation of *AIRE* gene expression or serum levels could have contributed further to the findings of the study. Second, including autoimmune and autoinflammatory patient control groups could be beneficial for comparison of our data. However, to the best of our knowledge, this is the first study to investigate the roles of both the *AIRE* and *IL-16* genes in BD and, therefore, we believe that the results would provide additional contribution to the literature.

In conclusion, although there are conflicting reports regarding whether BD is an autoimmune or autoinflammatory disease, we believe that the activation of the *AIRE* gene, which is involved in the primary step of autoimmunity, indicates a role of autoimmunity in the pathogenesis of BD. Interleukin-16, which is involved in the pathogenesis of chronic autoimmune diseases, does not appear to be involved in the pathogenesis of BD. This may be associated with BD not exhibiting the characteristics of a fully autoimmune disease. However, further large-scale, well-designed studies are warranted to fully elucidate the role of *AIRE* and *IL-16* gene in BD.

Data Sharing Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Author Contributions: Idea/concept: Z.B.Ç.; Design, control/supervision: D.Y.K.; Data collection and/or processing: M.A.; Analysis and/or interpretation: D.Y.K., M.Ö., S.G.; Literature review, writing the article: M.A., D.Y.K., M.Ö.; Critical review: H.K., M.A., D.Y.K., S.G., M.Ö., Z.B.Ç.; References and fundings: Materials: Hilal Koyuncu, M.A., D.Y.K.

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