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Promoter methylation of Bax and Bcl2 genes and their expression in patients with Behcet's disease

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Abstract

BCL2 and BAX genes are a group of signalling inducer and inhibitor genes playing a key role in the process of cellular physiological death (apoptosis). These genes, through the JAK/STAT signalling pathway, affect different cytokines on cell function and subsequently lead to the pathophysiology of diseases, especially autoimmune diseases. In addition, altering the methylation of genes can affect their expression. Since the aetiology and pathology of Behcet's disease is not fully understood, the aim of this study was to determine the methylation pattern of BCL2 and BAX genes in patients with Behcet's disease and compare it with those of control group. This was a case–control study on 51 patients with Behcet and 61 control subjects. Blood samples were received from all subjects. Subsequently, the peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll method and the methylation of the sites was investigated using quantitative methylation specific PCR (qMS-PCR) technique after extraction of DNA by salting out method and its examination with Nano drop. The results of methylation and expression of Bax gene suggest that the methylation level in the patient group significantly increased compared to the healthy individuals (p-value < .05). Furthermore, the results related to Bax gene expression revealed that the mean of gene expression in the patient group has decreased compared to the healthy group, and this decrease was statistically significant (p-value < .05). The rate of expression and methylation of Bcl2 did not indicate any change in the two patient and healthy groups. Given the results of this study, it can be guessed that perhaps DNA methylation is involved in certain conditions of the disease and it may result in regulation of the expression of the involved genes such as Bax gene, in the pathogenesis of the disease.

KEYWORDS

Bax gene, Bcl2, Behcet's disease, DNA methylation, qMS-PCR

1 | INTRODUCTION

As an autoimmune disease, Behcet's disease is characterized by recurrent oral ulcers jaw infection, oral uveitis and skin lesions. Since vascular manifestations are common in this disease, it is regarded as vasculitis. Nevertheless, the dominant histological pathological symptoms in inflamed tissues include infiltration of lymphocytes, monocytes and, in some cases, multi-core leucocytes. The disease is more prevalent in the Silk Road area from Japan to the Mediterranean countries and it is much more

prevalent in countries such as Iran and Turkey; hence, it led us to prioritize the disease in our studies (Hirohata & Kikuchi, 2003). Some studies have shown a strong relationship between HLA-B51 (human leucocyte antigen) and this disease in different races. The Mizuki group has recently suggested that the critical areas for human Behcet's disease in the MHC (major histocompatibility complex) area have been marked in the 46-kb range in MHC class I gene-A-dependent (MIC-A) and HLA-B gene (Mizuki et al., 1999). The prevalence of BD in Iran is 68 per 100,000 inhabitants, which is the second highest prevalence after Turkey (80–370) in the world (Davatchi et al., 2007).

Th1/Th17 (T helper cell) T-cell responses experience incremental regulation in Behcet's disease. Although signalling pathways are related to deviant immune responses, they are not yet fully clarified. Both CD4 + and CD8 + T cell types increase the production of inflammatory cytokines including IL-2 (Interleukin), IL-12 as well as interferon gamma in inflammatory tissues and peripheral blood of patients with Behcet (Imamura et al., 2005). Th-17 cells are a relatively new subset of T helper cells mainly producing IL-17A, IL-17F, IL-22 and TNF-α (Annunziato, Cosmi, Liotta, Maggi, & Romagnani, 2009). Recent data have proven the involvement of IL-17 and Th17 cell responses in the pathogenesis of Behcet's disease and shown the Behcet activated by high serum levels of interleukins 6, 10 17, 23, as well as Th17 cells (Abolhasani, Gholizadeh Ghaleh Aziz, Khabbazi, & Alipour, 2018; Ahmadi et al., 2019; Alipour, Nouri, et al., 2018a; Alipour, Sakhinia, et al., 2018b; Chi et al., 2008). Therapeutic methods such as interferon alfa and anti-TNF-α (Tumour necrosis Factor alpha) agents that are effective in Behcet's disease with eye contamination have revealed that they inhibit responses of Th17 cell (Liu, Yang, Wang, Li, & Kijlstra, 2010; Sugita et al., 2012).

Interferon gamma, GM-CSF, IL-2, IL-6, IL-12, IL-15, IL-17, IL-21, IL-22 and IL-23 are activated by means of various compounds of JAK/STAT (Janus kinase/signal transducers and activators of transcription) at cell surface and they are involved in the pathogenesis of infections, malignancies and autoimmune/inflammatory disorders such as rheumatoid arthritis, IBD and psoriasis (O'Shea, Holland, & Staudt, 2013). The JAK/STAT signalling pathways are very significant for activation of the inherent immune system, so that the interferon-gamma signal and interleukins 2 and 6 are activated by JAK1, JAK2 and JAK3; while interleukins 12 and 23 are activated by JAK2/Tyk2 pathway (O'Shea & Plenge, 2012). Anti-inflammatory cytokine interleukin 10 is activated by the JAK1/STAT3 pathway with the SOCS3 regulatory system (Murray, 2007).

Among the genes involved in apoptosis, the genes of BCL-2 family play a key role in the apoptosis regulation. BCL-2 is an anti-apoptotic gene promoting the cell survival; while BAX is a proapoptotic gene advancing the physiological cell death. Given the role of caspases in different cells, studies have revealed that Bcl-2 can also inhibit the process of apoptosis by inhibiting the synthesis and production of caspase. More specifically, by preventing the release of cytochrome C or through binding to Apaf-1, the Bcl-2 proteins in the mitochondria wall prevent the formation of apoptosomes and the emergence of caspase cascades (Cooney, 2002). In the study of Cui et al., who investigated the apoptosis pathways and expression of caspase and Bax genes in the patients with lupus nephritis, the rate of expression of these genes in glomerular parenchymal cells, tubular epithelial cells of kidney and interstitial inflammatory cells in patients with lupus nephritis increased compared to that in control group. These results suggested that apoptosis is induced by the pathway associated with these proteins in the pathogenesis of inflammatory diseases as lupus (Cui et al., 2012).

Despite the fact that the pre-inflammatory cytokines related to Th1/Th17 cells have been proven in the pathogenesis of Behcet's disease, the cause and pathology of Behcet's disease in the intracellular signalling apoptosis pathways have not yet been fully elucidated. Nevertheless, recent researches indicate considerable advances. Since rheumatologic diseases are from the diseases that, in addition to genetics, are associated with environmental factors like race, and since epigenetic processes affect the expression of genes due to the environmental factors, we decided to examine the effect of the expression of genes influencing the apoptosis pathways (Bax and Bcl2) in patients with Behcet.

2 | METHOD

All cases accessed their written informed agreement for this study, and the study protocol was allowed by the ethics committee in Tabriz University of Medical Sciences, Tabriz, Iran (Permit Number: TBZMED.REC.1396.640). In this case–control clinical trial, in order to determine the number of samples, 50 patients with Behcet's disease were estimated as patient group and 60 healthy subjects as control group under supervision of a statistical consultant, the confidence level of 95%, accuracy of 0.07 and level of type 1 error of 0.05; 47 patients and 61 healthy individuals were finally assessed. In this study, based on laboratory findings and medical record content and with a physician's opinion, patients with Behcet were randomly selected from the ones who were not being treated with immunosuppressive agents and referred to the rheumatologists. The selection of patients was based on the International Criteria for Behcet's disease (IBCD). In addition, the rate of disease activity was measured based on Iran Behcet's Disease Dynamic Activity Measure (IBDDAM; Davatchi, 1991; Shahram et al., 2009), according to which, the vascular, eye and joints threatening conflicts represented the disease severity. The control group included normal people with no history of autoimmune disease that have been randomly selected from conscious blood donors.

After receiving the informed consent from the subjects, 5 cc of venous blood was first received. After that, the single-core cells were isolated by the Ficoll method. Then, the required DNA was extracted from the blood samples using the salting out method (RGDE [Rapid Genomic DNA Extraction]; Ali, Mahnaz, & Mahmood, 2008). After determining the DNA concentration, ZymoResearch kit (*Catalog Nos: D5005 & D5006*) was used to measure the amount of methylation by qMS-PCR technique according to the desired protocol. To determine

the gene expression level, the RNA of all of the desired samples was first isolated based on the TRIzol™ reagent protocol and analysed by real-time PCR technique (as relative method). Using a nanometer device, the amount and purity of DNA was determined and optical density (OD) was defined at a wavelength of 260/280 and 260/230 Nanometer and specimens with the ratio of 260/280, ranging from 1.8 to 2.2 and the ratio of 260/230, ranging from 1.7 to 1.9 were used in order to evaluate real-time PCR and qMSP. Moreover, some extracted specimens were evaluated to evaluate the quality of DNA and RNA samples by gel electrophoresis. For this purpose, 1.5% agarose gel was used.

2.1 | Primer design

In order to design the desired primers, the sequences of Bax and Bcl2 genes were first extracted by NCBI site. Then, many primers were designed using OLIGO 7 software, and from which, the suitable primer pairs were selected (Table 1). In addition, both methylated and non-methylated primers were designed for the qMS-PCR technique. METHPRIMER and EPD (Eukaryotic Promoter Database) sites were referred to design methylated and non-methylated primers. Furthermore, the beta-actin gene was used in order to normalize the real-time data. Also, at the bottom shows the position of the promoter, which is full of CpG islands (Figure 1).

2.2 | Methylation measurement

Based on the kit's protocol, in this method DNA was first treated by sodium bisulphite that converts cytosine residues into a single-stranded DNA. Under these conditions, the 5mC sites remained unchanged; while the non-methylated cytosine sites were converted to uracil. Then, with certain MSP PRIMER design software, the primer was designed for both methylated and non-methylated sequences. In this technique, we have designed two primer pairs, one pair for methylated specific locus in the target genes, another pair for the non-methylated locus in the target genes. Then both primers will be added to the sample for the PRC. Eventually in this method, following the gel electrophoresis, the quantitative amounts of methylation and non-methylation sites were analysed by real-time PCR method in order to quantify the rate of methylation of the desired site. They were then analysed by the cycle threshold (CT) values provided by the apparatus.

The rate of expression in each sample was calculated for BCL2, BAX and β-actin genes by means of CT values. The expression of the BCL2 and BAX gene was measured and in fact normalized in proportion to β-actin gene expression. The normalized values (relative units) were standardized by control samples of each test. The changed values of BCL2 and BAX genes expression in the treated samples in proportion to the control sample normalized by expression of the β-actin gene expression were calculated by means of the $2^{-\Delta\Delta CT}$ method.

2.3 | Statistical analysis

Data were analysed using SPSS-23 software. The mean and standard deviation were used in order to describe the quantitative data. The diagrams were drawn using GraphPad PRISM software version 7. The qualitative data were reported as frequency (percentages). Data distribution was assessed using Kolmogorov–Smirnov test. For statistical analysis and to moderate the effect of confounding factors, the chi-square, paired sample *t* test, independent sample *t* test and one-sample *t* test were employed to respectively describe the qualitative data, intra-group comparison of quantitative variables, inter-group comparison of quantitative variables, comparison of genes expression with constant value. The significance level was considered to be $p < .05$.

3 | RESULTS

Data were collected based on data collection form in different groups of patients, and the results and data of the two groups were gathered through the mentioned tests and statistical analysis was done.

TABLE 1 PCR Primers AND Product size

Gene name	Sequences	Target size
QMS-PCR FOR BCL2	FM-bcl2: TATACGGTTAGAAAGGGTTTAGGC RM-bcl2: GAACGAACGACGAAATACGA	105
	FU-bcl2: ATATGGTTAGAAAGGGTTTAGGTGG RU-bcl2: AACCAAACAAACAACAAAATACAAA	108
QMS-PCR FOR BAX	FM-BAX: TACGTGACGGGATTAAATTTTTC RM-BAX: GTAAAAACCCCGCTAAACGTA	127
	FU-BAX: ATGTGATGGGATTAAATTTTTTGA RU-BAX: CACATAAAAACCCCACTAAACATA	129
BAX	F-BAX: TTCTGACGGCAACTTCAACT R-BAX: CAGCCCATGATGGTTCTGAT	117
BCL2	F-BCL2: GGGAATCGATCTGGAAATCCTC R-BCL2: GGCAACGATCCCATCAATCT	118

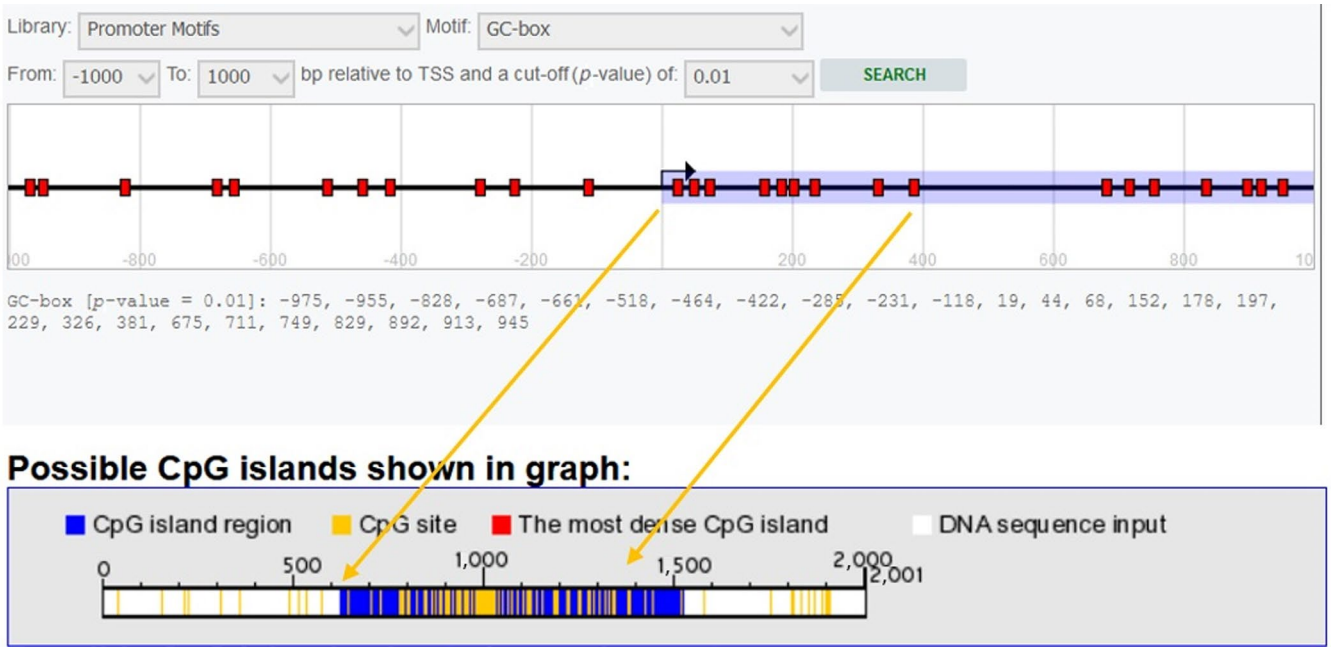

FIGURE 1 Bax promoter and CpG sites. CpGs figure around transcription start site (TSS) were predicted by EPD and CpG island finder software in −1,000 up +1,000 around primer

According to the analysis of demographic data, no significant difference was observed in the mean age and sex between the two groups of patients with Behçet's disease and healthy people; in the group of patients, there were 24 males and 16 females with a mean age of 38.02 ± 10.25. In the healthy group, there were 37 males and 23 females with an average age of 37.4 ± 8.5.

3.1 | Results for real-time PCR

After doing real-time, its results were analysed and evaluated and, they were repeated again if there were any errors. All specimens were performed three times.

In the case of Bax gene non-methylated specimens (more in control group specimens), given that the specimens of this gene are more non-methylated, their expression levels are high and their CTs are smaller.

The figure related to the melting temperature is considered as one of the most important outputs related to real-time. This figure indicates the specificity of primers used in PCR. As shown in the figure (Figures 2 and 3), in this experiment, two pairs of methylated and non-methylated primers were used for each specimen. For most specimens, non-methylated primers are sticking together and have sharp peaks. That is, most of the samples are non-reciprocal.

3.2 | Bax and Bcl2 gene expression and methylation

In order to compare the mean of gene expression and the amount of methylation due to the normal distribution of amounts of gene expression and the amount of methylation in Bax and Bcl2 genes, independent samples *t* test was used. In the case of Bax gene, the results showed that a significant difference was observed between the mean of the expression of the gene in the patient group (0.89 ± 0.13) and in the healthy group (1.93 ± 0.12; $p < .05$; Figure 4). Methylation level was also found in the patient group (5.68 ± 0.24) and in the healthy group (3.2 ± 0.17) which was statistically significant ($p < .001$; Figure 5).

In the case of Bcl2 gene, according to the results, the mean of the expression of the gene in the patient group was 2.77 ± 0.76 and in the healthy group was 2.62 ± 0.65, in which no significant difference was observed ($p > .05$). Also, the methylation level of Bcl2 gene was (1.77 ± 0.48) in the patient group and (2.08 ± 0.43) in the healthy group and no statistically significant difference was observed ($p > .05$).

3.3 | Relationship between gene expression and methylation with clinicopathologic characteristics of patients

In patient specimens, we examined the relationship between the expression of Bax gene and the methylation level with clinical characteristics. According to the results, the level of gene expression is different significantly in terms of the severity of Behçet's disease, so that in subjects with severe BD was decreased than in negative group (p-value < .05).

Table 2. Relationship between gene expression and methylation of BAX with clinicopathologic characteristics of patients.

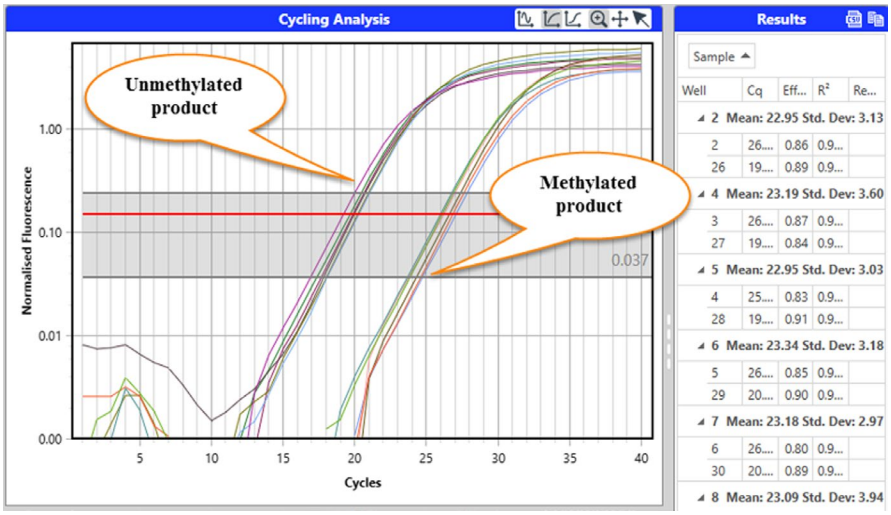

FIGURE 2 Curve for CT numbers (Cycling threshold) in specimens with methylated and non- methylated primers

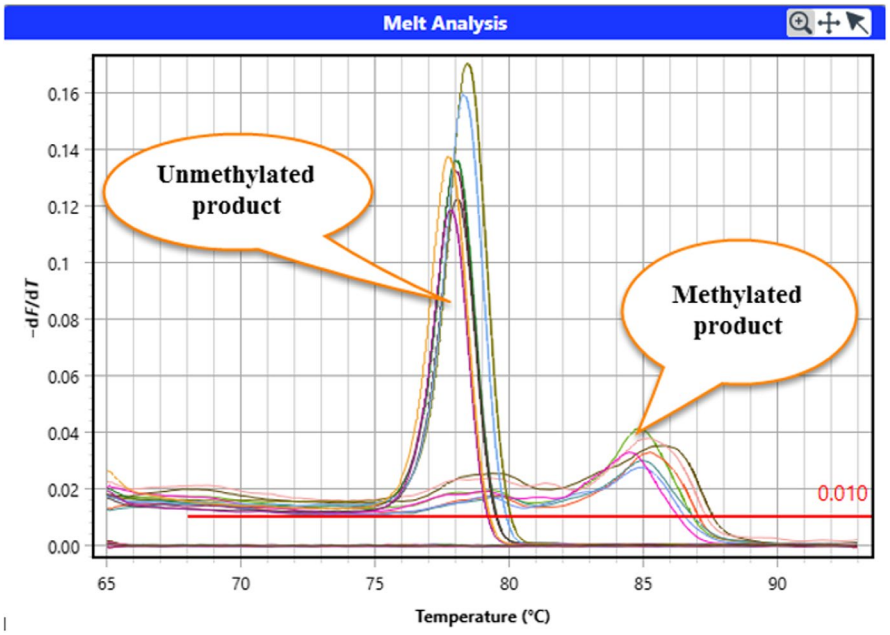

FIGURE 3 Melting Curve, indicating the specificity of primers and specimens

4 | DISCUSSION

Although the aetiology of this disease is still unknown, it is believed that Behcet's disease starts with environmental factors like microbial agents in individuals with specific genetic contexts. In people with this disease, the interaction between genetics and environmental factors can be the fundamental of pathogenicity (Karasneh, Gül, Ollier, Silman, & Worthington, 2005; Kaya, 2012). Recent studies have revealed that in addition to genetics, there is strong evidence showing the role of epigenetics in the pathogenesis of autoimmune diseases. Epigenetics helps understand and recognize complex diseases (Hatchwell & Greally, 2007). DNA methylation is one of the well-studied epigenetic biomarkers. Epigenetic studies among various communities have indicated a relationship between demographic factors and the genomic methylation pattern (Jiang et al., 2015).

Given the results of the expression rate and methylation of Bax in this study, gene methylation may be one of the regulatory mechanisms involved in regulating gene expression. Since, in our study, decreased expression of Bax gene was associated with increased methylation (hyper-methylation) of this gene in the patient group, while increased expression of this gene in the healthy group was associated with decreased methylation rate (hypomethylation). Similar results in terms of the relationship between gene expression and methylation are also seen in severe patients, as the expression level of this gene was decreased in those with more severe disease (positive subgroup for severe BD in Table 2), whereas those with lower severity of BD were higher (negative subgroup for severe BD in Table 2). In addition, as shown in the Table 2, concerning the age indicator, by increase in patients' age from 45, their Bax gene expression declined; however, methylation in these people did not increase, so that this increase was significant in proportion to the people who

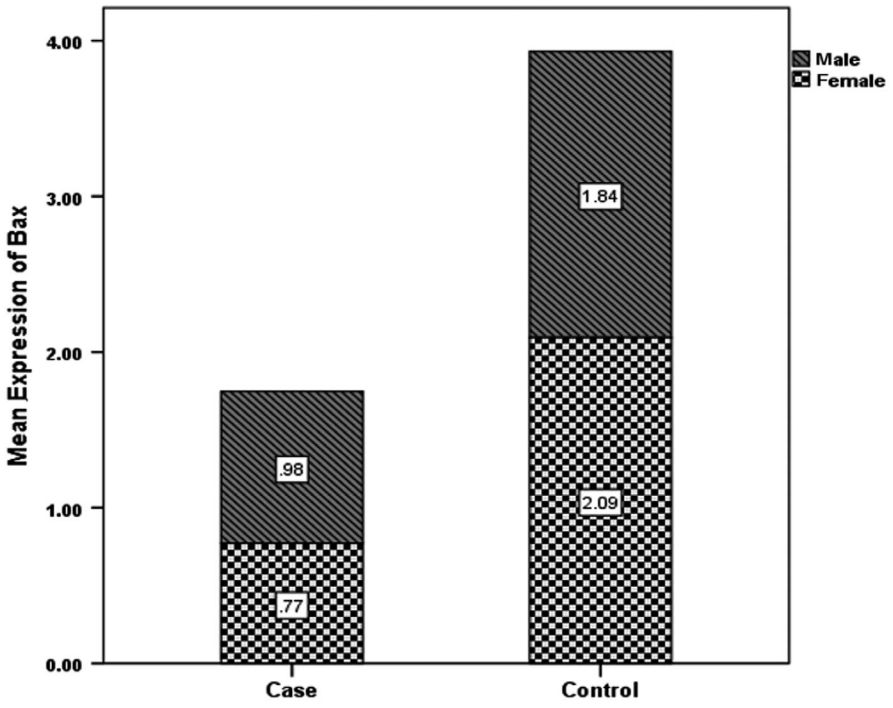

FIGURE 4 Bax gene expression. Comparison of Bax gene expression in two groups of people with Behçet's disease and healthy individuals based on gender showed that the expression of the gene in the patient group decreased compared to the healthy group

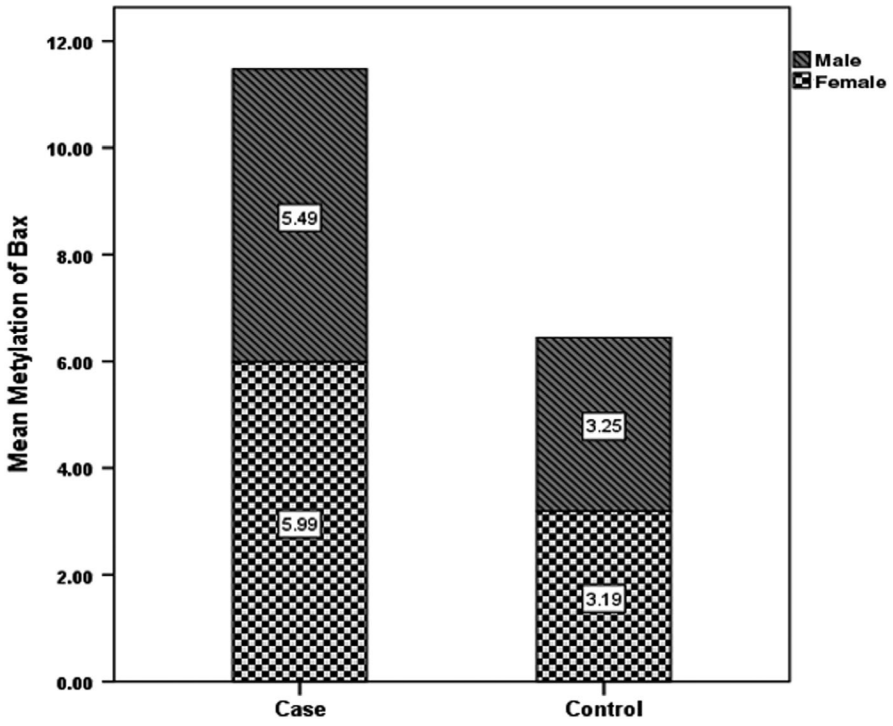

FIGURE 5 Bax Methylation level. Comparison of Bax methylation in two groups of people with Behçet's disease and in healthy people based on gender showed that the level of methylation in the patient group was higher than the healthy group

were lower than 45 years old. As for the bcl2 gene, it can be explained that perhaps the site chosen for methylation was not as effective for methylation, and perhaps by redesigning the primer and methylation for other locus in this gene, we could obtain better results. One of the limitations of the study may be its financial condition, and we were unable to re-analyse the bcl2 gene. Also strengths of the project were the presence of epigenetics and genetics specialists who provided much help during the project. Also, another strength of the plan may be that this study was conducted for the first time.

In a study performed by Itoh, Motte, Strong, Levine, and Fiocchi (2001), the results revealed that as one of the apoptotic proteins, the Bax expression rate was different in different T cells, that is the mucosal and peripheral blood cells, in patients with Crohn's disease and ulcerative colitis, so that it increased in peripheral blood cells compared to mucosal cells. Furthermore, the expression rate in Crohn's disease was lower than that in healthy cases, while it remained unchanged in patients with colitis (Itoh et al., 2001). In another study performed by Lee et al. (2013), it was revealed that as a

TABLE 2 Relationship between gene expression and methylation of BAX3 with clinicopathologic characteristics of patients

Characteristics and clinical features expression	Frequency	Change fold of BAX3 expression (Mean ± *SD*)	*p*-value (Mann–Whitney *U*)	Methylation level of BAX3 expression (Mean ± *SD*)	*p*-value (*t* test)
Age					
<45	25 (62.5%)	1.02 ± 0.85	.074	4.88 ± 1.07	**.001**[b]
≥45	15 (37.5%)	0.67 ± 0.81		7.02 ± 1.31	
Gender					
Male	24 (60%)	0.97 ± 0.84	.292	5.48 ± 1.34	.324
Female	16 (40%)	0.77 ± 0.86		5.98 ± 1.82	
HLA-B5-					
Positive	17 (65.4%)	0.95 ± 0.97	.916	5.77 ± 1.86	.688
Negative	9 (34.6%)	0.96 ± 0.94		5.52 ± 1.26	
HLA-B51					
Positive	8 (53.3%)	1.02 ± 1.05	.867	5.75 ± 0.87	.413
Negative	7 (46.7%)	0.74 ± 0.34		5.27 ± 1.30	
HLA-B27					
Positive	2 (8.3%)	1.55 ± 1.77	.652	4.66 ± 1.58	.371
Negative	22 (91.7%)	0.96 ± 0.95		5.69 ± 1.52	
Oral aphtha					
Positive	38 (95%)	0.88 ± 0.86	.352	5.72 ± 1.57	.471
Negative	2 (5%)	1.11 ± 0.53		4.91 ± 0.99	
Genital ulcer					
Positive	21 (52.5%)	0.92 ± 1.04	.432	6.09 ± 1.67	.081
Negative	19 (47.5%)	0.86 ± 0.56		5.23 ± 1.31	
Arthritis					
Positive	9 (22.5%)	0.64 ± 0.58	.517	5.75 ± 1.17	.889
Negative	31 (77.5%)	0.96 ± 0.90		5.66 ± 1.66	
Sever B.D					
Positive	22 (55%)	0.72 ± 0.89	**.019**	6.29 ± 1.61	**.005**[b]
Negative	18 (45%)	1.11 ± 0.75		4.94 ± 1.12	
Severe eye involvement					
Positive	8 (20.5%)	0.58 ± 0.57	.139	6.49 ± 1.65	.097
Negative	31 (79.5%)	0.98 ± 0.90		5.45 ± 1.51	
EN					
Positive	5 (13.2%)	1.68 ± 1.18	.066	5.49 ± 1.24	.771
Negative	33 (86.8%)	0.79 ± 0.75		5.71 ± 1.53	
Phlebitis					
Positive	4 (10.5%)	1.43 ± 1.15	.199	5.71 ± 0.65	.961
Negative	34 (89.5%)	0.85 ± 0.81		5.67 ± 1.56	
Ocular					
No eye involvement	9 (23.1%)	0.81 ± 1.01	.750	5.47 ± 1.41	.666[b]
One eye activity	12 (30.8%)	0.78 ± 0.49		6.01 ± 1.71	
Bilateral activity	18 (46.2%)	1.02 ± 0.98		5.53 ± 1.61	
Cataract					
Positive	8 (20.5%)	0.57 ± 0.58	.135	7.05 ± 1.61	**.004**[b]
Negative	31 (79.5%)	0.98 ± 0.90		5.31 ± 1.37	

(Continues)

TABLE 2 (Continued)

Characteristics and clinical features expression	Frequency	Change fold of BAX3 expression (Mean ± *SD*)	*p*-value (Mann–Whitney *U*)	Methylation level of BAX3 expression (Mean ± *SD*)	*p*-value (*t* test)
Vision loss					
One eye	4 (11.8%)	0.41 ± 0.31	.190	6.11 ± 1.41	.466
No eye	30 (88.2%)	0.95 ± 0.86		5.55± 1.41	

*According to the results, items that were significant at the level of less than 5%.

**That were significant at the level of less than 1%.

pro-inflammatory protein, Bax expression reduced, while as an anti-apoptotic factor, the expression of Bcl2 increased in fibroblasts of patients with rheumatoid arthritis compared to those with osteoarthritis (Lee et al., 2013). Moreover, in another study conducted by Wang (2017) et al., it was revealed that by the immunohistochemistry technique, the expression of Fas, Bax and Bcl2 genes in osteoarthritis group increased compared to the healthy group. The methylation of promoter sites in these genes reduced and this reduction in methylation may lead to the increased expression rate of the given genes and thus contributes to chondrocyte cells' apoptosis (Wang et al., 2017).

Given the results of our study and other studies, the genes involved in apoptosis may lead to the development of disease or its severity through interfering with the regulation of the planned death of the cells involved in the immune system and immune systems. The apoptosis effects are dependent to the environment where the planned death of the cell occurs. Various cytokines may mediate in launching this complex process, such as interferon, TNF-α and IL-1β (Takikita et al., 2001; Viard-Leveugle et al., 2013). Today, by means of apoptosis-inducing genes and, in some cases, the genes inhibiting this process, immune system direction is arbitrarily applied to balance between the cellular and humoral immune responses. Moreover, these genes are employed to treat cancer, autoimmune diseases as well as allergies (Castillo & Kowalik, 2002; Lebedeva, Su, Sarkar, & Fisher, 2003; Lin, Huang, & Juan, 2012; Nicholson, 2000).

5 | CONCLUSION

The results of the present research showed that the Bax gene promoter was hypermethylated in patients compared to the healthy individuals and the patients showed a higher methylation rate. In addition, the Bax expression rate in patients was lower than that in the healthy individuals. Given the results of the present study and other studies, one of the regulatory mechanisms for the Bax gene expression occurred through the methylation regulation. Nevertheless, no statistically significant difference was observed between the two patient and healthy groups for Bcl2 gene in terms of gene expression and methylation. Regarding the results of the disease, it can be interpreted that DNA methylation may be involved in certain conditions of the disease and lead to the regulation of the expression of the involved genes, like the Bax gene, in the pathogenesis of the disease.

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CONFLICT OF INTEREST

None of the authors report any conflicts of interest.

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