HLA-B gene methylation and expression in Behçet's syndrome: a potential role of epigenetics in the pathogenesis

M. Özkılınç Önen[1], E. Everest[1], T. Demirci[1], P. Köprülü Şen[1], E. Kızıltepe Kısakesen[1], Y. Özgüler[2], S.N. Esatoğlu[2], E. Seyahi[2], E. Tahir Turanli[3]

[1]Department of Molecular Biology-Genetics and Biotechnology, Graduate School of Science, Engineering and Technology, Istanbul Technical University, Istanbul;
[2]Division of Rheumatology, Department of Internal Medicine, Cerrahpaşa Medical Faculty, Istanbul University-Cerrahpaşa, Istanbul;
[3]Department of Molecular Biology and Genetics, Faculty of Engineering and Natural Sciences, Acıbadem University, Istanbul, and Graduate School of Natural and Applied Sciences, Molecular Biology and Genetics Program, Acibadem University Istanbul, Turkey.

Abstract

Objective

The HLA-B51 locus has the strongest association with Behçet's syndrome (BS). The presence of a CpG island in the HLA-B gene led us to examine the role of epigenetic regulation in BS.

Methods

HLA-B51 *genotyping was performed via sequence-specific PCR in 15 index familial BS cases, 17 affected relatives, 26 unaffected relatives, 46 sporadic BS cases, and 41 healthy controls.* HLA-B *methylation level was determined using the Zymo OneStep qMethyl kit, and* HLA-B51 *mRNA level was assessed by quantitative real-time PCR in 14 index familial BS cases, 15 affected relatives, 15 unaffected relatives, 11 sporadic BS cases, and 10 healthy controls.*

Results

HLA-B51 carrier ratio was 13/15 in index familial cases, 13/17 in affected relatives, 22/26 in unaffected relatives, 8/25 in healthy controls, and 35/47 in sporadic BS cases. HLA-B51 expression level in HLA-B51$^+$ BS cases was 2.2-fold higher than in their unaffected relatives (p=0.0149) and 1.3-fold higher than in healthy controls (p=0.0188), while sporadic BS cases had a 2.7-fold higher level than healthy controls (p=0.0487). HLA-B promoter methylation was significantly lower in HLA-B51$^+$ familial BS cases than in unaffected relatives (0.4-fold, p=0.01), affected relatives (0.36-fold, p=0.0219), and healthy controls (0.34-fold, p=0.0371) and slightly lower in HLA-B51$^+$ sporadic BS cases than in healthy controls (0.71-fold, p=0.2347). There was an inverse correlation between HLA-B promoter methylation and HLA-B51 expression in HLA-B51$^+$ sporadic BS cases (p=0.0164).

Conclusion

This study indicates epigenetic involvement associated with the HLA-B51 locus in BS, both in familial and sporadic cases. Further studies with larger sample sizes are needed to confirm our results.

Key words

Behçet's syndrome, *HLA-B* methylation, *HLA-B51* expression, epigenetics, familial Behçet's syndrome, sporadic Behçet's syndrome

Merve Özkılınç Önen, PhD,
Elif Everest, PhD
Turna Demirci, MSc
Pelinsu Köprülü Şen, MSc
Esra Kızıltepe Kısakesen, MSc
Yeşim Özgüler, MD
Sinem Nihal Esatoğlu, MD
Emire Seyahi, MD
Eda Tahir Turanlı, PhD

Please address correspondence to:
Eda Tahir Turanlı
Department of Molecular Biology and Genetics, Faculty of Engineering and Natural Sciences, Acıbadem University, 34469 Istanbul, Turkey.
E-mail: eda.turanli@acibadem.edu.tr

Received on August 2, 2023; accepted in revised form on November 9, 2023.

Competing interests: S.N. Esatoglu has received lecture fees from Roche, Pfizer, UCB Pharma and Merck Sharp Dohme. E. Seyahi has received honoraria or other financial support fron Pfizer, Novartis and UCB. E. Tahir Turanli has received grants from University Scientific Research Programs. The other authors have declared no competing interests.

Introduction

Behçet's syndrome (BS) is a chronic, multi-systemic, inflammatory disorder characterised by various clinical symptoms (1). It is characterised by recurrent skin-mucosa lesions and uveitis. Less often, other organ systems such as the locomotor, vascular, gastrointestinal, and central nervous systems are affected (1). The pooled prevalence of BS is 10.3 per 100,000 individuals worldwide (2), and Turkey has the highest prevalence (20–421 per 100,000) (3, 4). The aetiology of BS has not been clarified yet; both genetic and environmental factors are thought to contribute to disease development (5). A higher prevalence in countries along the Silk Road from Japan to the Middle East than in the rest of the world and a strong association with the *HLA-B51* allele in many ethnic groups constitute the basis of genetic heritability (6, 7, 8). In a study, familial aggregation was significantly higher in the paediatric BS group (12.3%) than in the adult BS group (2.2%) ($p<0.0001$), which suggests that genetic contribution may be stronger in children with BS than in adult BS cases (9). Although a basic Mendelian inheritance pattern has not been observed in most familial BS cases (5), a higher prevalence of early-onset BS in familial cases (25%) than in sporadic cases (8%) (10) and earlier disease onset in the children compared with their parents could be strong indications of genetic contribution in BS (11). In familial BS, the recurrence is seen mostly in the siblings, but there are also mother-child, father-child, first-degree cousins, and uncle-nephew familial cases (12). It was shown that the recurrence risk of BS in the siblings is 4.2% and that the recurrence risk ratio (λs) is approximately 11.4–52.5 in the Turkish population (13). In a Turkish study group, the frequency of *HLA-B51* positivity was 68% in familial BS cases, further supporting the contribution of genetics to BS development (14).

Several BS-associated genetic loci have been identified through genome-wide association studies (GWAS). The first GWAS conducted for BS included 1,215 BS cases and 1,278 healthy controls from Turkey and identified the association of the *HLA-B51* allele and another locus in the major histocompatibility complex class I region (15). However, it is unknown whether *HLA-B51* is responsible for the increased BS susceptibility directly or is associated with BS due to linkage disequilibrium with another gene responsible for the disease association (5). Although *HLA-B51* has been reported as the most strongly associated allele with BS in different ethnic groups, its estimated contribution to the overall genetic susceptibility to BS is less than 20% (16). *HLA-B51* is most commonly seen in Turkish BS cases (40–80%) and less frequently in Caucasians from western countries (~13%) (17-19). According to a meta-analysis including 80 independent studies comprising 4,800 BS cases and 16,289 healthy controls, the pooled prevalence of *HLA-B5*/*B51* was 55.0–63.5% in BS cases and 16.8–21.7% in controls in East Asia, the Middle East/North Africa, Southern Europe, and Northern/Eastern Europe populations (20). The random-effects pooled prevalence of *HLA-B51*/*B5* in BS cases and controls was 57.2% (95% CI 53.4–60.9%) and 18.1% (95% CI 16.1–20.3%), respectively (20). Masatlioglu *et al*. reported a higher concordance ratio in monozygotic twins (2/6) compared with dizygotic twins (1/8), and the estimated heritability was 41% (21). However, the phenotypic discordance in BS monozygotic twins, as well as the presence of BS cases without the *HLA-B51* allele (42.8%) (21) and healthy individuals possessing the *HLA-B51* allele, indicate the involvement of epigenetic and environmental factors in the development of BS.

In recent years, whether there is a link between epigenetic mechanisms and the pathogenesis of complex diseases has been frequently examined. Especially aberrant DNA methylation signatures are hypothesised to be involved in a range of complex chronic diseases. Identification of the presence of allele-specific methylation across the human genome has also led to the realisation of common pathways behind genetic and environmental effects in disease pathologies (22). Given the presence of

a CpG island, a hotspot region for DNA methylation, where the CG dinucleotide repeat ratio is >55%, in the *HLA-B* gene (spanning the promoter and exons 1–3 of the gene) (23) and previous data on phenotypic discordance, we hypothesised that *HLA-B51* may have an epigenetic role in BS aetiology. We also hypothesised that familial BS cases would have more pronounced epigenetic involvement compared to sporadic cases. Accordingly, we performed *HLA-B51* genotyping, *HLA-B* methylation analysis, and *HLA-B51* mRNA expression analysis in familial BS cases and their unaffected relatives, sporadic BS cases, and healthy controls to reveal the possible epigenetic involvement of *HLA-B51* in BS development.

Methods

Study participants

We included 15 (6M/9F) index patients with BS who have at least one relative diagnosed with BS, 17 (8M/9F) affected relatives, 26 (15M/11F) unaffected relatives, and 25 (13M/12F) age- and sex-matched healthy controls in the familial BS group. In the sporadic BS group, we included 47 (20M/27F) sporadic BS cases who have no family member with BS or other autoinflammatory syndromes and 17 (7M/10F) age- and sex-matched healthy controls. Study participants in the familial BS groups were assigned into subgroups based on their *HLA-B51* positivity status as follows: group 1, 11 *HLA-B51*$^+$ index BS cases; group 2, 11 *HLA-B51*$^+$ affected relatives; group 3, 11 *HLA-B51*$^+$ unaffected relatives; and group 4, 6 *HLA-B51*$^+$ healthy controls. Among the sporadic BS group, 11 BS cases and 10 healthy controls were included in the methylation and expression analyses. Study participants with inadequate DNA and cDNA quality were excluded from methylation and expression studies.

All patients fulfilled the International Study Group Criteria for BS (24) diagnosis and were registered in the multidisciplinary outpatient clinic at the medical faculty of Istanbul University-Cerrahpaşa. All patients were older than 18 years of age and had no apparently active disease. The study was approved by the Ethics Committee of Istanbul University-Cerrahpaşa Medical School (approval no.: 37260/2013) and carried out in compliance with the Helsinki Declaration. Written informed consent was obtained from all volunteers.

HLA-B51 genotyping

HLA-B51 genotyping was performed in the familial and sporadic BS groups with sequence-specific PCR using primers (F: 5'CCGGAGTATTGGGACCGGAAC-3' and R: 5'-GCGCGCTGCAGCGTCTCC-3') that produce a 622 base-pair DNA product, including all base substitutions for *HLA-B51*. To discriminate the PCR product of *HLA-B51* from non-specific sequences, a restriction enzyme specific to *HLA-B51* (BpU10I, New England Biolabs, Ipswich, MA, USA) was used. PCR products were treated with the restriction enzyme for 4 hours at 37ºC. Cleaved PCR products resulting in two bands (464 and 158 base pairs) were accepted to represent the *HLA-B51* allele.

RNA isolation and cDNA synthesis

RNA samples were isolated from the peripheral blood using the PureLink RNA Mini Kit (Thermo Fisher Scientific, Waltham, USA). RNA concentrations were measured using a NanoDrop spectrophotometer and adjusted to 1000 ng/µl for cDNA synthesis. Immediately after RNA isolation, cDNA synthesis was performed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA, USA) according to the manufacturer's protocol.

HLA-B methylation levels analysis

The CpG Island Searcher online tool was used to reveal possible CpG islands in the *HLA-B* gene (http://cpgislands.usc.edu). *HLA-B* region (Fig. 1) methylation levels were analysed using the Zymo OneStep qMethyl™ Kit by quantitative real-time (q-RT)-PCR in the familial and sporadic BS groups. This kit is designed for the detection of region-specific DNA methylation via selective amplification of methylated cytosines in CpG dinucleotides. The DNA to be tested is separated into two parts: the "test reaction" and "reference reaction." DNA in the test reaction is digested with methylation-sensitive restriction enzymes, while DNA in the reference reaction is not. DNAs from both reactions are then amplified via q-RT-PCR. Cycle threshold (Ct) values for the test and reference reactions vary due to different methylation levels; large Ct differences are characteristics of non-methylated DNA. At the end of the q-RT-PCR procedure, the *OneStep* qMethylTM Calculator (www.zymoresearch.com/qmethylcalc) was used to determine the methylation levels using the following equation: Percent Methylation = 100 x 2-ΔCt.

HLA-B51 expression level analysis

HLA-B51 expression level analysis was initially performed for the following 4 subgroups in the familial BS group: group 1, 11 *HLA-B51*$^+$ index patients; group 2, 11 *HLA-B51*$^+$ affected relatives; group 3, 11 *HLA-B51*$^+$ unaffected relatives; and group 4, 6 *HLA-B51*$^+$ healthy controls. The *HLA-B51* expression level was further assessed in 46 BS cases and 17 healthy controls from the sporadic BS group. Q-RT PCR experiments were performed using the HLA-B primers (F: 5'-TCTCTCCCTGGTTTCCAC-3' and R: 5'-GGGACTTTAGAACTGGGAC-3') and a UPL probe designed for the exon-3 region of the *HLA-B gene* on the Roche LightCycler 480 Instrument II platform.

Statistical analyses

Frequency differences in the *HLA-B51* allele among the study groups were analysed by Fisher's exact test. Expression and methylation level differences were analysed by the Student's t-test, and the correlation between *HLA-B51* expression and *HLA-B* methylation levels was analysed by Pearson correlation. All statistical analyses were performed using the GraphPad Prism 6 software (GraphPad Software Inc., USA).

Results

The family pedigrees of the 15 index patients in the familial BS group are given in Supplementary. Among the BS families, the patient distributions were as follows: 5 parents/children, 4 siblings, 4 third-degree relatives, 1 second-degree relative, and 1 fourth-degree relatives. Bioinformatics analy-

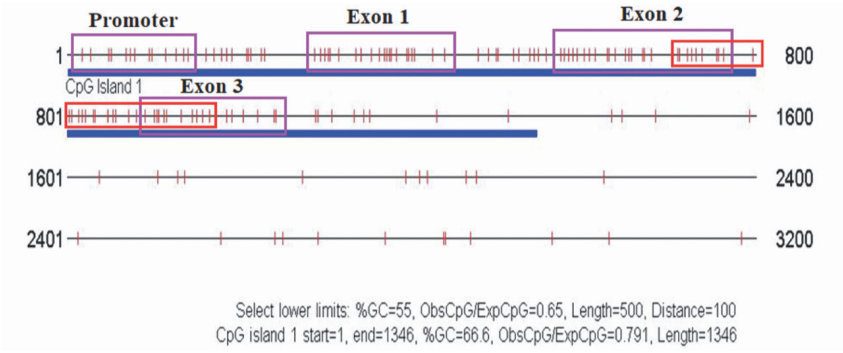

Fig. 1. CpG island in the *HLA-B* gene.
Blue bar represents the CpG island, and red boxes on exon 2 and exon 3 represent the regions where *HLA-B51* allele sequence differences are found.

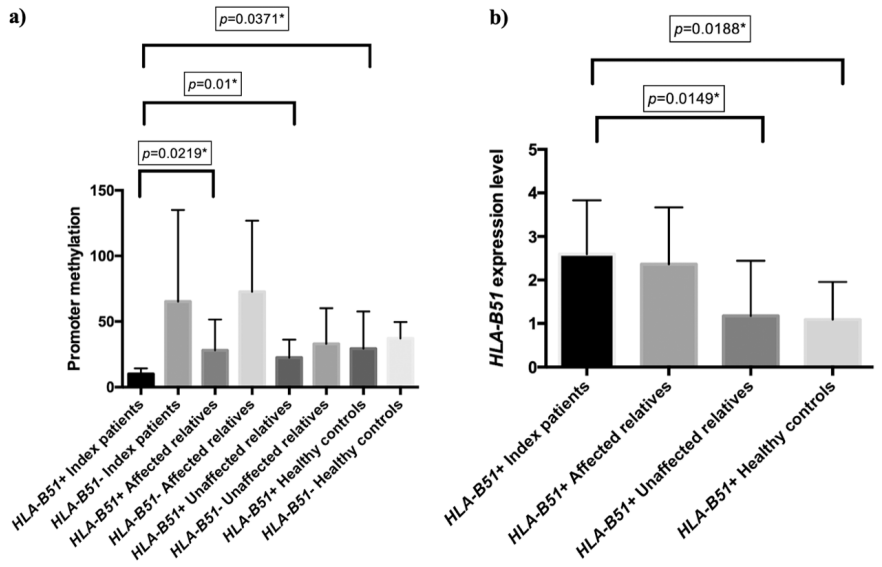

Fig. 2. *HLA-B* methylation level and *HLA-B51* expression level differences in the familial BS group.
a) Differences in the promoter region methylation level among all groups: 11 *HLA-B51*$^+$ index BS cases, 3 *HLA-B51*$^-$ index BS cases, 11 *HLA-B51*$^+$ affected relatives, 4 *HLA-B51*$^-$ affected relatives, 11 *HLA-B51*$^+$ unaffected relatives, 4 *HLA-B51*$^-$ unaffected relatives, 6 *HLA-B51*$^+$ healthy controls, and 6 *HLA-B51*$^-$ healthy controls. **b**) Expression level analysis of the *HLA-B51* allele in 11 *HLA-B51*$^+$ index BS patients, 11 *HLA-B51*$^+$ affected relatives, 11 *HLA-B51*$^+$ unaffected relatives, and 6 *HLA-B51*$^+$ healthy controls.
Student's t-test; $p<0.05$, statistically significant.

sis of the *HLA-B* region revealed the presence of a CpG island between -1 and -1346 nucleotides, with a GC ratio of 66.6% and 117 CG repeats (Fig. 1).

HLA-B genotyping

The *HLA-B51* allele carrier ratio was significantly lower in the total healthy control group (19/42) compared with the index familial BS cases (13/15, p=0.0066), affected relatives (13/17, p=0.0433), unaffected relatives (22/26, p=0.0019), and sporadic BS cases (35/47, p=0.0087). There was no significant difference between any of the other two groups.

HLA-B methylation and HLA-B51 expression levels in familial BS

Methylation profiles of *HLA-B* promoter and exonic regions were analysed in 11 *HLA-B51*$^+$ index BS cases, 11 *HLA-B51*$^+$ affected relatives, 11 *HLA-B51*$^+$ unaffected relatives, and 6 *HLA-B51*$^+$ healthy controls. We observed that the promoter methylation level was lower in the index BS cases compared with the affected relatives (0.36-fold, p=0.0219), unaffected relatives (0.4-fold, p=0.01), and healthy controls (0.34-fold, p=0.0371) (Fig. 2a). *HLA-B51* transcription level analysis was performed in 11 *HLA-B51*$^+$ index BS cases, 11 *HLA-B51*$^+$ affected relatives, 11 *HLA-B51*$^+$ unaffected relatives, and 6 *HLA-B51*$^+$ healthy controls. *HLA-B51* expression levels in the index BS cases (2.595±0.3725) were significantly higher than in the unaffected relatives (1.178±0.3801, 2.2-fold, p=0.0149) and healthy controls (1.090±0.3541, 2.3-fold, p=0.0188) (Fig. 2b). Overall, among the *HLA-B51* allele carriers, there was no significant correlation between *HLA-B* promoter methylation and *HLA-B51* transcript expression. The *HLA-B* exonic region methylation level also did not correlate with the *HLA-B51* expression level.

HLA-B methylation and HLA-B51 expression levels in sporadic BS

The methylation level of the *HLA-B* promoter region was analysed in 5 *HLA-B51*$^+$ sporadic BS cases, 6 *HLA-B51*$^-$ sporadic BS cases, 5 *HLA-B51*$^+$ healthy controls, and 5 *HLA-B51*$^-$ healthy controls. Among sporadic BS patients, those who were *HLA-B51*$^+$ had significantly lower promoter methylation levels than *HLA-B51*$^-$ ones (0.35-fold p=0.0208) (Fig. 3a) and slightly lower levels than *HLA-B51*$^+$ healthy controls (0.71-fold p=0.2347). *HLA-B51* transcription level analysis indicated that *HLA-B51* expression was significantly higher in *HLA-B51*$^+$ BS cases compared with *HLA-B51*$^+$ healthy controls (2.7-fold, p=0.0487) and *HLA-B51*$^-$ BS cases (35.5-fold, p=0.0017). *HLA-B51* expression was also significantly higher in *HLA-B51*$^+$ healthy controls compared with *HLA-B51*$^-$ healthy controls (41.4-fold, p=0.0224) (Fig. 3b). Overall, *HLA-B* promoter methylation had a significant negative correlation with *HLA-B51* transcript expression in *HLA-B51*$^+$ BS patients (Pearson correlation coefficient=0.881, p=0.0164) (Fig. 3c). *HLA-B* exonic region methylation did not correlate with *HLA-B51* expression in the sporadic BS group, either.

Discussion

DNA methylation is a significant epigenetic mechanism that affects the regulation of gene expression and disease pathogenesis. Several studies have suggested a potential role for DNA methylation in the pathogenesis of BS.

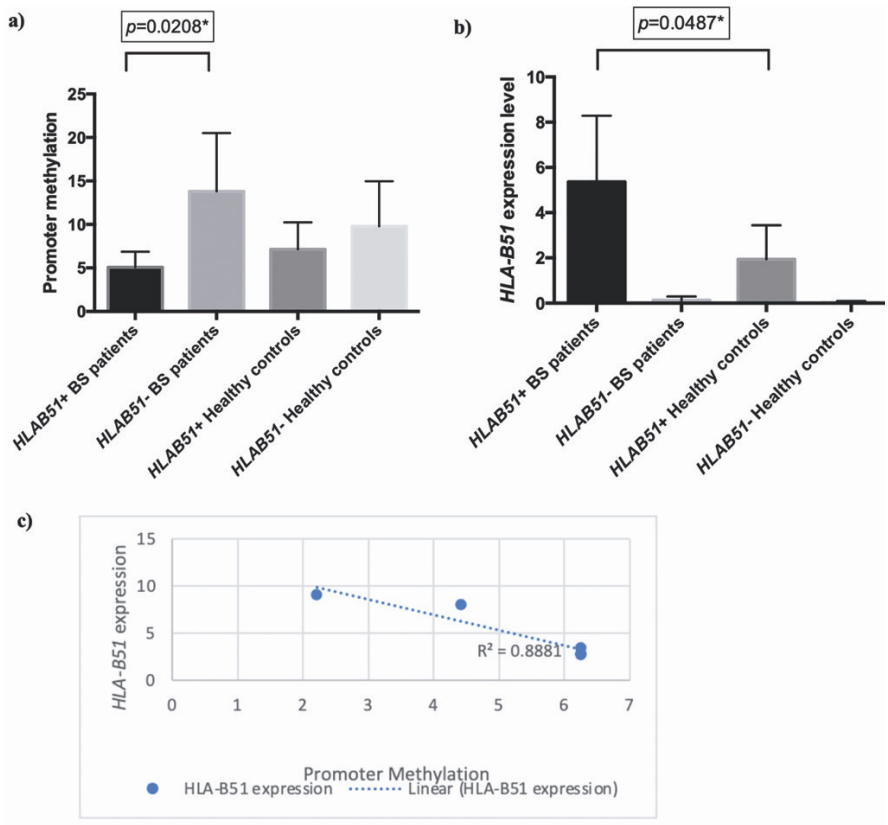

Fig. 3. Differences in and correlation between *HLA-B* methylation and *HLA-B51* expression in the sporadic BS group.
a) Differences in the promoter region methylation level among 5 *HLA-B51*$^+$ sporadic BS cases, 6 *HLA-B51*$^-$ sporadic BS cases, 5 *HLA-B51*$^+$ healthy controls, and 5 *HLA-B51*$^-$ healthy controls. **b**) Differences in the average exon methylation level among the same groups. **c**) Correlation graph between *HLA-B* promoter methylation and *HLA-B51* expression.
Student's t-test and Pearson correlation; $p<0.05$, statistically significant, R^2; correlation coefficient.

Some of the genes and/or pathways have been studied, including those related to IL-6, IL-10, toll-like receptors, CD4$^+$ T cells, CD14$^+$ monocytes and vitamin D (25-29). One study reported that patients with BS had high expression of the *IL6* gene with decreased promoter methylation, suggesting that changes in the methylation level of *IL6* may be associated with susceptibility to BS (25). Another study found that the expression of the *IL10* gene decreased in patients with BS with a high promoter methylation level. Interestingly, the high *IL10* methylation level was significantly associated with severe symptoms in patients with BS (26). Hughes *et al*. compared methylated CpG sites in CD4$^+$ T cells and CD14$^+$ monocytes among patients with BS and healthy controls (28). They found the presence of an abnormal DNA methylation pattern in genes that control the regulation of cytoskeletal dynamics. The authors suggested that the unusual DNA methylation of various categories of structural and regulatory proteins of the cytoskeleton may play a role in the development of BS. They also examined the methylation levels of patients with BS before and after treatment and found that the levels returned to normal levels after the treatment (28).

HLA-B51 is the most strongly associated allele with BS, yet the exact mechanism of its involvement in disease pathology is unknown and has been long debated (30). In this context, we attempted to investigate the relationship between *HLA-B* methylation as an epigenetic mechanism and BS. Initially, we found a high GC content (66.6%) involving 117 GC repeats between -1 and -1346 nucleotides in the *HLA-B* gene via bioinformatic analysis and focused on the *HLA-B* promoter and exonic methylation level and their correlation with *HLA-B51* expression level in familial BS cases, their affected and unaffected relatives, sporadic BS cases, and healthy controls. Then, we performed *HLA-B51* genotyping and found an unexpectedly high frequency of the *HLA-B51* allele in the unaffected relatives (22/26) of the familial BS cases, which strengthens the hypothesis of epigenetic regulation of the *HLA-B* locus in BS pathophysiology. Methylation level analyses of the *HLA-B* promoter region showed significantly decreased levels of methylation in *HLA-B51*$^+$ index familial BS cases than in their affected relatives, unaffected relatives, and healthy controls, which highlights the relevance of *HLA-B* promoter methylation in BS pathogenesis. However, in sporadic BS cases, we observed only a slight decrease in promoter methylation levels in *HLA-B51*$^+$ BS patients compared with *HLA-B51*$^+$ healthy controls. Further, in the expression analyses, we observed that *HLA-B51*$^+$ index BS cases had significantly higher *HLA-B51* expression levels than their unaffected relatives and healthy controls in the familial BS group and that *HLA-B51*$^+$ sporadic BS cases had significantly higher *HLA-B51* expression levels than *HLA-B51*$^-$ sporadic BS cases and *HLA-B51*$^+$ healthy controls in the sporadic BS group. A significantly higher *HLA-B51* expression level in *HLA-B51*$^+$ BS cases than in healthy controls supports the involvement of the *HLA-B51* locus in the pathogenesis of BS.

The term "MHC-I (major histocompatibility complex class I)-opathy" refers to a group of inflammatory diseases, including BS, psoriasis, spondyloarthritis, and birdshot uveitis, that have similar clinical symptoms and a significant genetic association to the MHC-I antigen presentation pathway (31). It is worth noting that MHC-I-opathies have been shown to be strongly associated with MHC-I alleles and to involve expression and methylation level changes in the corresponding loci. For example, in a study by Chen *et al*., the promoter methylation was measured for HLA-A, HLA-B, and HLA-C loci in patients with psoriasis (associated with *HLA-C*06:02*) and healthy controls, showing hypermethylation of only the *HLA-C* locus in psoriatic epidermis (32).

Correlation analyses between *HLA-B* promoter/exon methylation and *HLA-B51* transcript levels did not show a significant negative correlation in *HLA-B51*$^+$ familial BS cases. Although we observed lower *HLA-B* promoter methylation and higher *HLA-B51* expression levels in *HLA-B51*$^+$ index familial BS cases than in other groups, the lack of a significant correlation might be due to the variations in methylation and expression levels among different individuals. It is also possible that, alternative mechanisms to control the *HLA-B51* expression level might be operative. Our results might have been affected by the small sample size as well. In sporadic BS cases, *HLA-B* promoter methylation had a significant negative correlation with *HLA-B51* expression, while *HLA-B* exon methylation did not. Gene-body CpG islands can become extensively methylated; however, it is thought that this does not lead to the inhibition of transcription elongation (23), which might explain the lack of a correlation between *HLA-B* exon methylation and *HLA-B51* expression in all groups.

Although *HLA-B51* has the strongest association with BS among all proposed genetic contributors, the existence of *HLA-B51*$^-$ BS cases and *HLA-B51*$^+$ healthy controls strengthens the hypothesis of epigenetic involvement in BS pathogenesis. Some possible mechanisms underlying the involvement of the *HLA-B51* allele in BS pathogenesis have been proposed in the literature. One explanation is the abnormal peptide presentation since *HLA-B51* is a member of class I MHC molecules, which are responsible for antigen presentation to the T cells for immune responses. Certain polymorphisms in amino acid residues 97, 116, 152, and 67 of the HLA-B protein have been shown to influence the risk of developing BS significantly (33). Among them, residue 67 is important since it is one of the two known amino acids that differ between the *HLA-B51* and *HLA-B52* alleles. The *HLA-B52* allele is not associated with BS; therefore, this residue in the *HLA-B51* allele may lead to altered peptide presentation in BS pathology (33). This abnormal peptide presentation may occur through the interaction of *HLA-B51* with the inhibitory killer immunoglobulin-like receptor (KIR), KIR3DL, present on natural killer (NK) cells (34). NK cells were reported to be involved in BS pathology via two possible mechanisms: 1) NK cells lacking the inhibitory KIR cannot recognise self-MHC molecules, which can lead to autologous tissue damage; or 2) some defects in the NK cell repertoire may cause permanent viral infections, triggering chronic inflammatory responses (35). Regarding these data, an increase in *HLA-B51* allele expression, which is related to a low methylation level in the *HLA-B* promoter region, may result in inflammatory responses in BS.

We conclude that epigenetic regulation was involved in both familial and sporadic BS cases, but a significant negative correlation between promoter methylation and *HLA-B51* expression was only observed in *HLA-B51*$^+$ sporadic BS cases. This finding suggests that there may be different genetic predisposition factors in familial and sporadic BS cases, leading to different pathophysiological mechanisms since familial and sporadic forms of the same disease do not necessarily have the same genetic burden. For instance, in our previous study, we found that sporadic multiple sclerosis cases tended to have higher polygenic risk scores compared with familial multiple sclerosis cases, suggesting other rare susceptibility variants loading in the families (36). On the other hand, we do not have any evidence to support our assumption in the current study since familial and sporadic cases were similar regarding disease severity, major organ involvement and disease activity.

This study has some limitations. We did not have information on the symptomatic evolution of unaffected relatives who may have been diagnosed with BS later. Additionally, we might not have reliable information related to past medications, as it was reported that DNA methylation levels could be affected by treatment (27). We could not include all individuals in all analyses due to the inadequate amount or quality of some samples. As our study was cross-sectional, whether the methylation and expression levels of *HLA-B51* are consistent or fluctuate along the time course in a single individual is not known. It is also quite possible that some immuno-modulator agents, particularly type I interferon may influence the expression and methylation levels of *HLA-B51* through-out the disease course (37). Our study was not aimed to respond to whether there is such an evolution thought the course, either. Also, it must be noted that none of the patients (index BS cases or affected relatives) were using type I interferon or any other biological at the time of the study. Lastly, we cannot rule out the possibility of monoallelic *HLA-B51* expression in some individuals due to the genotyping method we used.

In conclusion, our study suggests the potential for epigenetic involvement linked to the HLA-B51 locus in both familial and sporadic cases of BS. However, to validate our findings, further research with larger sample sizes is imperative.

Acknowledgements

We would like to express our gratitude to Prof. Dr. Hasan Yazıcı for his valuable comments on the research design of this study. We also thank all patients who participated in this study, as well as the members of the Division of Rheumatology, Cerrahpaşa Medical Faculty, for their help in collecting the patient samples.

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