Research Article
Methylation Status of Alu and LINE-1 Interspersed Repetitive Sequences in Behcet’s Disease Patients

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Behcet’s Disease (BD) is a multisystem chronic inflammatory disease. The pathology is believed to involve both genetic susceptibility and environmental factors. Hypomethylation leading to activation of interspersed repetitive sequences (IRs) such as LINE-1 and Alu contributes to the pathologies of autoimmune diseases and cancer. Herein, the epigenetic changes of IRs in BD were evaluated using combined bisulfite restriction analysis-interspersed repetitive sequences (COBRA-IRS). DNA from neutrophils and peripheral blood mononuclear cells (PBMCs) of BD patients with ocular involvement that were in active or inactive states and healthy controls were used to analyze LINE-1 and Alu methylation levels. For Alu sequences, significant differences were observed in the frequency of $uC^uC$ alleles between PBMCs of patients and controls ($p = 0.03$), and between inactive patients and controls ($p = 0.03$). For neutrophils, the frequency of $uC^C^C$ was significantly higher between patients and controls ($p = 0.006$) and between inactive patients and controls ($p = 0.002$). The partial methylation ($C^uC + mC^C^C$) frequencies of Alu between inactive patients and control samples also differed ($p = 0.02$). No statistically significant differences for LINE-1 were detected. Thus, changes in the methylation level of IRS elements might contribute to the pathogenesis of BD. The role of Alu transcripts in BD should be investigated further.

1. Introduction

Behcet’s Disease (BD) is a complex systemic inflammatory disorder, generally characterized by recurrent oral aphthous ulcers, genital ulcers, and uveitis [1]. However the clinical spectrum is wide and the manifestations of the disease, such as the involvement of the nervous and gastrointestinal systems, and vasculitis in large veins and arteries vary considerably depending on gender, individual differences, and ethnicity and can lead to mortality and organ loss in severe cases.

BD shares many similarities with autoinflammatory diseases, which comprise a group of disorders caused by genetic mutations in the components of the innate immune system. Among these similarities are the nonspecific inflammatory response, which manifests itself as flares and remissions, with the main involvement of neutrophils, and clinical findings, such as fever, increased acute phase proteins, and overexpression of proinflammatory cytokines, such as IL-1B and TNF-alpha during the attacks [2–5]. Gene mutations in Familial Mediterranean fever, a prototypical autoinflammatory disease, have been found frequently in BD patients and are suggested to contribute to the severity of the disease [6]. BD also shows critical differences from classical autoimmune diseases, such as male dominance in severe disease [7–9], lack of association with autoimmune HLA class II haplotypes, and, more importantly, absence of disease-specific high titer autoantibodies or antigen-specific T cells [10].

The etiology of the disease is unknown; however, both genetic and environmental factors have been implicated in its pathogenesis. Occasional familial occurrence [11], genetic distribution along the ancient Silk Road, and an association
with HLA-B51 are some of the factors pointing toward genetic involvement [12, 13]. Environmental conditions, such as bacterial or viral infections, are thought to trigger the disease in genetically susceptible individuals [10]. To date, HLA-B51 shows the strongest association with BD but accounts for less than 20% of the risk [14], which suggests the involvement of other genetic factors. Genome wide association studies revealed other candidate genes, such as IL-10, IL23R, STAT4, CCR1, and KLRC4, that could contribute to BD pathogenesis [15, 16]. One genome wide association study also suggested the epistasis between HLA-B51 and ERAPI gene [17]. In another study, copy number variation in the DEFA1 defensin gene was associated with susceptibility to intestinal involvement in BD [18]. Other recent studies reported that more candidate gene polymorphisms involved in BD included ATG5, FAS, pre-miR-196a2, miR-182, and miR-146a [19–23]. In 2014, a genome wide methylation array study in monocytes and CD4+ T lymphocytes revealed the role of epigenetics in BD pathogenesis. The authors identified abundant aberrant methylation patterns of cytoskeletal element genes in monocytes and CD4+ T lymphocytes as a major contributor to disease pathogenesis [24]. Importantly, it was reported that, after treatment, when the patients were in remission, their methylation patterns reversed back to the patterns seen in healthy controls, suggesting that a better understanding of epigenetic alterations might help us to find new disease markers and treatment options for BD patients with different symptoms. Active transcription factors and specific proteins that affect the binding of methyltransferases in BD likely determine the specific genes that are hypomethylated. However, it is clear that the regulation of methylation is defective in BD.

Almost half of the human genome comprises interspersed repetitive sequences (IRSs) [25], which can be divided into DNA transposons and retroelements. There are two types of retroelements, non-long terminal repeats (LTRs) and LTRs; non-LTR retroelements are further classified into long interspersed nuclear element (LINE) retroposons, which are represented by LINE-1 (20.1%), and short interspersed nuclear elements (SINEs), which are primarily represented by Alu sequences (13.1%) [26]. Most IRS sequences are densely methylated in normal somatic cells. In this state, they are mostly inactive and remain silent. However, hypomethylation and reactivation of these sequences are suggested to have several functional roles, such as controlling the activity of genes by regulating enhancers and repressors, or acting as an alternative promoter upon mobilization, which could lead to insertional mutations and chromosomal instability.

There is growing evidence for the contributions of epigenetic alterations to the pathogenesis of cancer and autoimmune diseases [27]. Recently, hypomethylation and reactivation of IRSs were shown to be important in autoimmune disorders such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), and psoriasis. In SLE, LINE-1, but not Alu, was shown to be significantly hypomethylated in CD4+ T lymphocytes and neutrophils of active patients compared with inactive patients and healthy controls [28, 29]. Similarly, significant hypomethylation of LINE-1, but not Alu, in the epidermis of psoriasis patients was reported [30]. In RA, abundant LINE-1 transcripts were detected in synovial fluids from patients [31], as well as hypomethylation in LINE-1 promoters in synovial fibroblasts [32].

In this report, we investigated for the first time the methylation in LINE-1 and Alu repetitive sequences from active and inactive Behcet’s patients compared with healthy controls.

2. Materials and Methods

2.1. Patients and Controls. Patients who fulfilled the International Study Group criteria for Behcet’s Disease were recruited from the ophthalmology clinic of the Ankara Education and Research Hospital. BD patients with an ongoing severe ocular involvement (uveitis, retinitis, papillitis, or vasculitis) were defined as the active group (n = 12; eight male, four female). BD patients that were free of any active ocular involvement for at least 3 months (confirmed by microscopic and imaging findings) and any extracural involvement for at least 4 weeks were considered as the inactive group (n = 17; 12 men, five women). Fifteen ethnically matched healthy volunteers who were free of any acute or chronic immunemediated illness (chronic infection, allergic and autoimmune diseases) at the time of sample collection were used as the control group (n = 15; eight women, seven men). None of the healthy controls has family history of BD and any immunemediated diseases. The average and age range of the patients with active BD and inactive BD and healthy controls were 32.9 ± 9.0 (21–57), 36.8 ± 9.7 (18–49), and 33.8 ± 7.9 (20–48) years, respectively (mean ± SD). The details of the patient group are shown in Table 1. This study was approved by the Bogazici University Institutional Review Board for Research with Human Subjects. Informed consent was obtained from all patients and control subjects before entering the study.

2.2. Neutrophil and PBMC Isolation. Blood samples were collected into heparinized-Vacutainer tubes (Becton, Dickinson and Company, Plymouth, UK) and cell isolation was performed using density gradient centrifugation. In brief, the collected blood was carefully layered on top of lymphoprep (http://AXIS-SHIELD-PoC.com/, Oslo, Norway) at a blood:lymphoprep ratio of 2:1. The peripheral blood mononuclear cell (PBMC) layer was collected and washed once with Roswell Park Memorial Institute (RPMI) medium and stored at −80°C for DNA isolation. Remaining polymorphonuclear granulocytes (PMNs) or neutrophils were diluted 1:1 with RPMI and layered carefully on top of polymorphoprep (http://AXIS-SHIELD-PoC.com/), Oslo, Norway) at a 1:1 ratio. The PMN fraction obtained from the gradient centrifugation was collected and washed once with RPMI. The red blood cells were removed from the PMN fraction using hypotonic lysis buffer. PMNs were collected and centrifuged and the pellet was stored at −80°C for further experiments. The purity of the PMNs was confirmed by Giemsa-Wright staining.

2.3. Combined Bisulfite Restriction Analyses (COBRA). DNA was extracted from collected cells using a Qiagen EZ DNA
### Table 1: Patient demographics and medications.

| Group | Age  | Sex | Disease score | Medications                                                                 | Active extraocular findings | Total extraocular findings                                                                 | Active/inactive uveitis |
|-------|------|-----|---------------|-----------------------------------------------------------------------------|----------------------------|--------------------------------------------------------------------------------------------|-------------------------|
| 1     | Inactive | 32  | M             | 0                            | Interferon Alpha 2a (3 million) every other day                          | —                           | OA, GU, folliculitis, pathergy positivity, and joint involvement                          | Inactive                |
| 2     | Inactive | 25  | F             | 0                            | Cyclosporine-A 150 mg/day and Azathioprine 100 mg                        | —                           | OA, GU, and EN                                                                     | Inactive                |
| 3     | Inactive | 27  | M             | 0                            | Infliximab infusions (5 mg /kg) at 0, 2, and 6 and then every 4–8 weeks was administered Cyclosporine 2.5 mg/kg/day, Azathioprine 100 mg/day | —                           | OA, GU, and folliculitis                                                                | Inactive                |
| 4     | Inactive | 41  | M             | 0                            | Interferon Alpha 2a (3 million) every other day                          | —                           | OA, GU, and EN                                                                     | Inactive                |
| 5     | Inactive | 37  | M             | 0                            | Interferon Alpha 2a (3 million) every other day                          | —                           | OA, GU, EN, and joint involvement                                                   | Active U                |
| 6     | Inactive | 57  | M             | 0                            | Cyclosporine-A 2.5 mg/kg/day, Azathioprine 100 mg                        | —                           | OA, GU, EN, folliculitis, pathergy positivity, and joint involvement                  | Inactive                |
| 7     | Inactive | 26  | M             | 0                            | Unmedicated                                                              | —                           | OA, GU, EN, and joint involvement                                                   | Inactive                |
| 8     | Inactive | 46  | M             | 0                            | Interferon Alpha 2a (3 million) every other day                          | —                           | OA, GU, and EN                                                                     | Inactive                |
| 9     | Inactive | 40  | F             | 0                            | Unmedicated                                                              | —                           | OA, GU, EN, and joint involvement                                                   | Inactive                |
| 10    | Inactive | 57  | M             | 0                            | Unmedicated                                                              | —                           | OA, GU, EN, and joint involvement                                                   | Inactive                |
| 11    | Inactive | 21  | F             | 3                            | Interferon Alpha 2a (3 million) every other day                          | —                           | OA, GU, EN, and joint involvement                                                   | Inactive                |
| 12    | Inactive | 46  | M             | 0                            | Unmedicated                                                              | —                           | OA, GU, EN, and joint involvement                                                   | Inactive                |
| 13    | Inactive | 34  | F             | 0                            | Unmedicated                                                              | —                           | OA, GU, EN, and joint involvement                                                   | Inactive                |
| 14    | Inactive | 54  | M             | 0                            | Interferon Alpha 2a (3 million) every other day                          | —                           | OA, GU, EN, and joint involvement                                                   | Inactive                |
| 15    | Inactive | 50  | M             | 0                            | Interferon Alpha 2a (3 million) every other day                          | —                           | OA, GU, EN, and joint involvement                                                   | Inactive                |
| 16    | Inactive | 40  | M             | 0                            | Unmedicated                                                              | —                           | OA, GU, EN, and joint involvement                                                   | Inactive                |
| 17    | Inactive | 36  | M             | 0                            | Unmedicated                                                              | —                           | OA, GU, EN, and joint involvement                                                   | Inactive                |
| 18    | Inactive | 35  | M             | 0                            | Unmedicated                                                              | —                           | OA, GU, EN, and joint involvement                                                   | Inactive                |
| 19    | Inactive | 36  | M             | 0                            | Unmedicated                                                              | —                           | OA, GU, EN, and joint involvement                                                   | Inactive                |
| 20    | Inactive | 31  | M             | 7                            | Unmedicated                                                              | —                           | OA, GU, EN, and joint involvement                                                   | Inactive                |

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OA: Ocular Arthritis, GU: Gastrointestinal Ulcers, EN: Episcleritis, PP: Pathergy Test Positivity, GE: Gastroenteritis.
Table 1: Continued.

| Group | Age | Sex | Disease score | Medications | Active extraocular findings | Total extraocular findings | Active/inactive uveitis |
|-------|-----|-----|---------------|-------------|-----------------------------|----------------------------|-------------------------|
| 5     | Active 30 | M   | 7             | Interferon Alpha 2a (3 million) every other day | OA, PP | OA, GU, EN, folliculitis, pathergy positivity, joint involvement, and neurological involvement | Active |
| 6     | Active 37 | F   | 7             | Interferon Alpha 2a (3 million) every other day | OA, PP | OA, GU, EN, folliculitis, and joint involvement | Active |
| 7     | Active 49 | F   | 5             | Cyclosporine-A 2.5 mg/kg/day, Azathioprine 100 mg/day | OA | OA, GU, EN, folliculitis, pathergy positivity, and joint involvement | Active |
| 8     | Active 18 | F   | 5             | Interferon Alpha 2a (3 million) every other day | OA | OA, GU, and EN | Active |
| 9     | Active 31 | M   | 7             | Interferon alpha 2a (3 Million) every other day | OA, PP | OA, GU, folliculitis, pathology positivity, and vascular involvement | Active |
| 10    | Active 34 | M   | 5             | Cyclosporine-A 2.5 mg/kg/day, Azathioprine 100 mg/day | OA | OA, PP, and joint involvement | Active U |
| 11    | Active 27 | F   | 7             | Interferon Alpha 2a (3 million) every other day | OA, GU | OA, GU | Active U |
| 12    | Active 38 | M   | 9             | Cyclosporine-A 2.5 mg/kg/day, Azathioprine 100 mg/day | OA | OA, GU, folliculitis, and GI | Active U |

F: female; M: male
U: Uveitis; OA: oral ulcers; GU: genital ulcers; EN: erythema nodosum; GI: gastrointestinal involv.
amplified to generate Alu amplicons and then digested with TaqI and TasI enzymes to obtain fragments representing the four possible methylation patterns \((m^CmC, m^CmC, m^CmC, \text{ and } m^CmC)\), producing bands at 92, 60, 50, and 18 bp, respectively. (b) Bisulfite treated DNA was amplified to generate Alu ampiclons and then digested with \(m^CmC\) to obtain fragments representing the four possible methylation patterns \((m^CmC, m^CmC, m^CmC, \text{ and } m^CmC)\), producing bands at 133, 90, 75, and 32 bp, respectively.

isolation kit (Qiagen, Valencia, CA, USA), according to the manufacturer’s protocol. An EZ DNA methylation kit (Zymo Research, Orange, CA, USA) was used for the bisulfite conversion of 500 ng of the DNA, following the protocol supplied by the manufacturer. Global assessment of methylation levels in LINE-1 and Alu sequences was performed using the combined bisulfite restriction analysis–interspersed repetitive sequences (COBRA-IRS) technique, as previously described [28–30]. This technique enabled us to detect methylation levels in two CpG sites in LINE-1 and Alu repetitive elements among thousands of copies present in the human genome. The following primers were used for amplification of the bisulfite treated DNA: Alu forward 5’ GGY GYG GTT TAY GTT TGT AA 3’ and Alu reverse, 5’ CTAACCTTTTATATTTTTAATAAAA ACRAAATTTACCA 3’; LINE-1 forward 5’ GTTAAAGAAAAGGGGTAYGGGT 3’ and LINE-1 reverse 5’ AATACRCCRTTTC TTAAA CCRATCTA-3’. The 92 bp PCR product for LINE-1 was obtained using the following cycle conditions: initial denaturation at 95°C for 5 min; 35 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 45 s; and a final extension at 72°C for 7 min. The 133 bp Alu PCR product was obtained by using the following cycle conditions: initial denaturation at 95°C for 5 min; 40 cycles of 95°C for 30 s, 57°C for 30 s, and 72°C for 45 s; and a final extension at 72°C for 7 min. After amplification, the LINE-1 PCR products were digested with two units of TaqI (Fermentas International, Canada) and eight units of TasI (Fermentas International) restriction enzymes. The Alu PCR products were digested with four units of TaqI. Each digestion reaction was incubated overnight at 65°C and separation was performed the following day on 8% Tris-borate-EDTA (TBE) polyacrylamide gels. The gel was stained with SYBR green nucleic acid stain (Invitrogen, Carlsbad, CA, USA). The intensity of the staining of the DNA fragments was measured using a phosphorimagery equipped with ImageQuant software (Molecular Dynamics, GE Healthcare, Slough, UK). The samples were normalized against the data obtained from DNA samples of HeLa, Jurkat, and Daudi cell lines to eliminate interassay variation between each experiment.

2.4. LINE-1 Methylation Analysis. The intensity of the fragments generated by COBRA from LINE-1 depended on the methylation state of the two CpG dinucleotides and were represented by the following unique bands: fully methylated \((m^CmC)\) 50 bp; partial methylation \((m^CmC)\) 18 bp and \((m^CmC)\) 92 bp; and hypomethylation \((m^CmC)\) 60 bp (Figure 1(a)). To calculate the percentage methylation for each of the four possible states, initially, the intensity of each band was divided by the length (bp) of the double-stranded DNA: \(A = \) intensity of the 92 bp fragment/92, \(B = \) intensity of the 60 bp fragment/56, \(C = \) intensity of the 50 fragment/48, \(D = \) intensity of the 42 bp fragment/40, \(E = \) intensity of the 32 bp fragment/28, and \(F = [(D + E) - (B - C)]/2\). Note that the length of the double-stranded DNA was calculated due to the overhanging end from restriction enzymes. In addition, please also note that since we could not see 18 bp band clearly, we calculate intensity of 18 bp using \(F\) instead. The values obtained were then applied to the following formula; \(\% m^CmC = 100 \times C/(A + B + C + F)\), \(\% m^CmC = 100 \times F/(A + B + C + F)\), \(\% m^CmC = 100 \times A/(A + B + C + F)\), and

![Figure 1: LINE-1 and Alu methylation analysis using COBRA.](image)
Table 2: LINE-1 methylation frequency of BD patients and healthy controls. The results represented as the average ± standard deviation.

|                          | % 5mC | % 5mCmC | % 5mC+C | % 5mC+CmC | % 5mC+C+C | % 5mC+C+CmC |
|--------------------------|-------|---------|---------|-----------|-----------|-------------|
| PBMC control (n=15)      | 81.76 ± 2.04 | 81.76 ± 2.04 | 9.62 ± 3.39 | 16.42 ± 1.08 | 5.22 ± 1.07 | 26.05 ± 3.87 |
| PBMC active (n=12)       | 81.61 ± 1.63 | 68.93 ± 2.59 | 8.29 ± 1.92 | 17.07 ± 1.39 | 5.71 ± 0.80 | 25.37 ± 2.03 |
| PBMC inactive (n=17)     | 81.15 ± 1.18 | 68.21 ± 1.57 | 9.80 ± 1.97 | 16.07 ± 1.28 | 5.92 ± 1.59 | 25.87 ± 2.10 |
| PBMC all patients (n=29) | 81.34 ± 1.37 | 68.51 ± 2.04 | 9.18 ± 2.06 | 16.48 ± 1.40 | 5.83 ± 1.31 | 25.66 ± 2.05 |
| Neutrophil control (n=15)| 81.06 ± 1.94 | 67.73 ± 2.92 | 11.01 ± 2.03 | 15.64 ± 0.55 | 5.61 ± 1.20 | 26.65 ± 2.23 |
| Neutrophil active (n=12) | 82.07 ± 0.96 | 69.12 ± 1.39 | 10.06 ± 1.20 | 15.86 ± 1.32 | 4.97 ± 0.61 | 25.92 ± 0.98 |
| Neutrophil inactive (n=17)| 81.35 ± 1.25 | 67.82 ± 2.04 | 11.06 ± 2.54 | 16.01 ± 1.73 | 5.11 ± 0.89 | 27.07 ± 1.91 |
| Neutrophil all patients (n=29) | 81.65 ± 1.18 | 68.36 ± 1.89 | 10.64 ± 2.12 | 15.95 ± 1.55 | 5.05 ± 0.78 | 26.59 ± 1.67 |

\[ \% \text{5mC} = 100 \times B/(A + B + C + F), \% \text{Methylation (5mC)} = \left( \frac{5mC}{(5mC + \text{mC})} \right) = 100 \times \left( \frac{(A + 2C + F)}{(2A + 2B + 2C + 2F)} \right). \]

2.5. Alu Element Methylation Analysis. The COBRA method used in this study is based on the detection of methylation status of two CpG dinucleotides in the 133bp Alu amplicon by the restriction enzyme TaqI, as shown in Figure 1(b). The different methylation states of these two CpG sites were analyzed in four groups. Fully methylated loci (5mC5mC), represented by a 32bp fragment; unmethylated loci (5mC), represented by a 90bp fragment, and (mCmC), represented by a 75bp fragment (Figure 1(b)).

Enzymatic digestion of 133bp COBRA-Alu products with the TaqI enzyme generated bands of 133, 90, 75, 58, 43, and 32bp in length, with different intensities based on the methylation status of the two CpG dinucleotides. The frequency of each pattern was calculated according to the following formulas. First, the intensity of each band was divided by the length (bp) of the double-stranded DNA: \( A = \text{intensity of 133bp fragment/131} \), \( B = \text{intensity of 58bp fragment/56} \), \( C = \text{intensity of 75bp fragment/73} \), \( D = \text{intensity of 90bp fragment/88} \), \( E = \text{intensity of 43bp fragment/41} \), and \( F = \text{intensity of 32bp fragment/30} \). Next, the percentage of hypermethylated loci \( (mCmC) = 100 \times F/(A + C + D + F) \), the percentage of partially methylated loci \( (mC5mC) = 100 \times C/(A + C + D + F) \), \( (mC\text{mC}) = 100 \times D/(A + C + D + F) \), and the percentage of hypomethylated loci \( (5mC\text{mC}) = 100 \times A/(A + C + D + F) \), the percentage of methylated loci, and \% Methylation \( (5mC) = \left( \frac{5C}{(5mC + mC)} \right) = 100 \times (2D + F)/(2A + 2C + 2D + 2F) \) were calculated.

2.6. Statistical Analyses. Independent sample t-tests (two-tailed) were applied to compare various methylation patterns of LINE-1 and Alu among active, inactive, and healthy controls. In some groups where the data were not in normal distribution, we used Mann-Whitney test instead. A \( p \) value of <0.05 was considered statistically significant. Calculations were performed using GraphPad v5.0 (San Diego, CA) and SPSS software version 15.0 (SPSS Inc., Chicago, IL).

3. Results

3.1. LINE-1 Methylation Analysis. The differences in the frequency of methylation in genomic DNA obtained from PBMCs and neutrophils of patients with active and inactive BD as well as the controls were compared. The averages of the frequency of methylation are shown in Table 2. There were no statistically significant differences in the overall methylation between BD patients and healthy controls or between the active and inactive groups in both PBMC and neutrophil subsets. When we compared the differences between each methylation pattern (i.e., 5mC5mC, 5mCmC, mCmC, and mC5mC), we also found no significant differences among the groups in the cell types.

3.2. Alu Methylation Analysis. The frequency of Alu methylation and paired comparisons in the PBMC and neutrophil cell types are shown in Table 3. There were no statistically significant differences in overall methylation levels of PBMCs of active patients when compared with controls and inactive patients, as well as between controls and inactive patients. However, the frequency of 5mC5mC in PBMCs was significantly higher in BD patients compared with controls (\( p = 0.03 \)), particularly for the inactive patients group compared with the controls (\( p = 0.03 \)). In the neutrophil population, no statistically significant differences were observed between the groups in terms of the overall methylation. However, the level of 5mCmC was higher, indicating a hypomethylated status of Alu sequences in neutrophils of BD patients compared with those of healthy controls (\( p = 0.006 \)). This finding was more striking in the inactive group compared with controls (\( p = 0.002 \)). When we analyzed the 5mCmC + mCmC pattern, a significant difference was also found between inactive patients and controls (\( p = 0.02 \)).

4. Discussion

Hypomethylation of IRSs has been investigated extensively and shown to have a role in pathogenesis of complex diseases such as cancer and autoimmune related conditions [27, 33, 34]. The mechanism of how IRS hypomethylation contributes to the pathogenesis of autoimmune diseases is unclear; however, it has been suggested that it could cause aberrant regulation of neighboring genes that have a role in the regulation of the immune response [35, 36], as well as causing nonspecific autoimmune activation via the presence of viral dsRNA or ssRNA transcripts in the circulation [37–39]. In this study, we investigated the role of global hypomethylation in BD and found that levels of overall methylation were
were more ready to respond. However, previously, hypomethylation was found in inactive patients compared with healthy controls. In addition, the frequency of partial methylation was higher in the controls compared with patients. This finding is quite different from our previous finding in SLE [28, 29].

The mechanisms driving demethylation of IRs are complex and not fully understood; however, age-dependent and age-independent mechanisms accounting for the hypomethylation of IRs have been reported. Both age-related (possibly caused by malnutrition and/or oxidative stress) and unrelated loss of methylation events have been proposed to occur in Alu elements, whereas hypomethylation of LINE-1 sequences was reported to be age-independent [40, 41]. LINE-1, but not Alu, hypomethylation was reported in SLE and several other autoimmune diseases. Contrastingly, for many different types of cancer, both Alu and LINE-1 hypomethylation play a role in carcinogenesis [33, 34]. In particular, LINE-1 methylation has been suggested as a poor prognostic factor for several cancer types [42]. In this study, we observed a significant increase in the frequency of the $^{30}$C allele in both PBMCs and neutrophils in BD patients compared with controls. In addition, the frequency of partial methylation was higher in the controls compared with inactive patients in DNA obtained from neutrophils.

Although earlier reports proposed that Alu hypomethylation is age-related [40], the decrease was only significant for the 34–68-year age interval. The average ages of our control and patient cohort were comparable; therefore, the changes detected are unlikely to be related to the aging process. These findings indicated that there might be an increased tendency for hypomethylation of Alu sequences in BD patients. At present, it is unclear why there should be more change between the inactive group and the controls; however, previously, hypomethylation was found in inactive cells that represented a primed stage, suggesting that the cells were more ready to respond [29].

Interestingly, a recent report observed that the accumulation of Alu transcripts in retinal-pigmented epithelial cells induced NLRP3 activation through mitochondrial oxygen species generation, resulting in production of the proinflammatory cytokine IL-18, which contributed to the pathogenesis of age-related macular degeneration (AMD) [43]. Recently, another study showed that iron accumulation is responsible for Alu transcript accumulation and contributed to the inflammatory phenotype in AMD [44]. Thus, Alu transcript overexpression might induce inflammatory phenotypes in other diseases with similar etiologies. The BD patients who participated in this study were admitted to the eye clinic with severe eye complications, such as uveitis, in addition to oral and urogenital ulcers. An earlier report suggested that at low concentrations Alu transcripts were activators of dsRNA-dependent protein kinase R, which has an important role in the antiviral response, such as the activation of NF-KB, one of the key molecules in the initiation of the inflammatory response. It is not clear whether the slight hypomethylation of Alu seen in BD patients has any effect on the pathogenesis of BD. It should be kept in mind that BD is complex and manifests with different complications. Studies on BD have shown that different inflammatory cytokine profiles were present in different BD subtypes, such as dominant eye involvement, and vascular or neurological involvement [45]. It is possible that Alu-dependent inflammatory pathways play a role in a certain subset of BD profiles, but not in others. It should be noted that the sample size in this study was rather limited and the difference was marginal. Further analysis in larger patient groups, as well as studies on different patient profiles, will be useful to delineate whether Alu hypomethylation contributes to BD pathogenesis.

### Table 3: Alu methylation frequency of BD patients and healthy controls. The results are represented as the average ± standard deviation.

| Sample Type | % $^{30}$C | % $^{30}$C + $^{31}$C | % $^{30}$C + $^{32}$C | % $^{30}$C + $^{33}$C | % $^{30}$C + $^{34}$C | % $^{30}$C + $^{35}$C |
|-------------|------------|-----------------------|-----------------------|-----------------------|-----------------------|-----------------------|
| PBMC control (n = 15) | 42.14 ± 2.07 | 14.06 ± 2.96 | 31.90 ± 2.28 | 24.25 ± 1.08 | 29.79 ± 2.08 | 56.15 ± 2.99 |
| PBMC active (n = 12) | 41.57 ± 0.92 | 13.72 ± 1.21 | 31.25 ± 0.93 | 24.46 ± 0.76 | 30.58 ± 1.13 | 55.71 ± 1.45 |
| PBMC inactive (n = 17) | 41.50 ± 1.03 | 14.13 ± 1.51 | 30.62 ± 0.94 | 24.11 ± 0.82 | 31.13 ± 1.11 | 54.73 ± 1.66 |
| PBMC all patients (n = 29) | 41.53 ± 0.97 | 13.96 ± 1.39 | 30.88 ± 0.97 | 24.26 ± 0.80 | 30.90 ± 1.13 | 55.14 ± 1.62 |
| Neutrophil control (n = 15) | 42.88 ± 2.19 | 14.25 ± 3.68 | 32.39 ± 1.81 | 24.87 ± 1.40 | 28.49 ± 1.03 | 57.26 ± 3.16 |
| Neutrophil active (n = 12) | 42.89 ± 0.40 | 15.17 ± 1.58 | 30.83 ± 2.60 | 24.60 ± 0.83 | 29.40 ± 1.64 | 55.43 ± 3.11 |
| Neutrophil inactive (n = 17) | 42.43 ± 1.52 | 14.91 ± 1.88 | 30.88 ± 0.97 | 24.15 ± 0.69 | 30.06 ± 1.49 | 55.03 ± 1.50 |
| Neutrophil all patients (n = 29) | 42.62 ± 1.20 | 15.02 ± 1.73 | 30.86 ± 1.79 | 24.34 ± 0.77 | 29.78 ± 1.56 | 55.20 ± 2.26 |

*a, p = 0.03 for the frequency of the unmethylated allele ($^{30}$C) in PBMCs in inactive BD patients versus healthy controls.
*b, p = 0.03 for the frequency of the unmethylated allele ($^{30}$C) in PBMCs in BD patients versus healthy controls.
*c, p = 0.002 for the frequency of the unmethylated allele ($^{30}$C) in neutrophils in inactive BD patients versus healthy controls.
*d, p = 0.006 for the frequency of the unmethylated allele ($^{30}$C) in neutrophils in BD patients versus healthy controls.
*e, p = 0.02 for the frequency of the partially methylated allele ($^{30}$C + $^{31}$C) in neutrophils in inactive BD patients versus healthy controls.
*f, p = 0.055 for the frequency of the partially methylated allele ($^{30}$C + $^{31}$C) in neutrophils in BD patients versus healthy control.
to the findings for autoimmune diseases such as SLE, RA, and psoriasis, in which LINE-1, but not Alu, is significantly hypomethylated in patients compared with healthy controls. This result might be explained in part by the different pathogenesis between BD, which is more similar to the autoimmune diseases. Further study of the epigenetic alterations, and the role and regulation of Alu transcripts in BD and its clinical significance, should be pursued to gain a better understanding of the disease. Such research could lead to the discovery of uncharacterized mechanisms that could be useful as diagnostic biomarkers and therapeutic targets.

Competing Interests
The authors declare that there is no conflict of interests regarding the publication of this paper.

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