Research Article
Different Methylation of CpG-SNPs in Behcet’s Disease

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Received 18 January 2019; Revised 4 May 2019; Accepted 7 May 2019; Published 16 May 2019

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Purpose. We recently performed an Epigenome-Wide Association Studies (EWAS) study in Behcet’s disease (BD) and identified various cytosine–phosphate–guanine (CpG) loci that were aberrantly methylated. In the current study, we wanted to investigate whether these sites contained genetic polymorphisms and whether the frequency of these polymorphisms was altered in BD.

Methods. A two-stage study was performed. The first stage involved 358 BD patients and 704 healthy controls to investigate genetic variants of 10 CpG-SNPs (rs10454134, rs176249, rs3808620, rs10176517, rs11247118, rs78016579, rs9461624, rs10492166, rs34929465, and rs6507921) using an iPLEX Gold genotyping assay and a Sequenom MassARRAY. In the second stage, an additional 172 independent BD patients and 330 healthy individuals are to confirm trends found in the first stage.

Results. A higher frequency of both the rs10454134 AG genotypes (p = 0.008, OR = 1.413, 95% CI = 1.094-1.826) and a lower GG genotype frequency (p = 0.003, OR = 0.630, 95% CI = 0.465-0.854) were found in BD patients compared to the controls in the first stage. However, after correcting for multiple comparisons, all associations identified in the first stage lost statistical significance. The frequencies of the other CpG-SNPs investigated were not different between BD patients and controls. The second stage was designed using an additional cohort to confirm the association with CpG-SNP, rs10454134. The data failed to confirm the association between this CpG-SNP and BD.

Conclusions. This study did not show an association between BD and CpG-SNPs in genes that were earlier shown to be aberrantly methylated.

1. Introduction

Behcet’s disease (BD) is a chronic, relapsing, and multi-systemic inflammatory disorder. Its classical clinical characteristics include oral aphthae, genital ulcers, multiform skin lesions, and recurrent uveitis with hypopyon [1]. Its etiology and pathogenesis are not yet fully understood. Generally, it is thought that BD is caused by the interaction of genetic variation and environmental factors. To date, there are numerous genes shown to be associated with BD [2–4]. DNA methylation is a pivotal part of the epigenome and provides highly complementary data on the regulation of genomic regions [5, 6]. Previous study proved that epigenetic modification of some genes dynamics is involved in the pathogenesis of BD, such as cytoskeletal [7]. Furthermore, aberrant changes in DNA methylation have been shown to lead to abnormal gene expression in the pathogenesis of BD, including IL6, IL10, SOCS1, IRF8, GATA3, and TGF-β [8–12].

Sequence variations can change the CpG, which may result in indifferences in DNA methylation between individuals. Cytosine–phosphate–guanine single nucleotide polymorphisms (CpG-SNPs) are point mutated CpG sites [13] and may play a role in the methylation status of this gene region. DNA methylation is an important gene silencing mechanism and may play a role in controlling pathways of inflammation [14]. CpG-SNPs in the promoter regions have been found to be associated with various disorders, including type 2 diabetes [15], breast cancer [16], coronary heart disease [17], and psychosis [18]. However, SNPs located in CpG sites in patients with autoimmune diseases such as BD have not been reported and were therefore the subject of the study described here. Functional CpG-SNPs with an aberrant methylation status were selected from the Epigenome-Wide Association Study (EWAS) we performed earlier [19], and genotype frequency was compared between BD patients and healthy controls.
The study recruited a total of 530 BD patients and 1034 healthy individuals (Table 1), visiting the uveitis center of the First Affiliated Hospital of Chongqing Medical University from January 2009 to September 2017. All individuals were Chinese Han and all BD patients had uveitis. BD patients and controls were matched according to race which all from Chinese Han and geography. Healthy individuals who had systemic immune diseases or other chronic diseases were excluded. Diagnosis of BD was made according to the standard International Study Group for BD, requiring the presence of oral ulceration as well as any two of the following symptoms: genital ulceration, uveitis, multiform lesions, or a positive pathergy test [20]. The local research ethics committee of the First Affiliated Hospital of Chongqing Medical University (permit no. 2009-201008) approved the study. A written informed consent was obtained from all participants which abided by the tenets of the Declaration of Helsinki.

2.2. CpG-SNPs Screening. Target CpG-SNPs were selected according to our previous EWAS results [19] that included 60 BD patients and 60 matched healthy controls using the Illumina Human Methylation450K platform (Illumina, San Diego, CA, USA). A series of screening criteria was used to find target sites including a CpG-SNP methylation p value less than 0.05 and a Beta.Difference either less than -0.14 or more than 0.14 or p value<10^{-5}. Beta values used to score the methylation level ranging from 0 (unmethylated) to totally methylated [21, 22]. In addition, the minor allele frequency (MAF) in the Chinese Han population needed to be greater than 0.05, excluding the sites not included in the Han Chinese Hap Map database (https://www.ncbi.nlm.nih.gov/snp/). Furthermore, according to the UCSC (GRCh37/hg19) and HaploReg v4.1 (http://pubs.broadinstitute.org/mammals/haploreg/haploreg.php) databases [23, 24], the CpG-SNP sites located in potential functional regions (promoter region, enhancer region, CCCTC-binding factor (CTCF) binding region, first exon region, 5’UTR, TSS1500) [25] were selected. Finally, linkage disequilibrium (LD) data from the Han Chinese Hap Map database were also used to exclude SNPs in LD with each other. In total, 10 CpG-SNPs were selected based on these criteria and included rs10454134, rs176249, rs3808620, rs10176317, rs11247118, rs78016579, rs9461624, rs10492166, rs34929465, and rs6507921 (Table 3).

2.3. DNA Extraction and Genotyping. The two experimental groups including both BD individuals and healthy controls donated peripheral blood, which was applied to extract genomic DNA extraction with a QIAmp DNA Blood Mini Kit (Qiagen Inc., Valencia, CA, USA) and stored in 3.2% sodium citrate-treated tubes at -80°C. The iPLEX Gold genotyping assay and Sequenom MassARRAY (Sequenom, CA, USA) were performed to identify genotype of the 10 CpG-SNPs. iPLEX reactions primers were designed by SNP Assay Design software (version 3.0) (Table 2). All procedures were performed according to manufacturer’s instructions.

2.4. Statistical Analysis. The healthy controls should satisfy Hardy–Weinberg equilibrium (HWE) (p value<0.05). Hardy-Weinberg equilibrium analysis was using chi-square ($\chi^2$) test, while the genotype frequency was evaluated by direct counting. Fisher’s exact test or $\chi^2$ test was applied to estimate the differences in the allele and genotype frequencies of all CpG-SNPs between BD patients and healthy controls. P value was carried out by SPSS (version 170; SPSS Inc., Chicago, IL). Multiple comparisons were performed to correct P-value by using the Bonferroni method, whereby the p value was multiplied with the number of comparisons (P corrected (Pc)). When Pc< 0.05, it was considered to be significant.

3. Results

3.1. Clinical Features of the Subjects. The demographics and clinical symptoms of the BD patients and the healthy controls are displayed in Table 1. The healthy cohort consisted of 506 men and 528 women, who were on average 39.3 ± 10.5 years old. The BD patients comprised of 435 men and 95 women, with an average age of 34.3 ± 9.6.

3.2. CpG-SNPs Selected. SNPs located in the CpG loci with a CpG-SNP methylation level with a p value of < 0.05 and a Beta DIFFERENCE either less than -0.14 or more than 0.14 and MAF>0.05 were identified and eleven CpG-SNPs were included, according to the following criteria: (1) the MAF in the Chinese Han population was greater than 0.05 (https://www.ncbi.nlm.nih.gov/snp/); (2) CpG-SNP sites are located in potential functional regions according to UCSC (GRCh37/hg19) and HaploReg v4.1 (http://pubs.broadinstitute.org/mammals/haploreg/haploreg.php) databases [23, 24]; (3) linkage disequilibrium (LD) data from the Han Chinese Hap Map database was considered. Three CpG-SNPs were chosen: rs78016579, rs34929465, and rs6507921. The methylation levels of CpG-SNPs were considered with any sites with a p value<10^{-5} and the MAF of SNP in the CpG site
Table 2: Primers used in the analysis of restriction fragment length polymorphism (RFLP) in CpG-SNPs.

| rsID   | 1st-PCR                        | 2nd-PCR             | UEP     | SEQ                      |
|--------|--------------------------------|---------------------|---------|--------------------------|
| 10454134 | ACGTTGGATGAACGTACTCAGACTCGC     | ACGTTGGATGACTACGTCCAC | ATCACTGAATTTGCTCCG |
| 176249  | ACGTTGGATGCTACGTGCAAGTGGGGCTCTAC | ACGTTGGATGCTACGTGCAAGTGGGGCTCTAC | GAGCGCCCTCCAGCATGGCTCGCC |
| 3808620 | ACGTTGGATGTCAGTACGTGCAAGTGGGGCTCTAC | ACGTTGGATGTCAGTACGTGCAAGTGGGGCTCTAC | GAGCGCCCTCCAGCATGGCTCGCC |
| 1045134 | ACGTTGGATGCAACTCAGACTCGC     | ACGTTGGATGACTACGTCCAC | ATCACTGAATTTGCTCCG |
| 176249  | ACGTTGGATGCTACGTGCAAGTGGGGCTCTAC | ACGTTGGATGCTACGTGCAAGTGGGGCTCTAC | GAGCGCCCTCCAGCATGGCTCGCC |
| 3808620 | ACGTTGGATGTCAGTACGTGCAAGTGGGGCTCTAC | ACGTTGGATGTCAGTACGTGCAAGTGGGGCTCTAC | GAGCGCCCTCCAGCATGGCTCGCC |
| 1045134 | ACGTTGGATGCAACTCAGACTCGC     | ACGTTGGATGACTACGTCCAC | ATCACTGAATTTGCTCCG |
| 176249  | ACGTTGGATGCTACGTGCAAGTGGGGCTCTAC | ACGTTGGATGCTACGTGCAAGTGGGGCTCTAC | GAGCGCCCTCCAGCATGGCTCGCC |
| 3808620 | ACGTTGGATGTCAGTACGTGCAAGTGGGGCTCTAC | ACGTTGGATGTCAGTACGTGCAAGTGGGGCTCTAC | GAGCGCCCTCCAGCATGGCTCGCC |
| 1045134 | ACGTTGGATGCAACTCAGACTCGC     | ACGTTGGATGACTACGTCCAC | ATCACTGAATTTGCTCCG |
| 176249  | ACGTTGGATGCTACGTGCAAGTGGGGCTCTAC | ACGTTGGATGCTACGTGCAAGTGGGGCTCTAC | GAGCGCCCTCCAGCATGGCTCGCC |
| 3808620 | ACGTTGGATGTCAGTACGTGCAAGTGGGGCTCTAC | ACGTTGGATGTCAGTACGTGCAAGTGGGGCTCTAC | GAGCGCCCTCCAGCATGGCTCGCC |
| Target_ID | P.Value | Beta.Difference | ADDRESSA_ID | CHR | UCSC_REFGENE_NAME | Functional region | CpG-SNP | MAF |
|-----------|---------|----------------|-------------|-----|--------------------|------------------|--------|-----|
| cg0181180 | 3.65E-13 | -0.033524043 | 39744415 | 2   |                    | Promoter         | rs10454134 | 0.230 |
| cg15835500| 3.61E-12 | -0.035509286 | 51694401 | 6   |                    | CTCF-binding     | rs176249 | 0.276 |
| cg12480843| 1.8E-11  | -0.037228341 | 23659445 | 8   | UBE2W              | TSS200;TSS200\textsuperscript{d} | rs3808620 | 0.384 |
| cg08338478| 1.3E-10  | -0.042262334 | 10617308 | 2   | HECW2              | Enhancer         | rs10176517 | 0.162 |
| cg00062736| 3.55E-10 | -0.086936418 | 63727392 | 15  | MEF2A              | Enhancer         | rs11247118 | 0.392 |
| cg23296792| 0.0000000856 | -0.17564418 | 72682370 | 5   | FBXO38             | Enhancer         | rs78016579 | 0.135 |
| cg03157605| 0.0000000902 | -0.28707179 | 53675427 | 6   | MDC1               | 1stExon;5\textsuperscript{UTR} | rs9461624 | 0.059 |
| cg16479461| 0.00000003889 | -0.061269519 | 60757411 | 12  | CLECL1             | TSS200           | rs10492166 | 0.424 |
| cg26824678| 0.0000000976 | -0.15596614 | 49786417 | 1   | CAPZB              | Promoter         | rs34929465 | 0.258 |
| cg00254095| 0.004197255 | -0.14190315 | 69780487 | 18  | RPL17;SNORD58C;U58 | TSS1500          | rs6507921 | 0.478 |

\textsuperscript{a}: CHR = Chromosome containing the CpG; \\
\textsuperscript{b}: UCSC_REFGENE_NAME = Target gene name(s), from the UCSC database; \\
\textsuperscript{c}: MAF = Minor allele frequency of SNP(s); \\
\textsuperscript{d}: TSS = Transcription start site; \\
\textsuperscript{e}: UTR = Untranslated region
### Table 4: Genotype and allele frequencies of CpG-SNP polymorphisms in BD and healthy controls (first stage study).

| Target ID | CpG-SNP | Genotype | BD n (%) | Control n (%) | P value | Pc value | OR | 95% CI       |
|------------|---------|----------|----------|--------------|---------|-----------|----|-------------|
| cg0181180  | rs10454134 | total sample | 355 | 704 |          |          |    |             |
|            |         | AA       | 87 (0.245) | 173 (0.246) | 0.981 | NS | 0.996 | 0.741-1.340 |
|            |         | AG       | 195 (0.549) | 326 (0.463) | 0.008 | NS | 1.413 | 1.094-1.826 |
|            |         | GG       | 73 (0.206) | 205 (0.291) | 0.003 | NS | 0.630 | 0.465-0.854 |
|            |         | A        | 369 (0.520) | 672 (0.477) | 0.065 | NS | 1.185 | 0.989-1.420 |
|            |         | G        | 341 (0.480) | 736 (0.523) |          |          |    |             |
| cg15835500 | rs176249 | total sample | 357 | 702 |          |          |    |             |
|            |         | AA       | 13 (0.036) | 25 (0.036) | 0.947 | NS | 1.023 | 0.517-2.025 |
|            |         | AG       | 119 (0.333) | 207 (0.295) | 0.200 | NS | 1.196 | 0.645-1.976 |
|            |         | GG       | 225 (0.630) | 470 (0.670) | 0.203 | NS | 0.841 | 0.645-1.098 |
|            |         | A        | 145 (0.203) | 257 (0.183) | 0.266 | NS | 1.137 | 0.906-1.427 |
|            |         | G        | 569 (0.797) | 1147 (0.817) |          |          |    |             |
| cg12480843 | rs3808620 | total sample | 355 | 704 |          |          |    |             |
|            |         | GG       | 339 (0.955) | 670 (0.952) | 0.815 | NS | 1.075 | 0.585-1.976 |
|            |         | CG       | 16 (0.045) | 33 (0.047) | 0.895 | NS | 0.960 | 0.521-1.768 |
|            |         | CC       | 0 (0.000) | 1 (0.001) | 0.477 | NS | / | / |
|            |         | G        | 694 (0.977) | 1373 (0.975) | 0.742 | NS | 1.106 | 0.608-2.012 |
|            |         | C        | 16 (0.023) | 35 (0.025) |          |          |    |             |
| cg08338478 | rs10176517 | total sample | 354 | 703 |          |          |    |             |
|            |         | CC       | 190 (0.537) | 415 (0.590) | 0.096 | NS | 1.244 | 0.962-1.609 |
|            |         | CT       | 145 (0.410) | 253 (0.360) | 0.115 | NS | 0.810 | 0.624-1.053 |
|            |         | TT       | 19 (0.054) | 35 (0.050) | 0.787 | NS | 0.924 | 0.520-1.640 |
|            |         | C        | 525 (0.742) | 1083 (0.770) | 0.144 | NS | 1.169 | 0.948-1.441 |
|            |         | T        | 183 (0.258) | 323 (0.230) |          |          |    |             |
| cg03157605 | rs9461624 | total sample | 357 | 704 |          |          |    |             |
|            |         | GG       | 325 (0.910) | 619 (0.879) | 0.126 | NS | 0.739 | 0.484-1.130 |
|            |         | TG       | 32 (0.090) | 64 (0.091) | 0.818 | NS | 1.334 | 0.873-2.040 |
|            |         | TT       | 0 (0.000) | 1 (0.001) | 0.476 | NS | / | / |
|            |         | G        | 682 (0.955) | 1322 (0.939) | 0.122 | NS | 0.721 | 0.476-1.094 |
|            |         | T        | 32 (0.045) | 86 (0.061) |          |          |    |             |
| cg16744961 | rs10492166 | total sample | 358 | 696 |          |          |    |             |
|            |         | GG       | 123 (0.344) | 251 (0.361) | 0.584 | NS | 0.928 | 0.710-1.212 |
|            |         | GA       | 169 (0.472) | 344 (0.494) | 0.495 | NS | 0.915 | 0.709-1.181 |
|            |         | AA       | 66 (0.184) | 101 (0.145) | 0.098 | NS | 1.332 | 0.947-1.871 |
|            |         | G        | 415 (0.580) | 846 (0.608) | 0.212 | NS | 0.890 | 0.741-1.069 |
|            |         | A        | 301 (0.420) | 546 (0.392) |          |          |    |             |
|            |         | T        | 329 (0.459) | 582 (0.430) |          |          |    |             |

should be greater than 0.05. According to these criteria, 87 CpG-SNPs were included. However, after these CpG-SNPs were subjected to the aforementioned criteria, only 7 CpG-SNPs (rs10454134, rs176249, rs3808620, rs10176517, rs11247118, rs9461624, and rs10492166) were included in the study. Therefore, a total of 10 CpG-SNPs were included in this study (Table 3).

#### 3.3. Genotyping of the CpG-SNPs in BD.

Nine CpG-SNPs (rs10454134, rs176249, rs3808620, rs10176517, rs11247118, rs78016579, rs9461624, rs10492166, and rs6507921) were genotyped successfully in 370 BD patients and 704 controls. One CpG-SNP, rs34929465, was excluded, since it could not be analyzed successfully. Seven SNPs found in the healthy controls met the Hardy-Weinberg equilibrium and two SNPs (rs11247118, rs78016579), that deviated from the Hardy–Weinberg equilibrium \( p \text{-value} < 0.05 \), were excluded. Uncorrected values only showed a significant association of rs10454134 with BD (Table 4). A higher frequency of both the rs10454134 AG genotypes \( p = 0.008,\)
OR = 1.413, 95% CI = 1.094-1.826) and a lower GG genotype frequency (p = 0.003, OR = 0.630, 95% CI = 0.463-0.854) were found in BD patients compared to the controls. After correcting for multiple comparisons, these associations lost statistical significance. To further confirm the trend observed for the CpG-SNP, rs10454134, 172 additional independent BD patients and 330 healthy individuals were recruited. This second stage study also failed to demonstrate an association between this CpG-SNP and BD, even after combining both cohorts (Table 5). Stratified analyses were performed to investigate whether these CpG-SNPs might show an association with the primary clinical features. We chose genital ulcers since this feature has a frequency of approximately 50% in our BD cohort. After Bonferroni correction, no association was observed after stratification by genital ulcers.

4. Discussion

In this study, we failed to find an association between functional CpG-SNPs and BD. Functional CpG-SNPs were selected from data obtained in a previous study, whereby we identified various CpG sites with a different methylation status in BD patients [19]. The study presented here expanded these findings and investigated whether genetic polymorphisms in these sites might affect predisposition to BD. The fact that no association could be detected suggests that methylation of these sites may not be dependent on genetic variation of these sites themselves but may be regulated by other mechanisms. Further studies are needed to elucidate the exact mechanisms involved.

BD is considered an autoimmune or autoinflammatory disorder, characterized by chronic and recurrent episodes of posterior or panuveitis. It is generally thought that BD is caused by the combination of genetic variants and environmental factors. Accumulated evidences in previous study have unfolded epigenetic control of IL6, IL10, SOCS1, IRF8, GATA3 and TGF-β expression participates in the pathogenesis of BD [8–12]. The introduction or removal of CpG dinucleotides (possible sites of DNA methylation associated with the environment [6]) has been suggested as a potential mechanism through which SNPs can influence gene transcription and expression via epigenetics [26–29]. Many studies have reported that CpG-SNPs are associated with different diseases, such as type 2 diabetes, breast cancer, coronary heart disease, and psychosis which show a clear interaction between genetic (SNPs) and epigenetic (DNA methylation) regulation [15–18]. The role of SNPs located in CpG sites in autoimmune disease has not been widely addressed and we therefore expanded our earlier studies in this area. We chose BD, since this is a uveitis entity that is commonly observed in China allowing sufficiently large sample sizes to achieve adequate statistical power and allow meaningful conclusions. Identification of genetic variants may lead to novel therapies even in the absence of direct knowledge of the pathogenetic mechanisms involved.

A previous study, where a CpG-SNP MWAS (methylome-wide association studies) was performed, showed that CpG-SNP rs3796293 reached methylome wide significance in psychosis [18]. Rs3796293 is located in the gene encoding for interleukin 1 receptor accessory protein and supports the role of local inflammation in the pathogenesis of psychosis. In a genome wide association study (GWAS), rs3796293 was not found to be associated with the disease, suggesting that methylation of rs3796293 may not be the directly related to genetic variation of this site [18]. This result is consistent with our study in that genetic variation of significant methylation loci in BD did not show an effect on disease risk.

This study has several limitations. As SNPs were only selected if they satisfied the defined criteria, other unknown functional SNPs in CpG loci with a potential association with BD may have been missed and need to be investigated in future studies. Despite the fact that we had a large cohort of patients, it is possible that a weak but significant association might have been missed. The gene frequency of the SNPs analyzed in our study has only been tested in normal populations and no reference is available in clinical disease. It should also be noted that methylation changes at certain loci may be the result of the disease rather than its cause.

5. Conclusion

In conclusion, this study did not show an association between BD and CpG-SNPs in sites that have an aberrant methylation status.

Data Availability

All data generated or analyzed during this study are included in this published article.

Conflicts of Interest

The authors declare that they have no conflicts of interest.
Acknowledgments

Thanks to all donors enrolled in the present study. This work was supported by the National Key R&D Program of China (2016YFC0904000), Natural Science Foundation Major International (Regional) Joint Research Project (81720108009), Chongqing Key Laboratory of Ophthalmology (CSTC, 2008CA5003), Chongqing Science & Technology Platform and Base Construction Program (cstc2014ptsy10002), and Natural Science Foundation Project of Chongqing (cstc2017shmsA130073).

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