RHEUMATOLOGY

Original article

doi:10.1093/rheumatology/kez043

Epigenome-wide association study identifies Behçet's disease-associated methylation loci in Han Chinese

Hongsong Yu[1,*], Liping Du[1,*], Shenglan Yi[1], Qingfeng Wang[1], Yunyun Zhu[1], Yiguo Qiu[1], Yan Jiang[1], Minghui Li[2], Detao Wang[2], Qing Wang[2], Gangxiang Yuan[1], Qingfeng Cao[1], Aize Kijlstra[3] and Peizeng Yang[1]

Abstract

Objective. The aetiology of Behçet's disease (BD), known as a systemic vasculitis, is not completely understood. Increasing evidence suggests that aberrant DNA methylation may contribute to the pathogenesis of BD. The aim of this epigenome-wide association study was to identify BD-associated methylation loci in Han Chinese.

Methods. Genome-wide DNA methylation profiles were compared between 60 BD patients and 60 healthy controls using the Infinium Human Methylation 450 K Beadchip. BD-associated methylation loci were validated in 100 BD patients and 100 healthy controls by pyrosequencing. Gene expression and cytokine production was quantified by real-time PCR and ELISA.

Results. A total of 4332 differentially methylated CpG sites were associated with BD. Five differentially methylated CpG sites (cg03546163, cg25114611, cg20228731, cg23261343 and cg14290576) revealed a significant hypomethylation status across four different genes (*FKBP5*, *FLJ43663*, *RUNX2* and *NFIL3*) and were validated by pyrosequencing. Validation results showed that the most significant locus was located in the 5'UTR of *FKBP5* (cg03546163, $P = 3.81E\text{-}13$). Four CpG sites with an aberrant methylation status, including cg03546163, cg25114611, cg23261343 and cg14290576, may serve as a diagnostic marker for BD (area under the receiver operating curve curve = 83.95%, 95% CI 78.20, 89.70%). A significantly inverse correlation was found between the degree of methylation at cg03546163 as well as cg25114611 and *FKBP5* mRNA expression. Treatment with a demethylation agent, 5-Aza-2'-deoxycytidine resulted in an increase of *FKBP5* mRNA expression and a stimulated IL-1β production.

Conclusion. Our findings suggest that aberrant DNA methylation, independently of previously known genetic variants, plays a vital role in the pathogenesis of BD.

Trial registration: Chinese Clinical Trial Registry, chictr.org.cn, ChiCTR-CCC-12002184.

Key words: epigenome-wide association study, BD, FKBP5, methylation.

Rheumatology key messages

A total of 4332 differentially methylated CpG sites (DMCs) were associated with Behçet's disease (BD).
Four CpG sites with aberrant methylation status may serve as a diagnostic marker for BD.
Significant inverse correlation was found between the degree of methylation and FKBP5 mRNA expression.

[1]The First Affiliated Hospital of Chongqing Medical University, Chongqing Key Laboratory of Ophthalmology, and Chongqing Eye Institute, Chongqing, [2]Shanghai Biotechnology Corporation, Shanghai, China and [3]Department of Ophthalmology, University Eye Clinic Maastricht, Maastricht, The Netherlands

Submitted 11 November 2018; accepted 9 January 2019

*Hongsong Yu and Liping Du contributed equally to this study.

Correspondence to: Peizeng Yang, The First Affiliated Hospital of Chongqing Medical University, Chongqing Key Laboratory of Ophthalmology, and Chongqing Eye Institute, Chongqing 400016, China. E-mail: peizengycmu@126.com

Introduction

Uveitis is one of the leading causes of blindness in the world and most often affects the young adult, working age, population [1]. Behçet's disease (BD), which is known to be one of the most common uveitis entities encountered in China [2], is a systemic vasculitis characterized by a variety of symptoms including recurrent uveitis, oral aphthae, genital ulcerations, arthritis and skin lesions [3, 4]. Up to now, the exact aetiology and pathogenesis underlying BD have remained largely unknown.

Both genetic and environmental factors have been implicated in the pathogenesis of BD. Genetic distribution along the ancient Silk Road and an association with HLA-B51 are some of the factors pointing towards a genetic involvement [5, 6]. Environmental conditions, such as bacterial or viral infections, are thought to trigger the disease in genetically susceptible individuals [7]. To date, *HLA-B51* shows the strongest association with BD but accounts for $<$20% of the risk [8], which suggests the involvement of other genetic factors. Genome-wide association studies revealed that other non-HLA genes, such as *IL-10*, *IL23R*, *STAT4, CCR1*, *ERAP1* and *KLRC4,* are also associated with BD [9–13]. Other single nucleotide polymorphism (SNP) studies reported the involvement of *FAS*, *miR-182* and *miR-146a* in BD pathogenesis [14–16]. Besides the investigation of disease associations with gene polymorphisms, attention is now shifting towards a role for epigenetic regulation of disease-associated genes. Recently, we reported that the interferon regulatory factor 8 (IRF8) promoter region was hypermethylated in BD [17]. Moreover, a genome-wide methylation array study in monocytes and $CD4^+$ T lymphocytes revealed a variety of aberrant methylation patterns of cytoskeletal element genes in monocytes and $CD4^+$ T lymphocytes as a major contributor to BD pathogenesis [18]. However, the genome-wide methylation array study in monocytes and $CD4^+$ T lymphocytes, mentioned above, was performed in a relatively small number of patients (16 cases *vs* 16 controls) and has not yet been confirmed.

We therefore decided to repeat this study using a larger group of patients and performed a genome-wide methylation study in whole blood samples of 60 BD patients and 60 healthy controls using the Human Methylation 450K array (Illumina, San Diego, CA). Results of Human Methylation 450K array and pyrosequencing showed a significant association between differential DNA methylation and BD.

Methods

Patients and controls

One hundred untreated BD patients were selected from the BD patients seen at the Department of Ophthalmology of the First Affiliated Hospital of Chongqing Medical University (Chongqing, China) between May 2012 and November 2016. The diagnosis of BD was stringently based on the International Study Criteria for diagnosis of BD [19] and DNA samples of these patients were stored in our biobank. In parallel, a total of 100 unselected and consecutive control subjects were recruited from the same ethnical population and geographic area as the patients. We carried out a two-stage study. In the discovery stage, 60 active BD patients not yet receiving treatment and 60 normal subjects were enrolled. The replication stage consisted of 40 active BD patients without treatment and 40 healthy controls. The study followed the tenets of the Declaration of Helsinki and was approved by the Ethics Research Committee of the First Affiliated Hospital of Chongqing Medical University (Permit Number: 2009–201008). Before blood collection, a written informed consent was obtained from all patients and healthy controls.

Genomic DNA extraction and bisulphite conversion

Genomic DNA of all venous blood samples, including BD patients and healthy controls, was extracted using the QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's protocols. 500 ng of genomic DNA from each sample was bisulphite-converted using the EZ DNA Methylation-Gold Kit (Zymo Research, Orange, CA).

Genome-wide methylation profiling and DMCs identification

Illumina Infinium Human Methylation 450K BeadChip arrays (Illumina, San Diego, CA) were used to assess the genome-wide DNA methylation levels. This chip analyses ~485 000 CpG sites, covering 99% of all RefSeq genes with an average of 17 probes per gene, CpG shores, non-coding RNAs and DNase hypersensitive sites. The sex chromosomes (X, Y) and non-specific binding probes were excluded [20]. We also excluded the failed probes with a detection *P*-value $>$0.05 in $>$5% samples. Based on the principal components as proposed by Barfield *et al.* [21] (list of CpG sites is available at http://genetics.emory.edu/research/conneely/annotation-r-code.html), the probes with SNPs of minor allele frequency $>$5% within 10 base pairs of the CpG sites were also excluded. Finally, 401 020 DNA methylation probes passed quality control in the discovery stage, and then were included in further genome-wide analysis. Subset-quantile Within Array Normalization was used to normalize the DNA methylation probe intensity [22]. Quality control and genome-wide analysis were conducted with the Minfi [23] package from the Bioconductor platform in R [24]. In the discovery cohort, 60 patients with BD and 60 healthy control individuals were enrolled. The logical regression test was performed to identify BD-associated differentially methylated CpG sites (DMCs) using the Illumina Methylation Analyzer package in R [25].

Gene-specific DNA methylation validation by pyrosequencing

For the validation component of this study, the discovery cohort and a second replication cohort of 40 patients with BD and 40 healthy control individuals were enrolled.

Genomic DNA (500 ng) from each individual was bisulphite-converted as mentioned above. Based on a threshold of $P < 1.0E-05$ and delta $\beta > 0.14$ or being functional CpG site, five DMCs (cg03546163, cg25114611, cg20228731, cg23261343 and cg14290576) with a significant hypomethylation status across four different genes (*FKBP5*, *FLJ43663*, *RUNX2* and *NFIL3*) were tested by pyrosequencing in the discovery cohort first, and then validated by pyrosequencing in the replication cohort. For pyrosequencing, specific primers were designed for five CpG loci using PyroMark software (Qiagen, Hilden, Germany) (Supplementary Table S1, available at *Rheumatology* online), and PCR conditions were as described previously [26]. The methylation level for each CpG within the target region was quantified using the Pyro Q-CpG software (Qiagen, Hilden, Germany).

Biomarker analysis

In order to identify whether the five candidate DMCs can be used to distinguish BD patients from healthy controls, we conducted a biomarker analysis. Support vector machine (SVM) classifier [27] in the comprehensive R archive network (CRAN, found at http://cran.r-project.org/package=e1071) was applied to test and adjust the classifier using the pyrosequencing data for the discovery cohort (60 BD patients and 60 healthy controls) as the training phase. The predictors of the SVM classifier were then used to validate the pyrosequencing data of the replication cohort (40 BD patients and 40 healthy controls) and the combined cohort (100 BD patients and 100 healthy controls). The receiver operating curve (ROC) figures with area under the ROC curve (AUC) value in training set, validation set and combined set were drawn by the R package ROCR [28].

Network analysis

The Ingenuity Pathway Analysis software (Qiagen, Hilden, Germany) was used to construct a molecular interaction network of BD-associated DMCs, including canonical pathways, biological functions and upstream regulators, using all available interaction data in the Ingenuity Systems Knowledge Base.

Cell isolation and culture

Isolation of peripheral blood mononuclear cells (PBMCs) from heparinized blood samples was performed by Ficoll-Hypaque density-gradient centrifugation. The isolated PBMCs (2×10^6 cells per well) were then seeded in 24-well plates and cultured in 100 U/ml penicillin, Roswell Park Memorial Institute (RPMI) medium 1640 supplemented with 10% foetal calf serum (FCS, Greiner, Wemmel, Belgium) and 100 μg/ml streptomycin. In order to investigate the DNA demethylation effect, the isolated PBMCs were co-cultured with or without a DNA demethylation reagent, 5-Aza-2'-deoxycytidine (DAC, Sigma-Aldrich, St Louis, MO) at a concentration of 10 μM for 3 days, and then incubated with 100 ng/ml lipopolysaccharide (LPS, Sigma-Aldrich, St Louis, MO) at 37°C for 24 h. Both the cells and the supernatants were harvested for further assays.

Real-time PCR

The total mRNA was isolated from blood and cell samples, and was then used to synthesize cDNA with the cDNA synthesis kit (Takara, Japan). Real-time PCR was performed in the Applied Biosystems 7500 System based on the SYBR-Green method. The expression of *FKBP5*, *FLJ43663*, *RUNX2*, *NFIL3* and *β-actin* (the internal reference) was measured using the primers as listed in Supplementary Table S2, available at *Rheumatology* online. All tests were conducted in triplicate. The $2^{-\Delta\Delta Ct}$ method was applied to quantify the relative expression levels of the candidate genes.

Measurement of cytokines by enzyme-linked immunosorbent assay

The Duoset ELISA development kit (R&D Systems, Minneapolis, MN) was used to quantify the production of TNF-α, IL-1β and IL-6 by cultured PBMCs, according to the manufacturer's instructions.

Statistical analyses

The logical regression test was used to identify BD-associated DMCs in the discovery stage using DNA methylation level, age and sex as the determinants in a logical regression model. We considered a *P*-value $< 1.0E-05$ as genome-wide significant. Based on a threshold of $P < 1.0E-05$ and delta $\beta > 0.14$ or being a functional CpG site, we sought replication for five loci across four different genes (*FKBP5*, *FLJ43663*, *RUNX2* and *NFIL3*). In the replication samples, we implemented the same logical regression model with BD as dependent and methylation status, age and sex as indicators. The nonparametric Mann–Whitney *U* test, independent samples *t* test or paired-samples *t* test was used to compare gene expression as well as cytokine levels.

Results

Clinical features of the enrolled BD patients

The demographic characteristics and clinical features of the patients with BD enrolled in our study are shown in Supplementary Table S3, available at *Rheumatology* online. The distribution of clinical manifestations in the selected BD patients is as follows: 100% with vasculitis disclosed by fundus fluorescence, 94% with oral ulcer, 42.0% with genital ulcer, 82.0% with skin lesions, 20.0% with arthritis and 38.0% with hypopyon. All patients presented with uveitis and were not receiving immunosuppressive treatment at the time of blood sampling.

Identification of DMCs in BD

To identify DMCs in BD, we performed a stage I epigenome-wide association analysis in a group of 60 active untreated BD patients and compared data with a group of 60 healthy controls. After the filtering of probes that were ambiguously mapped and overlapped with single

nucleotide polymorphisms, we successfully detected 401 020 sites in all samples.

At a $P < 1.0E-05$, we detected 4332DMCs by logistic regression analysis (Fig. 1, Supplementary Fig. S1 and Supplementary Table S4, available at *Rheumatology* online). In the BD samples, 547 of these DMCs were significantly hypermethylated, whereas 3785 were hypomethylated (Fig. 1, Supplementary Fig. S1, available at *Rheumatology* online). The most significant differentially methylated site (DMS) was located in the 5'UTR of *FKBP5* (cg03546163, $P = 6.82E-07$) (Table 1). Other hypomethylated loci included the following four genes: *FKBP5* (cg25114611, $P = 1.74E-06$), *FLJ43663* (cg20228731, $P = 2.85E-06$), *RUNX2* (cg23261343, $P = 4.23E-06$), and *NFIL3* (cg14290576, $P = 5.19E-06$) (Table 1). As compared with the previously reported genome-wide methylation array study in monocytes and $CD4^+$ T lymphocytes [17], our epigenome-wide association study (EWAS) in whole blood confirmed 7 of the DMCs, including *FLJ43663*/cg20228731, *FLJ43663*/cg15928106, *WDR20*/cg08253808, *BRCA2*/cg12836863, *RNF19A*/cg13675051, *APP*/cg14414154 and *PFKFB3*/cg27545615 (Table 1, Supplementary Table S4, available at *Rheumatology* online). Except for *FLJ43663*/cg20228731, other loci with delta β values < 0.14, thus were not included in the next validation stage.

Validation of the methylation data

To further confirm our findings, we used pyrosequencing to validate the five sites identified during the stage I analysis in the discovery cohort (60 BD patients and 60 healthy controls) and the second independent replication cohort (40 BD patients and 40 healthy controls). For the discovery cohort, the same five tested sites were found to be significantly associated with BD as detected in the Illumina 450K analyses ($P < 0.05$, Table 1). For the replication cohort, the logical regression test revealed that four CpG sites showed a *P*-value of < 0.05, and the most significant locus was located in the 5'UTR of *FKBP5* (cg03546163, $P = 2.97E-06$, Table 1), whereas the locuscg20228731 lost significance in the validation stage ($P = 0.68$, Table 1). In the combined analyses, all five sites were significantly associated with BD, whereby the most significant locus was located in the 5'UTR of *FKBP5*(cg03546163, $P = 3.81E-13$, Table 1, Fig. 2).

Biomarker analysis

Biomarker analysis showed that four of the five differentially methylated loci, including cg03546163, cg25114611, cg23261343 and cg14290576, can be used as biomarkers to distinguish BD patients from healthy controls. Firstly, we used the pyrosequencing data for the discovery cohort (60 BD patients and 60 healthy controls) to build a classifier, and obtained a ROC with AUC of 94.94% (Supplementary Fig. S2a, available at *Rheumatology* online). In addition, we validated this classifier in the cohort of 40 BD patients and 40 healthy controls using the cytosine methylation data from pyrosequencing, and obtained a ROC with AUC of 82.12% (Supplementary Fig. S2b, available at *Rheumatology* online). Moreover, the ROC curve showed an accuracy of prediction in the combined sets (200 samples, AUC = 83.95%, Supplementary Fig. S2c, available at *Rheumatology* online).

Network analysis of BD-associated DMCs

To elucidate a pathway-based view of BD-associated methylome alterations, we used Ingenuity Pathway

Fig. 1 Manhattan plots of $-\log_{10}$ association *P*-values in the discovery cohort

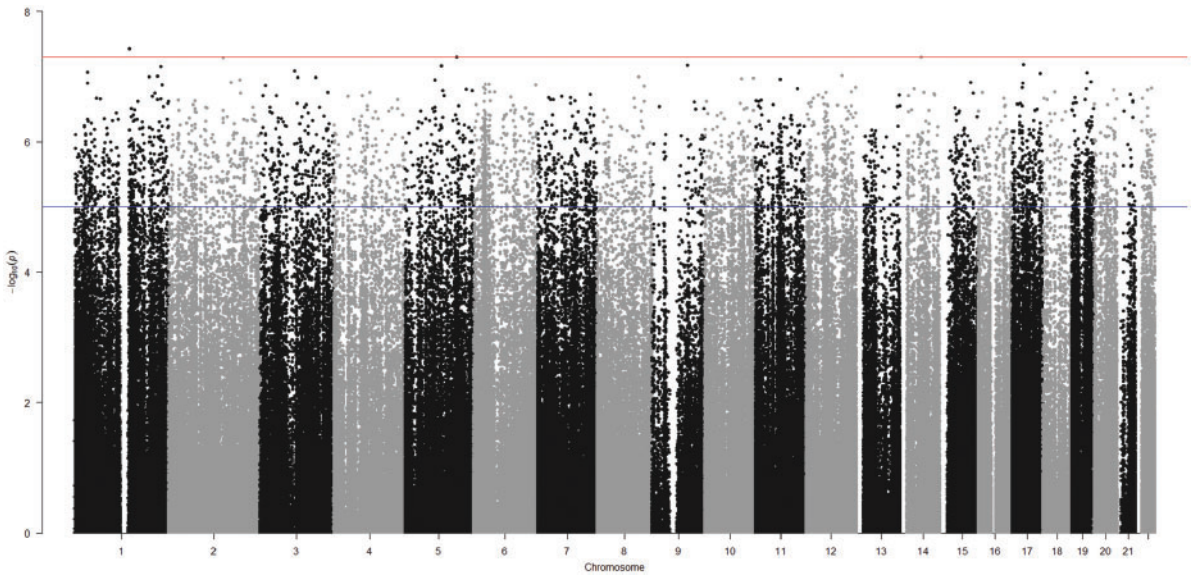


Table 1 Association between differentially methylated sites and BD

DMS	Gene	Chr	Location	Gene_context	Illumina 450K (discovery cohorts)		Pyrosequencing (discovery cohorts)	Pyrosequencing (replication cohorts)	Pyrosequencing (combined cohorts)
					Delta β	*P*-value	*P* -value	*P*-value	*P*-value
cg03546163	*FKBP5*	6	35654363	5'UTR	−0.14	6.82E-07	2.38E-07	2.97E-06	3.81E-13
cg20228731	*FLJ43663*	7	130646051	Body	−0.16	2.85E-06	3.13E-06	0.68	9.57E-05
cg23261343	*RUNX2*	9	45413792	Body	−0.15	4.23E-06	3.74E-05	6.08E-04	1.23E-07
cg14290576	*NFIL3*	7	94181612	5'UTR	−0.14	5.19E-06	8.44E-06	2.26E-04	9.65E-09
cg25114611	*FKBP5*	6	35696870	TSS1500	−0.06	1.74E-06	9.48E-05	6.00E-04	3.50E-08

BD: Behçet's disease; Chr: chromosome number; delta β: methylation differences between the cases and controls; DMS: differentially methylated site; Gene_context: location of the gene-associated CpG-site(s) with respect to the gene context.

Analysis tools to construct a molecular interaction network of DMCs-associated genes. The network included 137 genes organized around several nodes (Fig. 3). *FKBP5* is one of the most connected nodes. *FKBP5* can be regulated by *Akt*, *IL1B*, *IL10*, *STAT6*, *IL4*, *MECP2, NOD1*, *Histone h3* and *TCL1A*, and it can also regulate several genes, such as *Hsp90*, *NOS2* and *NF-κB* (Fig. 3).

Correlation between DNA methylation and respective gene mRNA expression

The result showed that the mRNA expression of *FKBP5* and *FLJ43663* genes was significantly higher in BD patients compared with healthy controls ($P = 0.0069$, $P = 0.0229$, respectively. Fig. 4a, b). No significant differences in the *RUNX2* and *NFIL3* gene expression were observed between BD patients and healthy controls ($P > 0.05$).

Correlation between DAC treatment and gene expression as well as cytokine production

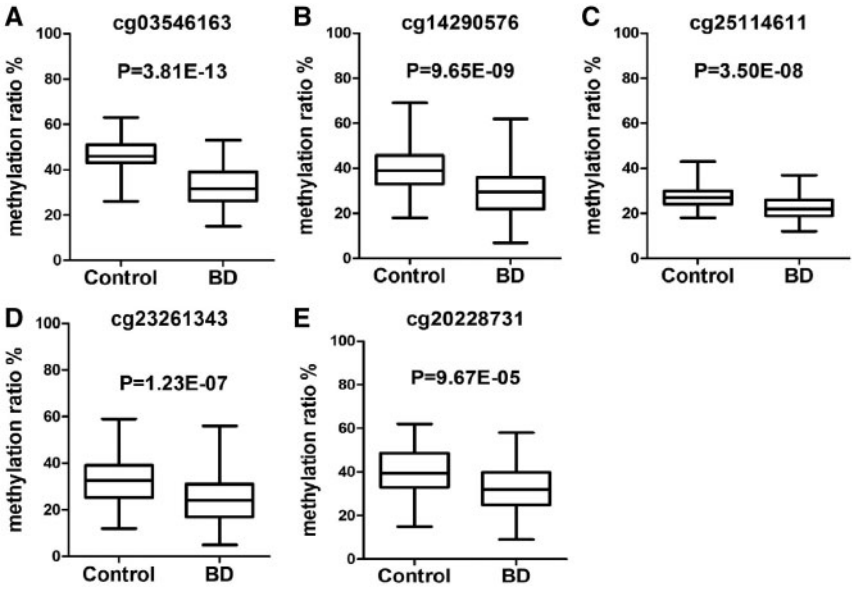
DAC was used to block methylation in cultured PBMCs. Using LPS as a stimulus, a significantly increased expression of *FKBP5* and *FLJ43663* genes was found in PBMCs treated with DAC as compared with cultures not treated with DAC ($P = 0.0037$, $P = 0.0005$, respectively; Fig. 4c, d). Moreover, a significantly increased production of IL-1β was observed in culture supernatants of PBMCs treated with DAC as compared with controls ($P = 0.0004$, Fig. 5a). However, no significant differences were observed in the production of TNF-α or IL-6 in PBMCs either treated with or without DAC (Fig. 5b, c).

Discussion

In the present study, we characterized the genome-wide methylation profiles of 60 BD patients and 60 healthy controls from a Han Chinese population, and found that 4332 DMCs were strongly associated with BD. Using pyrosequencing technology, five loci were further validated in the primary cohort and a second replication cohort of 40 BD patients and 40 healthy controls. Our results suggest that differential regulation of five DMCs, located in *FKBP5*, *FLJ43663*, *RUNX2* and *NFIL3,* may contribute to the pathogenesis of BD. To our knowledge, this is the first study performing an epigenome-wide analysis to investigate abnormalities in the whole blood DNA methylation status in Han Chinese BD patients.

The most significant differentially methylated site was located in the 5'UTR of the *FKBP5*gene, suggesting an important role for this gene in the development of BD. FKBP5, a member of the immunophilin protein family, plays a vital role in immunoregulation and basic cellular processes [29]. FKBP5 can bind to and negatively regulate the glucocorticoid receptor function, which subsequently reduces affinity of the glucocorticoid receptor to cortisol [30]. FKBP5 has been identified to be associated with a relative glucocorticoid receptor resistance, and polymorphisms have been shown to predict adult post-traumatic stress disorder following childhood abuse

FIG. 2 The methylation levels of five CpGs in 100 healthy controls and 100 BD patients



BD: Behçet's disease.

[31]. FKBP5 can bind to the immunosuppressants FK506 and rapamycin, and it is also involved in the TNF alpha/NF-κB signalling pathway, which plays an important role during inflammation [32]. Higher mRNA expression of *FKBP5* was observed in SLE patients as compared with healthy controls [33]. A previous study showed that *FKBP5* was expressed at higher mRNA levels in RA than in OA patients [34]. Some studies found that the DNA methylation of FKBP5 was increased in depression patients [35, 36]. A recent study suggested that hypomethylation of the *FKBP5* gene may be involved in the development and progression of chronic kidney disease [37], and may serve a potential mechanistic explanation for long-lasting glucocorticoid (GC)-induced psychopathology in Cushing's syndrome [38]. In addition to these findings, we now show that *FKBP5* was significantly hypomethylated in BD patients as compared with controls. As yet, no reports have described a role for FKBP5 in the development of BD and the exact role of *FKBP5* gene hypomethylation in BD awaits further investigation.

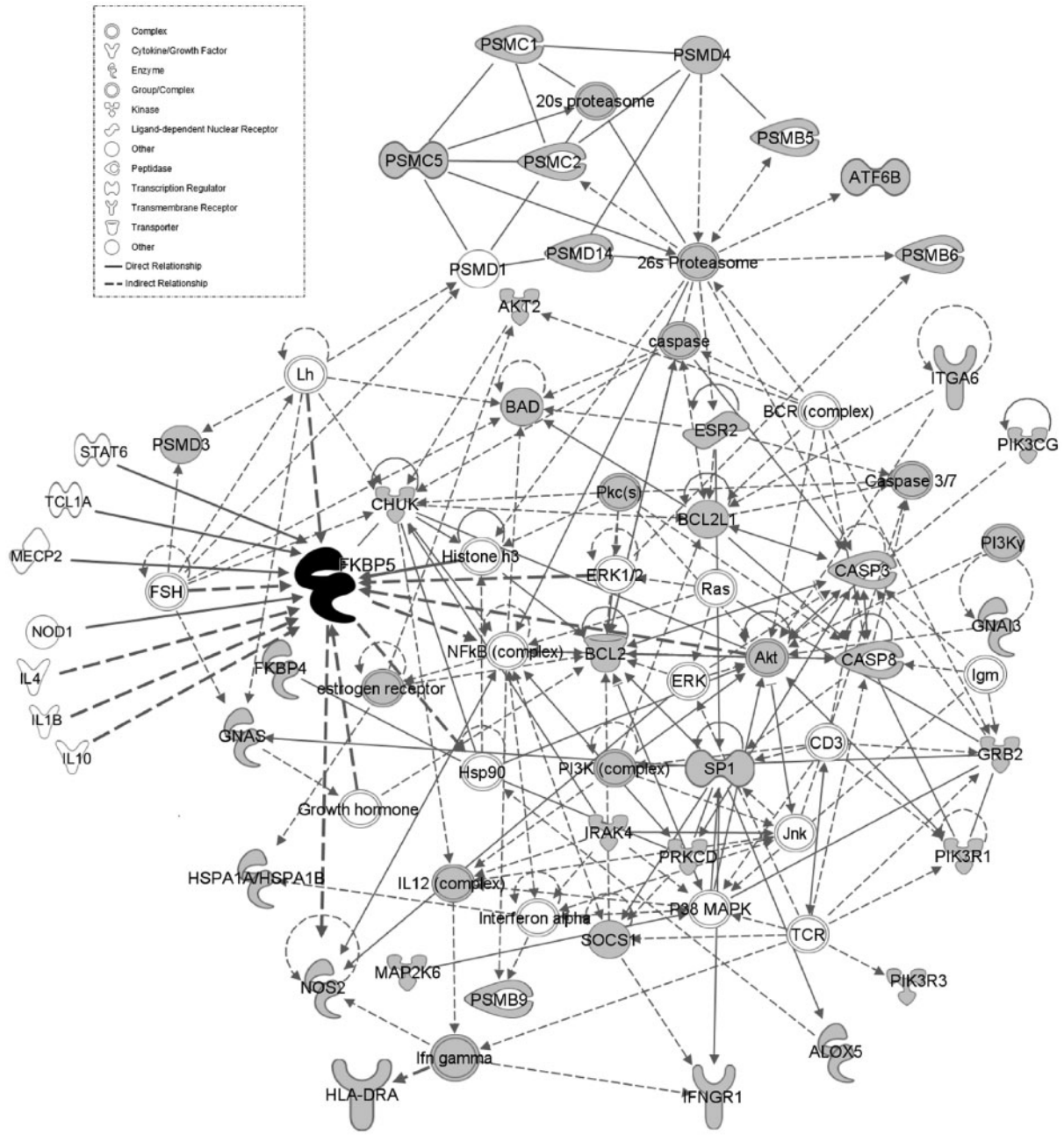
One of the other hypomethylated genes identified in our study is *FLJ43663.* To date, little research has focussed on *FLJ43663* in disease predisposition. A recent study showed that *FLJ43663* gene polymorphisms were associated with the risk of breast cancer in a Chinese Han population [39]. Our study is the first to show an involvement of *FLJ43663* as a risk factor for BD, although further investigation is needed to address the exact role of the *FLJ43663* gene in this disease.

RUNX2 was also shown to be hypomethylated in our BD patients. It is a member of the RUNX family, and is a key transcription factor associated with osteoblast differentiation [40]. Hypomethylation of *RUNX2* was earlier shown to be significantly associated with OA [41], and *RUNX2* was shown to be overexpressed in OA chondrocytes [42]. Recently, *RUNX2* was also shown to be hypomethylated in fibroblasts from both diffuse cutaneous SSc and limited cutaneous SSc patients as compared with healthy controls [43]. A role for *RUNX2* in the development of BD has not yet been proposed earlier and further studies are needed to confirm our findings.

NFIL3, also known as E4-binding protein 4 (E4BP4), is a transcription factor that regulates several immunologic pathways [44]. NFIL3 can regulate type 2 T helper (TH2) cell differentiation and TH2 cytokine production [45]. Moreover, NFIL3 can suppress Th17 cell development by directly repressing Rorγt transcription [46]. *NFIL3* polymorphisms were identified to be associated with human IBD [47]. Up to now, no reports have addressed an association between an abnormal methylation in the *NFIL3* gene with disease. Our findings are the first to show that hypomethylation in 5'UTR of *NFIL3* gene is associated with BD in Han Chinese. However, we could not show significant differences in *NFIL3* mRNA expression between BD patients and healthy controls. Further study is needed to investigate the exact role of *NFIL3* gene hypomethylation in the pathogenesis of BD.

A recent study from our group showed hypermethylation of the *IRF8* promoter and reduced mRNA expression of *IRF8* in dendritic cells (DCs) from active BD patients as compared with healthy controls [17]. However, abnormal methylation of *IRF8* was not detected in whole blood of BD patients in the current study. This may be due to the strict *P*-value threshold used in EWAS (1.0E-05), the cell types studied or the different sample size. It should be noted that our EWAS in whole blood confirmed seven

Fig. 3 Molecular interaction network of BD-associated DMCs

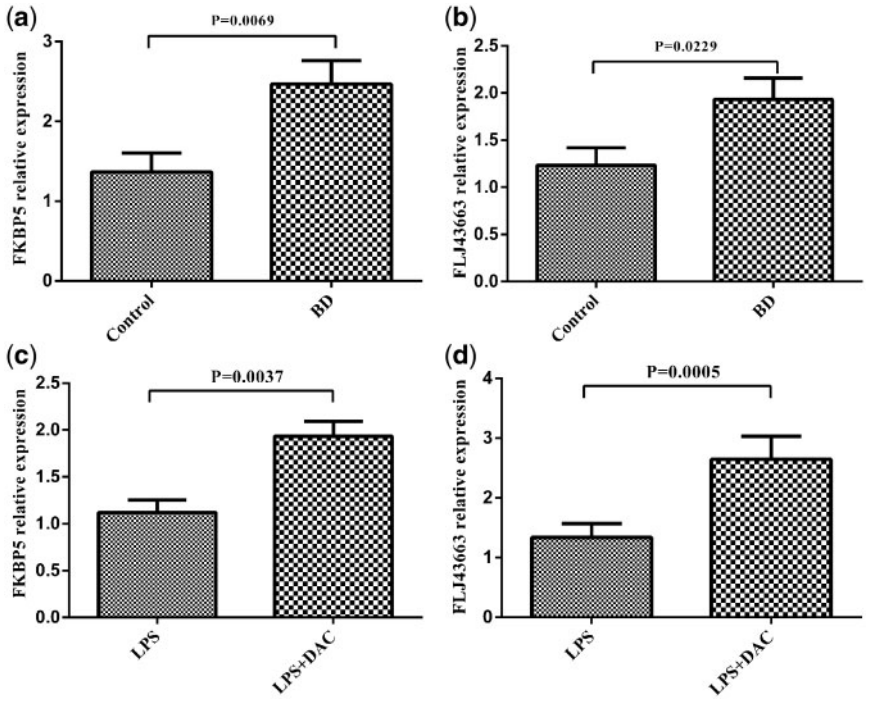


The network was constructed by using the available interaction data in the Ingenuity Systems Knowledge Base. FKBP5 is highlighted in black. Types of interactive molecules are defined in the legend. BD: Behçet's disease; DMCs: differentially methylated CpG sites.

DMCs, including *FLJ43663*/cg20228731, *FLJ43663*/cg15928106, *WDR20*/cg08253808, *BRCA2*/cg12836863, *RNF19A*/cg13675051, *APP*/cg14414154 and *PFKFB3*/cg27545615, reported in an independent previous genome-wide methylation array study by others in monocytes and $CD4^+$ T lymphocytes [18], suggesting their vital role in the pathogenesis of BD. Further investigations are needed to address these DMCs in the near future.

The recent epigenome-wide association studies have successfully identified numerous intriguing associations between aberrant DNA methylations and human diseases, such as in SS [48], psoriasis [49], RA [50] and asthma [51], suggesting that DNA methylation may play an important role in mediating the role of several genes during inflammation. Hypermethylation is always associated with gene silencing, whereas hypomethylation is often associated

Fig. 4 Correlation between methylation and mRNA expression



The mRNA level of FKBP5 and FLJ43663 in whole peripheral blood and PBMC cultures was detected by qRT-PCR. Comparison of the mRNA level between BD patients and healthy controls (a–b). Comparison of mRNA level of PBMC from healthy controls before and after DAC treatment (c–d). BD: Behçet's disease; DAC: 5-Aza-2'-deoxycytidine; PBMC: peripheral blood mononuclear cells.

with gene overexpression. In our study, hypomethylation in *FKBP5* promoter and the 5'UTR region was strongly associated with high *FKBP5* expression. Similarly, hypomethylation of *FLJ43663* was significantly associated with high *FLJ43663* gene expression. Our findings not only suggest a possible role of a DNA methylation defect in the overexpression of genes, but also suggest a possible role of DNA methylation in the pathogenesis of BD. We did not find a statistically significant association between the hypomethylation and the expression of *RUNX2* and *NFIL3*, which is similar to studies reported earlier by others [41, 43]. Besides methylation, other factors can also affect gene expression, such as microRNAs, gene copy number variation and regulation via upstream genes. The relative contributions of these various systems in comparison to DNA methylation deserves further study.

As mentioned above, the diagnosis of BD is made according to the characteristic clinical features, and sometimes it is difficult to make an early diagnosis because of heterogeneity in clinical manifestations. In this study, we provide preliminary evidence to show that an analysis of the methylation sites associated with BD might be used as a biomarker to distinguish BD patients from healthy controls. Further studies are needed to validate whether this method is feasible in clinical practice.

There are several limitations in our study. Firstly, our BD group predominantly includes male patients, and validation in a gender-matched population should be conducted in further studies. In addition, as our study was performed on Han Chinese, it is not certain whether our findings can be generalized to other ethnic populations. Furthermore, only five DMCs with a *P*-value $<1.0 \times 10^{-5}$ identified in the discovery stage were validated in a replication study, and it is possible that we missed genes due to this stringent *P*-value threshold and that other non-validated DMCs might also be involved in susceptibility to BD. Finally, although pyrosequencing has been considered as one of the gold-standard technologies for DNA methylation analysis [52], it also has certain limitations, including the short read length, homopolymer read errors and sensitivity due to background noise [53, 54].

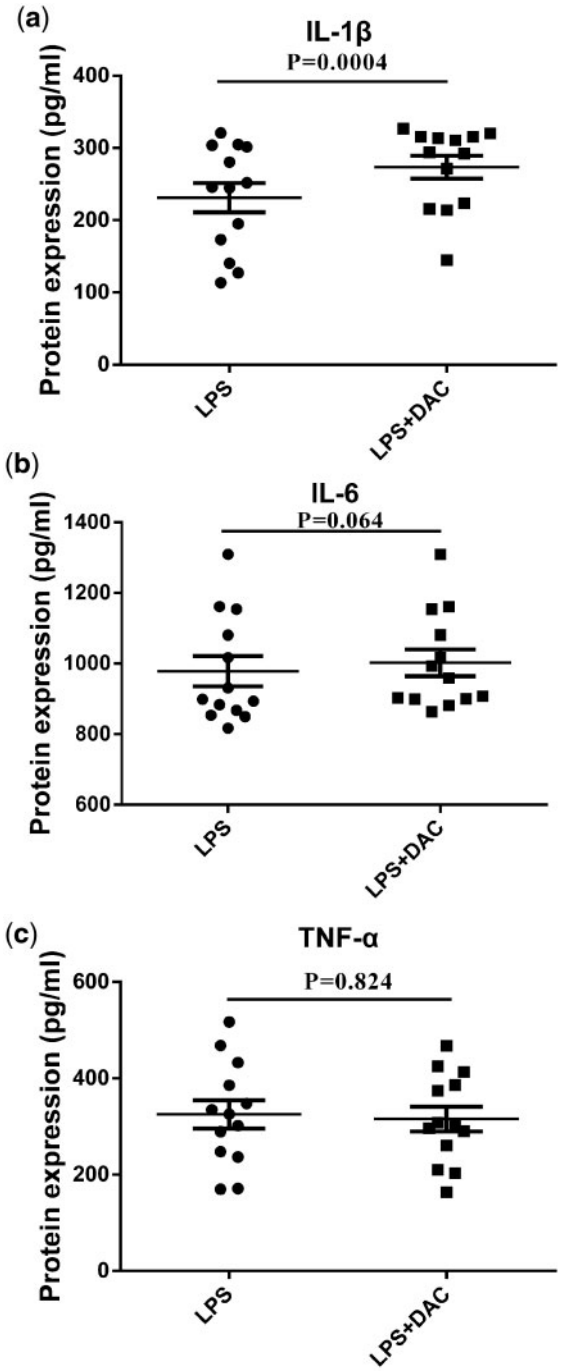
Conclusion

In summary, this well-powered EWAS provided strong evidence of a significant association between aberrant DNA methylation and BD in a Chinese Han population. Further studies are needed to investigate the exact mechanisms whereby an altered methylation affects the development of this disease.

Acknowledgements

The authors would like to thank all donors enrolled in the present study. H.Y. and P.Y. conceived the idea and designed the experiments. L.D., S.Y., Q.C. and P.Y.

FIG. 5 The role of a DAC on the production of inflammatory cytokines



The protein concentrations of IL-1β (a), IL-6 (b) and TNF-α (c) in the culture supernatants of LPS stimulated PBMCs treated with DAC as compared with those not treated with DAC as determined by ELISA. The data are shown as mean ± standard error of measurement (SEM). The paired-samples *t* test was performed for statistical analysis. BD: Behçet's disease; DAC: 5-Aza-2'-deoxycytidine; LPS: lipopolysaccharide; PBMC: peripheral blood mononuclear cells.

contributed to sample collection. H.Y., L.D., S.Y., Y.Z., Y.Q., Y.J. and G.Y. performed all the experiments. H.Y., M.L., D.W. and Q.W. analysed all data. H.Y. wrote the first concept of the manuscript. P.Y. and A.K. revised the manuscript critically for important intellectual content. All authors reviewed the manuscript. A total of 4332 DMCs associated with BD were listed in additional file 3: Supplementary Table S3, available at *Rheumatology*

online, and all specimens were deposited at the First Affiliated Hospital of Chongqing Medical University, China.

Funding: This work was supported by National Key R&D Program of China (grant numbers: 2016YFC0904000), Natural Science Foundation Major International (Regional) Joint Research Project (grant numbers: 81720108009, 81320108009), National Natural Science Foundation (grant numbers: 81670844, 81470620, 81770914), ChongqingKey Laboratory of Ophthalmology (grant numbers: CSTC, 2008CA5003), Chongqing Science & Technology Platform and Base Construction Program (grant numbers: cstc2014pt-sy10002), the Natural Science Foundation Project of Chongqing (cstc2017shmsA130073), and National Key Clinical Specialties Construction Program of China. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Disclosure statement: The authors have declared no conflicts of interest.

Supplementary data

Supplementary data are available at *Rheumatology* online.

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