Genome-wide DNA methylation profile analysis identifies differentially methylated loci associated with ankylosis spondylitis

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Abstract

Background: Ankylosing spondylitis (AS) is a chronic rheumatic and autoimmune disease. Little is known about the potential role of DNA methylation in the pathogenesis of AS. This study was undertaken to explore the potential role of DNA methylation in the genetic mechanism of AS.

Methods: In this study, we compared the genome-wide DNA methylation profiles of peripheral blood mononuclear cells (PBMCs) between five AS patients and five healthy subjects, using the Illumina Infinium HumanMethylation450 BeadChip. Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) was performed to validate the relevance of the identified differentially methylated genes for AS, using another independent sample of five AS patients and five healthy subjects.

Results: Compared with healthy controls, we detected 1915 differentially methylated CpG sites mapped to 1214 genes. The HLA-DQB1 gene achieved the most significant signal (cg14323910, adjusted \( P = 1.84 \times 10^{-6} \), \( \beta \) difference = 0.5634) for AS. Additionally, the CpG site cg04777551 of HLA-DQB1 presented a suggestive association with AS (adjusted \( P = 1.46 \times 10^{-3} \), \( \beta \) difference = 0.3594). qRT-PCR observed that the mRNA expression level of HLA-DQB1 in AS PBMCs was significantly lower than that in healthy control PBMCs (ratio = 0.48 ± 0.10, \( P < 0.001 \)).

Gene Ontology (GO) and KEGG pathway enrichment analysis of differentially methylated genes identified four GO terms and 10 pathways for AS, functionally related to antigen dynamics and function.

Conclusions: Our results demonstrated the altered DNA methylation profile of AS and implicated HLA-DQB1 in the development of AS.

Keywords: Ankylosing spondylitis, Methylation, HLA-DQB1

Background

Ankylosing spondylitis (AS) is a chronic rheumatic and autoimmune disease, mainly damaging the axial skeleton, spine, and sacroiliac joints [1]. AS leads to the imbalance between bone resorption and bone formation, ultimately resulting in ligamentous ossifications and vertebral joint fusion [2]. AS usually starts before 30 years of age, manifested as chronic pain and stiffness of the low back. With age, loss of spinal mobility and chest expansion, extension of the lumbar spine becomes evident [3, 4], resulting in severe disability in AS patients [1]. It was reported that AS affects between 0.5 and 14 per 100,000 people every year [5].

Previous studies have demonstrated that AS is an inheritable disease with over 90% of AS risk determined by genetic factors [6]. HLA-B27 is a well-known susceptibility gene of AS. Approximately 95% of AS patients express the HLA-B27 genotype [7–9]. However, less than 5% of HLA-B27-positive individuals develop AS. Twin and family studies have suggested that HLA-B27 accounts for less than 40% of the overall risk of AS [7–9]. In addition, tumor necrosis factor was implicated in the genetic mechanism of AS with limited genetic effect [10]. The missing heritability of
AS suggests unknown genetic factors that contribute to the development of AS.

DNA methylation is an important epigenetic mechanism, playing an important role in gene expression regulation [11–13]. Extensive studies have demonstrated dysfunction of methylation in the development of multiple rheumatic and autoimmune diseases [14, 15]. Genome-wide DNA methylation profile comparative studies are a powerful tool for interrogating methylation changes associated with disease status. However, to the best of our knowledge, although some studies of abnormal methylated loci in AS patients have been conducted [16, 17], no genome-wide DNA methylation profiling of AS has so far been conducted. Little is known about the potential role of DNA methylation in the genetic mechanism of AS, limiting our efforts to clarify the pathogenesis and develop effective treatments for AS.

In this study, we first performed a genome-wide DNA methylation profile comparative analysis of peripheral blood mononuclear cells (PBMCs) between five AS patients and five healthy controls. Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) was then conducted to validate the relevance of the identified differentially methylated genes with AS using another independent sample of five AS patients and five healthy controls.

**Methods**

**Human subjects**

Genetically unrelated male AS patients were recruited randomly from Xi'an 451 Hospital, Xi'an No.5 Hospital, and the Second Affiliated Hospital of Xi'an Jiaotong University in Xi'an city of Shaanxi province in China. All AS patients were diagnosed clinically and radiologically according to the modified New York criteria [18] by two AS experts. Specific for this study, 10 AS patients with bilateral grade 4 sacroiliitis and syndesmophytes were selected randomly from the recruited AS patients and used for genome-wide DNA methylation profile analysis and the qRT-PCR validation experiment, respectively (Table 1 and Fig. 1). Ten age-matched healthy males were recruited from Xi'an city as healthy control samples (Table 1). Clinical data for each participant were recorded by nurse-administered questionnaire, including self-reported ethnicity, lifestyle characteristics, health status, and family and medical histories. All study subjects are Chinese Han people. All patients were receiving only NSAIDs in the previous 3 months. Subjects with genetic bone diseases, cartilage diseases, rheumatic and autoimmune diseases, and undergoing biologic treatments were excluded from this study.

**Monocyte derivation, DNA extraction, and quality control**

Five milliliters of peripheral blood was drawn from each participant. Following the manufacturer's recommended procedure, blood specimens were diluted with phosphate-buffered saline (PBS), laid with Lymphocyte Separation Medium (TBD Sciences), and centrifuged under 2500 rpm/min for 15 min to separate PBMCs. DNA was extracted from PBMCs using the TIANamp Blood DNA Kit (TIGEN Inc., China). The quality and concentration of extracted DNA were determined by NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific Inc., USA). The DNA specimens with A260/280 values varying from 1.8 to 2.0 were used in this study.

**Genome-wide DNA methylation profiling**

Genome-wide DNA methylation profiling of PBMCs was conducted using the Illumina Infinium HumanMethylation450 BeadChip according to the manufacturer's standard procedure. Briefly, bisulfite treatment of 500 ng DNA specimens was performed by the EZ DNA Methylation Kit (Zymo Research, USA). The bisulfite-converted DNA specimens were then amplified, hybridized to a HumanMethylation450 array, stained, and washed. The raw image intensities of the hybridized arrays were scanned using an iScan SQ scanner (iScan System; Illumina). The obtained raw image intensity data were processed by GenomeStudio software (Illumina). The percentage of methylated cytosine at a given CpG locus was expressed as a β value, varying from 0 (completely unmethylated) to 1 (completely methylated).

**Statistical analysis**

The correlations of study samples were first evaluated by calculating the Pearson correlation coefficients of β.
values. The Empirical Bayes moderated $t$ test of the limma package from Bioconductor was used to compare the methylation profile difference between the AS group and the healthy control group. The Benjamini–Hochberg method was used to obtain an adjusted $P$ value for each CpG locus. For quality control, the CpG sites with missing values or detection $P > 0.05$ in more than 90% of specimens were eliminated. The CpG sites with both adjusted $P < 0.05$ and $|\beta$ difference$| > 0.20$ were identified as significantly differentially methylated loci.

Gene Ontology and pathway enrichment analysis
Gene Ontology (GO) and pathway enrichment analysis were conducted by DAVID (http://david.abcc.ncifcrf.gov/) with all genes and pathways available on the Illumina Infinium HumanMethylation450 platform as the background to identify the cellular processes and canonical pathways that were enriched in the identified differentially methylated genes. Molecular function (MF), cellular component (CC), and biologic process (BP) were considered for GO enrichment analysis. For pathway enrichment analysis, we employed all 192 KEGG (http://www.genome.jp/kegg/) biopathways in this study. The GO terms and pathways were considered significantly enriched with FDR-adjusted $P < 0.05$.

qRT-PCR validation
qRT-PCR was conducted to validate the relevance of identified differentially methylated genes for AS using an independent sample (Table 1). Total RNA was isolated from PBMCs using the TRIZOL kit (Invitrogen 15596-026). The quality and purity of the isolated total RNA were examined by NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific Inc.). A total of 100 ng of RNA was reverse-transcribed to complementary DNA (cDNA) using TaKaRa PrimeScript™RT Master Mix (Takara Bio Inc., Japan). The quality and concentration of cDNA were determined by NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific Inc.). The cDNA specimens with A260/280 values varying from 1.8 to 2.0 were used in this study. All primers were supplied by TaKaRa. The forward and reverse primers of $HLA-DQB1$ are 5'-GGTGCCAGAGGAGTTAGAA-3' and 5'-ACAGCCTCACCACACAAAG-3', respectively. Samples were run in triplicate using TaKaRa SYBR Premix® Ex Taq™ II (Tli RNaseH Plus). Glycerinaldehyde-3-phosphate dehydrogenase (GAPDH) was assayed simultaneously by qRT-PCR as an endogenous invariant control. Relative differences in mRNA expression level between AS patients and healthy controls were determined using the $\Delta\Delta C_t$ method as described previously [19].

Results
Identifying differentially methylated loci
Pearson correlation analysis results for methylation $\beta$ values of all study subjects are presented in Additional file 1: Figure S1. Additional file 2: Figure S2 shows the two-way hierarchical cluster analysis results for genome-wide methylation profiles. A total of 473,685 CpG sites passing the quality control procedure were analyzed in this study. Compared with healthy controls, we detected 1915 differentially methylated CpGs, including 1611 hypermethylated loci and 304 hypomethylated loci, in AS PBMCs. The 1915 differentially methylated loci were mapped to 1214 genes, including 1045 hypermethylated genes and 169 hypomethylated genes (Additional file 3: Table S1).

Across the whole genome, the CpG locus cg14323910 located on the $HLA-DQB1$ gene was the most significant for AS (adjusted $P = 1.84 \times 10^{-6}$, $\beta$ difference = 0.5634). Additionally, the cg04777551 locus of $HLA-DQB1$ presented a suggestive association with AS (adjusted $P = 1.46 \times 10^{-3}$, $\beta$ difference = 0.3594).

Gene Ontology and pathway enrichment analysis
We identified four GO terms significantly enriched in the differentially methylated genes, functionally related to antigen dynamics and function (Table 2). As presented in Table 3, pathway enrichment analysis detected 10 significant pathways for AS, such as antigen processing and presentation ($P = 1.76 \times 10^{-3}$), intestinal immune
network for IgA production \((P = 1.94 \times 10^{-4})\), and autoimmune thyroid disease \((P = 3.93 \times 10^{-5})\).

**qRT-PCR validation**

To further assess the functional relevance of the identified HLA-DQB1 gene for AS, we compared the mRNA expression levels of HLA-DQB1 in PBMCs between five AS patients and five healthy controls. The mRNA expression level of HLA-DQB1 in AS patients was significantly lower than that in healthy controls. The average expression ratio of HLA-DQB1 was 0.48 ± 0.10 (mean ± SD) \((P < 0.001)\).

**Discussion**

In this study, we observed a significant difference in DNA methylation profiles of PBMCs between AS patients and healthy control subjects, suggesting the implication of methylation in the development of AS. We also identified the significantly hypermethylated HLA-DQB1 gene in AS PBMCs. qRT-PCR further observed significant downregulation of HLA-DQB1 mRNA in AS PBMCs compared with healthy controls, supporting the functional relevance of HLA-DQB1 for AS.

One of the important findings of this study is that the HLA-DQB1 gene is involved in the development of AS. Human leukocyte antigen (HLA) genes are highly polymorphic gene clusters responsible for the regulation of the immune system in humans [20–24]. HLA-DQB1 belongs to the HLA class II molecules, which are involved in activating immune responses and recognizing self or foreign antigens [25]. HLA-DQB1 plays a central role in the immune system by presenting peptides derived from extracellular proteins [26]. The highly polymorphic amino acid chains of DQB1 are capable of forming the peptide binding groove that presents antigen to CD4+ T-helper cells [27], and controlling the immune response [28, 29]. Previous studies reported that HLA-DQB1 was associated with radiographic severity in AS [30, 31]. The different alleles of HLA-DQB1 were also associated with the age of AS onset [31]. In addition, HLA-DQB1 was closely associated with multiple autoimmune-related diseases [28, 29], such as multiple sclerosis [28] and type I diabetes [32]. Based on previous and present study results, it is reasonable to infer that the aberrant expression of HLA-DQB1 due to DNA hypermethylation contributed to the development of AS. Further biological studies are warranted to confirm our findings and clarify the potential mechanism of HLA-DQB1 involved in the pathogenesis of AS.

We found that the identified differentially methylated regions tended to be located in the genes functionally related to immune-related dynamics and function. The GO and pathway enrichment analysis of differentially

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**Table 2** GO enrichment analysis results of differentially methylated genes between AS patients and healthy controls

| GO ID         | Description                                                                 | Genes                                                                 | P value          |
|---------------|------------------------------------------------------------------------------|----------------------------------------------------------------------|-----------------|
| GO:0002504    | Antigen processing and presentation of peptide or polysaccharide antigen via MHC class II | HLA-DMB, HLA-DOA, HLA-DOB, HLA-DPB1, HLA-DQA1, HLA-DQB1               | 1.11 × 10^{-5}  |
| GO:0042605    | Peptide antigen binding                                                      | DHKR24, HLA-A, HLA-B, HLA-C, HLA-DQB1, HLA-DRB1, HLA-H, MAML1, SLC7A5 | 5.14 × 10^{-6}  |
| GO:0042613    | MHC class II protein complex                                                 | HLA-DMB, HLA-DOA, HLA-DOB, HLA-DPB1, HLA-DQA1, HLA-DQB1, HLA-DRB1    | 7.97 × 10^{-6}  |
| GO:0071556    | Integral to luminal side of endoplasmic reticulum membrane                  | CALR, HLA-A, HLA-B, HLA-C, HLA-DPB1, HLA-DQA1, HLA-DQB1, HLA-DRB1    | 8.79 × 10^{-5}  |

GO Gene Ontology, AS ankylosing spondylitis

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**Table 3** KEGG pathway enrichment analysis results of differentially methylated genes between AS patients and healthy controls

| KEGG ID       | Description                               | DMG | P value          |
|---------------|-------------------------------------------|-----|-----------------|
| hsa05332      | Graft-versus-host disease                 | 11  | 2.31 × 10^{-5}  |
| hsa05320      | Autoimmune thyroid disease               | 11  | 3.93 × 10^{-5}  |
| hsa04940      | Type I diabetes mellitus                 | 11  | 5.05 × 10^{-5}  |
| hsa05310      | Asthma                                    | 9   | 5.19 × 10^{-5}  |
| hsa05330      | Allograft rejection                       | 10  | 6.19 × 10^{-5}  |
| hsa04672      | Intestinal immune network for IgA production | 11  | 1.94 × 10^{-4}  |
| hsa04920      | Adipocytokine signaling pathway           | 13  | 4.47 × 10^{-4}  |
| hsa04514      | Cell adhesion molecules                   | 19  | 8.84 × 10^{-4}  |
| hsa04612      | Antigen processing and presentation       | 12  | 1.76 × 10^{-3}  |
| hsa05416      | Viral myocarditis                         | 12  | 1.76 × 10^{-3}  |

AS ankylosing spondylitis, KEGG Kyoto Encyclopedia of Genes and Genomes, DMG differentially methylated genes
methylated genes further revealed connections between the differentially methylated regions and the enrichment of immune-related genes. For instance, both GO and pathway enrichment analysis results support the implication of dysfunction of antigen processing and presentation (hsa04920) in the development of AS. Antigen processing and presentation is mainly mediated by HLA class I molecules. Zhu et al. [33] found that antigen processing and presentation is significantly associated with AS. As a high polymorphic member of HLA class I molecules, the important role of HLA-B27 in the pathogenesis of AS has been well documented. Briefly, HLA-B27 binds unique peptides of microbial or self-origin (antigen) and presents them to CD8+ T cells. Large multifunctional proteases and transporters associated with antigen presentation act as chaperones for peptide transport. Antigen peptides generated by proteasomal degradation can be further subjected to amino peptidase-mediated trimming before reaching the optimal size for HLA class I binding [34, 35]. The HLA-C of HLA class I molecules has also been confirmed to be associated with AS [36]. More interestingly, we found that the intestinal immune network for IgA production pathway (hsa04672), the autoimmune thyroid disease pathway (hsa05320), and the asthma pathway (hsa05310) were associated with AS in this study. Previous studies have observed the coexistence of abnormal IgA levels and AS [37, 38]. Compared with general populations, higher prevalence of autoimmune thyroid disease and asthma in AS patients have been reported by previous studies [39, 40].

There were some limitations in this study. First, we used five AS patients and five healthy controls as the study samples for DNA methylation profiling. This was a relatively small sample, which may lead to loss of statistical power caused by potential selection bias. Using another independent sample of five AS patients and five healthy controls, qRT-PCR was conducted to validate the functional significance of the hypermethylated HLA-DQB1 gene with AS. qRT-PCR observed significant downregulation of HLA-DQB1 in AS patients, supporting the accuracy of the DNA methylation analysis results. Further biological studies are warranted to confirm our findings and clarify the molecular mechanism of HLA-DQB1 involved in the development of AS. Second, it should be noted that AS patients usually underwent NSAID treatments. Because of the need for clinical AS treatments, it is difficult to exclude the possible impact of NSAIDs on DNA methylation in this study. Further biological studies are warranted to confirm our findings.

Conclusions
We conducted a genome-wide DNA methylation profile comparative study and observed significant difference in DNA methylation profiles between AS patients and healthy subjects. We also reported a set of differentially methylated genes and identified HLA-DQB1 involved in the development of AS. This study provides novel clues for clarifying the genetic mechanism as well as identifying biomarkers for AS.

Acknowledgements
Not applicable.

Funding
The study was supported by National Natural Scientific Fund of China (81472925, 81673112), the Technology Research and Development Program of in Shaanxi Province of China (2013KJXX-51), and the Fundamental Research Funds for the Central Universities.

Availability of data and materials
The genome-wide DNA methylation microarray data will be submitted to ArrayExpress (Additional file 4).

Authors’ contributions
FZ takes responsibility for the integrity of the work as a whole, from inception to the finished manuscript. FZ and XG were responsible for conception and design. JCH, YL, JWX, YW, and AWH were responsible for collection and analysis of data. JCH, WW, and FZ were responsible for interpretation of the data. JCH, YY, JWX, YW, WW, AWH, QRF, XG, and FZ were responsible for drafting, revising and final approval of the manuscript.

Authors’ information
Not applicable.

Ethics approval and consent to participate
This study was approved by the Institutional Review Board of Xi’an Jiaotong University. Written informed consents were obtained from all study subjects.

Consent for publication
This study has obtained consent to publish from the participant (or legal parent or guardian for children) to report individual patient data.

Competing interests
The authors declare that they have no competing interests.

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