**Genome-wide DNA methylation patterns in CD4+ T cells from Chinese Han patients with rheumatoid arthritis**

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**Conflict of interest statement**

No potential conflicts of interest were disclosed for all the authors

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**Abstract**

[Introduction] Rheumatoid arthritis (RA) is an autoimmune disease that causes chronic inflammation of the joints.  Recent evidence indicated the epigenetic changes may contribute to the pathogenesis of RA. [Method] To understand the extent and nature of dysregulated DNA methylation in RA CD4T cells, we performed a genome-wide DNA methylation study in CD4+ T cells in 12 RA patients compared to 12 matched normal healthy controls. Cytosine methylation status was quantified with Illumina methylation 450K microarray. [Result] The DNA methylation profiling showed 383 hyper- and 785 hypo-methylated genes in the CD4+ T cells of the RA patients (P<3.4×10-7) . Gene ontology analysis indicated transcript alternative splicing and protein modification mediated by DNA methylation might play important role in the pathogenesis of RA. In addition, the result showed human leukocyte antigen (HLA) region including *HLA-DRB6*, *HLA-DQA1* and *HLA-E* was frequently hypomethylated, but *HLA-DQB1* hypermethylated in CpG island region and hypomethylated in CpG shelf region in RA patients. Outside of the MHC region, *HDAC4*, *NXN*, *TBCD* and *TMEM61* were the most hypermethylated genes, while *ITIH3*, *TCN2*, *PRDM16*, *SLC1A5* and *GALNT9* are the most hypomethylated genes. [Conclusion] Genome-wide DNA methylation profile revealed significant DNA methylation change in CD4+ T cells from patients with RA.

**Keywords:**

[DNA Methylation](http://www.tandfonline.com/action/doSearch?Keyword=DNA%20Methylation)，rheumatoid arthritis，CD4+ T cells， Genome-wide，Illumina methylation 450k microarray

**Introduction**

Rheumatoid arthritis (RA) is an autoimmune disease that mainly attacks the synovial tissues and therefore causes chronic inflammation of the joints. Autoimmune diseases cause the body’s immune system to mistake its own tissues for foreign invaders. About 0.5-1% of the population in the world were effected by RA[[1](#_ENREF_1)] and the corresponding economic burden was heavy all over the world.

In the past decades, the etiopathogenesis of RA has been widely exploited. The most importance etiological source of RA comes from single nucleotide polymorphisms (SNP). Genome-wide association studies (GWAS) based on SNPs have identified multiple SNPs which were believed to be significantly associated with RA [[2](#_ENREF_2), [3](#_ENREF_3)]. However, as demonstrated in our previous work, even for high familial risk disease as thyroid cancer, a few of the significant SNPs could just have limited prediction power [[4](#_ENREF_4)]. As the expectation, large number of copy number variations were then demonstrated to be significantly associated with the [susceptibility to](http://www.ncbi.nlm.nih.gov/pubmed/23150419) RA [[5](#_ENREF_5), [6](#_ENREF_6)]. As the accumulating evidences from the genetics showed the systemic autoimmune rheumatic diseases, such as systemic lupus erythematosus (SLE), gout and systemic Sclerosis (SSc), usually shared some clinical characteristics and genetic risk factors. Therefore, theoretically, these complex diseases might share some analogous defects in the level of epigenome.

The current estimated heritability of RA was about 20%-50% and it is significantly different between RA patients with and without anti-citrullinated protein antibody [[7](#_ENREF_7)] which indicated that the epigenetic factors played more important roles in the etiology of RA. In addition, genome-wide DNA methylation of SLE [[8](#_ENREF_8), [9](#_ENREF_9)] and SjS [[10](#_ENREF_10)] have showed that large number of DNA methylation were changed in the CD4+ or CD8+ T-cells in the patients. Therefore, it could be postulated that DNA methylation would also be significantly involved in the pathogenesis of RA. DNA methylation is one of most important epigenetic modifications. In our previous study, it has been found DNA methylation played important role in gene [[11](#_ENREF_11)]and microRNA [[12](#_ENREF_12)] expression regulation, cancer initiation/progress and therefore has been play important role in the cancer diagnosis [[13](#_ENREF_13), [14](#_ENREF_14)] and prognosis.

Previous studies suggested several important immune-related genes were aberrantly methylated in RA genome [[15-18](#_ENREF_15)]. However, the genome-wide DNA methylation profile of RA patient was still very limited, especially in Asian population and cannot be access publically. It provided big problem for the candidate gene based research to discover more RA related DNA methylation regions and to identify missing heritability of RA. Considering of the evidence that treatment with monoclonal antibodies against the CD4 antigen leads to immunomodulation would significantly result in clinical benefits for RA patients [[19](#_ENREF_19)], in the present study, we conducted an investigation of the genome-wide DNA methylation profiles in the CD4+ T-cells of 12 RA and matched 12 healthy.

**Methods**

**Patients and controls.**

Twelve RA patients and 12 rigorously matched controls were studied, such as gender, age ([**Table 1**](http://www.tandfonline.com/action/showPopup?citid=citart1&id=T1&doi=10.4161/epi.6.5.15374)). All the sample that we collected were non-smoker and were first diagnosis RA patients without long-term medicine therapy. The mean age was 42.83 in patients and 43.75 in controls without significant difference (p = 0.95). All patients fulfilled the American College of Rheumatology classification criteria for RA. The research was approved by academic advisory board of Guanghua Hospital. Clinical data were collected at the time of sample collection. IgG class anti-CCP antibody ELISA kit (AESKULISA CCP, Germany). RF was measured by Siemen-s Dade Behring BN II Nephelometer. Erythrocyte sedimentation rate (ESR) was achiev-ed by Monitor-100 (VITAL DIAGNOSITICS, Italia) with routine test standards from the hospital laboratory.

**Isolation of PBMCs and CD4+ T cells**

(PBMCs) Mononuclear cells were prepared from blood specimens of RA and normal samples by Ficoll-Hypaque centrifugation (Amersham Biosciences) using the standard protocol and immediately processed for cell culture [[20](#_ENREF_20)]. CD4+ T cells were prepared from freshly isolated PBMCs by depleting cells expressing CD8, CD14, CD16, CD19, CD36, CD56, CD123, γ/δ T cell receptors, and glycophorin A using No-Touch T cell isolation kits (MiltenyiBiotec). The purity of the CD4+ T cells was 95%–98%, as determined by flow cytometry by being stained with Percp cy5.5-conjugated anti-human CD4 (BD Pharmingen, San Diego, CA, USA). Whole genomic DNA was then prepared using a Qiagen DNEasy kit (Qiagen, Germantown, MD) and bisulfite-treated using a Zymo EZ DNA Methylation Kit (Zymo, Orange, CA).

**Illumina Infinium Human Methylation 450.**

Bisulfite-converted patient and control DNA samples were prepared and quantified using a NanoDrop scanning spectrophotometer (Thermo, Wilmington, DE). For each sample, 500 ng of whole-genome bisulfite-converted DNA was denatured, fragmented, amplified and methylation signals were detected by [Infinium HumanMethylation450K BeadChip](http://support.illumina.com/array/array_kits/infinium_humanmethylation450_beadchip_kit.html) (Illumina, San Diego, CA, USA).

Standard DNA methylation 450K analysis pipeline (SMAP) was implemented to conduct the methylation microarray analysis. Genome Studio (Illumina) was used to generate signal intensities and detection p values with internal control normalization (ICN) and background subtraction (BS). Quality control and normalization were conducted with R package of "lumi”. Probes with SNPs or located in ChrX and ChrY were removed before further analysis. In addition, probes with a detection p value > 0.01 exceeding 5% of the samples were also filtered out while other probes less than 5% of samples were labeled as missing value (NA) to avoid further bias in the following statistic and bioinformatic analysis. And then the overall signal intensity, the distribution of M-values and the number of significantly detected sites were used to measure the quality of the beadchip. Significant outlier samples/beadchip were removed before the differential methylation loci identification. Color-bias adjustment and quantile normalization (QN) were performed on signal intensities with the package of "lumi". Finally, beta-mixture quantile normalization (BMIQ) to the β-values were conducted to adjust the bias caused by different types of probes (type I and type II).

**Statistical analysis.**

PCA and Hierarchical cluster analysis were applied to show the correlation between the samples. Two RA samples were filter out since they were obviously different with others in the PCA analysis. Differentially methylated loci were identified by paired t-test based on the beta value of the normalization data. The raw P-values were adjusted by false discovery rate (FDR<0.05) for the multiple test correction. Association between clinical characteristics and differential methylation loci were conducted with linear regression with the significant threshold of 0.005. Gene ontology analysis was conducted with DAVID bioinformatics resources[[21](#_ENREF_21)]. The interactions among the differential methylated genes were inferred by String (version 10.0) [[22](#_ENREF_22)]. Human references of GRCh37/hg19 were used in the bioinformatics analysis and result representation. All methods and analyses were performed in R (version 3.2.1). The data were deposited in the Gene Expression Omnibus (GEO accession: GSE71841).

**Results**

We studied genome-wide CD4+ T-cell DNA methylation in rheumatoid arthritis patients and controls using a high-throughput method based on bead microarrays that allow simultaneous screening of 482,421 CpG sites spanning the promoter region of 21,231 genes (99% RefSeq genes). Twenty-four biological replicates (12 rheumatoid arthritis patients and 12 matched healthy controls) were collected and enrolled in the project. ([Table 1](http://www.tandfonline.com/action/showPopup?citid=citart1&id=T1&doi=10.4161/epi.6.5.15374)). 1,067 probes were removed from the dataset filtered with detection p-value above 0.01. 443 probes were removed from the dataset with a bead count<3 in at least 5% of samples. 29,021 probes with SNPs (dbSNP version: 142) were removed to decrease the bias of the analysis. 8,510 and 11,245 probes were removed since multiple alignment or located in chromosome X or chromosome Y. Eventually, 435,226 probes were kept in the 24 samples.

In order to make sure the cells in our study were significantly derived from CD4+ T-cell, we projected our methylation signals into the PC1 and PC2 dimension of whole blood cell. The analysis showed our samples were clearly aggregated with CD4+ cells and was estranged with other kinds of cells, such as CD8+ T-cell, CD14+, CD19+ and so on which indicates the samples were successfully prepared in the process of sample collection (Figure 1A). What’s more, to ensure that differential methylation patterns identified in our study were not influenced by potential differences in T-cell subset populations between RA patients and controls, we detected the methylation profiles of a number of genes known to be related with particular T-cell subsets, such as  *IL4*, *IL13* (Th2), *IFNG* (Th1) and *IL17F* (Th17). None of these CpG sites were found to be significantly different between case and control, suggesting no difference in T-cell subsets between RA patients and controls.

Meanwhile, PCA analysis was conducted to our methylation 450K, the result showed PC1 and PC2 explained the total variance of 29.9% and 14%, respectively while the top 13 principle component could explain as high as 80% variance. These results indicated the information of the data composed by limited informative clinical or demographical signals and our data were creditable for the further bioinformatic and biostatistical analysis (Supplementary Figure 1).

**Genome-wide DNA methylation profile of rheumatoid arthritis**

We identified 810 hypo-methylated and 392 hyper-methylated CpG sites in RA CD4+ T cells compared to normal controls, representing 785 and 383 genes which were hypo-methylated and hyper-methylated in RA patients with P<3.4×10-7(Paired t-test, FDR<0.05, Supplementary Table 1).

Cluster analysis based on significantly differential methylated loci showed distinct separation between RA and normal controls (Figure 1B). More hypo-methylated CpG site than hyper-methylated CpG site indicated genome-wide hypo-methylation in the genome of rheumatoid arthritis. Interaction maps of differentially-methylated genes was constructed based on string 10.0 and the result showed these DMGs were highly interacted rather than functionally isolated (Figure 2). Gene ontology analysis showed alternative splicing (P=1.2×10-7, FDR) and phosphoprotein (P = 1.7×10-2, FDR) were significantly aberrant in RA patients (Table 2), indicating the abnormal of transcript alternative splicing and protein modification mediated by DNA methylation might play important role in the pathogenesis of rheumatoid arthritis. In addition, Immune response (P-value = 3.2×10-5) and White blood cells monocyte (P-value = 0.02) associated gene ontology was also significantly enriched.

What’s more, the result showed human leukocyte antigen (HLA) region was frequently hypo-methylated in RA patients, including *HLA-DRB6* (P=6.61×10-10), *HLA-DQA1* (P=7.09×10-9) and *HLA-E* (P=3.24×10-7), however, *HLA-DQB1* showed different methylation profiles with significant hyper-methylation in CpG island region and hypo-methylation in CpG shelf region(Table 3). Outside of the MHC region, the most hyper-methylated genes in RA included *HDAC4* (P=1.47×10-7), *NXN* (P=5.5×10-9), *TBCD* (P=4.48×10-8) and *TMEM61* (P=1.7×10-7) while the most significant hypo-methylated genes included *ITIH3* (P=1.16×10-7), *TCN2* (P=1.57×10-8), *PRDM16* (P=3.1×10-9), *SLC1A5* (P=2.94×10-7) and *GALNT9* (P=8.26×10-9).

**Correlation between DNA methylation and disease characteristics.**

As shown in table 1, large numbers of clinical characteristics were recorded for our patients. Identifying clinical relevant DNA methylation loci would provide important insight to the pathological mechanism of RA and valuable the corresponding clinical application. Association analysis between clinical characteristics and identified differential methylated loci were conducted (Table 4). We found the methylation level of *OR5A2* (cg02981094, P=2.6×10-4), *ALDH9A1* (cg03984859, P=2.8×10-4) and *C5orf32* (cg02070114, P=2.2×10-4) were significantly associated with the disease course of RA. In addition, the methylation level of *ZC3H11A* (cg02337583) was significantly associated with the level of [rheumatoid factor](http://www.webmd.com/rheumatoid-arthritis/guide/rheumatoid-factor-test) in RA patients (P=8.9×10-4). What’s more, the methylation level of *OAS2* (cg00085448) was significantly associated with the PGA in RA patients (P=4.1×10-4). Furthermore, *C16orf71* (cg04705084), *LOC100129716* (cg00598143) and miR-762 (cg02558026) were found to be significantly associated with DAS28-CRP with P-value of 5.8×10-3, 5.2×10-3, and 7.5×10-3, respectively. Five loci which included *SLC38A8* (cg01740650, P=3.0×10-3), *C18orf19* (cg00448482, P=3.0×10-3), *COL18A1* (cg04760448, P=1.9×10-3), *BAT3* (cg05649229, P=4.9×10-3) and *PLD3* (cg07071106, P=4.4×10-3) were significantly associated with ESR. In the end, we found *HSPA12A* (cg06942850) was significantly associated with tender joint count (TJC) with P-value of 3.2×10-3 (Table 4).

**Discussion**

In summary, we profiled the genome-wide DNA methylation changes of the CD4+ T-cell in the RA patients of Han Chinese with Illumina methylation 450K microarray. Based on stringent measurement and analysis, 1,202 CpG loci showed significantly different in the CD4+ T-cells between RA and controls. Gene ontology and interaction analysis showed these genes were functional related and interacted with each other. The hypo-methylated regions appeared dominant in RA patients which was consistent with previous report [[23](#_ENREF_23)].

Compared with DNA methylation change of cancer which usually contains thousand differential methylated loci, systemic autoimmune rheumatic diseases seems only have few differential methylation regions. PCA analysis to our methylation dataset also revealed there is no significant separation between RA and control indicating there would be not so many different methylation regions in CD4+ T-cells between RA and normal individual. Matlock and colleagues identified 341 differential methylation loci in CD4+ between SLE and health control [[24](#_ENREF_24)]. Nezam and colleagues identified 753 differential methylation loci in CD4+ between SjS and health control [[10](#_ENREF_10)]. Although multiple test correction has been conducted in these papers, we believe, there are still large number of differential methylated loci would be false positive. Therefore, the candidate differential methylated loci underlying the pathogenesis of RA would be limited. Compared with Kazuhisa’s study, there are 81 shared differential methylated CpG loci which might be very important in the pathogenesis of RA. More genome-wide DNA methylation profiles from different ethnic populations may provide ethnic specific information of methylation changes of RA patients.

In this study, we didn’t perform the validation in another independent cohort because previous studies shown the accuracy of the methylation 450K were very high [[25](#_ENREF_25)]. We also didn’t conduct the gene expression analysis because the function of the methylation to the complex disease not only in gene expression but also in some other important functions such as alternative expression [[26](#_ENREF_26)], genomic stability [[27](#_ENREF_27)] and interaction with genetic variation such as SNPs [[28](#_ENREF_28)]. On the other hand, we want to emphasis that the epigenetic mechanism of RA should be paid more precision not only PBMC [[29](#_ENREF_29)] but also the subset of the blood cells. In the next step we would complete the genome-wide methylation profile for CD8+, CD17+ T-cells and so on in RA to provide epigenetic contribution from each immune-cells. In addition, our data would provide an opportunity to compare the methylation profiles of CD4+ among SLE, Gout and other auto-immune diseases. We noticed Glossop and colleagues identified 761 differential methylated CpG loci between RA and normal individuals from Caucasia population [[18](#_ENREF_18)]. We compared the aberrant different methylation loci between our study and Glossop and found *GALNT9* was shared in both studies and *GALNT9* was demonstrated to be hypo-methylated in RA patients. Although only one gene were shared in our two studies, we think it is acceptable because the differential methylation loci identified by our and Glossop’s study are only a small subset of the total differential methylation loci since the small sample size and the low statistic power [[18](#_ENREF_18)]. What’s more, the DNA methylation was significantly influenced by large number of genetic, environment exposures and the clinical characteristics of the patients as shown in our study which might bring some difference between our study and Glossop [[18](#_ENREF_18)]. The roles of several of these genes in RA has been widely discovered in the previous studies. Numerous studies have indicated that decreased *HDAC* activity in patients with RA may contribute to local pro-inflammatory cytokine production and reduced the responses to corticosteroid treatment. HDAC activity can modify transcription factor activity, prevent expression of pro-apoptotic genes, and therefore terminate transcription following deacetylation of histones. These have been comprehensively described in Grabiec’s study [[30](#_ENREF_30)]. *ITIH3* was demonstrated as one of powerful biomarker for RA in the lasted study from Chou and colleagues [[31](#_ENREF_31)]. *ITIH3* could act as a carrier of hyaluronan in serum or as a binding protein between hyaluronan and other matrix protein, including those on cell surfaces in tissues to regulate the localization, synthesis and degradation of hyaluronan which were essential to cells undergoing biological processes (UniProtKB/Swiss-Prot). In term of other remaining genes, we still don’t have enough previous evidence to show they are playing roles in RA pathogenesis and development, including *MXN*, *TBCD*, *TMEM61*, *TCN2*, *PRDM16*, and *SLC1A5* and therefore they might be novel biomarkers for RA diagnosis. Our study demonstrate that the epigenetic-based association study or biomarker identification really need large samples in different populations that the shared epigenetic biomarkers could be found eventually.

**Conclusion**

Genome-wide DNA methylation patterns revealed significant DNA methylation change in CD4+ T cells from patients with RA.

**Authors’ contributions**

DH and GC, SG contributed to the conception, design and final approval of the submitted version. SG, QZ and TJ contributed to the integrated analysis of multiple microarray datasets, batch effect elimination and statistical analysis. TJ, YL, RW, YS, XZ, YW, FB, QD and XZ collected samples and helped to data cleaning, statistic and draft the manuscript. All authors read and approved the final manuscript.

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**References**

1. Firestein, G.S., *Evolving concepts of rheumatoid arthritis.* Nature, 2003. **423**(6937): p. 356-61.

2. Acosta-Colman, I., et al., *GWAS replication study confirms the association of PDE3A-SLCO1C1 with anti-TNF therapy response in rheumatoid arthritis.* Pharmacogenomics, 2013. **14**(7): p. 727-34.

3. Eleftherohorinou, H., et al., *Pathway-driven gene stability selection of two rheumatoid arthritis GWAS identifies and validates new susceptibility genes in receptor mediated signalling pathways.* Hum Mol Genet, 2011. **20**(17): p. 3494-506.

4. Guo, S., Wang, Y.-L., Li, Y., Jin, L., Xiong, M., Ji, Q.-H. and Wang, J, *Significant SNPs have limited prediction ability for thyroid cancer.* CANCER MEDICINE, 2014. **doi: 10.1002/cam4.211**.

5. Song, X., et al., *Association between HLA-DQA1 gene copy number polymorphisms and susceptibility to rheumatoid arthritis in Chinese Han population.* J Genet, 2014. **93**(1): p. 215-8.

6. Chen, J.Y., et al., *Association of FCGR3A and FCGR3B copy number variations with systemic lupus erythematosus and rheumatoid arthritis in Taiwanese patients.* Arthritis Rheumatol, 2014. **66**(11): p. 3113-21.

7. Frisell, T., et al., *Familial risks and heritability of rheumatoid arthritis: role of rheumatoid factor/anti-citrullinated protein antibody status, number and type of affected relatives, sex, and age.* Arthritis Rheum, 2013. **65**(11): p. 2773-82.

8. Javierre, B.M., et al., *Changes in the pattern of DNA methylation associate with twin discordance in systemic lupus erythematosus.* Genome Res, 2010. **20**(2): p. 170-9.

9. Richardson, B., et al., *Evidence for impaired T cell DNA methylation in systemic lupus erythematosus and rheumatoid arthritis.* Arthritis Rheum, 1990. **33**(11): p. 1665-73.

10. Altorok, N., et al., *Genome-wide DNA methylation patterns in naive CD4+ T cells from patients with primary Sjogren's syndrome.* Arthritis Rheumatol, 2014. **66**(3): p. 731-9.

11. Wang, X., et al., *Hypermethylation reduces expression of tumor-suppressor PLZF and regulates proliferation and apoptosis in non-small-cell lung cancers.* FASEB J, 2013. **27**(10): p. 4194-203.

12. He, Y., et al., *Hypomethylation of the hsa-miR-191 locus causes high expression of hsa-mir-191 and promotes the epithelial-to-mesenchymal transition in hepatocellular carcinoma.* Neoplasia, 2011. **13**(9): p. 841-53.

13. Guo, S., et al., *Identification and validation of the methylation biomarkers of non-small cell lung cancer (NSCLC).* Clin Epigenetics, 2015. **7**(1): p. 3.

14. Guo, S., et al., *Quantitative assessment of the diagnostic role of APC promoter methylation in non-small cell lung cancer.* Clin Epigenetics, 2014. **6**(1): p. 5.

15. Glossop, J.R., et al., *DNA methylation profiling of synovial fluid FLS in rheumatoid arthritis reveals changes common with tissue-derived FLS.* Epigenomics, 2015. **7**(4): p. 539-51.

16. Liebling, M.R., *Methylation of the CTLA-4 promoter and Treg cell dysfunction in rheumatoid arthritis: comment on the article by Cribbs et al.* Arthritis Rheumatol, 2015. **67**(5): p. 1406.

17. van Steenbergen, H.W., et al., *Differential methylation within the major histocompatibility complex region in rheumatoid arthritis: a replication study.* Rheumatology (Oxford), 2014. **53**(12): p. 2317-8.

18. Glossop, J.R., et al., *Genome-wide DNA methylation profiling in rheumatoid arthritis identifies disease-associated methylation changes that are distinct to individual T- and B-lymphocyte populations.* Epigenetics, 2014. **9**(9): p. 1228-37.

19. Horneff, G., et al., *Treatment of rheumatoid arthritis with an anti-CD4 monoclonal antibody.* Arthritis Rheum, 1991. **34**(2): p. 129-40.

20. Chen, G., et al., *Role of osteopontin in synovial Th17 differentiation in rheumatoid arthritis.* Arthritis Rheum, 2010. **62**(10): p. 2900-8.

21. Huang da, W., B.T. Sherman, and R.A. Lempicki, *Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources.* Nat Protoc, 2009. **4**(1): p. 44-57.

22. Szklarczyk, D., et al., *STRING v10: protein-protein interaction networks, integrated over the tree of life.* Nucleic Acids Res, 2015. **43**(Database issue): p. D447-52.

23. Karouzakis, E., et al., *Increased recycling of polyamines is associated with global DNA hypomethylation in rheumatoid arthritis synovial fibroblasts.* Arthritis Rheum, 2012. **64**(6): p. 1809-17.

24. Jeffries, M.A., et al., *Genome-wide DNA methylation patterns in CD4+ T cells from patients with systemic lupus erythematosus.* Epigenetics, 2011. **6**(5): p. 593-601.

25. Dedeurwaerder, S., et al., *Evaluation of the Infinium Methylation 450K technology.* Epigenomics, 2011. **3**(6): p. 771-84.

26. Lev Maor, G., A. Yearim, and G. Ast, *The alternative role of DNA methylation in splicing regulation.* Trends Genet, 2015. **31**(5): p. 274-80.

27. Rizwana, R. and P.J. Hahn, *CpG methylation reduces genomic instability.* J Cell Sci, 1999. **112 ( Pt 24)**: p. 4513-9.

28. Shoemaker, R., et al., *Allele-specific methylation is prevalent and is contributed by CpG-SNPs in the human genome.* Genome Res, 2010. **20**(7): p. 883-9.

29. Liu, Y., et al., *Epigenome-wide association data implicate DNA methylation as an intermediary of genetic risk in rheumatoid arthritis.* Nat Biotechnol, 2013. **31**(2): p. 142-7.

30. Grabiec, A.M., P.P. Tak, and K.A. Reedquist, *Targeting histone deacetylase activity in rheumatoid arthritis and asthma as prototypes of inflammatory disease: should we keep our HATs on?* Arthritis Res Ther, 2008. **10**(5): p. 226.

31. Liao, C.C., et al., *Comparative analysis of novel autoantibody isotypes against citrullinated-inter-alpha-trypsin inhibitor heavy chain 3 (ITIH3) peptide in serum from Taiwanese females with rheumatoid arthritis, primary Sjogren's syndrome and secondary Sjogren's syndrome in rheumatoid arthritis.* J Proteomics, 2016.

**Figure Legend**

**Figure 1. Genome-wide DNA methylation profile of RA and bioinformatics of differential methylation loci.** A, PCA analysis showed the CD4+ cells from our study were aggregated with CD4+ T-cells from GSE35069, indicating the samples were well prepared. B, hierarchical cluster analysis based on differential methylated loci separated samples into RA and control group.

**Figure 2. Gene-gene interaction analysis to differential methylated genes.**

The interaction was inferred by protein-protein interaction database of String 10.

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| **Table 1. Characteristic of the enrolled RA and control samples** | | | | | | | | | | | | | |
| SSID | Age | Gender | COD  (Year) | RF  (IU/mL) | Anti-CCP  (RU/ml) | ESR  (mm/h) | SJC | TJC | PGA | DAS28-CRP | Control | Age | Gender |
| RA0001 | 47 | Male | 6 | <20 | 158.21 | 29 | 4 | 6 | 70 | 5.27 | HP0001 | 47 | Male |
| RA0010 | 37 | Female | 0.8 | 22.2 | 792.81 | 14 | 0 | 0 | 10 | 1.99 | HP0010 | 35 | Female |
| RA0011 | 52 | Male | 0.2 | 2260 | 104.86 | 46 | 10 | 12 | 60 | 6.35 | HP0011 | 52 | Male |
| RA0012 | 56 | Male | 1.5 | 1000 | 1600 | 140 | 10 | 11 | 85 | 7.39 | HP0012 | 56 | Male |
| RA0002 | 40 | Female | 3 | 198 | 789.54 | 36 | 1 | 1 | 30 | 3.77 | HP0002 | 39 | Female |
| RA0003 | 47 | Female | 3 | 152 | 270.38 | 140 | 18 | 19 | 90 | 8.35 | HP0003 | 46 | Female |
| RA0004 | 23 | Female | 1.5 | <20 | 71.42 | 24 | 3 | 4 | 45 | 4.46 | HP0004 | 23 | Female |
| RA0005 | 28 | Female | 11 | 127 | 306.09 | 54 | 18 | 18 | 80 | 7.48 | HP0005 | 27 | Female |
| RA0006 | 39 | Female | 20 | <20 | <25 | 66 | 3 | 3 | 50 | 5.09 | HP0006 | 40 | Female |
| RA0007 | 25 | Female | 4 | 1020 | 1540.83 | 23 | 18 | 19 | 55 | 6.59 | HP0007 | 27 | Female |
| RA0008 | 57 | Female | 10 | <20 | <25 | 78 | 3 | 3 | 60 | 5.34 | HP0008 | 57 | Female |
| RA0009 | 39 | Female | 15 | 1200 | 178.88 | 75 | 22 | 26 | 70 | 8.17 | HP0009 | 38 | Female |

COD:  course of a disease, RF: rheumatoid factor; Anti-CCP: anti-cyclic citrullinated peptide antibody; ESR: erythrocyte sedimentation rate; SJC: swollen joint count; TJC: tender joint count; PGA: patient global assessment; DAS28-CRP: disease activity score in 28 Joints.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Term | Count | Freq(%) | P-Value | Fold Enrichment | Benjamini |
| Alternative splicing | 415 | 47.64 | 2.5×10-10 | 1.26 | 1.23191E-07 |
| Splice variant | 417 | 47.87 | 1.4×10-10 | 1.27 | 3.11674E-07 |
| Alternative products | 398 | 45.69 | 3.7×10-8 | 1.23 | 9.31881E-07 |
| [innate *immune* response](http://amigo.geneontology.org/amigo/term/GO:0045087) | 52 | 18.14 | 4.1×10-7 | 1.44 | 3.20146E-05 |
| [regulation of innate immune response](http://amigo.geneontology.org/amigo/term/GO:0045088) | 41 | 11.93 | 2.7×10-5 | 1.30 | 0.001039486 |
| Signaling by Rho GTPases | 16 | 1.84 | 3.0×10-4 | 2.88 | 0.013928772 |
| White blood cells monocyte | 72 | 8.26 | 1.7×10-3 | 1.43 | 0.028321431 |

**Table 2. Gene ontology analysis to differential methylation genes in RA**

**Table 3. Differential methylation status of HLA genes in RA**

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Gene | CpG site | P-Value | Delta-Beta | Ratio-Beta | Case | Control | Chrosome | CpG Shore | Type |
| HLA-DOA | cg00540941 | 4.70×10-8 | -0.18 | 0.65 | 0.34 | 0.52 | chr6:32974843-32974844 | N\_Shore | II |
| HLA-DQA1 | cg02919082 | 7.09×10-9 | -0.38 | 0.44 | 0.30 | 0.67 | chr6:32605694-32605695 | NA | II |
| HLA-DQA1 | cg04054303 | 9.83×10-12 | -0.47 | 0.40 | 0.31 | 0.78 | chr6:32606445-32606446 | NA | II |
| HLA-DQB1 | cg02902672 | 1.62×10-8 | -0.63 | 0.18 | 0.14 | 0.76 | chr6:32635360-32635361 | S\_Shelf | II |
| HLA-DQB1 | cg04777551 | 1.44×10-8 | -0.46 | 0.47 | 0.41 | 0.87 | chr6:32628953-32628954 | N\_Shelf | II |
| HLA-DQB2 | cg02964065 | 1.99×10-7 | 0.08 | 1.11 | 0.77 | 0.69 | chr6:32729545-32729546 | Island | I |
| HLA-DRB1 | cg00211215 | 1.60×10-7 | 0.66 | 3.39 | 0.94 | 0.28 | chr6:32552246-32552247 | Island | I |
| HLA-DRB1 | cg04026937 | 1.60×10-10 | -0.62 | 0.08 | 0.05 | 0.66 | chr6:32549361-32549362 | N\_Shelf | II |
| HLA-DRB1 | cg06032479 | 3.67×10-8 | 0.20 | 1.27 | 0.92 | 0.73 | chr6:32552026-32552027 | Island | I |
| HLA-DRB1 | cg06204447 | 4.05×10-8 | -0.18 | 0.76 | 0.57 | 0.74 | chr6:32546665-32546666 | NA | II |
| HLA-DRB6 | cg00103771 | 6.61×10-10 | -0.64 | 0.24 | 0.20 | 0.84 | chr6:32525805-32525806 | NA | II |
| HLA-DRB6 | cg04688450 | 5.38×10-9 | -0.34 | 0.44 | 0.27 | 0.62 | chr6:32526366-32526367 | NA | II |
| HLA-DRB6 | cg06559318 | 1.17×10-8 | -0.82 | 0.05 | 0.04 | 0.85 | chr6:32526260-32526261 | NA | I |
| HLA-E | cg02678305 | 4.74×10-8 | -0.05 | 0.89 | 0.39 | 0.43 | chr6:30460322-30460323 | S\_Shelf | II |
| HLA-E | cg03725115 | 3.24×10-7 | -0.02 | 0.72 | 0.05 | 0.07 | chr6:30458102-30458103 | Island | I |

Deta-beta represent the difference between RA and controls. Ratio-beta represent the fold-change of the average methylation level in RA compared with controls.

|  |  |  |  |
| --- | --- | --- | --- |
|  |  |  |  |
| **Table 4. Clinical characteristics associated methylation loci identified by HM450K in RA** | | | |
|  | Gene Symbol | CpG Loci | P-Value |
| Disease Course | *OR5A2* | cg02981094 | 2.6×10-4 |
| *ALDH9A1* | cg03984859 | 2.8×10-4 |
| *C5orf32* | cg02070114 | 2.2×10-4 |
| [Rheumatoid Factor](http://www.webmd.com/rheumatoid-arthritis/guide/rheumatoid-factor-test) | *ZC3H11A* | cg02337583 | 8.9×10-4 |
| Patient Global Assessment | *OAS2* | cg00085448 | 4.1×10-4 |
| DAS28-CRP | *C16orf71* | cg04705084 | 5.8×10-3 |
| *LOC100129716* | cg00598143 | 5.2×10-3 |
| *miR-762* | cg02558026 | 7.5×10-3 |
| ESR | *SLC38A8* | cg01740650 | 3.0×10-3 |
| *C18orf19* | cg00448482 | 3.0×10-3 |
| *COL18A1* | cg04760448 | 1.9×10-3 |
| *BAT3* | cg05649229 | 4.9×10-3 |
| *PLD3* | cg07071106 | 4.4×10-3 |
| Tender Joint Count | *HSPA12A* | cg06942850 | 3.2×10-3 |