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ORIGINAL ARTICLE

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

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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 ($p < 3.4 \times 10^{-7}$). Gene ontology analysis indicated transcript alternative splicing and protein modification mediated by DNA methylation might play an important role in the pathogenesis of RA. In addition, the result showed that 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 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

CD4+ T cells, DNA Methylation, Genome-wide, Illumina methylation 450k microarray, Rheumatoid arthritis

History

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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 are effected by RA [1] and the corresponding economic burden is heavy all over the world.

In the past decades, the etiopathogenesis of RA has been widely exploited. The most important etiological source of RA comes from single nucleotide polymorphisms (SNPs). Genome-wide association studies (GWAS) based on SNPs have identified multiple SNPs which were believed to be significantly associated

with RA [2,3]. 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]. As expected, large number of copy number variations were then demonstrated to be significantly associated with the susceptibility to RA [5,6]. 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 is about 20–50% and is significantly different between RA patients with and without anti-citrullinated protein antibody [7], which indicates that the epigenetic factors play more important roles in the etiology of RA. In addition, genome-wide DNA methylation of SLE [8,9] and SjS [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 the most important epigenetic modifications. Our previous study showed that DNA methylation played an important

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role in gene [11] and microRNA [12] expression regulation, cancer initiation/progress, and therefore has an important role in the cancer diagnosis [13,14] and prognosis.

Previous studies suggested several important immune-related genes were aberrantly methylated in RA genome [15–18]. However, the genome-wide DNA methylation profile of RA patient was still very limited, especially in Asian population and cannot be accessed publically. It created big problem for the candidate gene-based research to discover more RA-related DNA methylation regions and identify missing heritability of RA. Considering the evidence that treatment with monoclonal antibodies against the CD4 antigen leads to immunomodulation would significantly result in clinical benefits for RA patients [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 individuals.

Methods

Patients and controls

Twelve RA patients and 12 rigorously matched controls were studied, such as gender, age (Table 1). All the samples that we collected were non-smokers and were first diagnosed 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 was measured using ELISA kit (AESKULISA CCP, Germany). RF was measured by Siemens Dade Behring BN II Nephelometer. Erythrocyte sedimentation rate (ESR) was achieved by Monitor-100 (VITAL DIAGNOSTICS, Italia) with routine test standards from the hospital laboratory.

Isolation of PBMCs and CD4 + T cells

Mononuclear cells (PBMCs) 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]. 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 being stained with

Percp cy5.5-conjugated anti-human CD4 (BD Pharmingen, San Diego, CA). 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 (Illumina, San Diego, CA).

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 detect 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 located in ChrX and ChrY were removed before further analysis. In addition, probes with a detection of 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. 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. Obvious outlier samples and probes 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 filtered 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

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	35	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.

analysis was conducted with DAVID bioinformatics resources [21]. The interactions among the differential methylated genes were inferred by String (version 10.0) [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 RA 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 RA patients and 12 matched healthy controls) were collected and enrolled in the project (Table 1). 1067 probes were removed from the dataset filtered with the detection of p -value above 0.01. Four hundred forty-three 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. 8510 and 11,245 probes were removed since multiple alignments were 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+, which indicates the samples were successfully prepared in the process of sample collection (Figure 1A). Further, 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 RA

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 $p < 3.4 \times 10^{-7}$ (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 RA. 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 ($p = 1.2 \times 10^{-7}$, FDR) and phosphoprotein ($p = 1.7 \times 10^{-2}$, FDR) were significantly aberrant in RA patients (Table 2), indicating the abnormal that transcript alternative splicing and protein modification mediated by DNA methylation might play an important role in the pathogenesis of RA. In addition, immune response (p -value $= 3.2 \times 10^{-5}$) and white blood cells monocyte-associated (p -value $= 0.02$) gene ontology was also significantly enriched.

The result showed human leukocyte antigen (HLA) region was frequently hypo-methylated in RA patients, including *HLA-DRB6* ($p = 6.61 \times 10^{-6}$), *HLA-DQA1* ($p = 7.09 \times 10^{-9}$) and *HLA-E* ($p = 3.24 \times 10^{-7}$), 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 the MHC region, the most hyper-methylated genes in RA included *HDAC4* ($p = 1.47 \times 10^{-7}$), *NXN* ($p = 5.5 \times 10^{-9}$), *TBCD* ($p = 4.48 \times 10^{-8}$) and *TMEM61* ($p = 1.7 \times 10^{-7}$) while the most significant hypo-methylated genes included *ITIH3* ($p = 1.16 \times 10^{-7}$), *TCN2* ($p = 1.57 \times 10^{-8}$), *PRDM16* ($p = 3.1 \times 10^{-9}$), *SLC1A5* ($p = 2.94 \times 10^{-7}$) and *GALNT9* ($p = 8.26 \times 10^{-9}$).

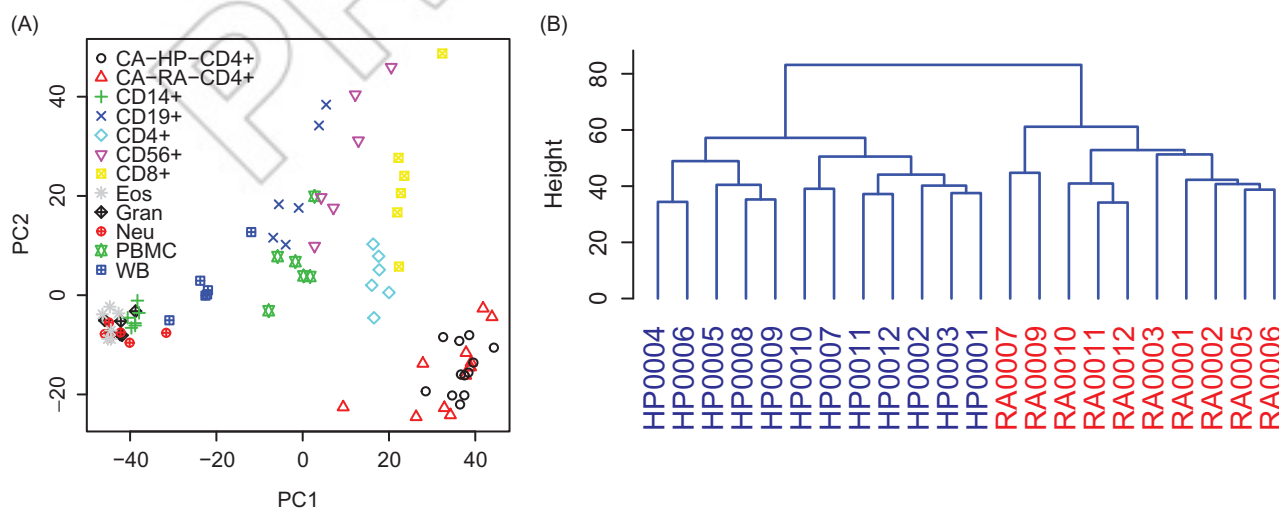


Figure 1. Genome-wide DNA methylation profile of RA and bioinformatics of differential methylation loci. (A) PCA analysis showed the CD4+ cells from our study were aggregated with CD4+ T-cells from GSE35069, indicating the samples were well prepared. (B) Hierarchical cluster analysis based on differential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

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

Gene	CpG site	p-Value	Delta-Beta	Ratio-Beta	Case	Control	Chromosome	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

Delta-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	<i>OR5A2</i>	cg02981094	2.6×10^{-4}
	<i>ALDH9A1</i>	cg03984859	2.8×10^{-4}
	<i>C5orf32</i>	cg02070114	2.2×10^{-4}
Rheumatoid factor	<i>ZC3H11A</i>	cg02337583	8.9×10^{-4}
Patient global assessment	<i>OAS2</i>	cg00085448	4.1×10^{-4}
DAS28-CRP	<i>C16orf71</i>	cg04705084	5.8×10^{-3}
	<i>LOC100129716</i>	cg00598143	5.2×10^{-3}
	<i>miR-762</i>	cg02558026	7.5×10^{-3}
ESR	<i>SLC38A8</i>	cg01740650	3.0×10^{-3}
	<i>C18orf19</i>	cg00448482	3.0×10^{-3}
	<i>COL18A1</i>	cg04760448	1.9×10^{-3}
	<i>BAT3</i>	cg05649229	4.9×10^{-3}
	<i>PLD3</i>	cg07071106	4.4×10^{-3}
Tender joint count	<i>HSPA12A</i>	cg06942850	3.2×10^{-3}

associated with tender joint count (TJC) with a *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, 1202 CpG loci showed significant difference in the CD4+T-cells between RA and controls. Gene ontology and interaction analysis showed these genes were functionally related and interacted with each other. The hypo-methylated regions appeared dominant in RA patients, which was consistent with the previous report [23].

Compared with DNA methylation change of cancer which usually contains thousand differential methylated loci, systemic autoimmune rheumatic diseases seems to have only 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 many different methylation regions in CD4+T-cells between RA and normal individual. Jeffries et al. identified 341 differential methylation loci in CD4+ between SLE and health control [24]. Altork et al. identified 753 differential methylation loci in CD4+ between SjS and health control [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 did not perform the validation in another independent cohort because previous studies have shown the accuracy of the methylation 450K were very high [25]. We also did not conduct the gene expression analysis because the function of the methylation to the complex disease is seen not only in gene expression but also in some other important functions such as alternative expression [26], genomic stability [27] and interaction with genetic variation such as SNPs [28]. On the other hand, we want to emphasize that the epigenetic mechanism of RA should be paid more precision not only for PBMC [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, etc., 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 autoimmune diseases. Glossop et al. identified 761 differential methylated CpG loci between RA and normal individuals from Caucasians population [18]. We compared the aberrant different methylation loci between our study and Glossop et al., and found GALNT9 was shared in both studies and GALNT9 was demonstrated to be hypo-methylated in RA patients. Although only one gene was shared in our two studies, we think it is acceptable because the differential methylation loci identified by us and Glossop et al. are only a small subset of the total differential methylation loci because of the small sample size and the low statistic power [18]. Furthermore, 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 et al. [18]. The roles of several genes in RA have 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 reduce the responses to corticosteroid treatment. Histone deacetylases (HDACs) activity can modify transcription factor activity, prevent expression of pro-apoptotic genes, and therefore terminate transcription following deacetylation of

histones [30]. They have pleiotropic effects within the immune system. At least 11 histone deacetylase (HDAC) exist in human genome and could be divided into four sub-families, including class I (HDAC1,2,3,8), II (HDAC4,5,6,7,9,10), III (Sirtuin 1-7) and IV (HDAC11) on the basis of size, homology, and assembly. HDAC4 belongs to the class IIa HDACs which are predominantly playing the role on regulating adaptive immunity. Previous evidence showed nuclear export of HDAC4 is quite important for induced expression of IL5 in activated T-cells, indicating the methylation status aberrant would cause compressive influence to immune-response. Meanwhile, methylation change of HDACs might also cause isoform-selective difference in immune cells, and therefore caused different response to the corresponding drugs for different RA patients [31]. ITIH3 was demonstrated as one of the powerful biomarkers for RA in the lasted study from Liao et al. [32]. 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 terms of other remaining genes, we still do not have sufficient evidence to show they play significant role in RA pathogenesis and development, including MXN, TBCD, TMEM61, TCN2, PRDM16, and SLC1A5, and therefore they might be novel biomarkers for RA diagnosis and would be helpful on personalized medicine and precision medicine in RA. Our study demonstrates 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.

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Conflict of interest

Authors declare no conflicts of interest.

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Supplementary material available online.

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