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Altered DNA Methylations May Contribute to Rheumatoid Arthritis

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ABSTRACT The purpose of this paper was to investigate roles of altered DNA methylations in rheumatoid arthritis (RA). To achieve this goal, firstly, DNA methylation data were recruited from the ArrayExpress database. Secondly, differentially methylated genes (DMGs) across RA patients and normals were detected based on *t*-test. Thirdly, hierarchical clustering analysis was performed to evaluate DMGs between RA patients and normal controls. Ultimately, functional enrichment analyses were conducted on DMGs to investigate significant biological functions in RA patients. A total of 45 DMGs (covering 50 CpGs) were obtained. Importantly, the DMGs could well classify RA patients and controls (accuracy = 0.9942), with well feasibility and confidence. Besides, the researchers identified 15 significant gene ontology (GO) terms and 10 significant pathways, respectively. In summary, the DMGs and their functional gene sets, which might shed new lights on the molecular mechanism of RA and provide potential biomarkers for prevention of this disease.

INTRODUCTION

It has been demonstrated that epigenetic mechanisms integrate genetic and environmental causes of diseases (Golla et al. 2017). Briefly, epigenetic refers to modifications of DNA and its packaging that alter the accessibility of DNA to potentially regulate gene expression and cellular function without changes to underlying genomic sequences (King et al. 2016). Meanwhile, DNA methylation is the most studied epigenetic modification in human beings, which represents the covalent attachment of a methyl group to the 52 position of cytosine, typically occurring in the context of cytosine-guanine dinucleotide (CpG) sites (Smith and Meissner 2013; Johnson et al. 2017). Furthermore, it has a variety of crucial functions, including control of gene expression, cellular differentiation, X-chromosome inactivation and genomic imprinting (Hermann et al. 2004). But changes in DNA methylation are dynamic and it is still largely unknown how they dictate spatial and temporal gene expression programs (Smith and Meissner 2013). Thus, a good understanding of aberrant DNA methylations in complicated diseases might point a new direction for revealing their pathological mechanisms.

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by joint swelling, joint tenderness, and destruction of synovial joints, leading to severe disability and premature mortality (Aletaha et al. 2010). Most commonly involved are the small joints of the hands, feet and cervical spine, but larger joints like the shoulder and knee can also be involved (Walker et al. 2014). However, the cause of RA is not clear, and it is believed to involve a combination of genetic and environmental factors. Coincidently, epigenome-wide association data implicate DNA methylation as an intermediary of genetic risk in RA patients (Liu et al. 2013). But the comprehensive and systemic analyses of DNA methylations in RA have not yet performed.

Objectives

Therefore, in the present study, abnormal DNA methylations were revealed for RA based

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on a series of bioinformatics analyses. First of all, differentially methylated genes (DMGs) between RA patients and normal controls were detected from the DNA methylation data. Subsequently, a hierarchical clustering analysis was conducted on these DMGs to access the classification performance of them. Finally, functional enrichment analyses were carried out to explore significant gene sets of RA. These results might provide evidence of the cumulative roles of epigenetic mechanisms in RA, and shed new lights on RA early diagnosis and treatment.

METHODOLOGY

Preparing DNA Methylation Data

DNA methylation data for RA patients (E-GEOD-42861) were downloaded from the public ArrayExpress database (http://www.ebi.ac.uk/arrayexpress/) using Illumina HumanMethylation450 BeadChip (HumanMethylation450_15017482_v.1.1) on 354 anti-citrullinated protein antibody associated RA cases and 335 controls. There were a total of 485,577 CpGs in the raw microarray dataset. Subsequently, these CpGs were obeyed to a set of rigorous quality controls. In brief, a probe would be removed if it satisfied with one of the following condition: the distance from CpG to single-nucleotide polymorphism (SNP) < 2; the minimum allelic frequency <0.05; and probes of cross-hybridising and on X and Y chromosomes. As a result, 426,758 CpGs were obtained for the subsequent analyses.

Identifying DMGs

Generally speaking, methylation at a CpG is denoted as a methylation β-value, which is a quantitative measure of methylation for each CpG site with range between 0 (no methylation) to 1 (completely methylated) (Wu et al. 2016a). Furthermore, the retained DNA methylation data were normalized according to the β-mixture quantile normalization method, which improves the robustness of the normalization procedure and reduces the technical variation and bias (Teschendorff et al. 2013). In this work, all CpGs for which one or more of the 689 samples displayed detection P values > 0.05 (indicating an unreliable site) or presented with missing β-values and the absolute difference of average β-value across RA patients and normal controls < 0.05 were excluded. Initial differential methylated CpGs between the two specific groups were determined through the *t*-test (Baldi and Long 2001) and the cut-off was set as $P < 0.05$.

Subsequently, the refinement of initial differential methylated CpGs was further filtering steps were applied to facilitate a more stringent analysis. For purpose of reducing the number of non-variable CpGs and improve the statistical power of subsequent analyses, the sits with β-values > 0.8 and < 0.2 were eliminated in all 689 samples. Moreover, CpG with the absolute difference of average β-value across RA patients and normal controls <0.1 was deleted. Consequently, differential methylated CpGs between RA patients and normal controls were investigated. The genes covered by differential methylated CpGs were considered to DMGs for RA patients.

Hierarchical Clustering Analysis

Hierarchical clustering analysis was conducted using the Cluster 3.0 software (Hoon 2002) to access DMGs classified performance between 354 RA cases and 335 controls. Ideally, the 689 samples should be classified into two major clusters: RA group and control group. The present study tested the method by measuring the percentage of test samples that could be correctly classified. Supposing that RA belonged to positive samples, and normal controls attributed to negative samples, accuracy was counted as followed formula (Mohammadi et al. 2011):

$$\text{Accuracy} = \frac{TN+TP}{TN+TP+FN+FP}$$

Where TP (true positive) represented the number of positive samples correctly predicted as positive; TN (true negative) stood for the number of negative samples correctly predicted as negative; FP (false positive) was the number of negative samples incorrectly predicted as positive and FN (false negative) referred to the number of positive samples incorrectly predicted as negative. Of note, a high accuracy indicated a good classification performance, and further validated the confidence of DMGs and the feasibility of the presented method.

Gene Ontology (GO) Enrichment Analysis

GO analysis has been widely utilized as functional enrichment researches for large-scale

genes (Ashburner et al. 2000). In the reseachers' study, the researchers implemented GO functional enrichment analysis for differentially methylated genes using Database for Annotation, Visualization and Integrated Discovery (DAVID, https://david.ncifcrf.gov) which is a soft tool providing a comprehensive set of functional annotation for researchers to understand the biological meaning behind a large number of genes (Huang da et al. 2009). Specifically, Fisher's exact test was utilized to classify the GO category. Then, P values were corrected using false discovery rate (FDR) using Benjamini and Hochberg (BH) method (Benjamini et al. 2001). Functional terms with $P < 0.05$ were considered statistically significant.

Pathway Enrichment Analysis

Kyoto Encyclopedia of Genes and Genomes (KEGG) database is a collection of manually drawn pathways mapped for metabolism, genetic information processing, environmental information processing, various other cellular processes and diseases (Kanehisa 2000). Hence, the KEGG pathway enrichment analysis was performed on DMGs based on the DAVID. Particularly, Fisher's exact test was utilized to evaluate the significance of pathways (Routledge 2005). The P values were corrected by FDR in BH method (Benjamini et al. 2001). Pathways with $P < 0.05$ were considered to be significant between RA patients and control samples.

RESULTS

DMGs

Following quality control and normalization to remove probes with SNP-CpG distance not more than 2, on X and Y chromosomes, with MAF < 0.05, and of cross-hybridising, a total of 426,758 methylated CpGs remained in the final dataset of 689 samples. A volcano plot exhibiting distribution of the 426,758 analyzed methylated CpGs was produced, as described in Figure 1. Among these 426,758 methylated CpGs, 5,422 CpGs (representing 3320 genes) were initial differentially methylated, when the absolute difference value of mean β-value between RA and normal groups was higher than 0.05 and P value was less than 0.05. Among 5,422 CpGs, 4,348 CpGs were hypermethylated, whereas 1,074 CpGs were hypomethylated in RA patients compared with normal controls.

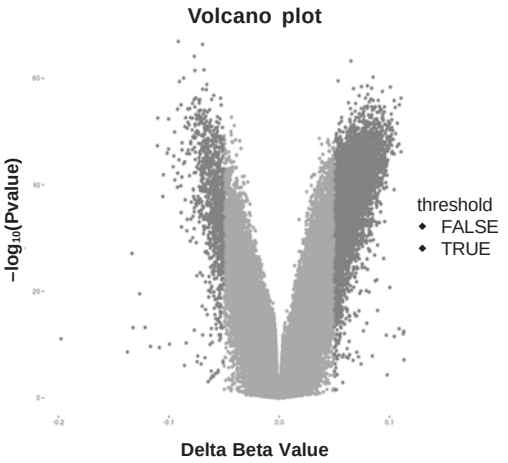

Fig. 1. Volcano plot exhibiting methylation data of rheumatoid arthritis (RA). X axis stood for the mean methylation differences between RA and normal. Y axis represented the log transformed P values
Source: Author

Subsequently, these initially differentially methylated CpGs were subjected to further filtering. The results showed that 5,421 CpGs (covering 3,319 genes) were detected after removing CpGs with β-values > 0.8 and < 0.2 were eliminated. Further, based on the cut-off threshold of > t0.1 average β-values difference across the two specific groups, total 50 differentially methylated CpGs were left, which referred to 45 DMGs (Table 1). ERCC3 (P = 4.48E-59), TNFRSF9 (P = 5.22E-57), HRH4 (P = 1.52E-56), PVT1 (P = 9.40E-56), and FOXP1 (P = 2.96E-55) were the most significant five DMGs for RA patients.

Assessment of DMGs

To assess the classification performance for 45 DMGs and validate the feasibility of the present method, a hierarchical clustering analysis was conducted. In consequent, 353 RA samples and 332 normal controls were correctly distinct, whereas 1 RA samples and 3 normal samples were wrongly attributed to normal samples. Thus, the accuracy for these DMGs was 0.9942, which suggested that the DMGs had a good classification performance across RA patients and normal controls and even enhanced the stability and confidence of the present study.

Table 1: Differentially methylated genes (DMGs)

Rank	*DMG*	*P value*	*Rank*	*DMG*	*P value*
1	ERCC3	4.48E-59	24	VCL	5.46E-47
2	TNFRSF9	5.22E-57	25	MYEOV	6.56E-47
3	HRH4	1.52E-56	26	CSNK1D	1.12E-46
4	PVT1	9.40E-56	27	ZFP36L1	1.21E-46
5	FOXP1	2.96E-55	28	WDR45B	1.59E-46
6	ZGPAT	5.20E-54	29	SORCS2	1.28E-42
7	RCAN3	2.91E-53	30	RUNX3	8.15E-40
8	IQCB1	4.53E-53	31	ATXN7	1.55E-38
9	SPTBN1	6.21E-53	32	TCF12	7.88E-28
10	PYURF	2.67E-52	33	FYCO1	1.91E-21
11	WDR49	2.71E-52	34	WDR20	2.85E-20
12	VRK2	1.78E-51	35	PDCD1	6.33E-14
13	GMDS	5.64E-51	36	CD1C	6.62E-14
14	LOC256880	6.33E-51	37	C6orf10	1.06E-13
15	PTEN	2.50E-50	38	MRGPRG-AS1	3.22E-13
16	DOCK2	3.39E-50	39	TMEM198	9.26E-13
17	TMCO3	7.56E-50	40	HLA-DQB1	2.85E-12
18	TRIM27	2.98E-49	41	MAGI2-AS3	8.17E-12
19	DRGX	3.32E-49	42	HLA-DRB6	2.19E-10
20	LINC00520	3.42E-49	43	DNAJB6	3.47E-10
21	RAD51B	5.56E-49	44	ASCL2	2.48E-09
22	FAM120B	2.23E-48	45	HLA-DRB5	7.44E-08
23	ADAMTS14	2.28E-48	46		

GO Enrichment Analysis

In an attempt to better reveal the potential biological functions of the DMGs, all these genes were annotated using GO annotation based on DAVID software. GO catergories with $P < 0.05$ were regarded as significant enriched. The result was illustrated in Figure 2, the researchers found that 15 significant GO terms were gained for RA patients, especially for regulation of B cell apoptotic process, lymphocyte costimulation, and regulation of leukocyte apoptotic process. Interestingly, among 15 significant GO terms, 3 were related to T cell, 3 were attributed to leukocyte, and 4 GO terms were correlated to lymphocyte.

Pathway Enrichment Analysis

Pathway enrichment analysis of all DMGs was conducted based on the KEGG automatic annotation server. Based on $P < 0.05$, DMGs were remarkably enriched in 10 KEGG pathways including Cell adhesion molecules (CAMs) (P=1.71E-03), Intestinal immune network for IgA production (P = 2.56E-03) and Asthma (P = 2.72E-03). The specific enrichment results were shown in Table 2.

DISCUSSION

DNA methylation, one of the most significant mechanisms involved in microRNA expres-

Table 2: Significant KEGG pathways with P < 0.05

ID	*Pathway*	*P value*
PATH:hsa04514	Cell adhesion molecules (CAMs)	1.71E-03
PATH:hsa04672	Intestinal immune network for IgA production	2.56E-03
PATH:hsa05310	Asthma	2.72E-03
PATH:hsa05320	Autoimmune thyroid disease	3.13E-03
PATH:hsa05330	Allograft rejection	3.43E-03
PATH:hsa05332	Graft-versus-host disease	4.07E-03
PATH:hsa05416	Viral myocarditis	4.94E-03
PATH:hsa04940	Type I diabetes mellitus	5.50E-03
PATH:hsa05150	Staphylococcus aureus infection	6.09E-03
PATH:hsa05321	Inflammatiory bowel disease (IBD)	7.58E-03

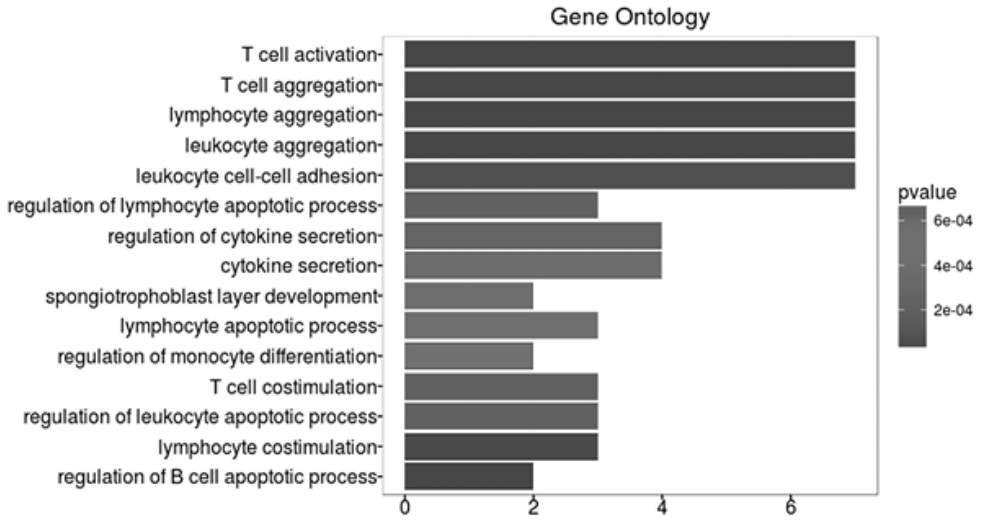

Fig. 2. Significant GO terms with P < 0.05 for rheumatoid arthritis (RA) patients
Source: Author

sion regulation (Nardone et al. 2016), gene silencing (Wu et al. 2016), and alternative gene splicing (Flores et al. 2012). Because DNA methylation is stable and easily detected qualitatively or quantitatively, it has been taken as the most promising diagnostic marker for the early detection of disease (Zhao et al. 2014). However, its dynamic patterns have not been analyzed at the genome scale in human pre-implantation embryos due to technical difficulties and the scarcity of required materials (Guo et al. 2014). Here pathogenesis in the development of RA was analyzed by bioinformatics systematically, including detection of DMGs, validation of DMGs and functional analyses for DMGs. On the basis of these results, potential mechanisms of RA were revealed, which might shed new lights on into OS diagnosis and therapy.

Based on the pretreated DNA methylation data, total 50 differentially methylated CpGs covering 45 DMGs were detected utilizing the *t*-test. Specifically, ERCC3, TNFRSF9 and HRH4 were more significant for RA patients compared with normal controls than the others. ERCC3 (excision repair cross-complementation excision repair 3), a subunit of basal transcription factor 2, encodes an ATP-dependent DNA helicase that functions in nucleotide excision repair (Ma et al. 2016). It showed a small but statistically significant increase of promoter DNA methylation in the exposed group compared with the unexposed group (Xing et al. 2013). Besides, alternative splicing of ERCC3 results in multiple transcript variants, and the dys-regulations even leading to disease of human, such as RA. TNFRSF9 (Tumor necrosis factor receptor subfamily 9) is a member of the TNF-receptor superfamily which contributes to the clonal expansion, survival, and development of T cells, and can also induces proliferation in peripheral monocytes, enhances T cell apoptosis induced by TCR/CD3 triggered activation, and regulates CD28 co-stimulation to promote Th1 cell responses (Blank et al. 2015). Coincidently, we explored 3 significant GO terms that related to T cell, which confirmed with it. Moreover, TNFRSF9 was reported to play a potentially important role in immune function (Eckstrum and Bany 2011). It had been demonstrated that DNA methylation could be identified as a potential biomarker response to anti-TNF therapies in RA (Webster et al. 2014).

Significantly, GO results of DMGs indicated that regulation of B cell apoptotic process, lymphocyte co-stimulation and regulation of leukocyte apoptotic process were more differentially expressed terms for RA patients comparing to normal controls. Regulation of B cell apoptotic process includes any process that modulates the frequency, rate, or extent of B cell apoptotic process (Xu et al. 2017). In addition, Cell adhesion molecules (CAMs) and Intestinal immune network for IgA production were the two important pathways for RA patients.

CONCLUSION

In summary, the researchers have revealed DMGs and their functional gene sets for RA patients, which might shed new lights on uncovering the molecular mechanism of RA and provide potential biomarkers for prevention and treatment of this disease.

RECOMMENDATIONS

Whereas, how the DMGs interacted with each other is still unclear, and the validations should be carried out in future.

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