Takayuki Sumida Tsukuba, MD, PhD

Editor-in-Chief

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Dear Dr. Tsukuba:

Thank you for your letter of Feb 23, 2016 regarding our manuscript entitled “Genome-wide DNA methylation patterns in CD4+ T cells from Chinese Han patients with rheumatoid arthritis”. Our appreciation also goes to the reviewers for their helpful comments. We have revised the manuscript following the reviewer’s comments and your instructions. All the update and change were red color bolded so that you and reviewers could recognize them quickly.

Enclosed please find the revised version of the manuscript along with a point by point description of our responses to the reviewer’s comments. We hope that the manuscript is now acceptable for publication in Modern Rheumatology. Thank you again for your letter and for your editorial assistance.

Sincerely yours,

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**Responses to Reviewer 1’s comments**

We first thank the reviewer for the helpful comments. In the revised manuscript, we have incorporated the reviewer’s comments.

The present study showed significant DNA methylation change in CD4+ T cells from patients with rheumatoid arthritis (RA) according to the genome-wide DNA methylation profiling. The results were relatively novel in this field, and its experimental technique was high-level. However, there are some concerns with the study design and text preparation.

Question: Page 4, paragraph 2: It would be better to explain why the authors have focused their attention on the CD4+ T-cells in the genome-wide DNA methylation study in the introduction.

Answer: Thank you so much for your suggestion. Definitely, it is a great comments. We add the following reason into the background section with red bold. Horneff and colleagues found that treatment with monoclonal antibodies against the CD4 antigen leads to immunomodulation would result in clinical benefits for RA patients (Arthritis Rheum, 1991).

‘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 (Arthritis Rheum, 1991), 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’

Question: Page 4, paragraph 3: Please include the information on smoking status and medication including glucocorticoids, because these have been reported to regulate DNA methylation levels. In addition, the authors did not show any methods and protocols for measurements of RF, anti-CCP, and ESR levels. Moreover, does the data of DAS28 in Table 1 mean DAS28-ESR or -CRP?

Answer: We apologies that we didn’t give comprehensive description to our sample information. We make these information more clear with current revision. We applied DAS28-CRP in our study. Thank you for your suggestion. The following content has been inserted into the manuscript. In addition, we added the RF, anti-CCP and ESR detection in the method section with red bolded.

‘All the samples that we collected were non-smoker and were newly diagnosed RA patients without long-term medication therapy.’

‘IgG class anti-CCP antibody ELISA kit (AESKULISA CCP, Germany). RF was me asureed by Siemen-s Dade Behring BN II Nephelometer. Erythrocyte sedim-entation rate (ESR) was achieved by Monitor-100 (VITAL DIAGNOSITICS, Italia) with routine test standards from the hospital laboratory’.

Question: Page 4, last paragraph: Please provide the more detailed information on the specific antibodies for determination of CD4+ T cells.

### Answer: Thank you so much for your suggestion. 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). We have added this sentence into the updated manuscript as the following with red bolded:

### ’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)’

Question: Page 6, paragraph 2: The authors should confirm and state that the confounding factors including age, gender, and smoking were comparable in their frequencies between the groups.

Answer: Thank you so much for your suggestion. We are serious to this question deeply. In this study, we spent long time to collect the samples so that we can highly guarantee that samples were rigorously matched including gender, age as well as race. In addition, in order to remove the effect of the smoking, we only selected all the individual without the smoking history. Finally, your mentioned the medicine consumption, it is really a great suggestion, we have realized that it was one of important confounders, however, it is hard to avoid that the patients have not taken as drugs before they were enrolled to our project in our hospital. Therefore, we enrolled the patients who didn’t take long-term medicine therapy before they come to our hospital with self-reports questionnaire. We forget to mention that since it is traditional operation in our methylation research. We hope it can be clear as the following sentence with red bolded:

‘All the sample that we collected were non-smoker and were newly diagnosed RA patients without long-term medication therapy’

Question: Table 1: It is difficult to understand the data of Table 1. Please add the column that indicates the RA and control groups, respectively, in the top stage. Please also include the mean and SD of parameter values in each group. In addition, statistical significance of differences in these mean values should be presented with P-value between the groups.

Answer: Thank you so much for your suggestion. You are right, the majority study they will show the samples as the way you mentioned in the question. However, in our study, we match the case and control one-by-one in age, gender, race and some other factors. And therefore, in the following analysis, we use paired-statistic test to make the difference inference. Therefore, here we descript our samples with pairwise style. Hope you can accept our study design and exhibition way.

Question: Page 8, last paragraph: It is recommended to indicate the correlations between DNA methylation levels and disease characteristics in additional new figures to improve readability. Otherwise, it is also fine to summarize all data in the new table.

Answer: Thank you so much for your suggestion. You are right. We didn’t do that in our first version manuscript since it is a very sparse matrix that it is hard to show it. In this version, we showed these significant association with Table 4.

|  |  |  |  |
| --- | --- | --- | --- |
| **Table 4. Clinical characteristics associated methylation loci identified by HM450K in RA** | | | |
|  | Gene | 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 |
| DAS-28 | *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 |

Question: Discussion: Please explain briefly what is novel in comparison to other similar studies in the first part of discussion. In addition, it would be appreciated to address the possible influence of significant hypomethylated HLA-DRB6, -DQA1, and -E on the pathogenesis of RA.

Answer: It is really a great question. We have known that genetic variation were quite different between different populations such as Asian, American and African. However, some evidence also found epigenetics such as DNA methylation have some population variations. Therefore, collecting different methylation data in different population is also very important. After the analysis to the dataset in Asian population, we did found the overlap of the significant differential methylated genes were quite small between two population. We admit that there are too many difference between two dataset, such as age, gender, race, smoking difference between two dataset and therefore the value for our manuscript is that we provided another material which can be used to find more accurate or specific RA related methylation loci. As to the second question, it is not very hard to understand since there are huge evidence which have been shown the relationship between HLA-genes with pathogenesis of RA, such as N Engl J Med 2011; 365:2205-2219. DNA methylation was also linked to gene expression, gene alternative splicing. Therefore, it might associated with RA through these pathway. In our following study, we would try to do the expression and gene alternative splicing analysis to provide more specific role of the methylation in RA pathogenesis. What’s more, the roles of the interaction between genetic variation and methylation variation can neither be omitted. We hope these data can be useful for our peers.

Question: Page 10, paragraph 2: Please add the reference number in the place of 'Jeffries' (lines 242, 245, and 249).

Answer: Thank you so much for your suggestion. We added the reference into the manuscript. Also, we replace ‘Jeffries’ with ‘Glossop’ to make it explicit.

**Responses to Reviewer 2’s comments**

In this article, authors identified differential DNA methylation sites in RA CD4 T cells. They also showed the association between DNA methylation and disease characteristics. Because a report based on the similar experimental design was already published by Glossop JR et al. (ref. 18), this article is not novel. However, the paper seems important for revealing the pathogenesis of RA. There are several issues that need to be addressed.

Major comments;

Question: Because they identified differentially methylated genes, such as HDAC4, NXN, TBCD, TMEM61, ITIH3, TCN2, PRDM16, SLC1A5 and GALNT9, in CD4 T cells, they should clarify the function of the genes in RA CD4 T cells. It would be interesting to reveal whether the genes have any important roles (e.g. activation, proliferation, cell survival, cytokine production etc.) in RA CD4 T cells.

Answer: Yes. We are quite agree with your idea. The roles of several of these genes in RA has been widely discovered in the previous studies, including:

1) *HDAC4*: 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, Tak et al. Arthritis Res Ther 2008](#_ENREF_1).

2) *ITIH3*: *ITIH3* was demonstrated as one of powerful biomarker for RA in the lasted study of [Liao, Chou et al. J Proteomics 2016](#_ENREF_2). 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 are essential to cells undergoing biological processes

3) *GALNT9*: *GALNT9* was showed aberrant methylated in RA patients in previous [John R Glossop](http://www.ncbi.nlm.nih.gov/pubmed/?term=Glossop%20JR%5Bauth%5D)’s study ([Glossop, Emes et al. Epigenetics 2014](#_ENREF_1" \o "Glossop, 2014 #1015)). GALNT9 could catalyzes the initial reaction in O-linked oligosaccharide biosynthesis, the transfer of an N-acetyl-D-galactosamine residue to a serine or threonine residue on the protein receptor.

4) The remaining 6 genes have not had enough previous evidence to show they were play roles in RA pathogenesis and development, including *MXN*, *TBCD*, *TMEM61*, *TCN2*, *PRDM16*, and *SLC1A5*.

Therefore, we added the following sentence to the updated manuscript:

‘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 diagnoses

Question: They mentioned the reason why they did not examine the gene expression in the discussion section. However, it would be interesting to investigate gene expression of differentially methylated genes, including HDAC4, NXN, TBCD, TMEM61, ITIH3, TCN2, PRDM16, SLC1A5 and GALNT9.

Answer: Yes. We are quite agree with your idea. It is a big flaw in our study design. We really should be extract the mRNA and conducted the RNA-seq simultaneously. We don’t want to conduct the RNA-seq or RT-PCR in another dataset since the expression were highly variated in different situation especially different genomic background, biological age as well as medicine consumption. We are publishing this study with shared microarray data so that other researcher can make full use of these data to make more accurate inference by combined different microarray data in different population. We hope you can understand and accept our motivation. As the expression mechanism investigation, we want to design another study which can detect the methylation and expression at the same time, especially in some well-accepted cell lines.

Minor comments;

1. In line 200, does DAS28 mean DAS28-CRP or DAS28-ESR?

Answer: Thank you so much for your suggestion. In current study, DAS28 is DAS28-CRP. We have replaced all DAS28 to DAS28-CRP.

2. In line 226-227, they compared DNA methylation between FLS and CD4 T cells. Because DNA methylation profiles as well as gene expression profiles are different in different types of cells, it seems meaningless to compare DNA methylation among different types of cells. I recommend that they should omit this sentence.

Answer: Thank you so much for your suggestion. We really happy to accept your comments and we have removed these sentence in the manuscript.