Author's response to reviews

Title: Genome-wide DNA methylation study of hip and knee cartilage reveals embryonic organ and skeletal system morphogenesis as major pathways involved in osteoarthritis

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Author's response to reviews: see over
19th August 2015  
Prof. Hiroshi Kawaguchi  
BMC Musculoskeletal disorders  

RE: MS: 1012042078176403 - Genome-wide DNA methylation study of hip and knee cartilage reveals embryonic organ and skeletal system morphogenesis as major pathways involved in osteoarthritis  

Dear Dr. Kawaguchi  

Thank you for your email of 10th Aug 2015. We have carefully considered the reviewer’s comments and suggestions and have revised the manuscript accordingly as outlined in the below responses to the reviewer’s comments. The changes in the revised manuscript are highlighted for your convenience.  

I confirm that the revision has been read and approved by all coauthors.  

My co-authors and I look forward to hearing from you.  

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Point by point responses to the reviewers’ comments  

Reviewer 1: Jose A. Riancho  

Authors are to be congratulated for their attempt to gather new information in the field of epigenomics of skeletal disorders. However, some issues need revision or clarification.  

Major compulsory revisions  

1. Age has a well-known influence on methylation. This is a critical point in the manuscript because of the difference between controls and patients with osteoarthritis. Authors must explore the possible association of methylation with age. Ideally they should repeat the analysis taking age into consideration as a covariate.
Response: We examined the association between the methylation of the 239 CpG sites that are potentially associated with OA and age. 28 of them showed an association with p<0.05, but none of these associations remained after correcting for multiple testing. Therefore we did not adjust for age in the analysis. We have now provided this information in the methods and results (lines 178-179, and line 219).

2. Authors state that the study is unique in identifying DMRs in developmental pathways. This seems to be an overstatement. In fact, their results are in line with other reports (for example, Delgado-Calle et al, Arthritis Rheum 2013) that should be included in the discussion.

Response: We have now added this point to the discussion and toned down the conclusion (lines 280-284).

Minor compulsory revisions

1. Please, expand figure legends to make them easier to understand without reading the text. For instance, in figure 1, specify the method used for dimension analysis (also in Methods section), as well as the general meaning of the axes and the figure as a whole, for the benefit of the non-expert reader.

Response: We have now provided more information to both the methods section and the figure legend (lines 189-201).

Discretionary revisions

1. Please, provide FDR values

Response: As stated in the manuscript, due to the small sample size we were not able to correct p-values for multiple testing. This was discussed as a limitation of the study. (lines 175-178, and 305-306).

Reviewer 2: Jesús Delgado Calle

In this manuscript Aref-Eshghi and col. describe changes in the methylome of cartilage tissue obtained from OA patients. The authors identified a total of 239 CpG sites differentially methylated between cartilage tissue from the hip or the knee of OA patients and cartilage from the hip of fractured patients. Pathway analysis revealed that enrichment in pathways associated with skeletal morphogenesis and development. There are some important issues that need to be addressed before the manuscript can be considered for publication. The authors need to highlight the novelty of their findings compared to previous similar publications. Moreover, validation and functionality of the differences found in some of the CpG sites differentially methylated in this study is needed. Specific comments are listed in the comments to the authors.

Major Compulsory Revisions

1. Authors use the term "healthy" in reference to the cartilage obtained from fractured patients. As discussed by the authors, this cartilage not necessarily represents healthy cartilage. Please change throughout the text.
Reponses: The term “healthy” was changed to “OA-free” throughout the manuscript, including the figures and tables. The term is now represented as “OA-“ in figures.

2. A better description of the samples used is needed. Were the samples obtained in each patient collected from the same area of the knee or hip? Was the status of cartilage degeneration similar between OA patients?

Response: We have now added to the methods section that the cartilage samples were collected from the articular surfaces of the tibial plateau or femoral head where the OA lesion occurred (lines 120-122). The cases had all end-stage OA, however, we did not perform histology scoring to comment on the severity of the cases. We have now discussed this as one of the limitations in the discussion section (lines 309-311).

3. Non-expert readers would benefit from references describing DNA methylation profiling (i.e. bisulfate conversion, beta value calculations)

Response: The requested references have now been provided to the methods (line 162)

4. Please include some references in the statistical analysis. How many genes were screened after quality control and distribution analysis? Authors should state in this section if they used test correction (i.e. FDR or Bonferroni). Would be more appropriate to analyze the differences between the three groups by one-way ANOVA?

Response: We have now provided the final number of CpG sites analyzed in the study to the results section (line 214). We did not correct for multiple testing as this was not realistic with the small sample size. Instead we only reported the sites with more than 10% beta difference and p<0.0005 (lines 175-178). Several references regarding the statistics were provided to the statistical analysis part of the methods (lines 164-168). Although ANOVA could be a good choice for identifying the sites varied across the three groups, we performed T-Test since we were mainly interested in the difference between the OA free and OA affected cartilage. We also examined the difference between knee OA and hip OA.

5. What is the rationale for including 800 loci in the phenotype clustering analysis? All these loci are differentially methylated? What were the criteria used for selection? Please describe in the text.

Response: The clustering is done for the subset of the CpG sites with the highest variation across the samples. It is not possible to show 400,000 sites in the heat map, and the majority of sites are not informative as they do not vary across the population. We calculated the cross population variance for each CpG site and performed the clustering for the top 200, 400, 600, 800, and above sites with the highest variance. The top 800 samples resulted in the best visual grouping of the three phenotypes while the numbers beyond this figure did not change the pattern. We have now added this information to the methods section (line 197-201)

6. Page 10, line 205. "sites with the highest methylation difference..." How was this group defined (i.e. above 10%)?

Response: Since it was not possible to show all the 239 sites in a table inside the manuscript, we selected the sites with more than15% difference from the comparison of all OA samples and OA
free samples and showed them in table 2, as we thought it might be of interest for the readers to get a sense of the difference of the sites and their characteristics in our study as they are reading the manuscript without the need of referring to the supplementary information which contains the full list of sites. We have now made it clearer in the manuscript (lines 228-229).

7. Authors mentioned that about one fifth of the sites found in this study were previously reported in other studies. Please include the manuscripts that were used as reference. In the current state of the manuscript, it is difficult to identify which are the new genes identified in the study. The paper would benefit from highlighting these novel findings (i.e. in a new column in the tables).

Response: The references discussed were added to the statement (line 258). We have now provided a new table in the supplementary material listing these CpG sites and their annotated genes (supplementary table 2).

8. Validation of the differences found in DNA methylation in at least one of the new CpG sites in more samples would benefit the paper (i.e. qMSP, pyrosequencing). Similarly, the functionality of the changes found needs to be tested; for instance, do the changes in methylation negatively correlated with gene expression?

Response: We agree with the reviewer that validation in all genomic studies is a crucial step. As well functional and translational studies will ideally reveal the mechanism of involvement. Unfortunately, we were not able to perform these steps due to budget issues. We have now discussed these as a limitation of our study (lines 311-314).

9. Previous publications by Delgado-Calle et al (2013), den Hollander et al (2015, 2014), and Moazedi-Furest et al (2014) described CpG sites differentially methylated in OA patients in Hox genes and other skeletal development related genes. Authors need to tone down their statements regarding the novelty of their findings and compare and discuss their results with those obtained in these studies.

Response: We have now toned down the conclusion on the novelty of the results and revised the discussion accordingly (lines 281-284, and lines 319-321).