Reviewer's report

Title: Genome-Wide Methylation Profiling of the Bronchial Mucosa of Asthmatics: Relationship to Atopy

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Reviewer: Jesús Delgado Calle

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Comments to the Authors

This is an interesting work suggesting a possible relationship between DNA methylation and atopy. The authors explored the methylome in the bronchial mucosa tissue of atopic asthmatics, non-atopic asthmatics and controls. They found 55 loci differentially methylated between atopic and non-atopic asthmatics. However no differences were observed between asthmatics and controls. Although data is potentially interesting the study has important limitations that must be stated and discussed in the text. Furthermore the manuscript needs to be revised to provide more general statements and updated to reflect the current thinking in epigenetics.

Major compulsory revisions

1. Methods, DNA methylation assay and differential DNA methylation analysis section.

More detail should be give of the array assay for non-expert readers. For instance what is interrogated by the assay, probes used, how beta values are calculated.

2. Statistical analysis.

a) Information regarding how DNA methylation results were analyzed should be included in a separate section, rather than in the DNA methylation assay and differential … section. Especially authors should also include information regarding how the clinical characteristics between subjects were analyzed. What kinds of tests were used? Did the variables follow a normal distribution? Please specify.

b) The method used to analyze the differences in DNA methylation is questionable. Parametric tests, such as the Student's test used in this manuscript, are suitable for continuous variables. However beta values range from 0 to 1. In this sense, beta values are usually transformed by using the following formula: beta/ (1-beta), thus transforming beta values into a continuous variable suitable to be analyzed by parametric tests. Additionally, since DNA methylation does not follow a normal distribution, data needs to be log transformed, in order to adjust to a Gaussian distribution. Lastly, significance levels should be corrected for multiple testing by the method of Benjamini to
control the false discovery rate (FDR). That being said, other authors have used similar methods to analyze similar DNA methylation data. This merits an acknowledgment in the discussion section.

3. Results, comparison of DNA methylation patterns of the BA and NC groups section. Authors analyze the differences between the NC and BA group. I assume that NC means normal controls. However, which patients are included in the BA group? Both atopic and non-atopic? This important information is missing. If the BA group includes both, atopic and non-atopic patients, the authors should analyze individually the methylome of the NC group against the methylome of the atopic group, and against the non-atopic group.

4. Figure 2C. Authors comment in the results section that this figure represents the heat map of the 55 loci showing differentially methylated regions between the atopic and non-atopic group. However, figure legend stated that the figure 2C displays the heat map of 2904 CpGs of chromosome 1. This needs to be corrected. A figure displaying the heat map of the 55 loci should be included in the manuscript.

5. Results, ontologies of the differentially methylated genes…section. Authors start the paragraph saying: “To examine the biological functions associated with the genes that had significantly hypomethylated and hypermethylated loci, we analyze…”. However, they perform gene ontology analysis using only the 48 hypomethylated genes. Why the 6 hypermethylated loci were excluded of this analysis? Could the exclusion of these genes affect the results? This should be clarified and discussed.

6. Discussion.

a) Paragraph 1. It is important to note that DNA methylation at regulatory regions is considered as one of the multiple determinants, but not the only, that define the expression profile of a given cell, at a given site and time. In fact, although generally DNA methylation at regulatory regions is inversely associated with gene expression, it has been demonstrated that several genes do not fit with this assumption, suggesting that other mechanisms, different from DNA methylation, has a major role in determining the fine tuning of gene expression. Therefore, the methylome, especially if a pooled sample is being analyze, may not directly reflect the global pattern of gene expression. Therefore, authors should revise this paragraph with this in mind.

b) Paragraph 2. This paragraph sounds very speculative and needs further development. First authors did not provide any evidence of changes in gene expression associated with those observed in DNA methylation. Second, they found some pathways overrepresented among those genes showing differentially methylated regions, however the role or influence of these pathways in asthma and/or atopy is not discussed. I recommend expanding this paragraph with new information in this late regard.

c) I think it would be helpful including a new paragraph to comment some of the
study limitations, such as the statistical analysis used, the sample size, absence of gene expression analysis, mix of cells present in the sample or whether these changes are the cause or the consequence of atopy.

Minor Essential revisions

7. In some places authors described 55 loci with differentially methylated regions and in other places 54. Please clarify.

8. Authors use the term Epigenesis instead of Epigenetics. Please correct.

9. Background section, paragraph 2 from line 14 until the end. Authors introduce the term Epigenetics and the role of these marks in the regulation of gene expression. It is important to note that epigenetic modifications induce stable changes in gene expression. Epigenetic mechanisms comprise DNA methylation, post-translational histone modifications and non-coding RNAs, not only deacetylation and DNA methylation. I suggest including a description of which regions are considered as CpG islands and their location (promoter, exon, intergenic…). DNA methylation usually is associated with gene silencing (instead of active coding regions), especially when DNA methylation occurs in regulatory regions such as promoters. This paragraph should be expanded and corrected to provide non-expert readers with this important information.

10. Methods, Subjects section. The English needs a little work in places, especially in the first 8 lines. This has several mistakes.

11. Methods, DNA methylation assay and differential...analysis section. The beta value is a quantitative measure of DNA methylation level of only a specific CpG per gene, and ranges from 0, when this CpG site is completely unmethylated to 1, when this CpG is completely methylated. This should be corrected.

12. Results, ontologies of the ….Df and Dp section. I recommend using Gene ontology analysis instead of ontologies.

13. Discussion, first paragraph, first sentence. The study only explores DNA methylation levels, thus methylome should be used instead of epigenome.

Discretionary revisions

14. Since authors comment that several environmental factors are associated with asthma, it would be interesting that the authors include some of the several studies suggesting that environmental influences may contribute to shaping the methylation pattern.

15. Authors found that among the 55 loci showing differentially methylated regions (DMRs), 33 were located in the gene body. Generally, DNA methylation at gene body is associated with DNA stability. Likewise, 31 loci showing DMRs were outside CpG islands. This kind of methylation is also generally associated with DNA stability. However, recent reports support the idea that DNA methylation outside CpG islands may have an important role (“shores”
methylation). It would be interesting that the authors take this into account when discussing their results.

16. What did the authors mean with the sentence: the extraction of RNA from airway mucosa tissue is not practical? It would be interesting that the authors explain what are the main difficulties/problems associated with RNA extraction in this samples.

17. Since authors do not provide any evidence that the microarray results are reproducible (validation by piroseq, qMPS,), the last sentence of the discussion section should be removed.

Minor issues not for publication

18. The text needs several language corrections (grammar and spelling) before being published.

**Level of interest:** An article whose findings are important to those with closely related research interests

**Quality of written English:** Needs some language corrections before being published

**Statistical review:** Yes, and I have assessed the statistics in my report.

**Declaration of competing interests:**

I declare that I have no competing interests.