Author's response to reviews

Title: Differential DNA methylation profiles in gynecological cancers and correlation with clinico-pathological data

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Author's response to reviews: see over
Response to Reviewer’s (Jayanthi Lea) comments

(1) What was the basis of testing the methylation profile of the genes mentioned in the manuscript?

Authors:
Gene silencing through DNA methylation is an important cause of cancer. We eagerly want to find molecular markers for better cancer management such as early diagnosis of cancer and disease prognosis. And usually, one particular marker may not be able to serve the purpose. Multiple markers are needed to achieve higher successful rate. So, when the idea of pooled DNA approach came to our mind, we just randomly picked genes that have been reported to be methylated and may have potential relationship to any human cancers. We carried out the analysis using more or less the same conditions as described in the literature and found that indeed the idea worked and potential markers for gynecologic cancers were identified. In consistent with other published data, we found that some genes were specifically methylated in a particular cancer while other might occur in more than one cancer.

(2) How are these genes relevant to cancer?

Authors: The genes selected in the present study have been reported to be methylated in human cancers. References of those genes in relation to cancer have been listed in the newly added Supplementary Table. The genes studied involve in multiple cellular pathways such as apoptosis (DAPK), DNA repair (MGMT) and cell cycle (p16).

(3) Why did the authors not to pursue the methylation status of CDH1 and MINT2 in cervical samples?

Authors
In this study, we did not design the primers by ourselves. We used the primers exactly same as those used by other investigators. In fact, in the literature, multiple research groups used the same primers for studying the methylation of many genes. While majority of the primers gave consistent result, just one to two gave variable results. CDH1 is the one with inconsistent result when pursuing the methylation status in cervical samples. For the MINT2, we could not trace back the position of the primers in the Ensembl Genome Browser database. The same happened to the MINT1, MINT31, MINT32 and FHIT. For the FHIT, using the same primers, Lea et al. (J Soc Gynecol Investig 2004; 11:329-337) reported that the aberrant methylation assay was not concordant with the FHIT gene expression. Thus, we should be very caution when using biomarker for cancer.

(4) Why were only cervical cancers included in the survival analysis?
For the endometrial cancer and ovarian cancer, the sample sizes are still too small for a meaningful survival analysis. In another study, we have studied the methylation status of nine genes in a larger number of ovarian cancer samples including benign, borderline and invasive tumors. A manuscript of the results of the above has been submitted for publication.

(5) The authors must clearly disclose the references that contain the primer sequences for the genes tested.

Authors:
The primer sequences and references have been listed in the newly added Supplementary Table.

(6) The manuscript needs more detail about the position of primers for each gene tested.

Authors:
The annealing temperatures, product sizes and positions of the primers for each gene have been tabulated in the newly added Supplementary Table.

(7) The discussion is too long and choppy. A succinct discussion of the results would suffice.

Authors:
The discussion has been revised.
Response to Reviewer’s (ALFONSO DUENAS-GONZALEZ) comments

(1) The background in the abstract just provides the objective of the study and repeat what is stated in the method section. A short paragraph on the methylation field is required.
In this sense, the methods section should describe the patient population studied, the techniques employed and the statistical analysis used for study correlations and survival.

Authors:
The abstract has been revised according to the reviewer’s comments. A short paragraph on methylation was added to the background. The original description was moved to the method section to rectify the repetitive statement. (Page 2, under Background and Methods sections)
In Conclusions section of the Abstract, the final conclusion is tuned down as suggested by the reviewer “More studies are needed to define the potential role of DNA methylation as marker for cancer management.” (Page 3, under Conclusions section)

(2) Background.
The basis on which authors decided to study these three gynecological cancers can not be justified on the “correlation in the embryonic development of ovarian epithelium, endometrium and cervix”. The etiology, and molecular physiopathology are quite different for these tumors, for instances, cervical cancer has a clear viral etiology; the endometrial cancer is clearly a hormone-dependent neoplasm and ovarian has other potential causes. The fact that authors wanted to compare the role of CpG methylation for the development and progression of these tumors on the basis that tumors share a similar embryonic development is not therefore supported.
The last sentence in the Background section “Potential epigenetic markers for diagnosis, prognosis and prediction of treatment outcome in the three cancers were also identified” is too strong. It was tried for cervical cancers but not for endometrium and ovarian.

Authors:
The reviewer is absolutely right that the etiology, and molecular physiopathology are quite different for these tumors. However, since these epithelial cancers all arise from the mesothelium of the embryo, this still supports the basis of our study to compare the role of CpG methylation for the development and progression of these tumors.
We agreed with the reviewer that the last sentence in the Background section is too strong. We replaced it by just stating that “Markers identified are used to correlate with
clinico-pathological data of tumors.” (Page 5, lines 4)

(3) Methods.
Authors should clearly specify whether the “normal tissue” was from blood in all cases or included “adjacent healthy tissues”. A description of the PCR controls should be added.

Authors:
The “normal tissue” has been clarified. This has been described in the paragraph under the heading “Patients” on Page 5.
The description of the PCR controls has been added to the end of the paragraph under the heading “DNA methylation analysis” on Page 6.

(4) Results.
It is intriguing that FHIT was found methylated in “all” normal DNAs at least in the representative cases showed in figure 1. Authors should point out this finding in the discussion. Authors report that 4, 4 and 5 genes were aberrantly methylated in cervix, endometrium and ovarian cancer and that “for other loci aberrant methylation was not indicated in any of the three cancers because methylated alleles were present in both tumoral and normal DNA or methylated allele was not detected in both. This should be presented in detail as it is not clear at least for this reviewer. This reviewer also wonders why if authors found genes specific for each of these tumors, they chose to study other such as p16, APC and PTEN in cervical cancer and the same for the other tumors. The analysis of the methylation and therapeutic outcome and prognosis in cervical cancer should be better described. Because cervical cancer was studied more in deep a separate table should be added for cervical cancer, and of course, treatments must be described more in detail and also included in the multivariate analysis. Is not clear for this reviewer what means “clear information of recurrence”. To include only 100 patients having this tag could be a source of bias. It is also confusing that for the OS and DFS survival, 116 patients were analyzed, this means that 16 patients with “unclear” information of recurrence were also analyzed?
Regarding the evaluation of response prediction, which were the pathological findings to consider a tumor radioresistant or radiosensitive? Was the biopsy taken after external radiation or after brachytherapy if done? Which was the treatment delivered?
Authors should also better describe the endometrium and ovarian cancer patients and the analysis for supporting the lack of association between methylation and recurrent disease in those patients.
Authors:
Concerning the FHIT, we have pointed out the discrepancy between the present findings and other studies in the last paragraph on Page 14.
Concerning the unclear part: “for other loci aberrant methylation was not indicated in any of the three cancers because methylated alleles were present in both tumoral and normal DNA or methylated allele was not detected in both”, we have described it in more detail in the first section of the Results section under the heading “Identification of CpG island methylation profiles in gynecologic cancers” on Page 8.
Concerning the statistical analysis for findings in cervical cancer, the reviewer did make a very good point. If we included all the 127 cases, then the DAPK lost its significant association with recurrence and DFS. Under this circumstance, we have re-written the whole section in the Results section under the heading “Methylation-based prediction for therapeutic outcome and prognosis” on Pages 11 and 12. We have also deleted Table 5 and Figure 4 from the manuscript.
Concerning the evaluation of response prediction, the answers to the reviewer’s questions have been answered and described in the last paragraph on Page 11 and Page 12.
Concerning the relationship between methylation and endometrial and ovarian cancers, more description was made in the last paragraph (on Page 12) of the Results section under the heading “Methylation-based prediction for therapeutic outcome and prognosis”.

(5) Discussion
The findings of the methylation at the endometrium and ovarian cancers should be discussed with other published results on the methylation at these genes in these tumors. The same applies for the findings in cervical cancer. There are at least four studies on cervical cancer analyzing methylation at these genes that should be discussed. The discussion on the meaning of DAPK gene for predicting response and prognosis should remain only if authors clarifies the issues raised on the “radioresistance” and on the recurrence assessment, also should also discuss on the marginal significance on prognosis (DFS) (p=0.041) in the multivariate analysis. On this regard, it could be useful to have an expert statistician reviewing the manuscript.

Authors:
As mentioned above, we have repeated the statistical analysis and confirmed that DAPK lost its significant association with recurrence and DFS when included all the 127 cases. The statement on the relationship between DAPK methylation and recurrence and DFS was deleted. In order to compare our own results with the methylation studies done by others, we are analyzing larger panel of methylated genes in individual samples of each cancer types. For
example, the frequencies of occurrence of methylation of nine genes were analyzed in a large panel of ovarian diseases including benign tumor, borderline tumor and malignant tumor. Concurrent methylation of 2 to 3 genes showed stage association and could be potential be used as an independent predictor of survival. We have submitted another manuscript to publish the above data.

(6) Conclusion
As stated before, the last sentence of the conclusion “This epigenetic event might be a potential molecular marker in diagnosis, prognosis and treatment of gynecological cancers” is essentially correct, however, this can not be assumed from this work. I would better suggest something like “more studies are needed to define its potential role.......

Authors:
We agreed with the reviewer comment and has tuned down the conclusion as suggested by the reviewer by adding the following sentence at the end of the conclusion “More studies are needed to further define the potential role of methylation DNA marker in cancer management.”

(on Page 16)