Author's response to reviews

Title: DNA methylation changes in ovarian cancer are cumulative with disease progression and identify tumor stage.

Authors:

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Version: 3 Date: 23 July 2008

Author's response to reviews: see over
23 July 2008

Dear Dr. Koutsos,

Please find enclosed a revised version of our manuscript titled “DNA methylation changes in ovarian cancer are cumulative with disease progression and identify tumor stage”. We are submitting our revision of the manuscript following the comments of the reviewers and in consultation with you. After receiving the reviewer’s comments July 7, we have taken two weeks to develop our response to the reviewer’s critiques. The changes made to the manuscript are detailed point by point below. The major changes to the manuscript are removal of the section in which we predict survival time, and re-analysis of the class prediction of benign (normal and LMP) versus Stage III cancer. When we re-analyzed the class prediction, we switched to less computationally intensive gene-selection and cross-validation methods that allowed us to perform analysis of the permutated p-value of the cross-validated misclassification error rate. This permutated p-value represents the odds of finding a random classifier that performed as well as ours on our data. We have also addressed the issue of bias raised by the statistical reviewer (Dr. Xiao). We were pleased with the reviewer’s enthusiasm for our study and its results. We believe the comments of the reviewers have led us to improve and focus the manuscript and hope that it will be approved for publication.

Thank you for your personal attention in getting this revision back out to the reviewers for a timely consideration following the 6-month initial review period due to problems finding a statistics reviewer.

Sincerely,

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Title: DNA methylation changes in ovarian cancer are cumulative with disease progression and identify tumor stage.

Version: 2 Date: 15 February 2008
Reviewer: Kenneth Nephew

Reviewer's report:
This is a well conceived and novel study. The study is unique in scope, as no such analysis has been reported for ovarian cancer (and few other cancers in that regard). The study is thorough and well done. The results are important and should be published.

Discretionary Revisions:

1) All tumors are classified as <2 or >4 years. Did the authors examine tumor samples falling between 2 and 4 years? If so, what were the results of those analyses? If 24 loci are tested, by chance alone, at least one marker would have a p-value of 5% (vs. no difference in methylation between short and long groups). The authors may want to comment on this.

Response: We have removed the section of the paper dealing with survival time prediction based on another reviewer’s recommendation and in agreement with the editors.

2) page 9-multiple periods in red text should be removed.

Response: Corrected

What next?: Accept after discretionary revisions
Level of interest: An article of outstanding merit and interest in its field
Quality of written English: Acceptable
Statistical review: Yes, but I do not feel adequately qualified to assess the statistics.
Declaration of competing interests:
I declare that I have no competing interests.

Title: DNA methylation changes in ovarian cancer are cumulative with disease progression and identify tumor stage.
Version: 2 Date: 8 April 2008
Reviewer: Robert Brown

Reviewer's report:
The manuscript by Watts et al describes the analysis of 137 benign and malignant ovarian tumour samples for DNA CpG methylation using a 6,560 element array containing CpG rich sequences. Differences in methylation have been identified using Differential Methylation Hybridisation based on differential restriction enzyme cleavage using the methylation specific restriction enzyme MceRbc. Samples analysed are: 9 benign, 17 Stage I, 15 Stage II, 54 Stage III, 15 Stage IV and 23 Low Malignant Potential (LMP) tumours. Prediction Analysis of
Microarrays identified a class predictor of 3 CpG rich sequences which distinguished Stage III from benign and LMP samples, which become hypomethylated in the Stage III tumours. More detailed analysis of NXK-2-3 CpG island using bisulphite sequencing and re-expression with a demethylating agent is presented.

Major Compulsory Revisions:

1. The analysis with clinical outcome has several deficiencies or lack of clarity. The authors note that the study was not designed to address this issue and it would perhaps be better to remove this section or provide greater detail. Specific problems with this part include:
   a) Whether the original classifier used a test and validation set (currently it reads as though this was done subsequent to the analysis)
   b) The power of the analysis is limited and is not commented on: only 47 samples.
   c) The analysis has not been stratified for types of chemotherapy or histological subtype of tumour. A multivariate analysis, using known prognostic markers for ovarian cancer in the model, would be best.
   d) The clinical relevance of <2 and greater than >4 year survival (a one year cut-off would be more clinically relevant, as this is used clinically to distinguish platinum sensitive and resistant relapse disease). Using survival as a continuous variable would be more appropriate. Also, does this mean that patients with 3 year survival are excluded? What is the rational for this?
   e) These types of prognostic studies should follow REMARK criteria (e.g. see Nature Clinical Practice Oncology (2005) 2, 416-422).

Response: After careful consideration of Dr. Brown’s comments we have decided that the best course of action is to follow his recommendation above, and simply remove the section in which we attempt to predict patient survival time. The great majority of patients in our study had progression free survival times longer than 6 months. Thus, we cannot conform our study to the expected standard analysis. Given that the results of our analysis were marginal, and the fact that our study was not designed to address patient survival time, we agree with Dr. Brown and have removed the section.

2. The differential methylation of the 3 class predictors of Stage III versus benign/LMP samples should be confirmed by bisulfite sequencing of a cohort of samples.

Response: We ask Dr. Brown to reconsider requiring this additional confirmation for publication. We showed confirmation that the CpG island microarray data is reliable in the manuscript when we confirmed differential methylation of the NKX2-3 CpG island. In addition, we have long experience using microarrays to study DNA methylation and have several publications in which we have confirmed microarray analysis of CpG methylation with bisulfite sequencing including:


Ignatenko NA, Yerushalmi HF, Watts GS, Futscher BW, Stringer DE, Marton LJ, Gerner EW. Pharmacogenomics of the polyamine analog 3,8,13,18-tetraaza-10,11-[(E)-1,2-


Given the long delay in the review process due to problems finding a statistics reviewer, we feel further delay as we obtain bisulfite sequencing data is undue. We are currently in the process of developing the classifiers described in our manuscript further using next generation sequencing technology and given our previous validation of the microarray data, we do not feel bisulfite sequencing is necessary.

3. It would be useful for the authors to discuss the limitations of the methods used to determine the false discovery rate. The method used simply uses repeat hybridisations of the same DNA, rather than repeat DNA extractions. This means that possible differences due to heterogeneity in the tumor sample are not accounted for.

Response: Both reviewers Drs. Brown and Xiao noted the issue with determining FDR based on technical replicates. As noted by Dr. Xiao, the selection of elements by statistical analysis followed by a fold-change cut-off while un-necessary from a statistical point of view, is common from a practical point of view to allow researchers to focus on the largest changes. The reason for using a fold-change cut-off was to focus on only those CpG islands with the largest changes in methylation. Since any fold change cut-off following statistical selection, however useful, is arbitrary and not required, we have re-written the section to emphasize the reviewer’s points and removed reference to FDR determination.

4. While for tumour types such as colo-rectal cancer there is clear evidence that late stage tumours can evolve from earlier precursor lesions, it is not clear that late stage ovarian tumours arise from Stage I disease. Indeed some authors have argued that these are Stage III tumours arise independently rather than progressing from Stage I disease. The authors should discuss whther their data addresses this issue.
**Response:** The question of whether tumor progress from Stage I through to Stage IV is an interesting one. Our data certainly suggests this is a possibility. We bring up the possibility suggested by our results in the discussion which has been modified and expanded to address the possibility of developing early detection markers from our work.

**Minor Essential Revisions:**

1. Authors should detail numbers of different types of subgroups examined in abstract and results. Some of the subgroups are small (e.g. 9 benign) and this at least allows the reader to appropriately interpret the data.

**Response:** We have added the numbers of sample subtypes to the abstract and results section.

2. The authors should describe clearly how NKX 2-3 was identified and chosen for further study.

**Response:** We have added our rationale for studying NKX2-3 to the results and discussion sections.

3. The authors should clarify whether it is known if NKX 2-3 is methylated in the cell lines examined.

**Response:** We have clarified this point in the discussion.

**Discretionary Revisions:**

1. The authors use the term “genome-wide” on a number of occasions, however only a subset of loci (6,560 elements) are in fact examined and the authors should at least comment at some point on the genome representation of CpG islands and/or CpG rich sequences this represents.

**Response:** We have modified the use of “genome-wide” throughout the paper when referring to the coverage of our CpG island microarrays

2. Given that mitochondrial sequences are used to normalise the data, whether any of the mitochondrial sequences have any homology on BLAST searches to nuclear genomes should be reported.

**Response:** Mitochondrial sequences are well established controls for DNA methylation and have been used by many lab, as well as Affymetrix, to control and normalize methylation microarray data.

Level of interest: An article of importance in its field
Quality of written English: Acceptable
Statistical review: Yes, but I do not feel adequately qualified to assess the statistics.
Declaration of competing interests:
Work in our laboratory is also conducting genome-wide analysis of methylation patterns in ovarian cancer. This work has been funded by Cancer Research UK,
Title: DNA methylation changes in ovarian cancer are cumulative with disease progression and identify tumor stage.
Version: 2 Date: 9 April 2008
Reviewer: Pearly Yan

Reviewer's report:

Minor Essential Revisions:

Please provide the rationale and any additional information for selecting NKX2-3 for the in-depth validation.

Response: We have added the rationale for studying NKX2-3.

As ovarian tumor histology was a confounding factor for the predictive power of hypomethylation in repeat sequences in the progression of disease stage, did it affect the correlation between promoter hypermethylation and gene expression?

Response: We feel that analysis of this aspect of the study in which we compared our methylation analysis with another lab’s is beyond the scope of the paper and would not change the conclusions of our study.

Please comment on how the outcomes of this study can be used for disease diagnosis/prognosis.

Response: We have added this to the discussion.

Level of interest: An article whose findings are important to those with closely related research interests.
Quality of written English: Acceptable
Statistical review: Yes, but I do not feel adequately qualified to assess the statistics.
Declaration of competing interests:
I declare that I have no competing interests' below. If your reply is yes to any, please give details below.

Title: DNA methylation changes in ovarian cancer are cumulative with disease progression and identify tumor stage.
Version: 2 Date: 20 June 2008
Reviewer: Yuanyuan Xiao

Reviewer's report:
The manuscript by Watts et al. investigates CpG methylation changes in ovarian cancer using a CpG island-based microarray. As my role of the statistical reviewer of this manuscript, I found it to be very well written and its publication will be of interest to a wide range of readers.
I have a few comments, however, which will only require a limited amount of extra efforts from the authors:
1) Using replicated hybridizations to estimate fold change FDR is a biased approach as the error structure in the real experiment concerns with biological variation not hybridization variation. So instead of examining pairwise replicate hybridizations, the authors should examine among the 9 independent ovarian samples. However, since the p-values derived from the ANOVA F-test is already multiple testing corrected by the Benjamini-Hochberg method, it seems unnecessary to estimate FDR separately for fold-changes and it is not uncommon for array studies to apply double criteria of significance and fold changes.

Response: This issue was raised by another reviewer as well. Dr. Xiao is correct that the fold-change is simply an after-thought since rigorous filtering is provided by the statistical analysis. The fold-change cut-off we used was simply a way to focus on those CpG islands with the largest changes. We have changed the wording of the results section to reflect this fact, and no longer make any reference to the technical replicates or any estimate of the FDR they may provide. Accordingly, we have removed figure 1 from the manuscript.

2) Typo: "Benjamini Hochburg False Discovery Rate" should be "Benjamini Hochberg..."

Response: Corrected.

3) I have concerns about how cross validation is applied in building the classifiers and estimating prediction errors in this study. The authors select discriminative features at the outset instead of within each cross-validation fold, for instance for the SVM classification of normal/LMP and stage III cancer, the authors selected genes satisfying p-value cutoffs of 0.01, 0.005, 0.001 and 0.0005 using all samples and before classification is even applied. This results in data re-use and will result in an under-estimation of classification error, because the "validation" set is also used in feature selection and therefore influences classifier construction. For references on this "honest" cross validation issue, please consult (i) Dudoit S. et al. In Microarray Data Mining (G. Piatetsky-Shapiro and P. Tamayo (eds)), Special Issue of SIGKDD Explorations, 5: 56-68. and (ii) West M et al, Proc Natl Acad Sci 2001, 98(20):11462-7. I think this problem at least needs to be mentioned and discussed in the Discussion section.

Response: We used BRB ArrayTools, developed by Richard Simon at the NHGRI, for the class prediction analysis. The cross-validation as implemented in BRB ArrayTools performs the entire analysis for each cross-validation of the training set from scratch – including selection of significant genes for all class prediction methods. In this way, the bias described above is avoided. We failed to clearly state this in the text, and have added sections to the methods and results to clarify the fact that only data from the training set was used to determine members of the classifier and that for each iteration of the cross-validation the significant genes were re-evaluated.

In addition, we have expanded our analysis by including additional class prediction methods in our analysis. In our re-analysis, we changed the gene selection and cross-validation methods to the more computationally manageable selection of sequences at a p<0.001 and the leave-one-out method of cross-validation. This has allowed us to perform an additional analysis: the permuted p-value of the cross-validated mis-classification error rate. In this
calculation, the class labels were randomly scrambled 2000 times and entire class-prediction and cross-validation re-done from scratch. In this manner we were able to estimate the proportion of the random permutations that gave as small a cross-validated misclassification rate as was obtained with the real class labels. Our results indicate that less than 0.001 random permutations performed as well as the that obtained with the real labels.

4) I think an M vs A plot for an exemplary slide is needed in the Additional Data section to give the readers a sense of data representation and quality.

*We have added three representative M vs A plots as Supplemental Figure 4.*

Level of interest: An article of importance in its field

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