Reviewer's report

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

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Reviewer: Robert Brown

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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 McrBc. 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)

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.

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.

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.

• 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.

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

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

• 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.

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.

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,
Ovarian Cancer Action and Orion Genomics (St Louis, USA).