Author's response to reviews

Title: Gross genomic alterations and gene expression profiles of high-grade serous carcinoma of the ovary with and without BRCA1 inactivation

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Author's response to reviews: see over
August 11, 2010

To,
Dr. Mellissa Norton
Editor-in-Chief,
BMC Cancer

Dear Madam,

We would like to submit our revised manuscript “Gross genomic alterations and gene expression profiles of high-grade serous carcinoma of the ovary with and without BRCA1 inactivation” for publication as an original article in your journal, BMC Cancer.

We would like to thank you and the reviewers for the very useful comments and we believe that the changes we have made to the manuscript based on these comments have significantly improved the readability of the paper. This is the first paper demonstrating that gross genomic alteration in high-grade serous carcinoma of the ovary is not associated with BRCA status, which is of particular interest given the central role of BRCA genes in both preservation of the structural and numerical stability of chromosomes, and in the pathogenesis of ovarian cancer. In addition, near identical gene expression profiles are observed in the three groups of cases defined based on BRCA1 status.

RESPONSES TO REVIEWERS’ COMMENTS

(Responses in Bold)

Reviewer: Jose Palacios
Reviewer's report:
This article observed no differences in gross genomic alterations and gene expression profile between high grade serous ovarian carcinomas with and without BRCA1 inactivation. The study is interesting because it suggests alternative pathways to BRCA1 dysfunction to produce genomic instability in a high proportion of high grade serous carcinoma.
Minor clarifications:
Had all cases without genetic/epigenetic BRCA1 alterations normal levels of BRCA1 mRNA expression? There are not alternative mechanisms of BRCA1 down-regulation, such as ID4 expression?

RESPONSE: As described in the Results section, there was a trend for BRCA1 to be more highly expressed in the group with no demonstrable BRCA1 loss compared to the other groups (web supplemental Figure 1). Therefore, cases without genetic/epigenetic BRCA1 alterations do not show BRCA1 down-regulation.

Should BRCA1 pathway functionally altered in spite of average BRCA1 mRNA expression?

RESPONSE: Our current analysis showed that a subset of high-grade serous carcinomas demonstrated preserved BRCA1 mRNA expression with no demonstrable genetic/epigenetic abnormality. It is plausible that defects in other components of BRCA1 DNA repair complex may be involved in these cases lacking demonstrable BRCA1 abnormalities, which would ultimately result in similar genetic instability. However, this notion remains speculative as no additional mutations related to BRCA1 functions have been implicated in high-grade serous carcinomas at the present.

Reviewer: Kwong-Kwok Wong
Reviewer's report:
Major Compulsory Revisions

1. The quality of the samples was not discussed. What percentage of tumor cells in each of the frozen tumors? The presence of normal cells in the tumors will greatly affect the expression profiles.

RESPONSE: Prior to RNA extraction and gene microarray analysis, frozen sections were prepared from on all frozen high-grade serous carcinomas tumor samples. All samples were confirmed to contain histological viable and representative tumor tissues with no contaminating normal structures/tissues present; the neoplastic epithelial cells in these samples typically comprise of >90% of the cell population as the non-neoplastic
stromal cells constitute a small minority of overall cellular fraction. This sample quality check step has now been described in the revised Methods section – “Prior to RNA extraction, frozen section analysis was performed and all tumor samples were confirmed to contain viable and representative tumor with no contaminating normal tissue structures.”

2. The authors failed to acknowledge other expression profile studies of ovarian tumor with BRCA1 such as by Tone et al., Clin Cancer Res 2008, 14:4067-4078 (raw data is available at GEO database as GSE10971) or Bellacosa et al., Cancer Prev Res 2010, 3:48-61. These prior studies did find genes with differential expression in normal ovarian epithelial cells with or without BRCA1 mutations. Microdissected tumor cells or low-passage ovarian epithelial cells were used for study. The authors should discuss their data.

RESPONSE: The findings from these prior studies that examined the gene expression pattern of non-malignant fallopian and ovarian surface epithelial tissues/cells in BRCA mutation carriers are now alluded to in the revised Discussion section. The primary focuses of these studies (and hence the study designs) are however somewhat different from ours as they compared the gene expression pattern of putative pre-neoplastic precursor tissues in BRCA mutation carriers to high-grade serous carcinomas, while our study was focused on exploring potential differences between groups of high-grade serous carcinomas with different BRCA1 status. The comparison between non-neoplastic normal ovarian epithelial cells with or without BRCA1 germline mutation is less relevant to our aim since 1) the normal epithelial cells without BRCA1 germline mutation most likely have not acquired the equivalent defect akin to BRCA1 germline mutation in the tumorigenesis pathway that lead to sporadic high-grade serous carcinoma and 2) these normal cells are all expected to possess normal diploid karyotype, hence a normal genetic landscape that is entirely different from the complex genetic changes seen in high-grade serous carcinomas.

“This is in keeping with an earlier finding made by Tone et al on a smaller series of 13 high-grade serous carcinomas (of either ovarian or tubal origin), where highly overlapping gene expression profiles were observed between cases with known BRCA1/2 mutation and/or family history and cases with unknown familiar status [30].”... “As such, their gene expression profiles irrespective of BRCA1 status would all show significant
dysregulation/difference from that of the putative tissues of origin in normal ovarian surface epithelium and normal fallopian tube as demonstrated previously [30,34].”

3. The authors concluded that all ovarian high-grade serous carcinomas arise through oncogenic mechanism that result in chromosomal instability irrespective of BRCA status is questionable. Probably some ovarian high-grade serous carcinomas arise through a BRCA dependent pathway while other are not and may through p53 pathway. For those samples with wild type BRCA, do they carry another p53 mutation?

RESPONSE: This question indicates that our conclusions were not clearly stated, with potential for misunderstanding. Our main observations in this paper were 1) all high-grade serous carcinomas display DNA ploidy abnormalities, hence genetically unstable, irrespective of BRCA1 status (i.e. whether BRCA1 is mutated, epigenetically silenced or intact) and 2) high grade serous carcinomas with different BRCA1 status show no significant difference in global gene expression profiles. These findings have led us to the conclusion that high-grade serous carcinomas arise through oncogenic mechanism that result in chromosomal instability irrespective of BRCA1 status, because tumors lacking demonstrable BRCA1 abnormalities exhibit the same degree of genomic instability (DNA ploidy abnormalities) and similar extent of gene dysregulation as seen in tumors harbouring BRCA1 mutation or epigenetic silencing. We do not imply that all high-grade serous carcinomas arise through a BRCA-associated or conversely a BRCA-associated pathway but rather those tumors with BRCA1 abnormalities and tumors without BRCA1 abnormalities seems to exhibit the same degree of genetic instability/dysregulation. We have clarified this more in the revised Discussion section – “While chromosomal instability can be accounted for in the BRCA1 mutant and BRCA1 epigenetically silenced groups, it will be important to identify the mechanism in the large group of tumors that lack BRCA1 or BRCA2 abnormalities and these may involve BRCA1/2-related mechanism(s) or non-BRCA1/2 related mechanism(s).”

Mutation in TP53 is a very common event in high grade serous carcinomas. In our previously published TP53 mutation analysis performed on a largely identical series of
the high-grade serous carcinomas, we have identified TP53 mutations in 88% of the cases and no difference was observed between tumors with different BRCA1 status.

4. The number of samples is still small and should be addressed. The expression level of BRCA1 should be measured by real-time RT-PCR in all the samples as another indication of the mutation status and epigenic status before SAM analysis should be performed.

RESPONSE: Our study sample size is as pointed by the reviewer somewhat limited in size and we have acknowledged this potential limitation in our revised Discussion section – “While the relative small sample sizes (n=8~9) of the different BRCA1-defined groups examined here may contributes to the paucity of consistent differences identified, it does represent the largest series examined to date and a larger number of differentially expressed genes can usually be identified between different tumor types with similar sample sizes. Therefore, the paucity of differences observed between these groups of serous carcinomas with different BRCA1 status is likely a reflection of intra-group non-uniformity and inter-group overlap in the gene expression patterns.”

With regards to BRCA1 mRNA levels, we have previously published the expression levels of BRCA1 by qRT-PCR analysis in a series of ovarian/tubal carcinomas that included the current series of high-grade serous carcinomas (reference 16 in the manuscript) and tumors lacking demonstrable BRCA1 mutation also showed higher BRCA1 levels compared to tumors with epigenetic BRCA1 silencing or tumors with BRCA1 mutation, similar to the findings of the current gene expression profile data. This previously reported observation is now described in the revised Results section.

5. In the gene filtering, gene with less than 1.5 fold of signal to background will be removed. In such case, if the gene is not expressed in reference RNA and BRCA mutation but in wt type BRCA will be filtered out and not used for analysis. In other word, when genes with the signal for reference RNA and BRCA mutated sample are close to background signal, but with strong signal in the wild BRCA samples, those genes will be missed.

RESPONSE: The reviewer points out an inherent limitation related to the quality-check step of microarray-based gene expression profiling platform in that the hybridized
signals need be discernible from background signals in order to be considered as real measurements. In the current analysis, in order to maximize our chance of identifying a difference in gene expression levels between different comparison groups, we have used a relatively relaxed quality check step in that the signal quality is considered acceptable if its intensity is 50% or more of the background. With this relaxed quality filter, the main concern is not under detection of true difference (false negative finding), but rather false positive detection. The fact that very little differences in gene expression profiles between groups with different BRCA status were identified with this relaxed quality filter supports the notion that these different groups indeed shows very little difference between them. We have also employed more stringent quality check threshold and no additional differences were identified. These issues with quality check are now better clarified in the revised Methods section - “To ensure that the measured signals reflect true readings, only spots with a ratio of signal over background of at least 1.5 in the Cy5 or 1.5 in the Cy3 channel were included.”

6. "Genes were filtered retaining only those whose expression levels differed by at least 4-fold in at least 3 samples" is not clear. Is the fold change between the tumor sample and the reference RNA?

RESPONSE: The 4-fold variation in at least 3 samples refer to the degree of variability in the expression level for individual gene across the entire series (n=26), with respect to the series average for the respective gene. With the more stringent filtering criteria used in the current study (4-fold difference in at least 3 samples), at least 3 tumor samples should exhibit a 4-fold difference from the series average in expression level for the gene to pass the filter. As described in the Results section, we also employed less stringent filtering criteria (2-fold difference in at least 3 samples). This gene filtering step is intended to eliminate genes whose expression levels remain relatively constant across the study samples (therefore not going to be a gene of interest since our focus is to identify genes whose expression levels between different groups). The purpose of this gene filtering step is now explained in more detail in the revised Method section.

7. The use of FDR of 5% is arbitrary. Have you tried to use higher FDR and validate the differential gene expression by RT-PCR?
RESPONSE: The use of a false-discovery rate of 5%, though arbitrary, is the widely used standard (some studies use even a more stringent FDR, particularly if a large number of genes were identified to be significant). As described in the report, only two genes in total (across the three separate comparison analyses) showed a significant difference between groups with different BRCA status with a FDR of < 5%. Even if we relax the FDR to <10%, the results still remain the same. We therefore do not believe there is a need to relax the FDR cut-off beyond the usual 5%. We however have added a supplemental Table (web supplemental Table 3) that displays all the genes with a FDR < 20%.

We additionally wish to point to a few changes made in the manuscript:

1. In page 10 line 1 “table 2” changed to “table 3”
2. In page 28 table 3 “FDR <0.5%” changed to “FDR <5%”

We declare that each author has seen and approved the final draft.

We look forward to hearing from you.

Sincerely yours,

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