Title: Identification of the IGF1/PI3K/NFkB/ERK gene signalling networks associated with chemotherapy resistance and treatment response in high-grade serous epithelial ovarian cancer

Authors:

Madhuri Koti (kotim@queensu.ca)
Robert J. Gooding (gooding@physics.queensu.ca)
Paulo Nuin (nuin@genedrift.org)
Alexandria Haslehurst (9ah33@queensu.ca)
Colleen Crane (cocrone@ohri.ca)
Johanne Weberpals (jweberpals@ottawahospital.on.ca)
Timothy Childs (childst2@kgh.kari.net)
Peter Bryson (bryson@queensu.ca)
Moyez Dharsee (mdharsee@ocbn.ca)
Kenneth Evans (kevans@ocbn.ca)
Harriet E. Feilotter (hf4@queensu.ca)
Paul C. Park (pcpark@gmail.com)
Jeremy A. Squire (squirej@queensu.ca)

Version: 2 Date: 13 August 2013

Author's response to reviews: see over
Reviewer's report
Title: Identification of the IGF1/PI3K/NFkB/ERK gene signalling networks associated with chemotherapy resistance and treatment response in high-grade serous epithelial ovarian cancer
Version: 2 Date: 26 March 2013
Reviewer: Trevor Shepherd

Reviewer's report:
Summary:
The majority of women with ovarian cancer are diagnosed with late stage disease. Although most initially respond to carboplatin and paclitaxel combination chemotherapy, a significant number of patients will relapse quickly with platinum-resistant disease. The purpose of the present study by Koti et al is to determine if there are prognostic gene expression networks that differentiate between tumours that will respond well to chemotherapy (longer time to recurrence) compared to those that did not respond sufficiently to chemotherapy (shorter time to recurrence). Affymetrix gene expression microarray analysis was performed using RNA isolated from tumours surgically removed prior to chemotherapy treatment, with one group of samples from patients who relapsed under 8 months and the other group over 18 months. Ingenuity Pathway Analysis identified several potential gene networks, which include IGF1/PI3K/NFkB/ERK and Myc-Rb, that are differentially expressed between the two cohorts. Gene expression differences were validated using four genes. The authors conclude that these networks may be implicated in differences in chemotherapy response and could be integrated into biomarker-based clinical trials.

Overall criticism:
This report represents a very limited depth of analysis and investigation into the differences in gene expression observed between their two datasets. There is no subsequent nor sufficient experiments performed to validate the pathways they have identified merely by IPA. With such small sample numbers in each set, it is unclear how statistically powered the study actually is. Given that, it would be imperative to compare their gene expression data with the numerous larger datasets which are publically available and have clinical data, particularly TCGA and Tothill datasets. In addition, if the authors are strongly convinced of the significance of the IGF1/PI3K/NFkB/ERK network as possessing important prognostic significance, these need to be supported by more evidence, such as IHC for pathway markers using archived tissues on the same specimens, and by systematically assessing these genes/pathways using larger datasets. Further specific criticisms are listed below. All in all, this study is far from complete for publication.

Response: The authors would like to thank this reviewer for his critical review of the manuscript and for his comments. This reviewer had overall concerns about several issues that are hard to address given the complexities of performing translational research in a rare tumour such as ovarian cancer.

He believes this sample size to be too small to provide adequate statistical power. The definition of a “small” sample size is clearly not appropriate given the exploratory
nature of this study, and the difficulty in obtaining high quality patient tumours with matched detailed clinical information. In addition, the patient sample size is comparable to similar investigative publications in ovarian cancer with similar objectives of addressing chemoresistance mechanisms (Bachvarov D, Int J Oncol. 2006;29:919-33; Bernardini M, Neoplasia 2005; Selvanayagam ZE, Cancer Genet Cytogenet 2004;154:63-6). Moreover the reviewer himself has indicated that he is not well qualified to assess the statistical methodologies.

This reviewer was also concerned that the tumour cohort cannot be described as “homogeneous”. The reviewer has clearly missed the point of the paper and we detail below how we have reworded the cohort description highlighted on page 2 of the revised manuscript to make this aspect of the experimental design clearer. Our overall goal was to identify gene expression differences primarily associated with progression free survival (PFS) as a surrogate of chemotherapy response. The only major distinction between the 12 patients with poor response in comparison to the 16 more favorable responders was their PFS. Otherwise the samples were “homogeneous” belonging to the same histological serous subtype, they were all high grade and they all underwent a similar chemotherapy regimen.

The reviewer is concerned that we “merely used IPA analysis”. In the field of ovarian cancer microarray analysis many recent similar papers (reviewed in Helleman et al., Gynecologic oncology 2010) on gene expression profiling have only provided a list differential expressed genes. Having a pathway context for experimental validation is a significant step towards future therapeutic interventions. The input gene list allowed us to generate network/pathway data with high significance in terms of the differential expression in the two cohorts. Similar earlier published papers include, Tao F et al., BMC cancer, 2012, 12:619; Yang et al., PLoS One, 2012; Jovov et al., PLoS One, 2012; van Beveran et al., PLoS One. 2012; Kong et al., BMC Genomics, 2011, 23:571; Wognum et al., PLoS One, 2009; have all used IPA to identify pathways of interest.

As per the reviewer’s suggestion, we performed an in silico validation of our results using selected patient samples from the TCGA database, and using PFS to make comparisons. Although we could not directly validate individual gene expression data in the TCGA cohorts we found that IPA analysis of genes sets derived from the TCGA data identified NFkB and ERK pathways. Significantly these pathways were also identified as over-represented in a recent publication (Barlin et al., 2013) that also interrogated the TCGA dataset to examine associations with disease recurrence. We have included the results of these new validations in the supplementary material (Supp. Table S2). In the discussion on page 12 of the revised manuscript we discuss recent findings from Barlin et al, (2013) in comparison to ours and suggest that multiple routes affecting different genes in the NFkB and ERK signaling axes lead to deregulation of both pathways in tumours with shorter PFS.

The Tothill dataset suggested by this reviewer does address the question of molecular
subtyping. Unfortunately there is no clinical outcome classification provided, so that it was not possible to use this dataset for validation purposes.

Compulsory Revisions:
1. Abstract: how is IGF1 the most strongly over/under expressed gene? It can only be one or the other.

**Response:** This statement reflects the role of IGF1 as the most differentially expressed genes in the two groups. Because we are not comparing absolute gene expression levels to any “normal” reference tissue we are not able to describe the expression as either under-or overexpressed. By using the term “over/underexpressed gene”, we were indicating that this gene is over expressed in the resistant but relatively under expressed in the sensitive group. On page 2 (abstract) of the revised manuscript we have modified this statement to clearly indicate that the overexpression of IGF1 observed in the drug resistant group is derived from comparisons to the levels present in the sensitive group.

2. Introduction, page 4: serous ovarian cancer should not be defined as a homogeneous group. The best examples are the TCGA and Tothill studies which clearly demonstrate the complexity and heterogeneity within and among patients with this histotype. In addition, high-grade serous epithelial ovarian cancer must be clearly defined as such (HG-SOC) to distinguish from low grade.

**Response:** We have detailed above why we describe our selected cohort as “homogenous”. The TCGA and the Tothill studies have included diverse (heterogeneous) tumors with limited outcome data and they are actually not good examples for performing a study of the type we present in this manuscript. The gynecologist oncologist (JW) who is an author on this paper has confirmed that all tumors in the study were correctly described as high-grade as stated in the original version of the paper.

3. Methods, page 4: these tumours should not all be classified as locally advanced, since only Stage II would fit this category.

**Response:** Almost all samples in this multidisciplinary study were collected by a gynecologist oncologist (a coauthor on this manuscript) who classified the samples using standard international guidelines.

4. Methods, page 5: Less than 8 months comes across as a vague classifier for trying to encompass partially-resistant. Have other groups used this classifier and time point?

**Response:** JW (Gynecologic oncologist) has responded to the reviewer’s question. Based on the PFS after completion of chemotherapy patients in this study are classified as platinum-sensitive (PFS > 18 months) or platinum-resistant (PFS < 6 months). The women who progress between 6-12 months post treatment were considered to have tumors with decreased sensitivity to platinum and were characterized as partially
platinum-sensitive (or partially resistant) (Markman, M. Gynecol Oncol, 69; 91-92, 1998). The intermediate group (6-12mo) is a poorly defined subgroup and as suggested by Markman et al, “a precise definition of the minimum required duration of the “treatment-free interval” to define resistance, versus potential residual chemosensitivity to a platinum agent, has never been provided based on the results on well-designed controlled clinical trials.” Certainly, it is accepted clinically that the progression from sensitive to resistance is a spectrum. Those patients who recur <6 mo can possess inherent sensitivity to platinum and those who recur >6mo can be inherently resistant. In our 2 groups of patients, the <8 mo and >18 months are two very distinct subgroups with a clear separation in their recurrence interval. Thus, we considered it best for the purpose of this study to include the 2 tumors in the partially-resistant group since their PFS interval was relatively short.

5. Methods, page 6: you should provide a source that recommends this approach of performing both the Welch two-sided t-test and Mann-Whitney non-parametric U test when using small sample numbers. Is the Welch test even valid if the distribution is not normal?

Response: We have described the statistical rationale in the methods section of the paper. As the second referee has noted, given the sample size we have employed the most reliable methods available. (N.B. The second referee has indicated he can assess statistical methods).

6. Methods: a significant amount of the methods section regarding the microarray analysis reads as results and should be re-organized into its proper section.

Response: We have modified the methods and results sections to address this comment.

7. Methods, page 7: “Therefore, on average there is an under expression of differentially regulated genes in the resistant cohort relative to the sensitive.” This statement does not make any sense and should be removed.

Response: The majority of gene expression differences in the two groups were such that the resistant cohort showed an overall average underexpression of genes relative to the sensitive. We have revised the text on page 8 to make this important concept clearer to readers.

8. Methods, page 7: Four genes were validated by qRT-PCR, this is a ridiculously small number of targets to validate. This needs to be increased, with a focus on representative genes in the networks.

Response: Selection of small number of genes for technical validation is common in the field of microarray based gene expression studies (For example this paper published in BMC cancer, used only a set of 5 genes for technical validation, “Expression profiling identifies genes involved in neoplastic transformation of serous ovarian cancer: BMC Cancer 2009, 9:378). Since the two groups are basically subgroups of ovarian cancer,
large differences in gene expression are not expected. Therefore, four genes (2 over and 2 under expressed) that showed the most gene expression differences were selected. Moreover, the technique of real time PCR, although efficient for cross validation is not ideal for differentiating minor changes <1.5 of expression. We also validated two other genes, MEIS1 and SYNPO2 that were present in the gene list with a p-value <0.05 (results not included in the manuscript). As discussed above both the Barlin study and our own analysis of the TCGA cohort validate the NFkB and ERK pathways involvement, therefore PCR based technical validation is not indicated.

9. Results, page 8: The subsection Correlations is vague and extraneous and should be removed, unless much more extensive comparisons are made with additional publicly available serous ovarian tumour gene expression datasets.

Response: Correlation plots are usually generated in gene expression studies to inspect sample-to-sample proximity and similarity (Verhaak et al., BMC Bioinformatics 2006, 7:337). Gene expression studies comparing two groups employ this tool to visually integrate the findings. In this section of the paper we intended to emphasize the robustness of the differentially expressed gene set that classify tumors based on PFS. The inter-sample correlation was performed to demonstrate the rigorous accuracy of the classification of the cohorts based on their mutually exclusive patterns of gene expression. On page 8 of the revision we have emphasized this aspect more clearly and the figure 2 legend has been improved to include the rationale for the correlation analysis.

Most of the publicly available datasets are derived from diverse platforms and they do not provide a distinct or comparable classification of chemotherapy response. Whilst this idea is interesting for future directions, it is not practical to provide a meta-analysis using existing public domain data.

10. Results, page 8: “Further, the chemo-resistant cohort is seen to be particularly homogeneous.” How was this homogeneity defined?

Response: As already discussed and highlighted on page 4 of the revised manuscript the rationale for describing the cohorts as homogeneous has now been detailed.

11. Results, page 8: Even with such few number of genes validated by qRT-PCR, there is no description of the results obtained.

Response: The genes for qRT-PCR validation were selected from the robust list of 32 genes that emerged by application of multiple normalizations. Depending on the fold change differences as well as based on their appearance in top network by IPA analysis (IGF1 and ZFP36). These results show concordance with the microarray data and provide us with information on the directionality of the fold changes resulting from microarray analysis. As per the reviewer’s suggestion, this section has been now modified to describe the results.
12. Results, page 9: overall, this is a poor description of the results regarding IPA from the gene expression data. It merely lists the few networks and the number of genes represented by each without even so much as reference to specific figures.

**Response:** The results section has been modified on page 9 to draw readers’ attention to genes that are commonly seen to deregulate the pathways.

13. Results, page 9: “The appearance of this gene [IGF1] in multiple analyses highlights its putative role in understanding the biology of the chemo-resistant cohort.” This is very weak argument and needs to be substantiated with a more rigorous set of additional experiments to back up this claim.

**Response:** This statement has now been modified to reflect the conclusions of the gene expression profiling as per the reviewer’s suggestion.


**Response:** As already discussed above and for point 10 and highlighted on page 4 of the revised manuscript the rationale for describing the cohorts as homogeneous has now been detailed.

15. Conclusions, page 10: These are all vague descriptions describing data from other studies looking at gene expression changes with respect to platinum resistance. The authors must try to make firm/direct connections to previous data or describe why their data may be different with specifics. For example, did any of these previous reports identify IGF1, or the PI3K pathway or NFkB signalling? This should be very straightforward.

**Response:** A number of earlier published papers that suggest similar findings (including one most recent paper based on TCGA ovarian dataset by Barlin et al., 2013, detailed in the discussion section) to address various questions have been included in the discussion section.

16. Conclusions, page 11: In the final sentence, the authors use the term “adapt”, but this implies changes in gene expression after a selective pressure such as chemotherapy treatment. Their study did not test this capacity since they looked at gene expression differences already present in tumours prior to chemotherapy administration. Unless they are suggesting that the pathways already present in the future resistant tumours provide an advantage to the adaptive response, then this should be better described as such.

**Response:** We were discussing the “intrinsic ability to adapt” in the conclusions, but the reviewer is probably correct that this may suggest “adapt to selective pressure”. We have therefore changed the word “adapt” to “respond” on page 13 of the revised
17. Figure 2: is this correlation map necessary?

**Response:** *The need for the correlation map is detailed in point 9 above.*

18. Figure 3: this is not an acceptable number of genes to validate from the microarray. Several more must be added and those should include ones that match genes within the identified networks.

**Response:** *We have already addressed this comment in point 8.*

19. Figure 4: should it not be the reverse that red is representing over-expressed genes and green are under-expressed genes? For example, IGF1 is red in Figure 4a.

**Response:** *Ingenuity pathway analysis uses its own color-coding to represent over and underexpressed genes. This feature is inbuilt in the software and thus cannot be modified. The exact fold change values are presented as gene lists (attached at supplementary file). Red is overexpressed in resistant compared to sensitive. Similarly, green is underexpressed in the resistant group compared to sensitive.*

20. Table 1: Should sample 1224 be excluded? Was this patient unstaged? Was HGSOC confirmed for this patient?

**Response:** *This patient is a High grade/stage case, which was confirmed by pathologist. Also, this patient belonged to the serous epithelial histologic classification.*
Reviewer 2: Ajit Narayanan

Reviewer's report: This is an interesting paper that studies resistance to platinum-based therapy for epithelial ovarian cancer. Data comes from 28 patients divided into shorter or longer progression free survival. Analysis of gene expression data indicates that IGF1 is the most strongly over-expressed/under-expressed gene, while pathway construction and analysis indicate a potential gene signalling network involving IGF1 and other genes. The research question is well defined.

The authors acknowledge the small number of samples from which they have drawn their conclusions. The paper describes a series of statistical procedures for ensuring that the data is reliable and of sufficient quality for drawing inferences. A variety of methods are used to reduce the interesting genes to, first, 434 probe sets, then 310 and then 219 before a final set of 204 genes is identified. A relatively tight p value of less than or equal to 0.01 was used together with a log2 fold change between 0 and 1 (except for IFG1) to result in a final set of 32 differentially expressed genes. Summarising the data analysis, it is difficult to see what else the authors could do to try to overcome the 'curse of dimensionality' (the large number of probes versus the small number of samples). Unfortunately, even with a 0.01 p value, this means that about 700 probes, genes and transcripts may be falsely identified as significant using the U133 Plus chip, but there is nothing the authors can do about that. I am satisfied they have done what they can with the data available to them. In particular, using qRT-PCR to support the measurements made by the arrays for four specific genes (Figure 3) also provides added confidence in the results.

On a statistical point, there are no non-ovarian cancer patient profiles included in the analysis. The authors may wish to make clear that, while the 32 differentially expressed genes separate the two groups, these may not be sufficient for distinguishing between ovarian cancer and non-ovarian cancer patients. That is, for some of the differentially expressed genes, there may be overlap with non-ovarian cancer patients also. Only the inclusion of non-ovarian cancer patient data may help us identify whether the 32 genes are specific to ovarian cancer treatment and separation using PFS.

Nevertheless, some basic cross-validation may have helped, such as leave one out, to add confidence that the final set of genes and signalling network are predictive as well as fit the data. But with such a small sample size, the removal of even a single sample for testing may lead to different reduced gene sets and signalling networks.

My comments (minor essential revisions) below are mainly requests for clarification that may help the material reach a wider audience.

(a) A brief description of the different stages of advanced ovarian cancer (IIa-IV) will help readers identify the severity of ovarian cancer in the patients sampled as well as understand the cancer better.

Response: The authors would like to thank the reviewer for providing helpful comments to improve the manuscript. A brief description on the different stages of ovarian cancer
has been added to the introduction section on page 3 of the manuscript.

(b) It is not clear who performed the histological classification. One pathologist was used to confirm greater than 70% tumour in all samples. It may be useful for the authors to say how confident they are that the groupings and confirmations are correct, given what appears to be one expert's evaluation. I think some further information here as to how initial classification was done would be helpful.

**Response:** Histopathological classification of all the samples was performed by a pathologist at the time of primary debulking surgery (prior to tumor sample banking) at the hospitals where samples were collected. Following sample accrual from the Ontario tumour bank and the Ottawa health research institute, Dr. Timothy Childs (TC; Pathologist at Kingston General Hospital and co-author on this paper) performed a histopathological analysis of all the samples.

(c) Some clarification is needed (please see comments above) that the purpose of the study is mainly to find models that fit the data rather than be used for predictive purposes. This could lead to some additional statements in the Conclusion as to what would be required to generate predictive models in this area in the future.

**Response:** Distinct gene lists emerged from gene expression analysis of our discovery cohort and the independent validation cohort from TCGA. However, alterations in the affected gene networks/pathways showed some overlap. This indicated that pathway alterations could occur by multiple routes along the signaling network. Similar findings concerning the diversity of gene expression affecting common pathways were also reported in earlier meta-analysis studies. The conclusion section has been modified to include these findings.

(d) The identification of IGF1 as playing a critical role in cancer is, as the authors point out, now becoming well established. The authors claim (p10) that the role of IGF1 in chemoresistance in ovarian cancer has not yet been reported. This may well be true, but IGF1 can be expected to play a role in chemoresistance if its role is as important as previous cancer studies have indicated. The authors explore in some detail other pathway genes (pp 10-11) and then infer that, according to their analysis, IGF1 may activate the PI3/Akt and ERK pathways. However, this linkage of IGF1 with these two pathways comes across as a leap of faith and not founded on any previous research linking IGF1 and the genes involved in these two pathways. It would greatly help the authors if they could cite any evidence from any source that shows that there may be a link between IGF1 and the genes on the two pathways. If they have already searched and not found any such link, perhaps this could be made clear in the paper in addition to their reference to the need for further work. It would also be of interest to readers if the authors could identify possible research designs (in very general terms) that would test the hypothesis that IGF1 was related to genes on the two pathways identified, bearing in mind that non-platinum drug treatments may also need to be tested against IGF1.

**Response:** The role of IGF1 in activation of PI3K/Akt and ERK pathways has been reported earlier (discussed in the conclusion section). Previously published papers establishing this role of IGF1 are included in the discussion section of the manuscript.