Author's response to reviews

Title: Carboplatin-induced gene expression changes in vitro are prognostic of survival in epithelial ovarian cancer

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Author's response to reviews: see over
Dear Editor,

We would like to thank the reviewers for their thoughtful evaluation of our manuscript and for their helpful comments. We have now revised our manuscript in accordance with ALL reviewers’ suggestions (see below), with inserted changes being underlined and in bold font for clarity.

Specifically, we have now added:

1. One figure presenting the deregulated genes between treatment and control for each time point

2. One figure illustrating that treatment with 100µM resulted in early apoptotic changes at 36 hours, followed by significant cell death at 48 hours

3. One supplementary document that discusses the theoretical principles of time series analysis.

4. Excel document including all genes that are up- or downregulated between treatment and control for each time point.

Furthermore please see our detailed responses to all the reviewers’ comments below.

We would like to thank you for your consideration

Dimitrios Spentzos,
Corresponding Author
Reviewer 1 (Dr Hasan Otu)

Major compulsory revisions:

1. We appreciate the reviewers comment that 3 data points are the bare minimum to achieve statistical significance with time regression analysis. We have raised this issue with Dr Richard Simon’s group at the NCI, which developed the Array Tools software, and they have confirmed that 3 data points with duplicates can indeed suffice. To address the reviewer’s question about referencing the method, we now include the BRB software’s user manual section describing in detail the principles of time series analysis (Time Series Plug-in, as a supplement to this reply). We have also added the Storey et al reference (“Significance analysis of time course microarray experiments”), which indeed has relevance to our analysis, as suggested by the reviewer. However, given the limitations of time series regression with 3 data points, we have now strengthened our analysis and present the deregulated genes between treatment and control for each time point (Results Section), as the reviewer requested. For this purpose we have added another Figure (Figure 2) illustrating the deregulated genes between treatment and control for each time point and these genes are included as a supplement to our manuscript (supplementary excel file). Of note the deregulated gene list overlap between the two analyses, was very high, specifically 88%, or 281 out of 317.
2. The time course analysis identifies genes whose variation of expression over time is different between carboplatin and control treated cells. The reviewer is correct in pointing out that genes that have similar variation of expression between carboplatin and control treated cells are not identified by time-course analysis. However, the primary goal of our study was precisely to capture genes with different patterns of variation between carboplatin and control treated cells – as genes with stable differential expression between platinum treated and control cells have been previously reported in the literature (references provided in the manuscript).

3. a) in Figure 1, only treated cells are included and fold change (FC) values are in reference to baseline-0 hours. The baseline-0 hours timepoint was not involved in determining the upregulated genes. We have now adjusted Figure 1 legend to reflect this; b) in Table 2, we present the top 10 genes (among the 317 genes identified by time-course analysis) with the highest upregulation (highest fold change compared to baseline 0) after carboplatin exposure at 36 hours. This explanation is now included in manuscript where reference to Table 2 is made.

4. The pathway analysis was not performed at each timepoint; rather we combined all time points for carboplatin and control treated cells. Since there has been no algorithm or software to apply time regression analysis and pathway analysis simultaneously, we recognize that by performing pathway analysis time-
dependent changes are not accounted for. This is the reason why we have separately performed pathway and time-course analysis so that information that is not captured by one approach is effectively captured by the other.

5. The 270 genes included in the pathway gene signature include all the genes of the 40 signaling pathways. The cytokine network is shown to have 5 genes (instead of the 24 genes included in the Biocarta pathway) because 5 out of 24 genes remain after filtering. Genes were filtered out if their log intensity variation percentile was less than 25% and/or if they were absent in more than 85% of the experiments. These filtering criteria are now included in the materials and methods section of the manuscript.

Minor Essential Revisions:
1. We have now added another supplementary figure illustrating that treatment with 100µM resulted in early apoptotic changes at 36 hours, followed by significant cell death at 48 hours (supplementary Figure 5).

2. All samples were included in the two risk groups in a leave one out cross validation. The data were informative of survival and the high risk samples had appropriately unfavorable survival values. As requested by the reviewer, we increased the number of permutations from 100 to 1000 for 2 survival analyses and the results remained robust. Specifically, the U133A-mapped subset of the pathway signature distinguished between patients with unfavorable and favorable
OS in Dataset 2 (median OS 31 vs 112 months, log-rank p<0.001, permutation p=0.04 (100 permutations), permutation p=0.036 (1000 permutations) and the U95A2-mapped subset of the pathway signature distinguished between patients with unfavorable and favorable DFS in Dataset 1, (median DFS 11 versus 17 months, log-rank p=0.01, permutation p=0.08 (100 permutations) versus permutation p=0.076 (1000 permutations).

3. Figure 1 legend has now been appropriately adjusted to state ‘denote fold expression changes’ instead of ‘denote expression’. We decided to leave time point ‘0 hours’ in the figure because we think that it is more illustrative for the readers.

4. As suggested by the reviewer, in order to support the validation of the carboplatin related genes we investigated the expression of the 10 most upregulated genes identified in our analysis in another dataset that included gene expression data (Affymetrix U95Av2 platform) from A2780 ovarian cancer cells treated either with cisplatin or control (Varma et al. Cancer Chemother Pharmacol 2007, 59(6): 711-723). Despite the different experiment design, and the different cell line, and after mapping probesets from U133 to U95, 7 genes (GDF15, GADD45A, ATF3, IL8, IL6, MAFF and TNFAIP3), were also upregulated in cisplatin treated cells versus control. This information is included in the Results section of the manuscript.
Reviewer 2 (Dr Ulrich Steidl)

Minor Essential Revisions:

1. Both pathway and time-course signatures were independently associated with DFS and OS when tested in multivariate analysis that included known prognostic factors of ovarian cancer including age and debulking status. Specifically, multivariate analysis showed that pathway signature was associated with DFS (adjusted Hazard Ratio: 2.04, 95% C.I. 1.1-3.78) and OS (adjusted Hazard Ratio: 2.2, 95% C.I. 1.05-4.57), while time-course signature was associated with DFS (adjusted Hazard Ratio: 2.52, 95% C.I. 1.36-4.67) and OS (adjusted Hazard Ratio: 1.96, 95% C.I. 0.92-4.2). This analysis has been incorporated in the results section of the manuscript.

2. We analyzed whether the combined expression levels of the genes that were differentially expressed at baseline between unfavorable and favorable prognosis tumors were sufficient for prognostic separation, and found that they were not associated with OS or DFS in either datasets. As postulated by the reviewer, this finding further strengthens the value of the approach of assessing dynamic expression signature. This is now included in the results section of the manuscript.

Discretionary Revisions:
1. We appreciate the reviewer’s comment but functional in vitro evidence was outside the scope of this study. However, the fact that some of the pathways identified from our analysis have been shown to be deregulated after platinum exposure (i.e. the ATM, FAS and epidermal growth factor receptor (EGFR) pathways) provides credibility to the notion that these pathways are related to chemoresistance.

2. In order to demonstrate the tight reproducibility of our array duplicates, we estimated the correlation between the two replicates for each time point, and found that it was very high (Pearson correlation coefficient >0.95, p<0.001 at all time points). This information has now been added to the Figure legend, in lieu of error bars.