Author’s response to reviews

**Title:** Identification of a candidate prognostic gene signature by transcriptome analysis of matched pre- and post-treatment prostatic biopsies from patients with advanced prostate cancer

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**Author's response to reviews:** see over
19 November 14

Dr Dafne Solera
Executive Editor
BMC Cancer

Dear Dr Solera,

Re: Manuscript ID: 1069870281140402 - “Identification of a candidate prognostic gene signature by transcriptome analysis of matched pre- and post-treatment prostatic biopsies from patients with advanced prostate cancer”

Thank you for your e-mail regarding the above manuscript. We thank the reviewers for their suggestions, which significantly strengthen the manuscript, and are pleased to re-submit with our detailed point-by-point response to the reviewers comments given below.

Yours sincerely,

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Reviewer 1:

MAJOR COMPULSORY REVISIONS:

1. The selection of differentially expressed genes for downstream knowledge-based examination is insufficiently explained (both in the main text and in the figure legends) and appears to involve some level of arbitrariness.

Response: We have clarified our selection strategy as follows:

Page 10, Lines 229-234 Results: “Based on the full gene list (Log2 fold change ≥ 2/≤ -2; FDR<0.05) (Table 2 and Supplementary Table S3), we ranked genes according to the magnitude of their fold changes, regardless of whether they were up- or down-regulated. The 10 top-ranking genes differentially-regulated by docetaxel plus ADT were selected (range of fold changes -9.96 to 9.86) for further downstream knowledge-based validation. From these 10 genes, we selected genes that exhibited expression changes consistent in direction in at least 3 out of 4 patients.”

Page 25, Lines 574-576 Figure 2: “Differential expression of genes affected by docetaxel chemotherapy plus ADT. (A) Log2 fold change of 6 of the 10 top-ranking differentially-expressed genes (Log2 fold change ≥ 2/≤ -2; FDR<0.05) consistent in the direction of expression changes in at least 3 out of 4 individual patients.”

The authors mention that differential expression was tested with a paired linear model using edgeR (lines 172-173, page 8). One therefore assumes that the up- and down-regulated genes mentioned in lines 214-215 (page 10) are differentially expressed between the group of 4 post-docetaxel samples and the group of 4 pre-docetaxel samples. However, in lines 223-225 (page 10) a selection of genes with consistent expression changes in at least 3 patients is mentioned. How was differential expression for each individual patient tested?

Response: When selecting candidates for cBioPortal analyses, we applied additional criteria: that these genes should also have a consistent direction of change of gene expression in three of the four patients as well as having an average change across all patients of greater than two-fold and for that change to be judged as significant in the edgeR model (Log2 fold change ≥ 2/≤ -2; FDR<0.05). This is clarified as discussed above.

The authors mention selecting the top 10 differentially-regulated genes but then state they identified 6 differentially expressed genes. How were the genes ranked for differential expression (LFC or FDR?)? What happened to the other 4? Is it that those 10 were significantly differentially expressed but only 6 of them showed consistent expression changes in at least 3 patients? Or is it that no statistical significance was used in ranking the genes and only 6 of the top 10 were significant? Whatever the case, it is not what is conveyed by the way it is phrased.

Response: We have clarified our selection strategy in the Results section as discussed above.

It is also not clear why only 10 (6) genes were chosen, given that cBioPortal can handle longer user-defined lists of genes. This immediately limits the number of biomarker candidates to be found.

Response: We ranked genes from the full gene list according to the magnitude of their fold changes, regardless of whether they were up- or down-regulated. The 10 top-ranking genes by fold change were selected for further downstream knowledge-based validation. From these 10 genes, we selected genes that exhibited expression changes consistent in direction in at least 3 out of 4 patients. This is clarified as discussed above.
Again, what was the criterion for deeming FAM72B and ADAM7 particularly impactful in terms of survival (line 236, page 11)? Were all the combinations involving any of the 6 genes tested and that proved to be most significantly associated with survival?

Response: We have clarified our survival analyses as follows:

Page 11, Lines 249-254 Results: “Survival analysis identified a statistically-significant reduction in disease-free survival of patients with tumours exhibiting alterations in expression of this geneset (p=0.023) (Supplementary Figure S1A) which was lost when FAM72B and ADAM7 were removed from the geneset (p>0.05) (data not shown). Survival analysis using only FAM72B and ADAM7 demonstrated a statistically-significant disease-free survival advantage in patients with no alterations in gene expression (p=0.001) (Figure 2C).”

Similarly, how were the 5 genes in Figure 3B selected from the 11 in Figure 3A? The authors mention that those 5 are the ones “which were significantly altered” (line 272, page 12) but so should the other 6, given that they were deemed to be up- or down-regulated (line 248, page 11).

Response: We have clarified our survival analyses as follows:

Page 12-13, Lines 312-317 Results: “Survival analyses did not identify any statistically-significant associations between disease-free survival time in patients with tumours exhibiting alterations in expression of these genes as compared with patients with tumours exhibiting no alterations in expression (p>0.05) (data not shown). However, when genes exhibiting alterations in high (>25%) proportion of tumours only were included in this geneset (Figure 3B), we observed statistically-significant reduction in disease-free survival of patients with tumours exhibiting alterations in expression of this geneset (p=0.024) (Figure 3C).”

2. In lines 207-212 (pages 9-10), the authors state they have observed 3 novel inter-chromosomal gene fusions. Apart from the inherent usefulness of identifying new disease biomarkers, what could be the relevance of these findings? Can something be said about the functionality of the resulting products? Moreover, given that the seminal reference on gene fusions in prostate cancer dates from the distant 2008, wouldn't it be straight forward, for instance, to run the gene fusion identification pipeline on a large number of samples from the TCGA dataset?

Response: The main aim of our study was to undertake expression profiling using RNA-Seq on prostate tumours pre- and post-treatment with docetaxel plus ADT. Although RNA-Seq yields information on recurrent gene fusions, our findings was merely observations of gene fusions in prostate cancer, and our study was neither designed nor powered to determine the wider biological significance of the gene fusions in prostate cancer. A recent (2014) TCGA-based analysis published by Stransky el al (Nat Commun. doi: 10.1038/ncomms5846) examined reported fusions involving kinases only. We feel that a further validation of our observations in this dataset is beyond the scope of our study.

MINOR ESSENTIAL REVISIONS:

3. The second paragraph of the Background (line 96, page 5) omits which treatment confers a survival advantage, although one guesses it is docetaxel based on the first paragraph. This ends up slowing down the reading with somewhat misconveyed information that is already included in the first paragraph. I recommend rephrasing accordingly.

Response: We have amended this sentence as follows:
Page 5, Lines 96-97 Background: “Early trials demonstrated an overall median ~2-3 month survival advantage for docetaxel-based therapies over standard treatments for CRPCa [3, 4], supporting its recommendation as first-line standard of care for CRPCa [1]. “

4. Lines 217-218 (page 10) and Figure 1B: the statistical significance of the correlation (and its actual value) between KLK3 expression and PSA levels should be stated.

Response: Both the correlation coefficient and a test of its significance have been added to the main text, figure, and legend as follows:

Page 8, Lines 175-6, Methods: “Correlations were identified using Pearson’s product moment correlation coefficient (p<0.05).”

Page 10, Lines 222-4, Results: “The levels of expression of KLK3, which encodes PSA (Prostate Specific Antigen), detected by RNA-Seq of the docetaxel plus ADT arm correlated as expected with serum PSA levels (r² = 0.992; p<0.05) (Figure 1B).”

Page 25, Lines 568-70, Figure Legends “(B) Correlation between KLK3 (encodes PSA) mRNA expression levels (X-axis) normalized by trimmed means of M-value (TMM) in normalized counts per million (ncpm) and serum PSA levels (ng/ml) (Y-axis) (r² = 0.992; p<0.05).”

DISCRETIONARY REVISIONS:

5. Figure 1B: are there zero values or wouldn't a log-log scale show the trend more emphatically?

Response: We feel that presenting the data on a log-log scale would artificially inflate the significance of the trend.

Reviewer 2

2. Are the methods appropriate and well described?

All the methods, both wet-lab and bioinformatics, are described in full, including software versions etc. One issue I think I should mention is that gene length bias is an issue in RNA-seq data, in which longer genes have higher counts (at the same expression level) than shorter genes with the same ‘nominal’ expression level. One method that takes this into account is GoSeq and provides the probability that a gene will be differentially expressed based on its length alone. None of their enrichment methods take gene length into account. It may be worth their while to investigate this (Discretionary Revision).

Response: We have substituted the p-values generated using the hypergeometric statistic with those generated with GoSeq. This has led to the identification of an addition enriched KEGG pathway (Steroid Biosynthesis) in the down-regulated gene list. We have updated the text to reflect this as follows;

Page 8, Line 176-180 Materials and Methods: “Enriched KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways [26] were identified by downloading gene pathways associations and testing each pathway for enrichment in significantly up- and down-regulated genes (FDR<0.05) with a transcript length-corrected Wallenius approximation as implemented by the GOSeq package for Bioconductor”

Page 11 & 12, lines 264-291 Results: The KEGG terms “Cell Cycle” (n=11/124; enrichment=5.89-fold; FDR=0.0014) and “Steroid Biosynthesis” (n=5/19; enrichment=17.63-fold; FDR=0.0014) were enriched greater than 2-fold in the down-regulated gene list
(Supplementary Table S4), while no pathways were significantly enriched in the up-regulated gene list. Genes within the KEGG term “Cell Cycle” included the key positive cell cycle regulators CCNB1, CCNB2, CDK1 and CDC25A (Figure 3A and Supplementary Table S6), the expression of which was down-regulated following docetaxel plus ADT. “

3. Are the data sound?

Yes, the data are sound. They have followed a good quality control assessment and they also mentioned their attrition rate (two samples were removed due to bad quality). They have submitted their data to GEO. I was unable to find any of the supplementary tables and therefore I am unable to comment on these (Minor Essential Revisions).

Response: These were submitted using the on-line system, however we should be happy to provide again if required.

6. Are the discussion and conclusions well balanced and adequately supported by the data?

Overall the discussion is balanced and well-supported by the data but they could discuss the sample size issue in more detail. One suggestion could be to use their data to estimate what sample size they require for more meaningful results. The paper reports mainly on how feasible it is to perform RNA-seq analysis on needle biopsies and a third of their samples failed. Could they discuss reasons for this and how the attrition rate could be lowered? Also, there is no mention on whether there is information on treatment response. It would be interesting to compare those that do but I do understand that sample size is a limitation here. Also, for the post-treatment biopsies, were there any changes in cellularity when compared to pre-treatment? This may correspond to differences between pre- and post treatment. Could they comment on this (Discretionary Revisions).

Response: We have amended the first paragraph of our discussion as follows:

Page 13 Lines 328-337 Discussion: “The limitations of our study include a targeted TRUSS-guided needle-core biopsy strategy that may result in heterogeneous tissue sampling with variable cellularity and small sample numbers due to the high quality RNA required for RNA-Seq (RIN>6 and total RNA>500 ng). Despite using fresh-frozen tissue samples, the high sample attrition rate (33%) from analyses prevented more meaningful clinical outcomes, such as treatment response, to be extrapolated from our results. Nonetheless, we clearly demonstrate the feasibility of this in vivo approach to obtain informative transcriptomic data from small tissue samples pre- and post-treatment with cytotoxic chemotherapy. As tissue sample processing and RNA-Seq methodologies are further refined, it may become possible to obtain reliable sequencing information from low input and/or degraded clinical samples [33]. “