Author’s response to reviews

Title: Dickkopf-1 is a potential novel mediator of cisplatin-refractoriness in non-small cell lung cancer

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

We are very grateful for having the possibility to submit our revised MS 2048730946141488 entitled “Dickkopf-1 is a potential novel mediator of cisplatin-refractoriness in non-small cell lung cancer” by Hogir Salim, Dali Zong, Petra Häåg, Metka Novak, Birgitta Mörk, Rolf Lewensohn, Lovisa Lundholm and Kristina Viktorsson. The reviewers’ comments and points have been of great value for us in improving our study. Please find below our comments to these points in an itemized way and with annotation to the manuscript where changes have been introduced. For the revision an additional author contributed to the study, Dr Metka Novak. Dr Novak is therefore added to the list of authors.

On behalf of the authors,

Kristina Viktorsson and Lovisa Lundholm

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Responses to reviewers’ questions for MS: 2048730946141488

Reviewer 1: Fredrik Jerhammar
Reviewer's report:
Major compulsory revisions

The discrepancy of the microarray results and lack of robustness is a major concern. In three replicates authors find only one overlapping gene. This gene is not studied at length. The authors have noted the discrepancy between replicates but even so decided to proceed. Optimization of the protocol and further analysis of three replicates with less discrepancy would have been preferred.

Comment: We agree with the reviewer that the heterogeneity among the replicates may be a concern. As we see it and also stressed in the manuscript (p.4, p.13 p.21) this reflects that cisplatin treatment can result in the expansion of different resistant clones in different experiments, giving rise to the observed heterogeneity. If this is the case, we would not get any better overlap even if we performed new replicate experiments using gene expression analysis. The reason is probably that using our setup, we are not studying any primary events occurring after cisplatin treatment, but instead the long term effects after a pulse treatment which in fact are very heterogeneous.

Nevertheless, it was still possible to identify interesting targets using this approach as illustrated by DKK1. In relation to DKK1 we have indeed found it to confer cisplatin sensitivity when ablated with siRNA in non-small cell lung cancer (NSCLC) and ovarian carcinoma. With respect to FMN1, we have in relation to the reviewer’s point also studied its role in cisplatin refractoriness by overexpressing it using a plasmid in NSCLC cells as indicated on p. 10, with results presented on p. 14, and in supplementary figure 2 in the manuscript. Albeit we do obtain overexpression, it did not confer cisplatin sensitivity. Our interpretation of these data is given on p. 22: This data suggests that either the observed down-regulation of FMN1 in cisplatin refractory clones is not directly associated with resistance, or FMN1 down-regulation acts in concert with other signaling components in order to regulate cisplatin responsiveness which not is recapitulated when forced overexpression is used.

Further evaluation of DKK-1 in tumor samples would strengthen the data significantly.

Comment: We do agree with the reviewer that further studies of DKK1 in tumor samples would be interesting. However, as pointed out in the submitted manuscript, DKK1 has already been demonstrated to be connected to NSCLC. Thus in NSCLC patients high serum and tumor tissue level of DKK1 has been detected and found to be associated with tumor progression and poor outcome (Ref 20: Yamabuki T, et al., Dikkopf-1 as a novel serologic and prognostic biomarker for lung and esophageal carcinomas. Cancer research 2007, 67(6):2517-2525.) By using the cBioPortal for Cancer Genomics (cbioportal.org) which integrates data from several databases including The Cancer Genome Atlas, we also found DKK1 to be altered in up to 10% of NSCLC patient samples, p. 24.

Yet, DKK1 expression in relation to cisplatin response in vivo remains to be shown. However, in order to carry out such analyses it would require a highly controlled cohort where samples can be taken just prior to treatment and also during the treatment course. In NSCLC such analyses are challenging since sampling of tumor is an obstacle. It would of course be possible to monitor DKK1 on primary tumor material taken at surgery however it is associated with several caveats as the protein expression of DKK1 may have changed during the course of the tumor from its localized growth to spread and metastasis, the stage at which...
Minor essential revisions
The title suggests that Dickkopf-1 is a potential novel mediator of cisplatin-refractoriness in non-small cell lung cancer, which is a farfetched conclusion given that this gene is not deregulated even in the three replicates.

Comment: The title is based on the fact that siRNA towards DKK1 indeed sensitized NSCLC cells to cisplatin. Thus albeit only deregulated in one of the biological replicates of gene expression analyses, siRNA in fact demonstrate a role for DKK1 in sensitizing to cisplatin.

Discretionary revisions
Including more cell lines reflecting both sensitivity and resistance would have been a useful approach. The genetic background and deregulation of genes of only one cell line makes the data of limited value for the clinical situation.

Comment: When it comes to the conclusions regarding DKK1, we have already tested the siDKK1 in two NSCLC cell lines, U-1810 included in Fig 5 and A549 in Suppl.fig. 2, representing large cell and adenocarcinoma, respectively. It would of course be interesting to further examine it in other NSCLC cell lines. Given the limited time of the revision we however in response to this comment evaluated the effect of DKK1 siRNA in another tumor type, ovarian carcinoma, where cisplatin is part of the standard regimen and where an isogenic cell system of platinum responsiveness and resistance was at hand (i.e. A2780 and A2780 cis) as indicated on p. 6. We have added a new Figure 5F-G where we show that A2780 cells also can be sensitized to cisplatin in the presence of DKK1 siRNA. The acquired resistance of A2780 cis was however more difficult to target at the tested conditions, but these cells also had a higher baseline DKK1 which fits with our data of the involvement of DKK1 in cisplatin resistance. The results and discussion sections were updated on the following places p.10, p.19 and p.23.

A resistance mechanism of FMN1 is discussed and speculated upon in the paper but not tested experimentally which would have been interesting. A simple cell adhesion assay with or without cisplatin treatment in cells with differing expression patterns of FMN1 could have been informative.

Comment: With respect to FMN1, we have in relation to the reviewer’s point also study its role in cisplatin refractoriness by overexpressing it using a plasmid in NSCLC U-1810 cells as indicated on p. 10, with results presented on p. 14, and in supplementary figure 2 in the manuscript. Albeit we do obtain overexpression, it did not confer cisplatin sensitivity. Our interpretation of these data is given on p. 22 and described above.

Reviewer 2: Vildan Bozok Cetintas

Reviewer's report:
This paper reports DKK1 as a possible target to cisplatin response in NSCLC cells and emphasis the 2-fold down regulation in expression of the anti-apoptotic protein BCL2 in cisplatin-treated samples with ablated DKK1.
According to the figure 5C there is no significant difference between just siRNA treated and siRNA+ cisplatin treated samples. I think further studies should be performed for apoptosis. 

Comment: The difference in the scanned plates of Figure 5C is perhaps is not so clear, this is however only an illustration of the data summarized in Figure 5D. We chose to only include the statistics for the comparison si+cis vs NT+cis. There is however a significant difference between DKK1 siRNA alone and siRNA+cisplatin, for both si1 (p<0.005) and si2 (p<0.05), using a t-test.

We agree with the reviewer that it would be of interest to further confirm that DKK1 targets apoptotic pathways, and not other death modes which are included when we use clonogenic assay to detect the response. In the cell viability data from siDKK1 in A2780 the effect is however measured after 72 h, where the majority of cell death events might be suspected to be due to apoptosis. These new results therefore support the BCL2 data. We have included a sentence in the results on p. 19 and in the discussion on p. 26, “Yet the importance of this down-regulation and the role of DKK1 in regulating cisplatin-induced apoptotic signaling would require further studies”.

There are some figures without standard deviations and p values (3 and 5) Are the RT-PCR experiments performed for single sample?

Comment: Yes, for Figure 3, the RT-PCR data is from single samples only. This was done to confirm the RNA expression changes from the microarray data in the same sample using RT-PCR for the replicate specified. Still, technical duplicates were performed. For Figure 5A, the data presented is from two separate experiments. Therefore, neither of these graphs display standard deviations.