Author's response to reviews

Title: Increased expression of Transcription Factor TFAP2 alpha correlates with chemosensitivity in advanced bladder cancer

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Author's response to reviews: see over
BMC Cancer
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Dear Editor-in-Chief:

Thank you for reviewing our manuscript entitled “Increased expression of Transcription Factor TFAP2\(\alpha\) correlates with chemosensitivity in advanced bladder cancer” and for giving us the opportunity to submit a revised version.

We have now included a ‘Competing interests’ section and correctly placed the Author’s contributions and Acknowledgement section.

We have found the comments from the reviewers very constructive and inspiring and have tried to address all points in the revised manuscript. The manuscript was corrected for language and the abstract section has been reformulated.

We hope you will find the manuscript suitable for publication in BMC Cancer.

Respond to referee 1:
Reviewer: Srikala Sridhar

Reviewer’s report:
Overall this paper by Nordentoft and colleagues attempts to understand chemosensitivity in advanced bladder cancer by studying the expression of a transcription factor known as TFAP2 alpha. Since response rates to cisplatin based chemotherapy are only about 50\%, this is an important area of study.

However, this paper was somewhat difficult to follow.

Major compulsory Revisions
1. Introduction
Page 3, Line 3: Further details should be provided about the other predictive markers BIRC5 and BSG.

Author rebuttal:

We have added further details about this and we now write:

“\textit{BIRC5} (survivin) and \textit{BSG} (emmprin) were validated by immunohistochemistry in an independent material of 124 patients with locally advanced (T_{4b} and N_{2-3}) or metastatic (M_1) disease receiving cisplatin-containing therapy as independent predictive markers for response and survival after cisplatin-containing chemotherapy”
Reviewer:

2. Discussion

a) Line 4: There needs to be discussion as to why high levels of TFAP2alpha protein expression predict a better outcome in lymph node invasive dz vs. non-lymph node invasive subgroup. For example, other than actual involvement of the lymph nodes are there other molecular differences known to exist between lymph node+ and negative cancers that could explain this? Specifically with respect to to p53/p21?

b) There is a fair amount of information about other cancers, as opposed to a more detailed discussion about bladder cancer itself.

c) There should be some discussion about future directions of this research, and how these results could be further validated.

a) We added the following sections to the discussion:

1

Interestingly, node positive colorectal cancers showed significant losses for p21 and E-cadherin compared to node negative cancer. TFAP2α directly binds to the promoter of E-cadherin, where it has been previously reported to act as a transcriptional activator. High E-cadherin expression has been reported to increase cisplatin and gemcitabine sensitivity in pancreatic cancer.

2

In non-small cell lung cancer, expression of p53 and p21(Waf1) in mediastinal lymph node specimens were significantly related to the response to platinum chemotherapy

b) We have added the following information to the introduction:

Presently, there are two standard chemotherapeutic regimens: MVAC (methotrexate, vinblastine, doxorubicin, and cisplatin) or GC (gemcitabine and cisplatin). Median survival is 14 to 15 months, and 5-year overall survival rate is between 13% and 15% (von der Maase H, ClinOncol 2005). Although the gemcitabine and cisplatin combination has a significantly better toxicity profile, both regimens still carries risk for significant toxicity and toxic deaths (von der Maase H, ClinOncol 2000) and a substantial fraction of patients will suffer from adverse reactions without achieving any benefit.

c) We have added the following section to the conclusion

Future studies are needed to further validate the predictive potential of TFAP2α expression in bladder cancer. We are collecting bladder tumor samples (locally advanced T3-4, N1-3 and/or metastatic M1) from patients that have been treated with cisplatin based chemotherapy and which have been characterized according to the RECIST response.
criteria. This cohort will be used to evaluate if TFAP2α staining and expression are predictive for cisplatin response in addition to survival.

We had selected a total number of seven molecules from the signature and tested antibody specificity on protein extracts from cell lines overexpressing the respective recombinant protein. Immunohistochemical analyses of the tissue microarrays using the specific antibodies did not show significant clinical correlation and were thus excluded from in vitro functional analyses. However, some of the molecules were borderline significant. In light of these findings we conclude that a transcript signature obtained on archival material cannot be transferred to the protein level. We suggest that future studies should focus on the analyses of ribonucleic acids including microRNAs which can be accessed from archival material. In addition, we will use a larger number of patient samples from more defined patient cohorts.

Reviewer:

Introduction:
Page 3, Line 2. Punctuation after "resistance" should be removed.
Line 19. Punctuation before [6] should be removed
Line 19. Currently many studies...is a run on sentence and should be divided.
Discussion:
7th line from the end: Punctuation after Furthermore should be removed.

Author rebuttal:
Punctuations have been removed.
Furthermore, we have now reformulated the sentence and now write:
"Currently many studies have linked deregulated TFAP2α activity to malignant transformation. The TFAP2α gene locus at 6p22 is frequently lost in various cancers"

Reviewer's report (2)
Reviewer: Alexandre Zlotta

Reviewer's report:
The authors found that high levels of nuclear and cytoplasmic TFAP2alpha were predictors of increased overall and PFS in BC patients treated with cisP based therapy. TFAP2alpha silencing increased Cis and Gem sensitivity.
siRNA directed knock down of TFAP2alpha stimulated SW780 cell line proliferation whereas in p53 mutated T24 it did not affect proliferation.
TFAP2alpha induces p21 dependent transcriptional activation.

General comment
Studies improving our understanding of CisP responsiveness or absence of response to chemo in BC patients are utterly needed and therefore the authors
should be commended for trying to bring an additional piece of information.

1. There was no association between TMA staining and overall and PFS when applying the analysis to the entire cohort of patients whereas it did reach statistical significance in patients with lymph node invasion. Although the authors refer to 2 previously described multicenter adjuvant chemotherapy trials using 2 different chemo regimens, it does not appear very clearly how many patients had node positive disease, what the extent of the lymph node dissection was. A table summarizing the data, even if these have been previously described would help the manuscript to be more reader-friendly.

**Author rebuttal:**

We completely agree and a table characterizing the patient samples has been added. This was uploaded as Table 1.

**Reviewer:**

1. The kappa for TFAP2 alpha between researchers is far from breathtaking (0.75 and 0.68).

**Author rebuttal:**

Naturally we would have preferred higher kappa values, however Landis and Koch, characterized Cohen's kappa coefficient values in the range of .61–.80 as substantial and Fleiss's characterized kappa's of .40 to .75 as fair to good and over .75 as excellent and therefore we are confident that the calculated TFAP2 alpha kappa values are within an acceptable range.

**Reviewer:**

Any comment about any applicability of this marker in future Studies

**Author rebuttal:**

We are trying to obtain a new cohort with bladder tumor samples from patients that have been treated with cisplatin based chemotherapy and have been characterized according to the RECIST response criteria. This cohort could in the future be used to evaluate if TFAP2α staining and expression can predict cisplatin sensitivity. Moreover, we soon expect to obtain 8 bladder cell lines (from the BLA40 bladder library) with different sensitivity against cisplatin which will be used for TFAP2α and p53 over-expression and down regulation experiments.

We have added the following section to the conclusion

**Future studies are needed to further validate the predictive potential of TFAP2α expression in bladder cancer. We are collecting bladder tumor samples (locally advanced T3-4, N1-3 and/or metastatic M1) from patients that have been treated with cisplatin based...**
chemistry and which have been characterized according to the RECIST response criteria. This cohort will be used to evaluate if TFAP2α staining and expression are predictive for cisplatin response in addition to survival.

Reviewer:
2. Several series have outlined the benefit of adjuvant chemotherapy even in the absence of lymph node positivity. Was there any trend regarding the predictive value of TFAP2 alpha in T3 BC where one would anticipate a subset of patients to harbor micro-metastatic disease and therefore potentially benefit from adjuvant chemo if their cells are responsive.

Author rebuttal:
When the pT3 pN+ (89 samples) and pT3 pN0 (95 samples) were analyzed together there was no significant predictive value of TFAP2α staining for survival. For the pT3 N0 group, TFAP2α nuclear staining was inversely correlated to survival time as was the case for the analysis of all N0 samples in agreement with pT3 N0 constituting 79% of the lymph node negative samples.

Reviewer:
3. It would make sense to think of a study using neoadjuvant chemotherapy and TFAP2 alpha. Do the authors have access to any of these patients. Linking complete or partial response to TPAP2 alpha expression would greatly enhance our understanding of this marker and support the concept.

Author rebuttal:
TFAP2α staining in combination with neoadjuvant chemotherapy would probably be very helpful in order to establish the predictive value of TFAP2α. However, neoadjuvant chemotherapy is not part of the standard treatment regimes for advanced urothelial TCC or as part of radical cystectomy in Denmark so it is difficult to obtain a sufficient number of samples.

We added the following section to the discussion:
Future studies are needed to further validate the predictive potential of TFAP2α expression in bladder cancer. We are collecting bladder tumor samples (locally advanced T3-4, N1-3 and/or metastatic M1) from patients that have been treated with cisplatin based chemotherapy and which have been characterized according to the RECIST response criteria. This cohort will be used to evaluate if TFAP2α staining and expression are predictive for cisplatin response in addition to survival.
Reviewer:

4. was there any reason as the authors had access to TMAs not to stain for p53,

Author rebuttal:
In this project, we had stained a number of tissue microarrays to investigate the expression of a total of seven molecules from the previously published signature. For each molecule, several antibody dilutions were applied to different TMA slides. As access to these TMAs was limited we had chosen to focus on the TFAP2α staining. We agree, in the future it would be interesting to stain for other molecules of interest as p21, p53 etc.

Reviewer:

5. The authors state that TPFAP2 alpha silencing augmented gemCis sensitivity and did not stimulate proliferation in the p53 mutated and non tumorigenic cell line, corresponding to clinical findings in lymph node negative patients. However, a percentage of patients with lymph node negative actually harbor node positive disease and a subset of BC non metastatic to the nodes is clearly TP53 mutated but tumorigenic...
The authors conducted functional chemosensitivity studies but did not reintroduce TFAP2 into a TFAP2 negative bladder cancer cell line to add robustness to their findings although data had been shown in colon cancer cell lines.

Author rebuttal:
We agree that overexpression of the recombinant wild-type p53 in T24 cells combined with +/- TFAP2α silencing +/- cisplatin/Gemcitabine would be highly informative to analyze whether this may “restore” the SW780 phenotype. With regard to this, we have made several attempts for this transient overexpression in T24, however the transfection efficiency was consistently too low to be used (<20%). As T24 seems to be notoriously difficult to transfect with high efficiency, future approaches investigating the effect of TP53/p21/TFAP2α overexpression should be performed in a panel of bladder cell lines with p53 wild-type background. Currently we do not have a retroviral classified cloning laboratory that probably would be the best way to go. Again we hope to be more successful when obtaining a selection of the BLA40 bladder cancer cell lines.

We added the following section to the discussion:
Within our study, re-introduction of TFAP2α in T24 and SW780 was performed by transient and stable transfection, Transient transfection was very low (<20%) as monitored by QPCR/WB and the selected clones seem to loose TFAP2α as no increased in transcript was measured (QPCR) compared to the mock transfected.
Reviewer:
Minor comments
1. A couple of typos should be corrected throughout the manuscript and in the legends of the figures: for instance Fig 5 axis is labelled as ralativ expression. It should read relative

Author rebuttal:
The typographical errors in Figure 5 have been corrected and we have carefully checked spelling and settings throughout the whole manuscript.

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The authors declare that the manuscript has not been published previously and is not under consideration for publication elsewhere. All of the authors are aware of and agree to the content of the paper and their being listed as an author on the paper.

Best Regards
Iver Nordentoft