Author's response to reviews

Title: TGF-beta isoforms and receptors mRNA expression in breast tumours: prognostic values and clinical implications

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Author's response to reviews: see over
Answers to the reviewers’ comments

We sincerely thank the editors and reviewers for their interest in our work and for the valuable comments that have greatly helped us to improve this manuscript.

We have taken all the comments into account in the revised version of the manuscript to clarify our research objectives and results.

Sincerely,

Chenfeng Chen (on behalf of all authors)

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Reviewer's report 1

Title: Prognostic Values of TGF-Beta Isoforms and Receptors mRNA levels in Breast Cancer

Version: 1 Date: 27 January 2014

Reviewer: wilma mesker

Reviewer's report:

The manuscript provides nice work relating the TGFB signalling pathway and its impact for clinical application.

Although the authors describe the different factors of the pathway accurately and dedicated they do not come with an overall suggestion to apply the markers in daily clinical use. Could a combination of the markers help substantially to select patients for additional or more intensified therapy?

Reply 1: Thank you for the comments. We have rewritten the Discussion and Conclusions; please refer to the revised paper for the new interpretation for our results. We also believe that a combination of the markers will improve the selection of patients for TGFB signalling pathway targeted therapies that are under development. We have tried to test the prognostic values of a few combinations of the 5 genes. Unfortunately, we did not observe a much improved significant association. We would like to try a broader range of genes to test this hypothesis in the future.

Major Compulsory Revisions

Male breast cancer is considered as different to female breast cancer. These patients should have been excluded from the analysis.

Reply 2: As suggested, we have removed the male samples (n = 6) from the analysis. There is no significant change of the results by omitting the 6 samples.

The tumor microenvironment is very important in tumor progression. By selecting patients >50% tumor cells a selection is performed for "good prognosis" patients (see de Kruijff et al, Breast Cancer Res Treat. 2011 Feb;125(3):687-96.). There has thus been a selection bias on basis of patients with predominatly tumor cells and a good prognosis.
Reply 3: We agree with this opinion. However, it seems that the “> 50% tumour cells” has been commonly used as one of selection criteria.

Why was the TGFB3 not correlated with Her2 to analyse the triple negative subgroup. Currently only information is available about the ER, PR. Her2 is available and could be applied?

Reply 4: Thank you for this comment. The associations between the mRNA expression levels and the triple-negative status of tumours have been added to Figure 2 in the revised manuscript.

What could a cut of value of 7 years mean for clinical management in case of TGFB1 mRNA expression?

Reply 5: At the time of analysis, the maximum follow-up time in the TCGA cohort was 19.5 years, however, there were less than 10% of patients whose follow-up time were longer than 7 years. To avoid distortion of the results that might be introduced by the small risk set of participants remaining at the far right tail, we restricted the follow-up time to the first 7 years for the statistical analyses. However, in the revised manuscript, we have visualized the results of survival analysis from 1 year to 10 years in Figure 4B and Figure 5B, so the problem can be avoided.

Kaplan Meier curves could be less. It is not indicative to demonstrate the not-significant curves. This can be displayed in a short table or in the results section. Just give some informative images.

Though, the M status gives a beautiful curve!

Reply 6: Thank you for this suggestion. We have removed the unnecessary Kaplan Meier curves and it is more visual to understand the results based on Figure 4B and Figure 5B now.

Minor Essential Revisions

It is more indicative to have the P values also in the abstract.

Reply 7: We were trying to add all the p values for our results. However, it appears to be difficult to read, as there are around 20 p values in total. As a result, we expressed the p values as < x, where x is the largest p values in a set of results.
**Level of interest:** An article whose findings are important to those with closely related research interests

**Quality of written English:** Acceptable

**Statistical review:** Yes, but I do not feel adequately qualified to assess the statistics.

**Declaration of competing interests:**

I declare that I have no competing interests
Reviewer's report 2

**Title:** Prognostic Values of TGF-Beta Isoforms and Receptors mRNA levels in Breast Cancer

**Version:** 1  **Date:** 6 February 2014

**Reviewer:** Rosemary Akhurst

**Reviewer's report:**

1. **Is the question posed by the authors well defined? The authors need to formulate a question in the background. As it stands there is not hypothesis or question,**

   **Reply 1:** We have clarified our question in the background section as suggested. “Much of our knowledge about TGFβ signalling in breast cancer is based on the studies characterizing the proteins involved. A few pioneer studies have evaluated the mRNA levels [11, 12], whereas whether the mRNA levels of the key components of the TGFβ pathway also have prognostic values and clinical implications is inconclusive.”

2. **Are the methods appropriate and well described? Concerns about multiple testing and slight cherry picking in Fig 3. The need to cherry pick in the first place is not clear, as the outcome seems fairly robust regardless of thresholds for high and low expression. Maybe the authors could comment on this,**

   **Reply 2:** The original Figure 3 has been replaced by Figure 5 in the revised manuscript. The “low” and “high” groups were defined as: x% of patients with the lowest expression of y gene vs. x% of patients with the highest expression of y gene. It is apparent, if the mRNA expression is associated with the patients’ survival, then the smaller is the x, the bigger is the effect size. However, it is also true that the smaller is the x, the fewer patients are compared, and thus it is less likely to get a significant P value which may lead to a false negative result. As a result, to avoid arbitrary grouping, we showed all the results for x ranged from 10 - 50. The results of the calculated Cox regression coefficients were shown in a heatmap format now, so it should not be considered as a cherry picking. The Cox proportional hazards regression model used for survival analysis needs to specify the length of the years of follow-up, however, we also wanted to know if the differentially expressed mRNA levels had different effects in the early and late years after the patients were diagnosed with breast cancer,
thus we calculated the hazard ratios over different periods of follow-up from 1 to 10 years in the new Figure 4 and 5.

3. Are the data sound? RT-PCR validation data is not shown.

Reply 3: The RT-qPCR dataset did not contain survival data, and was used for validating the associations between the gene expression and the tumour characteristics that was derived from the TCGA dataset. In the revised manuscript, we have added another cohort compiled from 5 GEO datasets with relapse-free survival data as a means to validate the TCGA dataset and to extend the scope of the research.

4. Does the manuscript adhere to the relevant standards for reporting and data deposition? N/A

5. Are the discussion and conclusions well balanced and adequately supported by the data? No

Reply 4: The Discussion and Conclusions have now been re-written in the revised manuscript.

6. Are limitations of the work clearly stated? They could be more clearly stated, and direct comparison to general concepts and other manuscripts comparing TGF beta levels in breast cancer should be discussed.

Reply 5: The limitations of the work have been mainly addressed in the second paragraph in the Discussion section. We have also compared our results with other papers in the Results section.

7. Do the authors clearly acknowledge any work upon which they are building, both published and unpublished?

8. Do the title and abstract accurately convey what has been found? Not certain until reviewers address questions about prognosticative value of TGFB1 TGFB2 and TGFB3.

Reply 6: We have changed the title to “TGF-beta isoforms and receptors mRNA expression in breast tumours: prognostic values and clinical implications”.
This manuscript would be of interest to individuals interested in targeting TGF beta for breast cancer, since it is a survey of RNA expression levels of the three ligands and two receptors in published data sets from TCGH, and correlation with nodal status and with overall survival. There is some validation of data by qRT-PCR using additional BCa tumors from an Australian cohort (n=71).

The overall conclusion is that high TGFBR1 and low TGFB2 mRNA levels in tumours were associated with a worse prognosis for patients, which appears like a sound and useful observation. The authors also state that “the prognostic value of lymph node metastasis status can be significantly improved by assessing the TGFB1, TGFB2 and TGFB3 mRNA levels of the primary tumours”. However they do not explain the magnitude of this improvement, nor what they are using as their base level for prognostication e.g. PAM50? This needs more clarification and a quantitative approach to the added value of including the assessment of TGFB1, TGFB2 and TGFB3 mRNA levels of the primary tumours.

Reply 7: Thank you for the comments. We have completely rewritten the Discussion section in order to more clearly interpret our results. The mRNA levels provided additional prognostic values for patients stratified by lymph node status; however, we had not compared the additional prognostic values with the classical prognostic predictors. We have now realized that the additional prognostic values may only limited to the patients who participate in a clinical trial using drugs targeting the TGFβ pathway and serve as a means to select suitable patients. Thus we have taken off the statement.

The authors do not adequately address the limitations on the study of RNA only. They do not sufficiently survey the literature of the prognostic value of markers of TGF beta signaling in cancer e.g. that by Reiss group Cancer Res 2002 Alterations of Smad signaling in human breast carcinoma are associated with poor outcome: a tissue microarray study. The authors need to undertake a thorough literature search, and place their data in the light of others, including de Kruijf et al 2013.

Reply 8: Thank you for the suggested references. We have included the results of de Kruijf et al 2013 in the Results to compare with our results. Reiss group’s work had a focus on the Smad protein. Unfortunately, we could not find a direct association with our study. However, a reference by Gobbi et al. that was cited by them is consistent with one of our result about the expression of TGFBR2 and thus has been included. We have now addressed the limitations of the study in the second paragraph in the Discussion.
The RT-PCR validation data should be shown graphically.

Rather than presenting Table 3, it would be more informative have a histogram of distribution of expression levels (med low high ) for each gene within the primary tumor of each BCa class. This could simple be:

\[ y \text{ axis = number of tumors and x axis = tumor class and gene. Each bar could include number of tumors categorized as low, med and or high for that gene. This gives a visual of how many tumors examined in each class and the relative distribution of expression levels within and between tumor classes. Alternatively a box and whisker plot of log transformed RNA levels for each tumor class. Indicate number of tumors within each class.} \]

Reply 9: Thank you for this constructive comment. We have added Figure 2 to replace Table 3 according to the suggestion. However, it might not be necessary to visually show the med low and high groups as mentioned in Figure 4 and Figure 5 (Originally Figure 3), as the comparison between the “low” and “high” groups were: x% of patients with the lowest expression of y gene vs. x% of patients with the highest expression of y gene. There should be no overlap between the “low” and “high” groups.

Figure 1 B. This should be omitted for clarity, and include only those correlations r2 > 0.5. Moreover, the p values need to be corrected for multiple comparisons. Figure 2 . What do the P values calculated by Kruskal-Wallis test refer to: i.e. what is the null hypothesis?

Reply 10: Figure 1B has been removed. There were originally 28 comparisons in either tumour or normal samples. The adjusted significant threshold p value should be 0.05/28 = 0.0018. The positive correlation between TGFB1 and TGFB2 mRNA levels (Spearman’s rho = 0.51, P = 5.6×10^{-5}) that was observed in the tumours had a much smaller P value. It worth to be noted that the Spearman’s rho for the correlation of the mRNA levels of ESR1 (estrogen receptor) and PGR (progesterone receptor) was 0.66 with P = 3.1×10^{-8}, which was much lower than we expected as the correlation between ER and PR is usually around 0.9. The null hypothesis for Kruskal-Wallis test was that there was no difference in mRNA levels of each gene across different PAM50 subtypes.
Figure 3A is very difficult to comprehend, as multiple manipulations of the data are shown in the same graph, which also raises the issue of multiple comparisons. What is there to gain by using any value other than the 40th percentile for high versus low expression (i.e. the right hand data point in each graph)? The most significant values are found predominantly in this larger group, since smaller sample sizes (on the left) lower significance.

Why have the authors selected to show Kaplan Meier plots in B-F using different percentile cut-offs for inclusion of low and high expression? This seems like cherry-picking?

Reply 11: Figure 3 has now been replaced with Figure 4 and 5. Please refer to Reply 2 for answers to the questions for multiple comparisons and the grouping. The representative survival curves have been changed in Figure 5. It should be noted that each survival curve only represent one data point in the Figure 5 A and B. The rationale for the selection of representative survival curves was that we wanted to show by which percentage was the best to group the patients into high, mid and low according the mRNA expression for prognosis. Depending on the distribution of the contribution of the three groups to the prognosis, the mid group is likely to vary for different genes.

It would be valuable to visualize a Kaplan Meier for TGFβ2 by nodal status.

Reply 12: It has been added as Figure 5C.

Regarding the conclusion that higher levels if TGFβ1 TGFβ3 are associated with poorer outcome, the authors should discuss their findings in the light of the consensus view that expression of TGF beta ligands, particularly TGFβ1, are generally thought to increase with tumor progression and be associated with higher risk for progression. Since the data in the manuscript appear to go against this consensus view, the authors should discuss this in more depth. Or are the studies on ligands, apart from TGFβ2, inconclusive due to multiple testing issues? The authors might also like to speculate on the therapeutic significance of high TGFBR1 levels correlating with worse prognosis.

Reply 13: Our data showed that TGFβ1 did increase in the tumours compared with the matched normal tissues and in the tumours from lymph node-positive patients compared with the tumours from lymph node-negative patients, which implicated the expression of TGFβ1 was up-regulated in the more malignant tumours. Interestingly, our data implicated that the expression of TGFβ3 was up-regulated in the less malignant tumours. These data suggested the isoform-specific biology of TGFβ1 and TGFβ3. Paiva et al 2010 had shown that “TGF-beta1-positive tumours were associated
with increased disease-free survival.”, while Ciftci et al. 2014 had shown that “High serum transforming growth factor beta 1 (TGFB1) level predicts better survival in breast cancer.” Our results show the TGFB1 expression is differentially associated with patients’ clinical outcome in different subgroups of patients. As discussed in the revised paper, tumours can be grouped into three groups according the types of responsiveness to TGFβ: “those that respond to TGFβ and are suppressed (X1), those that do not respond to TGFβ (X2) and those that respond to TGFβ but progress (X3).” Tumour cohorts from different studies may have different proportion of the three groups, thus the association between the mRNA levels and clinical outcomes of the patients may significantly different.

Validation by some protein data (IHC?) would be helpful, although not essential because, as the authors point out, there is considerably post-transcriptional control of TGF beta ligands.

Reply 14: It would be great to have additional quantitative protein data from the same tumour samples, which might not only help to validate the data but also help us to better understand the possible post-transcriptional control involved for these genes. However, we did not have the resource conduct this study at the moment.

Discussion: the authors state that “Our results suggest that the prognostic value of lymph node metastasis status can be significantly improved by assessing the TGFB1, TGFB2 and TGFB3 mRNA expression levels of the primary tumours, i.e. high TGFB1 and TGFB3 mRNA levels provide better prognosis for patients diagnosed with regional lymph node metastases, and high TGFB2 mRNA levels provide better prognosis for patients with lymph node negative diseases”. Significantly improved compared to what? And how more accurate would this predictor be compared to standard predictors, and Pam50?

Reply 15: We also felt that the original statement was too strong, which was only based on the fact that the mRNA levels provided additional prognostic values for patients stratified by lymph node status. It has been taken off from the revised paper.

All requests are mandatory. The suggestion of undertaking some IHC is optional.
**Level of interest:** An article of importance in its field

**Quality of written English:** Acceptable

**Statistical review:** Yes, but I do not feel adequately qualified to assess the statistics.

**Declaration of competing interests:**

I am on the SAB or Isarna Therapeutics. However, I do not feel that this affects my judgment of the data.