Author's response to reviews

Title: Low expression of TFPI-2 associated with poor survival outcome in patients with breast cancer

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Author's response to reviews: see over
The Biomed Central Editorial Team

Object: MS: 1460848862790513 - Low expression of hTFPI-2 associated with poor survival outcome in patients with breast cancer

Thank you for consideration of our manuscript for publication in your journal.

We have reviewed the above manuscript according to your reviewer’s comments. We have improved the quality of written English and done some language corrections in version 2 of our manuscript.
Reviewer's report
Title: Low expression of hTFPI-2 associated with poor survival outcome in patients with breast cancer
Version: 1 Date: 18 October 2012
Reviewer: Nina Iversen
Reviewer's report:
Cheng Xu and colleagues have studied the relationship between hTFPI-2 expression and clinical and pathological parameters in 156 breast cancer patients and 40 controls, to find out if TFPI-2 expression had any prognostic value in breast cancer. They have used immunohistochemical staining and stratified the patients according to the degree of TFPI-2 expression level. 118 of the patients were followed up for survival analysis.
The authors reported that the TFPI-2 expression was correlated with several of the clinicopathological parameters, and that TFPI-2 expression was associated to disease free survival in the patients. Low expression of TFPI-2 is associated with cancer progression, recurrence and poor survival outcome after breast cancer surgery. The authors therefore suggest that TFPI-2 expression may be a useful prognostic marker in breast cancer.
In general I think the manuscript is of general interest and importance in cancer biology. I find the results very interesting and convincing.

Minor essential revisions:
The manuscript could benefit from some language improvements, as for instance:
- The subheading in results on page 8 should be corrected to: Immunohistochemical tissue staining.
  The subheading has been changed as the reviewer indicates.
- “Meandensity” should be corrected several places from page 9.
  We have corrected the word and changed all ‘meandensity’ to ‘mean-density’ in our paper.
- “Homeostasi” at page 11 must be corrected.
  We have corrected the word in our paper as the reviewer indicates.
- Include a space before all brackets.
  We have corrected these words in our paper as the reviewer indicates.

Level of interest: An article of importance in its field
Quality of written English: Needs some language corrections before being published
Statistical review: Yes, but I do not feel adequately qualified to assess the statistics.
Declaration of competing interests:
I declare no competing interests of personal or financial affairs in relation to the paper.
Reviewer’s report
Title: Low expression of hTFPI-2 associated with poor survival outcome in patients with breast cancer
Version: 1 Date: 10 December 2012
Reviewer: Santhi Konduri

Reviewer’s report:
The authors wanted to investigate the relationship between hTFPI-2 expression and breast cancer clinicopathological features and to identify the prognostic value for TFPI-2 expression in breast cancer patients. The authors used immunohistochemistry to evaluate hTFPI-2 expression in breast cancer patients. Kaplan-Meier method and COX’s Proportional Hazard Model was used to investigate hTFPI-2 prognostic value in breast cancer patients. They also showed clinicopathologic factors in breast cancer patients. The manuscript was written well and the minor corrections required.

Discretionary Revisions: No discretionary revisions required.

Minor Essential Revisions: Please change the sentence construction in discussion last paragraph: In our study, we indicate..............
   We have changed the sentence construction as the reviewer indicates.
Same paragraph: higher expression hTFPI-2.......at the end ‘cum’. Please expand that.
   The reviewer is correct and we have expanded the word as 'cumulative'.
Figure 3: cum survival – please expand cum.
   We have changed the legend of Figure 3 as ‘Kaplan–Meier analyses of the effect TFPI-2 expression on disease-free survival’.

Major Compulsory Revisions: No major compulsory revisions required.

Level of interest: An article of importance in its field
Quality of written English: Needs some language corrections before being published
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
Title: Low expression of hTFPI-2 associated with poor survival outcome in patients with breast cancer
Version: 1 Date: 11 December 2012
Reviewer: jianfei huang

Reviewer's report:
Xu et al analyzed the expression of hTFPI-2 in malignant breast cancers as well as benign breast tumors. They found hTFPI-2 expression was correlated with common cancer progression clinicopathologic features, such as tumor size, lymph node metastasis, histologic grade, clinical stage, and vessel invasion. By using computer digital image analysis, the study got relatively objective and accurate results. And more interestingly, hTFPI-2 expression was also associated with disease-free survival (DFS) of breast cancer. Breast cancer remains to be the most mortal malignancy in women worldwide and has become a global health concern. Reliable prognostic factors and effective prediction methods for breast cancer are urgently required for improving the diagnosis and treatment of breast cancer. This work seems to provide a new clue for more accurate prognosis. The experiments are well-designed and carried out in good quality. However, the potential interest and significance of the finding was enshrouded by poor English writing. The authors need to put big effort in polishing the writing of the manuscript.

Minor Essential Revisions:
1. The labels in the Figures are tiny and faint, please make the fonts bigger.  
   We have changed the font's size as the reviewer indicates.

2. The authors defined that no staining or staining less than 10 % of tumor cells as negative. The authors should describe the reason. And why the negative staining slices were excluded in the calculation of the IOD values?
   The reasons why we used a hybrid approach and set a cut-off as 10% are described as below.
   1). The antibody we used in immunohistochemical staining is polyclone, and the sensitive of our second antibody system (EnVision Detection Kit), make some negative cases having traces of stain signal. This is the reason why we would get IHC (±) sometimes. In our digital image analysis, we used image pro plus (IPP) which was so sensitive that tiny staining signal could be detected and analyzed. However, we set a filtering value to exclude the noisy spots less than 50 pixels according to the manual, which would interfere the analysis of tiny signal. All these factors may disturb calculation of area value in slides with weaker staining.
   2). There were 19 cases which were judged to be absolutely TFPI-2 negative by pathologists, these cases were so few that cannot become a separate group in statistical analysis.
   3). We have tried multiple values around 10% as cut-off and obtained the

The area value of staining area of negative staining slide is close to zero. It would not be appropriate to use this value as denominator of the division 3. The authors should discuss thoroughly the biological contribution of hTFPI-2 expression with respect to other factors, and give emphasis to how its evaluation would improve prognosis prediction.

We have added relevant content in discussions according to the reviewer's advice.

**Level of interest:** An article of importance in its field

**Quality of written English:** Needs some language corrections before being published

**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

Title: Low expression of hTFPI-2 associated with poor survival outcome in patients with breast cancer

Version: 1 Date: 13 December 2012

Reviewer: Vagner Bernardo

Reviewer's report:

1- Patients Section

The authors randomly selected 156 breast cancer patients and assessed h-TFPI-2 expression by immunohistochemistry. The follow-up data were available for 118 patients. So, 38 losses are expected. However the authors stated that only 33 patients were lost to follow up.

We followed up the selected patients through phone or and outpatient visits by ourselves. 38 patients were lost in the follow-up. We have corrected wrong number in our manuscript.

2- Digital Image Analysis Section

A- The main purpose of digital image analysis is to increase reproducibility. All slides are imaged under optimal conditions and a continuous variable is produced (e.g. IOD, area index, labeling index), being most often used in survival curves. The authors used a hybrid approach. Slides were assessed by two independent pathologists and only tumors showing more than 10% of positive cells were assessed by DIA. Why 10% of tumor cells was the cut-off point? Why were neither 11.5% nor 20% of tumor cells chosen? This must be clarified.

How will borderline “negative” tumors (e.g. 9% of positive cells) be distinguished from borderline “low-expression” tumors (e.g. 11% of positive cells)? Remember that manual counting is tedious and time-consuming. Visual estimates are biased and non reproducible.

The reasons why we used a hybrid approach and set a cut-off as 10% are described as below.

1). The antibody we used in immunohistochemical staining is polyclone, and the sensitive of our second antibody system (EnVision Detection Kit), make some negative cases having traces of stain signal. This is the reason why we would get IHC (±) sometimes. In our digital image analysis, we used image pro plus (IPP) which was so sensitive that tiny staining signal could be detected and analyzed. However, we set a filtering value to exclude the noisy spots less than 50 pixels according to the manual, which would interfere the analysis of tiny signal. All these factors may disturb calculation of area value in slides with borderline staining.

2). There were 19 cases which were judged to be absolutely TFPI-2 negative by pathologists, these cases were so few that cannot become a separate group in statistical analysis.
3). We have tried multiple values around 10% as cut-off and obtained the same statistical result. Based on the suggestion from pathologists and reference paper (Wang H, Zhang Z, Li R, et al. Overexpression of S100A2 protein as a prognostic marker for patients with stage I non small cell lung cancer. Int J Cancer. 2005 Aug 20; 116(2):285-90.), we set less than 10% tumor cells as the cut-off point of negative group.

B- The stoichiometric principle must be followed when Integrated Optical Density is used. Walker states: “…One problem with using diaminobenzidine as a chromogen is that there is only a linear relationship between the amount of antigen and staining intensity at low levels of the latter. Image analysis systems assess the amount of staining by measuring absorption, so the non-linear relationship that occurs at higher levels between amount of antigen and intensity can result in inaccurate readings.” (Walker RA - Histopathology 2006, 49, 406–410)

DAB is not a stoichiometric stain. So IOD is not proportional to the amount of the target protein in the whole range of protein expression. It is further hampered by signal enhancers (e.g. Envision).

We agree the referee's opinion about in using diaminobenzidine as a chromogen, there is only a linear relationship between the amount of antigen and staining intensity at low levels of the latter. Though we used an EnVision Detection Kit as the second antibody detect system in immunohistochemistry staining, may further hampered by signal enhancers. The TFPI-2 staining in breast cancer tissues in our study were not very strong staining. So IOD suppose can be used in our study.

C- More details about DIA must be supplied. Was Koehler illumination implemented before image acquisition? Was shading correction used? The area of the microscopic field (in #m2) must be supplied, allowing other researchers to reproduce these findings. Field area can vary enormously between different DIA systems under the same magnification.

The pictures were taken in the imaging center, the microscope was adjusted by the technologist, including do shading correction and white balance. All the pictures were shoot in the same condition and the same magnification (×200), keeping the illumination stable, using same time of exposure, only adjust focus button when changing areas or slides. We realized the heterogeneous in breast cancer cells, we shoot six pictures in each slide, for the purpose to acquire the staining information of the tissue in the slides, we randomly move the slide from left to right, and from up and down, evenly pick up six areas of pictures. When analysis groups of these pictures, we use one typical picture to do calibration, using the same condition in analysis, averaging the density of six visions on each slide.

D- I think that the best approach to assess h-TFPI-2 expression by
immunohistochemistry is:

1- Negative cases are cases without staining
   The reasons why we defined <10% positive staining as negative group are elaborately explained in the answer of question A.

2- Quantify all remaining cases assessing the stained area (area index = stained area/total area).
   TFPI-2 staining was observed mainly on the cytoplasm of cells in breast glandular tissue or breast tumor tissue. Only sporadic positive staining was found on stroma areas, most of these areas showed negative staining. The area of IOD referred to positive staining of tumor area. The stroma areas were not the area of our interest. So area index is not appropriate for our calculation.

3- Ensure that all slides are imaged under optimal conditions (Koehler illumination, shading correction…)
   As described details above, we ensure that all slides are imaged under optimal conditions.

• Minor Essential Revisions
1- Images are dark. Ensure Koehler illumination before image acquisition.
   We have adjusted the figures as the reviewer indicates.
2- IOD stands for integrated optical density and not for integrated option density
   The reviewer is correct and we have corrected the word.
3- Several spelling mistakes are present in the manuscript
   We have improved the quality of written English and done some language corrections in version 2 of our manuscript.
4- Table 2 – The following variables (LN metastasis, ER, PR and HER-2) do not sum 121 patients
   There are several cases of patients with incomplete medical records in each group, we have marked these cases as 'unknown'.

Level of interest: An article whose findings are important to those with closely related research interests

Quality of written English: Not suitable for publication unless extensively edited

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

Title: Low expression of hTFPI-2 associated with poor survival outcome in patients with breast cancer

Version: 1 Date: 14 December 2012
Reviewer: Pascale Reverdiau

Reviewer's report:
In this manuscript, Dr Cheng Xu and his colleagues studied the relationship between Tissue Factor Pathway Inhibitor-2 expression and survival rate in 196 patients with breast cancer or benign tumor. They demonstrated that the expression of TFPI-2 in patients with breast malignant tumor is lower than in patients with breast benign tumor and suggested that a low level in TFPI-2 is associated with a poor prognosis.

The purpose of the study is well defined and this paper brings new information concerning the expression of a proteinase inhibitor, TFPI-2, in breast cancer when compared to the previous data published in this field. Methods are appropriated and clinicopathologic features are well documented. Moreover, statistical analysis strengthens adequately the data. Finally, the paper is well written with only some points that remain to be elucidated.

Discretionary Revisions

Introduction:
- p4 line 8, the authors list one reference (Whitley et al., 2004) about the tumor migration and invasion in the breast and ovarian cancer cell lines that is reduced by active PAI-1. However, in the 6th sentence “active plasminogen” is disturbing and authors should explain this expression.

  Breast cancer cells express uPA-PAI-1 complexes, which converts plasminogen into plasmin, Urokinase-type plasminogen activator (uPA) is regulated by plasminogen activator inhibitor-1 (PAI-1), active PAI-1 can reduce invasive phenotype in breast cancer cell lines. In order to avoid disturb, we have added a reference.

- The authors should list other references demonstrating that hypermethylation of TFPI-2 gene promoter is a factor for poor prognosis in patients with other cancer (lung cancer, gastric carcinoma, colorectal cancer).

We have listed some references in background as the reviewer indicates.

Results:
- Positive staining on stroma areas were observed. However this data is not discussed. Which cells are positive? (endothelial cells, macrophages)

  Positive staining on stroma areas was sporadic (endothelial cells), most of these areas showed negative staining. We mainly studied TFPI-2 expression of breast cancer cells. The stroma areas were not the area of our interest. So we excluded stromal staining in computer-aided analysis.
- In figure 2D: negative staining was obtained in breast cancer, which clinical stage?
  We reviewed the clinical data of this slide: female, 61 years old, IDC, tumor size 2.0cm, LN 5/14 (LN matted), vessel invasion (+), Histologic grade II~III, T1N2M0, clinical stage IIIA.

- In table 2: How was evaluated the vascular invasion?
  Vessel invasion was defined as the presence of neoplastic emboli in two or more blocks, we clarified the definition in Specimen cohorts.

Discussion
The authors should add a comment about the direct inhibition of MMP activity by TFPI-2 that is discussed by some authors.
  We have added relevant content in discussions according to the reviewer's advice.

In conclusion, this paper is of importance in its field and could be accepted after minor revision.

**Level of interest:** An article of importance in its field  
**Quality of written English:** Acceptable  
**Statistical review:** No, the manuscript does not need to be seen by a statistician.  
**Declaration of competing interests:**  
I declare that I have no competing interests