Author's response to reviews

Title: Oxidative stress and counteracting mechanisms in triple-negative and basal-like breast carcinomas

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Version: 2 Date: 8 May 2011

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

We thank you for your interest in our manuscript “Oxidative stress and counteracting mechanisms in triple-negative and basal-like breast carcinomas”.

We would also like to thank the reviewers for their highly constructive and useful comments. We have incorporated the requested changes and additions to the manuscript. Hopefully the manuscript now fulfills your requirements and is ready to be published in BMC Cancer.

We would be pleased to answer any questions related to this manuscript.

Sincerely,

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**Reviewer's report:**

**General comments:**

The manuscript describes the role of several redox state markers in breast cancer. The novelty of this study relies on the joint evaluation of the role of this set of markers, which has not been performed in the subtypes of breast cancer here analyzed.

The manuscript is concise and well written. The results are well presented and support the conclusions.

**Minor Essential Revisions**

1. Abstract: the rationale and novelty of the study are not clear in the abstract. The authors should change the text accordingly.

**Reply:** We have added the following sentence to the Abstract: “This is the first study to assess oxidative stress and or cell redox state-regulating enzymes in TNBC and BLBC.”

2. Results, page 9, second paragraph, line 4: “... had a nearly significant association with BCSS (p=0.06)”. There is some word missing before “BCSS”. The mentioned association is with “better” or “shorter” BCSS?
Reply: That sentence reads now “Cytoplasmic PRDX III immunostaining had a nearly significant association with poorer BCSS (p=0.06).”

3. The supplementary Tables are important and should be part of the main manuscript.

Reply: All tables (1-3) are now part of the main manuscript.

Discretionary Revisions
Results: analysis of the results described in paragraphs 3-5 would benefit from presentation in the Table format.

Reply: Due to the length of paper and because the key results are duplicated in the Discussion, we would like not to add this Table to the manuscript.

Reviewer's report:
In the present manuscript, Karihtala et al. aimed to investigate whether or not oxidative stress and/or cell redox state-regulating enzymes have special roles in triple negative and basal-like (BLBCs) breast cancers. To address this question the authors assessed the immunohistochemical expression of 8-OHdG, Nrf2, Keap1, PRDX III and PRDX IV in a small series of 79 invasive ductal carcinomas, including 37 triple negative tumours, of which 12 were classified as BLBCs. This is a simple study with few significant findings, mostly related to non-triple negative tumours despite the title of the paper. Association with survival independent of other prognostic factors has not been demonstrated, limiting the significance of the results.

Below are specific comments:

Major Compulsory Revisions
1) It seems that the authors misunderstood the Nielsen immunohistochemical surrogate panel for BLBCs. According to Nielsen et al. (Clin Cancer Res 2004) and Cheang et al. (Clin Cancer Res 2008), BLBCs are defined as triple negative tumours expressing EGFR and/or CK5/6. Therefore, to classify BLBCs as “the triple negative tumours that also expressed both EGFR and CK5/6” may be incorrect and is certainly not very sensitive, considering that the panel as described by Nielsen et al. has a sensitivity of 76%. Moreover, requiring expression of both EGFR and CK5/6 would not increase the specificity, which is 100% when considering EGFR and/or CK5/6. This should be amended in the study and all analysis comparing the BLBCs re-done.

Reply: We have now modified the criteria of BLBC as the Referee suggests and re-analyzed the results. The proportion of basal-like subtype showing cancers in our material is now 83%, which is more in line with previous reports (reviewed in e.g. Foulkes et al. NEJM 2010). According to the new definition of BLBC, there is no association with PRDX IV, but instead we found larger T-class in BLBC group compared to non-BLBC triple-negative cancers (p=0.005).

2) There is large amount of evidence to suggest that at least the great majority of BLBCs do not originate “from stem cell precursors of basal myoepithelial cells”, but actually from ER-negative luminal progenitor cells. Please see Molineux et al. Cell Stem Cell 2010 and Lim et al. Nat Med 2009 and amend the introduction.
Reply: We have modified this sentence: “It has been suggested that BLBCs may have a different pathogenesis, originating probably from mammary epithelial luminal progenitor cells (Molyneux et al. 2010).”

3) In 2010, the ASCO/ CAP guidelines for evaluation of ER and PR were published (Hammond et al J Clin Oncol 2010). According to those guidelines, a cut-off of more than 1% (and not 10% as used in the present study) of positive cells should be used for considering a tumour ER/PR positive. Therefore, the study has not been “updated to match the latest criteria concerning ER- and PR-negative tumors”. This should be updated in the manuscript.

Reply: We are aware that several guidelines have recently suggested that any amount of positive ER staining should be considered as ER-positive cancer and ER-negative breast cancers as those without any ER-staining. As mentioned in the Materials and Methods section, the TNBC group did not show any ER- or PR-positivity, but tumors exhibiting more than 10% of invasive tumor cells were considered as steroid receptor-positive. The latter decision was made since 1-10% of positive cells may be “grey zone”, where estrogen responsiveness is uncertain.

We have now modified the mentioned sentence in the Discussion: “Here we reassessed the steroid receptor status and HER2 status of tumors and updated them to match the latest criteria concerning ER- and PR-negative tumors [14], tumors exhibiting more than 10% of invasive tumor cells were considered as steroid receptor-positive.”

4) How specific and sensitive are the antibodies used in the study? The authors failed to demonstrate how were the not commonly used antibodies PRDXIII, PRDXIV, 8-OHdG, Nrf2 and Keap1 optimised. What has been used as positive controls? Adequate optimisation and positive controls ought to be demonstrated as this is of utmost importance for an immunohistochemistry-based study.

Reply: We have used these commercial antibodies in numerous studies previously, also in breast carcinomas. As positive controls we used bladder and ovarian cancer cases known to be positive for the studied markers (Pylväs et al. Eur J Cancer 2010, Soini et al. Int J Clin Exp Pathol 2011).

5) Expression of both 8-OHdG and Keap-1 were significantly associated with prognosis. Given that both markers were significantly associated with other prognostic factors, such as histological grade and triple negative phenotype, the authors ought to perform a multivariate analysis and describe the results in the manuscript.

Reply: We have now made Cox regression multivariate analysis. Unfortunately, neither 8-OHdG nor Keap-1 were independent from traditional prognostic factors, especially from N-class. We have mentioned this both in the Materials and Methods and the Results sections.

6) The authors mention in the discussion that “8-OHdG was a marker of good BCSS also in the current population and this difference was similar in all studied subgroups”. Data in relation to the distinct studied subgroups has not been provided in the manuscript. Considering the small number of cases in the subgroups, it is unlikely that the differences are significant.

Reply: Although there was a similar trend of 8-OHdG expression and better prognosis in all studied subgroups, these associations were not statistically significant. We have therefore modified the
above mentioned sentence as follows: “The current data confirm previous results, as 8-OHdG was a marker of good BCSS also in the current population."

7) The title and the conclusion of the abstract are misleading. The title should mention breast cancers in general as the positive findings of the study and most of the discussion are in relation to ER+/PR+/HER2- tumours. Moreover, it does not seem to this reviewer that the results of this study have helped “elucidating the pathogenesis of TNBC”. Although the abstract conclusion may indeed hold true, this has not been demonstrated in this study. Likewise, the conclusion of the manuscript does not match with its title.

Reply: The title reads now “Oxidative stress and counteracting mechanisms in hormone receptor positive, triple-negative and basal-like breast carcinomas”. The conclusion of Abstract is now: “Cellular redox state markers may be promising targets when elucidating the pathogenesis of TNBC.”

Discretionary Revisions:
1) If the rationale that oestrogen induces oxidative stress and anti-oxidant defense via Nrf2/Keap1 pathway is correct, one should expect higher nuclear expression of Nrf2 in ER+/PR+/HER2- tumours. However, no difference was detected in the levels of expression of Nrf2 between ER+/PR+/HER2- tumours and triple negative tumours. It would be interesting to discuss those contradictory findings.

Reply: This may be indirectly true since estrogen levels in tissues of ER positive tumors are higher (Drafta et al. J Steroid Biochem 1983). However, estrogen is not the only factor causing increased oxidative stress in tissues and other things (such as inflammation etc.) may influence the results.