Author's response to reviews

Title: Co-expression of putative stemness and epithelial-to-mesenchymal transition markers on single circulating tumour cells from patients with early and metastatic breast cancer

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Version: 4 Date: 20 May 2014

Author's response to reviews: see over
Dear BMC Editorial Board,

Thank you for having reviewed our manuscript entitled “Co-expression of putative stemness and epithelial-to-mesenchymal transition markers on single circulating tumour cells from patients with early and metastatic breast cancer” by Papadaki et al, and providing us the experts’ remarks. Please find attached the revised version of our manuscript which we would like to resubmit for publication in your journal. We have considered carefully all comments and all changes are highlighted in the revised manuscript. The following modifications were performed according to the editor's and reviewers’ comments:
Editor's comment

I would suggest the Authors to revise the statistical analysis used to evaluate in Tables 2 and 3 the incidence of the different phenotypes as a function of tumor stage (left columns): such analyses are based on chi square test, and the overall outcome is a p value derived from the overall analysis of (for example) a 2x2 contingency table like this one:

<table>
<thead>
<tr>
<th>early metastatic total</th>
<th>ALDH1 high</th>
<th>4 9 13</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALDH1 low/neg</td>
<td>20 5 25</td>
<td></td>
</tr>
<tr>
<td>total</td>
<td>24 14 38</td>
<td></td>
</tr>
</tbody>
</table>

The two-tailed P value equals 0.0085

According to the editor’s suggestion, we have now revised the statistical analysis of the manuscript. As requested, we now provide all p values concerning the incidence of the various phenotypes as a function of stage by using Continuity Correction, which gives an Asymp.Sig. (2-sided) p value. Analysis was performed by the use of IBM SPSS version 20 software in the revised manuscript. All changes in p values are highlighted in the revised form of the manuscript (Abstract; Results; Tables).
Reviewer: Vera Cappelletti

Version:3 Date:1 April 2014

Reviewer's report:

I thank the authors for taken into consideration my comments and suggestions. I feel satisfied with the answers to all my points

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

Quality of written English: Acceptable

Statistical review: No, the manuscript does not need to be seen by a statistician.

Declaration of competing interests: I do not have any competing interests
Reviewer: Giorgio Stassi  
Version:3 Date:15 April 2014  
Reviewer's report:

As I have reported in the prior round of review, the overall message contained in the manuscript is not innovative because the same concept has been abundantly demonstrated in several other studies over the last few years.

My replies to the rebuttal letter.

1. To be classified as a CTC, the cell should be positive for cytokeratin, negative for CD45, and positive for a nuclear staining. Although the methodology used for breast CTC isolation is based on the expression of epithelial markers, authors should show that the isolation procedure excludes hematopoietic cells.

The reviewer is right that in order to characterize a cell as a CTC, cytokeratin expression along with absence of CD45 expression is required. Since in our study the ARIOL system was used for CTC detection, the addition of CD45 antibody to cytokeratin/ALDH1/TWIST/dapi staining was not possible due to the limitation of this system for using up to four immunofluorescence filters.

However, as described in previous studies published from our group, all patients’ cytospins were first double-stained with pan-cytokeratin and CD45, in order to exclude possible ectopic expression of cytokeratins on hematopoietic cells [1-3]. After verifying that all cytokeratin-positive cells were CD45-negative, we subsequently performed triple immunofluorescence experiments using cytokeratin/ALDH1/TWIST/dapi. We have now added a sentence to further clarify this procedure in page 7 in Methods Section - Immunofluorescence assay. In addition, as already described in Results Section - Sensitivity and specificity of CTC detection, no cytokeratin-positive cells could be detected among PBMCs’ cytospins from healthy donors although, expression of both ALDH1 and TWIST could be identified in all samples analyzed.

2. Although authors are currently evaluating the potential of breast CTC to generate tumors in vivo, they could show this behavior at least in vitro by performing different experimental procedures such as an invasion assay, in
order to confirm that breast CTCs identified as ALDH1high/TWISTnuc are more aggressive than the others.

The reviewer is right that functional assays are required to confirm that breast CTCs bearing the ALDH1high/TWISTnuc phenotype have increased metastatic potential. However, as we have previously stated, this was beyond the objectives of the current study, which aimed in the evaluation of the expression patterns of two putative stemness and EMT markers on CTCs of patients with breast cancer. The higher incidence of ALDH1high/TWISTnuc CTCs observed in metastatic compared to early disease, suggests that this phenotype may be associated with disease progression in breast cancer. We thank the reviewer for his suggestion which could be evaluated in a future study e.g by using a cell invasion assay to confirm that CTCs selected by their invasive potential are more of the ALDH1high/TWISTnuc phenotype.

3. my concern was addressed
4. my concern was addressed

Level of interest: An article of limited interest
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

References cited

