Author's response to reviews

Title: Relationship between circulating tumor cells and epithelial to mesenchymal transition in early breast cancer.

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Version: 4  Date: 13 May 2015

Author's response to reviews: see over
Dear Dr. Solera,

Thank you very much for the opportunity to submit our revised manuscript named “Relationship between circulating tumor cells and epithelial to mesenchymal transition in early breast cancer.” and for the valuable suggestions/comments of the reviewers. We would like to respond to their questions.

Reviewer's report
Reviewer: Vera Cappelletti
The manuscript by Mego et al presents a well-written study, which builds on robust data. However, the authors did not finalize their study, which makes it weak from a translational perspective. Nonetheless, in the manuscript the authors define their study as ‘translational’ adding confusion. The role of CTC in early breast cancer definitely merits to be further explored, but the simple observation of patients with pure epithelial, mesenchymal or mixed CTC does not represent a novelty: again, the translational relevance is not clear. Indeed, the abstract itself lacks an informative conclusion and simply underlines the need of future studies.

Major compulsory revisions:
Results
This referee is a bit confused with the reported numbers: CTC are claimed to be present in 24.5% of patients, but when detailed, the authors report 11.8% of CTC with epithelial only features, 14.7% of patients with mesenchymal CTC and 2% of patients with CTCs co-expressing both markers. The sum is 28.5 and not 24.5.

Thank you very much for this point. We checked the data for consistency between abstract (incorrect data), text and Tables (these data were correct) and we corrected them. We detected CTC in 25 (24.5%) of patients, CTC with only epithelial features were detected in 9 (8.8%) patients, mesenchymal only CTC were detected in 13 (12.8%) of patients, while in 3 (2.9%) of patients we detected CTC co-expressing both markers. The sum is 24.5%.

Line 144: probably the Authors did mean ‘identification of gene transcripts in D45-depleted subsets’

Thank you very much for this point. We agree with reviewer and we changed this sentence.

Material and methods No details on the stroma sampling are reported. How far was the stroma used for IHC from the tumor lesion?
Thank you very much for this point. As tumour associated stroma, the stromal cells between tumour nests, adjacent to tumour cells were evaluated. Cancer associated stroma was indicated by vimentin-positive (Dako, Monoclonal mouse anti-vimentin clone V9, code IR630) and pan-cytokeratin-negative (Dako, Monoclonal mouse anti-human clones AE1/AE3, code M3515). We added clarification to the manuscript.

It is very important to define the timing of blood withdrawal for CTC. Was it pre-or after surgery?

Thank you very much for this point. We added clarification to the manuscript. “The blood was drawn in the morning on the day of surgery, before surgical procedure.”

The positivity criteria for defining the CTC status need to be better described. Is expression of just one of the markers (either epithelial or mesenchymal) at levels above the defined cutoff enough to define a sample as CTC positive?

Thank you very much for this point. Expression of at least one of the markers (either epithelial or mesenchymal) at levels above the defined cutoff was sufficient to define a sample as CTC positive. We added clarification to the manuscript.

No data are reported on the retro-transcription preventing from understanding the amount of RNA from which the assay started.

Thank you very much for this point. We added these data to the manuscript.

Discussion, line 299: the explanations reported for the lack of correlations between IHC data and CTC should be considered as study limitations rather than true explanations.

Thank you very much for this point. We agree with the reviewer and we added limitations of the CTCs definition to the manuscript.

Conclusion: This referee feels that the conclusion reported at the end of Discussion: ‘these results suggest that expression of EMT proteins in unselected tumor tissue is not a surrogate marker of tumor invasivity and its metastatic potential’ is not supported by the data. In fact evaluated biomarkers (CTCs and tissue EMT proteins) were neither correlated with any clinical outcome (as it is probably not available for this patient series), nor CTC status (as evaluated in this study) can be considered a surrogate marker of outcome.

Thank you very much for this point. We agree with the reviewer and we change the conclusion in the manuscript as well as in the abstract. “These results suggest, that expression of EMT proteins in unselected tumor tissue is not surrogate marker of CTC with either mesenchymal or epithelial features.”
The authors claim the need of future studies to identify ‘expression of proteins in tumor tissue associated with presence of CTC in peripheral blood’. Since studies reporting CTC status/enumeration and gene expression profiles are available in the literature (e.g. Molloy et al PlosOne 2012 7, e32426), this referee would suggest to use them for a preliminary exploration of tissue genes associated with presence of CTCs, possibly separately evaluating early and metastatic tumors.

Thank you very much for this suggestion. We added these points to the discussion.

Minor revisions Line 125: the sense of the sentence ‘no children, a parent or guardian were involved into the study’ is not clear.

We agree with the reviewer that this statement is confusing and we deleted it from the manuscript.

How many pathologists were reviewing samples? Contrasting data are reported, see line 175 vs line 210

Thank you very much for this point. Two pathologist (ZC and PJ) were reviewing the samples. We corrected the inconsistency.

Lines 240-242, please rephrase to make the sense clearer

Thank you very much for this point. Based on reviewer 2 suggestion we added new Table to present data more clearly.

Line 308, please correct: ‘didn’t observed’

Thank you very much for this point. We changed this sentence according to reviewer suggestion.

In the tables report the statistical test used for calculating p-values.

Thank you very much for this point. We added statistical tests used for calculating p-values.

Reviewer's report
Reviewer: Nadia Dandachi
Reviewer's report:
In this manuscript, Mego and colleagues correlated the presence of CTCs with the expression of EMT transcription factors in tumor tissues of breast cancer patients. Overall, CTCs were detected in 24% of patients with 12% expressing only epithelial markers, 15% expressing only EMT markers and 2% expressing both epithelial and EMT markers. They found no correlation between EMT transcription factors TWIST and SLUG in breast tumors and presence of CTCs suggesting that EMT markers do not play a
The authors have addressed an interesting topic, however technical details on CTC enrichment are not clear and need to be addressed together with some minor issues:

1) In the methods section, the authors state that they used CD45 depletion for CTC enrichment as previously described (Line 133). In the paragraph “RNA extraction” (Line 135) they state that they used RosetteSep kit to perform CD45 depletion. However, the paper cited used first EPCam-depleted PB followed by Ficoll-hypaque. Then they performed CD45 depletion using magnetic beads coated with CD45. So it is not clear, which technique was used for the CD45 depletion in the present manuscript. For clarification please describe in more detail which technique was used in this paper.

Thank you very much for this point. For CD45 depletion we used RosetteSep kit, we added correct references to the manuscript (Mego M et al. Breast J. 2015 Mar-Apr;21(2):155-60 and Cierna Z et al. BMC Cancer. 2014 Jun 28;14:472.)

2) In our own experience RosetteSep has a low sensitivity compared to other CTC enrichment techniques. Do the authors have any data on sensitivity of this method? The major problem with this technique is that CD45 depleted cells do not necessarily contain only CTCs. So, concluding that EMT expression defines the presence of CTC with EMT phenotype is critical. In this same context, CTC definition (LINE 162) is not clear. Again the definition of CTCs with EMT phenotype is questionable. The authors need to address this issue and discuss it.

Thank you very much for this point. Unfortunately, we don´t have data related to sensitivity of this methods. We agree with the reviewer and we added limitations of the CTCs definition to the manuscript.

3) Line 175 The authors write that two pathologist performed review of slides, but three initials are listed.

Thank you very much for this point. Two pathologist (ZC and PJ) were reviewing the samples. We corrected the inconsistency.

4) Highest expression levels of CK19 and EMT markers are not informative. Please add median and range.

Thank you very much for this point. We checked and added suggested data to the manuscript.

5) In the result section, the paragraph “CTC detection” is not clear, at times even confusing and therefore needs revision. An additional table with analyzed genes and results might be helpful to better understand results.
Thank you very much for this point. We added suggested data to the manuscript.

More specifically:
Line 242-244. The number of genes listed is four, but results are presented for 5 genes?

Thank you very much for this point. We corrected the data and added new Table to better visualize the results.

Line 244-247. Numbers presented in this section do not correspond with numbers in the abstract.

We checked the data for consistency between abstract (incorrect data), text and Tables (these data were correct) and we corrected them. We detected CTC in 25 (24.5%) of patients, CTC with only epithelial features were detected in 9 (8.8%) patients, mesenchymal only CTC were detected in 13 (12.8%) of patients, while in 3 (2.9%) of patients we detected CTC co-expressing both markers. The sum is 24.5%.

Line 247: What do the authors mean with the term overlap in expression of EMT markers?

In one patient samples, there was overexpression of two EMT-inducing TF gene transcripts (Slug and Twist1), e.g. expression of both genes were higher than the cut-off value in the same sample. We added this clarification to the manuscript.

6) In the discussion section, the authors describe the limitations of their study. However, they also should include the fact that mRNA and protein levels do not always correlated (e.g. post translational modifications)

Thank you very much for this suggestion. We added this point to the discussion.

7) In the tables, it would be helpful for the reader to include which method was used for presented data (protein versus mRNA data)

Thank you very much for this point. We added suggested data to the manuscript.

8) For IHC staining, please add information on which controls were used (negative and positive).

Thank you very much for this point. We added suggested data to the manuscript.
Samples of breast carcinoma with high expression of TWIST1 served as the positive control for TWIST1 as described previously (Int J Biol Sci. 2014; 10(4): 396–403) and placental tissue served as a positive control for SLUG. As negative control, breast tissue was subjected to the same procedure without staining with the primary antibody.
9) The authors mention in the discussion section that intratumoral heterogeneity could have influenced mRNA expression results. In this context, did the authors notice any staining differences of EMT markers when using IHC that could support this assumption?

Thank you very much for this point. Intensity of positivity in tumour tissue was not homogenous, but it showed only focal heterogeneity. In spite of it we suppose, that intratumoral heterogeneity could influence correlation between CTCs in peripheral blood and protein expression in tumour tissue.

10) There are some spelling errors that need to be checked. For example, Line 288, 310, 318, etc.

Thank you very much for this point. We tried to correct spelling errors.

We believe that your journal meets the expectation we address here.

Thank you in advance.

Kind regards,

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