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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Author's response to reviews: see over
Dear Dr. Maria Grazia Daidone

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. We would like to thank the reviewers for their constructive comments and suggestions. 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 the reviewers’ comments. All changes are highlighted yellow in the revised manuscript. The following modifications were performed according to the reviewers’ comments:
Reviewer 1

Reviewer's report

The manuscript submitted by Maria Papadaki is well written and adequately introduces the field of CTCs in breast cancer and the urgent need to flank the simple quantification of CTCs with a biological characterization able to describe the heterogeneity within the CTC population.

The methods are well described and data are sound.

My major concern relates to the message that the manuscript wants to convey to the scientific and clinical community. It is already known (which does not mean that additional studies must not be done) that CTCs can ‘swing’ between a fully epithelial and a fully mesenchymal phenotype and that epithelial and mesenchymal features can co-exist in the same cells. The paper by Dr Papadaki confirms this important observation on an adequate number of breast cancer patients belonging to two different clinical settings. However, unfortunately no data are given to support the clinical meaning of the prevalence of certain CTC-phenotypes over others. Why do not show, at least for some index patient in the metastatic setting, how the CTC phenotypes relate to progression?

The absence of data trying to address the clinical meaning of this well-done and important observations on CTC phenotypes, pushes the entire manuscript from a clinical field into a more methodological area. In such a case however, to mainly convey a technical message the manuscript lacks some important data (eg. On repeatability, on possibility to transfer the method to other laboratories, data on healthy donors, data on yields with spiked cells, etc). I would therefore suggest the Authors to clarify what is in the area that they want to mainly address (clinical or technical) and to improve/complete the manuscript accordingly.

In the current study we focused in the development of a methodology to evaluate the co-expression of two putative stemness and EMT markers at the single CTC level. Given the heterogeneity of CTCs, we aimed to evaluate the presumed differential expression of these markers among CTCs in two separate groups of patients with
early and metastatic breast cancer. The reviewer is right that we do not provide any clinical information regarding the outcome of patients according to the expression of these markers. However, we show a significant difference in the expression pattern of these markers as disease progresses from the early to the metastatic stage.

We are currently in the process of investigating the co-expression of ALDH1 and TWIST on CTCs in a large cohort of patients with metastatic disease, both before and after chemotherapy, in order to evaluate the clinical significance of these findings regarding patient prognosis and response to therapy. We have now described this in more detail in the last paragraph of Discussion (pages 19-20).

According to the reviewer’s suggestion, we provide in the revised manuscript control experiments performed in order to evaluate the sensitivity and specificity of our methodology. Three representative breast cancer cell lines were spiked into peripheral blood (whole blood or isolated PBMCs) obtained from healthy female donors and results on the yield, as well as on the sensitivity and specificity of the assay are now included (page 9 in Methods; “Evaluation of sensitivity and specificity of CTC detection” and pages 11-12 in Results). In addition the specificity of the CTC detection was evaluated in samples prepared from healthy blood donors and processed as patients’ samples. Moreover, a figure representative of the control experiments performed to evaluate the specificity of the antibodies (marked in the initial manuscript as additional file 1) is now included in the revised version as Figure 1.

The isolation of PBMCs by Ficoll-Hypaque density gradient centrifugation is a widely used method for CTC enrichment whereas, the antibodies used, as well as the ARIOL system are commercially available. Therefore we consider that our methodology is reproducible and can easily be transferred to other labs.

**Minor essential Revisions**

**Abstract**

In the Methods section please specify which breast cancer cells were used and when mentioning ‘CTC detection’ (line 7) shortly give the criteria for defining a cell as a CTC. In the Results section avoid using ‘great majority’ without giving a percentage value.
According to the reviewer’s suggestion, we have now added in the methods section of the Abstract that we used SKBR-3, MCF-7 and MDA.MB.231 cell lines for control experiments. Moreover, in the results section of Abstract we added the definition of a CTC and only percentage values are now presented (pages 3-4).

**Introduction**

Last paragraph claims:

‘Although co-expression of such markers has been shown on CTCs using molecular techniques this has not been demonstrated on individual CTCs’. This is in my opinion a very important statement which deserves a better description of the data obtained in this study, focusing more on the description of CTC heterogeneity.

Indeed, the reviewer is right that an advantage of our methodology over molecular techniques, such as AdnaTest®, is that it allows the characterization of individual CTCs. Thus, in this report we showed that individual CTCs isolated from patients with breast cancer present differential levels of ALDH1 expression and differential TWIST localization patterns. However, according to the reviewer’s suggestion we have now added in the results section more data emphasizing on the intra- and inter-patient heterogeneity of individual CTCs in each disease setting regarding the expression of the markers evaluated (Two last paragraphs in Background; page 6 and Results; pages 14-15).

**Methods**

Are CTCs obtained from fresh or from frozen PBMCs?

A clear definition of what is considered as a CTC is lacking. It appears to be a cytokeratin positive cell fulfilling additional cytomorphologic criteria which are not detailed.

In the current study, prospectively collected cytopsins were retrospectively analyzed for the presence of CTCs. Therefore, as it is referred at page 7 (Patient samples and Cytospin preparation), PBMC cytopsins were prepared and stored at -80°C till further
use. In addition, according to the reviewer’s suggestion we have now defined the cytomorphological criteria for CTC characterization, as proposed by Meng et al (page 8) [1].

**Discretionary Revisions**

**Results**
I would suggest to the Authors to organize the Results section in a different way to emphasize the description of the heterogeneity of the CTC population. Now Results are organized by marker. This means separate paragraphs are used for each marker (ALDH and TWIST) in the two settings and a third paragraph is addressing the co-expression of the markers (which is the real nodal point about CTC heterogeneity) again in each setting. Probably a subdivision in paragraphs by clinical setting analyzing the heterogeneous composition of the CTC population, followed by a paragraph making comparisons, would be more efficacious.

According to the reviewer’s suggestion, we have now re-organized the results section and provided the evaluation of the markers according to the clinical setting.

It would be probably better to describe CTCs heterogeneity referring to the total number of CTC positive cases rather than the total number of patients (both in the text and in the Figures).
In the current study a total of 80 and 50 patients with early and metastatic breast cancer, respectively, were evaluated for the expression of ALDH1 and TWIST; 13 out of 80 and 25 out of 50 patients were CTC-positive. All results, both in text and tables, are referred to the CTC-positive patients only. We have now added a sentence to further clarify this in page 9, last sentence in *Immunofluorescence*) and in the legends of Tables 2 and 3.
Why not consider a pie chart or a single bar with a different color-code for each subtype, or even more than one bar detailing subdivision by one marker and followed by additional bars going more and more in detail taking into account the other markers? (always referring to CTC positive samples only).

In the revised form of the manuscript we now include a condensed presentation of the different CTC phenotypes, grouping low and negative ALDH1 versus high ALDH1 expression and cytoplasmic or absent TWIST expression versus nuclear TWIST localization. According to this subdivision, four CTC phenotypes (instead of the nine previously shown) are now described which we believe is easier for the reader to follow. Importantly, we were still able to demonstrate the heterogeneity of CTCs and also the enrichment of the population presenting high ALDH1 and nuclear TWIST expression during disease progression. The different CTC phenotypes are now presented in Tables 2, 3 and 4. The abstract has been also adjusted accordingly.

**Discussion**

Avoid repeating data (and even p values) in the Discussion unless absolutely necessary. Try to convey a clear message. Now it is mainly a repetition of Results.

According to the reviewer’s suggestion we avoided the repetition of results in the Discussion section and tried to present the clear message stemming from our observations.
Reviewer 2

Reviewer's report

Although the subject of this proposed manuscript is potentially interesting, the data presented are not sufficient to speculate general conclusions. Moreover, an EPCAM+CD44+CD47+MET+ subpopulation has been already identified in breast cancer patients. In particular, these metastasis-initiating cells was able to give rise to a fenocopy of the parental tumors and were correlated with a worst prognosis in a cohort of metastatic breast cancer patients. The manuscript data are incomplete and based only on a simple identification and characterization of ALDH1 and TWIST expression in CTC from breast cancer patients.

Major concerns

1. According to the Epithelial-to-Mesenchymal transition (EMT), a cancer cell acquires the mesenchymal trait in order to give rise to a metastasis. For this reason authors identified a TWIST nuclear localization principally in CTC of metastatic patients. This is in contrast with the staining of the same cells with anti-pancytokeratin. The analysis of TWIST localization is not sufficient to support the authors conclusions. Other mesenchymal markers should be investigated.

The reviewer is right that co-expression of an epithelial marker along with an EMT marker on a single cell seems contradictory, however this has been previously shown in several reports. Thus, co-expression of cytokeratin and Vimentin was found to characterize cancer cells with even higher metastatic potential [2]. In addition, we have recently shown the expression of TWIST and Vimentin in the majority of Cytokeratin-positive CTCs of breast cancer patients [3]. In accordance, Armstrong et al demonstrated simultaneous expression of epithelial and mesenchymal markers on single CTCs of advanced prostate and breast cancer patients [4]. Moreover, a recent
article by Yu et al. reported that CTCs of breast cancer patients exhibit dynamic changes in epithelial and mesenchymal composition [5]. In this study EMT state was defined by the ratio between several epithelial and mesenchymal markers among CTCs.

However, the detection of CTCs using our methodology, similarly to other systems such as CellSearch® or AdnaTest®, is based on the expression of epithelial markers. Therefore, CTCs with complete loss of epithelial markers, bearing exclusively mesenchymal characteristics cannot be detected by these systems, since mesenchymal markers are commonly expressed also in normal hemopoietic cells.

We were not able to analyze further markers in the single CTC level due to the limitation of four immunofluorescence filters of ARIOL system. Nevertheless, the objective of this study was not to prove that CTCs with nuclear TWIST localization are indeed cells undergoing EMT; we simply evaluated the expression of a putative EMT marker on CTCs of patients with breast cancer. We showed a significant inter- and intra-patient variability in TWIST localization on CTCs. In addition, we showed that nuclear TWIST prevailed in the metastatic compared to early disease setting. TWIST is a transcription factor which induces EMT through repression of E-cadherin, by binding to the E-box elements of its promoter region [6]. Since efficient nuclear localization is essential for a protein to function as an activator and/or repressor of transcription of target genes [7], we suggested that CTCs with nuclear TWIST localization could potentially be in EMT state. We have now clarified our suggestions regarding the significance of TWIST localization, in the Discussion Section (page 18, second paragraph).

2) In order to identify a subpopulation with a metastatic potential authors should perform an in vivo model in which CTC co-expressing ALDH and TWIST are able to give rise to a tumor that resemble the parental.

The reviewer is right that functional assays are required to investigate the ability of CTCs co-expressing ALDH and TWIST to regenerate the primary tumor and therefore to prove their metastatic potential. However, this was not among the objectives of the current study, which aimed in the evaluation of the co-expression of
ALDH1 and TWIST at the single CTC level in patients with breast cancer. Indeed there is a study in which EPCAM+CD44+CD47+MET+ CTCs from breast cancer patients could initiate tumors in mice [8]. We are also currently evaluating the potential of CTCs to generate tumors in mouse models, aiming in the determination of specific CTC phenotypes capable of generating tumors.

3) Authors reported that all cell lines showed an heterogeneous population for both ALDH1 and TWIST stainings. Considering that the cell lines analyzed are representative of the three different breast cancer subtypes: luminal, basal and HER2 pos; data obtained do not provide any novelty. Moreover, all the images should be showed with a lower magnification.

The three representative breast cancer cell lines were included as controls for the investigation of the expression pattern of ALDH1 and TWIST. We have shown that ALDH1\textsuperscript{high}, ALDH1\textsuperscript{low} and ALDH\textsuperscript{neg} cell subpopulations could be detected within each cell line, with comparable ranges and median expression values. Similarly heterogeneous subpopulations according to TWIST expression (TWIST\textsuperscript{nuclear}, TWIST\textsuperscript{cytoplasmic} and TWIST\textsuperscript{neg}) were observed among all cell lines. These experiments verified the objectivity of our method irrespectively of the specific breast cancer subtype and allowed its application on patient samples. This is explained in Discussion, in the second paragraph of page 17.

Moreover we tried to change the magnification of images, though in many cells low ALDH1 expression and nuclear localization of TWIST could not be obvious. However, lower magnification is now presented in Figure 2B, which depicts a Cytokeratin+ CTC bearing high ALDH1 and nuclear TWIST, between normal hemopoietic cells.

4) Results reported in the text do not always correlate with the images. For example, authors report that HepG2 cells have a TWIST nuclear localization while in the Figure 1 it seem cytoplasmatic. Authors should better check the correlation between Figures and text.
Actually two HepG2 cells with cytoplasmic TWIST localization and one cell with nuclear TWIST are depicted in this Figure, which has now been changed to Figure 2. As it is mentioned in the Methods (Evaluation of ALDH1 and TWIST expression in cancer cell lines using the ARIOL system”), in this study TWIST was characterized as cytoplasmic when localized exclusively in the cytoplasm, and as nuclear when localized in the nucleus, regardless of its co-localization in the cytoplasm. However, we have now marked indicatively one cell with nuclear and one with cytoplasmic TWIST expression in MCF7 cell line (Additional file 1). High and low ALDH1 expressing cells were also marked in this figure.

**Minor revisions**

1) Several grammar errors

We apologise for the mistakes observed. We have carefully re-edited the revised manuscript.

**EDITORIAL REQUEST**

Please add the full name and affiliation of the ethics committee that approved the study.

The ethics committee that approved this study was the Local Ethics Committee of University Hospital of Heraklion, Crete, Stavrakia-Voutes 71110, Heraklion.
References cited


