Author's response to reviews

Title: Selection of Cancer Stem Cells: a Role in Acquisition of Resistance to EGFR Inhibitors in Lung Cancer

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Version: 2 Date: 3 November 2011

Author's response to reviews: see over
RESPONSE TO REVIEWERS

We thank the two expert reviewers for their evaluation and constructive suggestions for improving our manuscripts. We were pleased to note that both reviewers found the work interesting and important to the field. To address the reviewers’ concerns we have performed additional experiments over the past month to provide additional repeats of experiments in the first draft and to assess expression of additional stem cell markers. Also, we added substantial statistical analysis to better support our conclusions. Finally, we attempted to clarify our approach and conclusions through text edits.

Our specific responses to the reviewers are provided in the context of the original reviews, shown below. Changes to the manuscript are noted by red text in the manuscript file.

Reviewer's report
Title: Selection of Cancer Stem Cells: a Role in Acquisition of Resistance to EGFR Inhibitors in Lung Cancer
Version: 1 Date: 6 September 2011
Reviewer: Raj Batra
Reviewer's report:
Ghosh et al present an important report to add to the sparse literature on “Cancer Stem Cell” (CSC) properties in lung cancer. They utilize a paired model of H1650 and H1650 EGFr-blocker-resistant cells to characterize “CSC” properties. The rationale for the study is that clinical treatment of EGFr-blockers is transiently effective in selective subsets of patients with EGFr mutations, and that the recognized mechanisms for that resistance need to be better understood. They potentially have a model in which they can deduce those mechanisms.

The MAJOR issues and inquiries I have with the report are as follows:
MATERIALS AND METHODS:
1) I believe that the cell model used (H1650 cells) are derived from an advanced stage lung cancer model (a malignant pleural effusion). If that is the case, please discuss that derivation phenotype might impact the outcome measures where one is counting aggregates in suspension or anchorage independent growth as functional attributes of “CSC”?

Derivation or immortalization of H1650 cells enables them to grow for a prolonged period of time in vitro when cultured under adherent conditions in the serum supplemented media. However, non adherent and serum free culture conditions lead to the death of most of the cells (anoikis). Only cells with stem cell like properties can survive under these stem cell selective conditions and give rise to tumor spheroids (Zhang, S., et al., Identification and characterization of ovarian cancer initiating cells from primary tumors. Cancer Research 68 (2008) 4311; Lee, J. et al., Tumor stem cells derived from glioblastomas cultured in bFGF and EGF more closely mirror the phenotype and genotype of primary tumors than do serum-cultured cell lines. Cancer Cell 9 (2006) 391). The ability of H1650 cells to form tumor spheroids under non adherent conditions suggests existence of cells with stem cell properties. Enhanced tumor spheroid formation ability of erlotinib resistant subline, H1650-ER1, also suggests that the resistant subline is enriched with putative CSCs.

It is, however, possible that conferring of stem cell like properties to H1650 cells is related to the derivation of these cells, as suggested by Reviewer 1. In order to exclude
the possibility of existence and enrichment of cell populations with cancer stem cell properties only in H1650 cells, we investigated CSC properties in SCC-1 and EGFR TKI refractory sublines (SCC-1-Erl-R and SCC-1-Gef-R). We demonstrated that EGFR TKI selects for cells with stem cell traits in resistant sublines. Based on our results we cannot conclude erlotinib treatment selects for CSC-like cells in all cancer or lung cancer cells, but we have shown this result in more than one cell line. In future work we plan to investigate mechanisms by which this selection occurs and determine if properties of the cancer cell affects the selection process.

We added the following discussion to p. 14 to attempt to clarify this point: “To ascertain that the existence of CSC like cells in H1650 and corresponding enrichment upon erlotinib treatment in H1650-ER1 cells is not specific to H1650 cell line, presence of cells with CSC traits was also investigated in human head and neck squamous carcinoma cell line SCC-1 and EGFR TKI refractory sublines (SCC-1-Erl-R and SCC-1-Gef-R). We also demonstrated the existence of putative CSCs in SCC-1 as well as SCC-1-Erl-R and SCC-1-Gef-R cells via side population analysis and tumor spheroid formation assay.”

2) With respect to spheroid formation assay, how were cells dissociated for serial transfer?

The spheroids were collected via centrifugation. The collected spheres were treated with trypsin and passed through cell strainer to obtain single cells. The details have been added to page 5 of the manuscript: “In order to assess self renewal through formation of secondary spheroids, the spheroids were collected by centrifugation, dissociated into single cells by treating with trypsin and passing through 40 µm cell strainer, and then cultured under conditions described above.”

3) With respect to soft agar assays, what was the “growth medium” used for infiltrating the agar?

Growth medium used was RPMI 1640 supplemented with FBS and glutamine. This has been included on page 7: “Growth medium (RPMI1640 supplemented with 10% FBS and 2 mM glutamine) was changed every 3 days.”

RESULTS AND DISCUSSION:

4) The text on page 8 does not corroborate the reported observations in Figure 1A.

The text did not correlate with the figure 1A due to an error in labeling the figure. The mislabeling of the vimentin and occluding data points in the figure has been corrected.

5) Exactly what is being measured in figures 1A and 1B? How are these measures normalized? If these are measures related to qRT-PCR, then please include the primers used and data analyses undertaken to make and report these measures.

Figure 1A reports mRNA expression of E-cadherin, vimentin, occludin and fibronectin, and Figure 1 B reports mRNA expression of and Snail, Twist and Zeb1. In both panels, qRT-PCR was used to quantify mRNA levels in H1650-ER1 and H1650 cells. ∆Ct was calculated to normalize the gene expression in the cells with respect to the expression of GAPDH. ∆∆Ct was calculated to determine the fold change in H1650-ER1 as compared to H1650 cells. A clearer description of this method has been included in the manuscript.
Gene expression in H1650, H1650-ER1 cells, H1650-ER1 spheroids and adherent cells was initially normalized against GAPDH to obtain ∆C\textsubscript{t} values. Relative fold change in gene expression was then compared between H1650-ER1 and H1650 or H1650-ER1 spheroids, adherent cells and H1650-ER1 cells using ∆∆C\textsubscript{t} method of quantitation. ∆C\textsubscript{t} values of different cell populations were used to perform statistical analysis. p-value < 0.05 was considered significantly different.

The oligonucleotide primer sequences are now provided in Table 1.

6) With respect to Figure 1c, please provide the rationale for using beta-catenin (as opposed to E cadherin) membrane localization as a measure of epithelioid properties. Please discuss whether anything be deduced from the absence of nuclear b-catenin in 1C?

mRNA expression (Fig 1A) and western blot analysis (data not shown) indicated significant reduction in E-cadherin expression in H1650-ER1 cells as compared to H1650 cells. So, to correlate E-cadherin downregulation with β-catenin localization, immunofluorescence for β-catenin was performed. Even though membrane localization of β-catenin in H1650-ER1 cells was reduced and cytoplasmic localization was increased as compared to H1650 cells, little nuclear localization was observed in ER1 cells. In case of H1650 cells, virtually no nuclear localization was detected. This observation is consistent with a partial EMT in H1650-ER1 cells.

7) With respect to Figure 1D, please describe the details of how the motility assay was conducted (it is not described in methods).

A wound was inflicted in the cell monolayers via scraping and wound healing was calculated from images captured at time points t=0 and t=12h. Details of this method are now included on pg 5-6 of the manuscript: “Cell Migration Assay: H1650 and H1650-ER1 cells were seeded in each well of 6 well plates and allowed to reach confluence. Once confluent, a wound was inflicted in the monolayer by scraping with a sterile 200 µL pipette tip. The cell monolayer was then washed three times with DPBS to remove the cell debris and incubated with the growth media. Pictures of the wound were captured at time points t=0 and t=12 h to calculate the wound area. Migration of the cells was calculated fractional closure of the wound area.”

8) With respect to Figures 2, 3, 4 and 6, can one derive a standard error about the mean (SEM) from 2 measures?

In case of Figure 2, additional experiments have been carried out and the error is calculated from 3 independent measures. However, in case of Figures 3, 4 and 6, the error bar represents the standard deviation of 6 replicates. This point has been clarified in the figure captions.

9) Again, exactly what is being measured in Figure 2B and how was this normalized? (I suspect that constitutive expression profiles are being compared, but neither the primers used for amplification nor the analytical details for normalization are presented).
As described in detail in response to comment 5, expression levels of target genes were normalized with respect to GAPDH and then fold change calculated using the \( \Delta \Delta CT \) method. Primers have been included in Table 1.

10) What does the author mean by the term "self-renew"? Is this term synonymous with the ability to efficiently form soft agar colonies? and/or with possessing the ability to serially form soft agar colonies? And if so, then what is the molecular basis for verifying this as a "self-renewal" property?

The term self-renewal means that the cell progeny retain the cancer stem cell-like properties, including spheroid formation in soft agar, following cell division. Molecular evidence of self-renewal can be found in Figure 3E as upregulation of the stem cell markers OCT3/4, NANOG, and BMI-1 in third generation spheroids.

11) What is the basis of making the statement that culture in RPMI 1640 with 10% FBS induces "differentiation"? Are adherent cells more "differentiated" than an "aggregate cluster" of cells in suspension?

The term differentiation refers to loss of stem cell markers and phenotypes. Studies have demonstrated serum induces differentiation in tumor stem cells and reduces expression of stem cell markers (Lee, J. et al., Tumor stem cells derived from glioblastomas cultured in bFGF and EGF more closely mirror the phenotype and genotype of primary tumors than do serum-cultured cell lines. Cancer Cell 9 (2006) 391). So, when cultured under differentiation condition (i.e. addition of FBS, withdrawal of growth factors, and adherent conditions), dissociated single cells from spheroids can give rise to differentiated progeny. As discussed in Fig 3E, expression of self-renewal genes was reduced in adherent cells, suggesting that these cells are undergoing differentiation from a CSC-like state to a non-CSC-like state.

12) What is the surface phenotype of the parental versus erlotinib resistant cells with respect to CD44/CD24 status?

A higher fraction of H1650-ER1 cells displayed CD44\textsuperscript{high}/CD24\textsuperscript{low} phenotype as compared to H1650 cells, supporting the conclusion that the H1650-ER1 contains a larger population of CSC-like cells than the H1650 population. Flow cytometric plots have been included in Fig 2. A discussion of these data are provided on p. 9: “As demonstrated in Fig 2A, approximately twice as many H1650-ER1 cells displayed CD44\textsuperscript{high}/CD24\textsuperscript{low} expression patterns as compared to H1650 cells. The CD44\textsuperscript{high}/CD24\textsuperscript{low} cells comprise a small fraction of the total H1650-ER1 population, representing less than 2% of the cells.”

EDITORIAL COMMENT (DISCRETIONARY)
1) It would be interesting (and perhaps predictable) that the changes induced by drug exposure may contribute to therapeutic resistance above and beyond erlotinib exposure. The paper would be made much stronger by the inclusion of such data.

We thank Reviewer 1 for this interesting suggestion. Our goal with this manuscript was to identify and perform \textit{in vitro} characterization of subpopulations of cancer cells that develop upon exposure to EGFR TKIs. In future work we plan to investigate more deeply the behavior of this subpopulation in animal models more relevant to cancer
development and treatment, as discussed below in response to Reviewer 2, concern 1. In this study we will implement Reviewer 1’s suggestion to investigate therapeutic responses to treatments other than erlotinib.

**Level of interest:** An article of importance in its field  
**Quality of written English:** Acceptable  
**Statistical review:** Yes, but I do not feel adequately qualified to assess the statistics.  
**Declaration of competing interests:** I declare I have no competing interests.

**Reviewer’s report**  
**Title:** Selection of Cancer Stem Cells: a Role in Acquisition of Resistance to EGFR Inhibitors in Lung Cancer  
**Version:** 1  
**Date:** 12 September 2011  
**Reviewer:** Brigitte Gomperts  
**Reviewer’s report:**  
Major compulsory revisions:  
1. The gold standard in the field to identify a cancer stem cell (CSC) population is the performance of serial xenografting of tumors. This needs to be done comparing the cell line vs cell subline and side population (SP) vs non-SP.

We agree that serial xenografting is the most widely accepted functional assay for demonstrating the two properties that define cancer stem cells, self-renewal and differentiation. Serial xenografting has imperfections, including a complex and poorly defined microenvironment, low throughput, and difficulty in assessing temporal dynamics in the cell population. In this study we profiled the cell populations through *in vitro* assessments of stem cell markers and phenotypes that suggest a stem cell character, including spheroid formation in soft agar.

Several studies have made conclusions of cancer stem cell populations solely based on *in vitro* characterization (e.g. Sung, J.M., et al., Characterization of a stem cell population in lung cancer A549 cells. *Biochemical and Biophysical Research Communications* 371 (2008) 163–167; Tirino, V., et al., Detection and characterization of CD133+ cancer stem cells in human solid tumors. *PLoS One* 3 (2008) e3469). While there is debate as to what assays are necessary or sufficient to conclude a cell is a CSC, we agree with the reviewer’s concern that it is not valid to conclude that the cell population obtained in our study represents CSCs without serial xenografting. The goal of our study was to identify and characterize populations of cells selected by EGFR TKI treatment. Our results demonstrate that this subpopulation has cancer stem cell markers and phenotypes. We believe this result is of interest to the EGFR therapeutic research community.

To address this concern we have changed the title and text throughout the manuscript to remove any indication that these are cancer stem cells, and instead refer to them as cells possessing cancer stem cell properties, cancer stem cell-like cells, or putative cancer stem cells. A primary goal of our ongoing work in this area is to determine whether these truly are CSCs by collaborating with an expert in serial xenografting. We thank Reviewer 2 for highlighting this point and assisting us in not making conclusions that cannot be justified by the data.
2. Statistical analysis is missing from large parts of the data and is essential to support some of the authors' conclusions.

Statistical analyses had been performed and p-value had been reported throughout the manuscript. In addition, we have repeated several experiments to increase the statistical significance of the data, as described in response to Reviewer 1, Concern 8. All statistical analysis support the corresponding conclusions with p less than 0.05.

Minor essential revisions:
This is an interesting article on an important clinical problem. The issue of resistance to EGFR targeted therapy in lung cancer is of great interest to the field and the question being asked is whether this resistance is specifically in a population of cells that represents a tumor-initiating or cancer stem cell population. In order to examine this question, the authors cultured a non-small cell lung cancer cell line with erlotinib to generate a resistant subline and then examined features of this resistant subline. The deletion mutation #E746-A750 within the EGFR kinase domain of the EGFR gene was notably present in both the cell line and subline. There were no additional mutations observed in the EGFR open reading frame in the subline.

The following concerns should be addressed:
1. No statistical analysis was performed to demonstrate whether there truly is a difference in expression of e-cadherin, occludin, snail and twist between the cell populations by Q-RT-PCR. A quantitative assessment of localization of beta-catenin is needed. Again a statistical analysis is needed to assess the significance of the motility differences between the cells of the line and subline.

Statistical analyses have been performed to indicate significant difference in expression of E-cadherin, occludin, fibronectin, vimentin, snail and twist between the cell populations. We performed quantitative assessment of beta-catenin localization using images captured following immunostaining and statistical analysis of cell migration. These data are now included in pg 9 of the manuscript: “Immunofluorescence analysis showed that beta-catenin remained localized at the membranes in 68% of H1650 cells as opposed to 33% of H1650-ER1 cells (p-value <0.01), whereas there was greater cytoplasmic localization of beta-catenin in H1650-ER1 cells (51% of H1650-ER1 cells vs. 18% of H1650 cells, p-value< 0.01) (Fig 1C). In addition, resistant cells also displayed enhanced motility (p-value< 0.05) measured as the ability to heal a defect in a cell monolayer (Fig 1D).”

We appreciate this suggestion which strengthens our conclusions regarding beta-catenin localization and altered motility in the H1650-ER1 cell line.

2. It is not clear how cells were counted to assess differences in expression of surface markers CD133, SSEA-3, SSEA-4, Tra-1-60 and Tra-1-81. If it is flow cytometry, flow cytometry plots should be shown. Also, it is not clear how the relative mRNA expression is calculated and no statistical analysis is performed.

Representative flow cytometric dot plots and histograms are now shown (Figures 2A and 2B). mRNA expression was calculated by ΔΔCt quantification method as indicated in our response to Reviewer 1, comment 5. Statistical analysis had been performed and reported in the manuscript as discussed above.
3. For the SP data, the flow cytometry plots look similar. Are there really only 4% of SP cells in the gate in fig 4A-, H1650? The actual percentage of cells in the SP gate for that particular plot needs to be shown and not for all the plots with the SEM. No p-value is reported to know whether there is a difference between the cell line and subline. Fig 4B should show the flow cytometry plots of sorted SP cells vs non-SP cells from the cell subline. Either this is incorrectly labeled or the plots are incorrect.

We indicate the percentage of cells in the SP gate in the plots shown in Figure 4A. In case of H1650 and H1650-ER1 cells, the SP populations are comprised of 7% and 15% of cells respectively. The p-value for multiple replicates of this experiment has been included in page 11 of the manuscript (p<0.05), indicating a statistically significant difference in the SP fraction between H1650 and H1650-ER1 cells. We corrected the label on Figure 4B to indicate SP populations in the sorted SP and non-SP cells.

4. Fig 5A– the images of the soft agar colonies are of very poor quality.

We have increased the resolution of the phase contrast images in Figure 5A. Since these are in 3D many of the colonies are out of the focal plane and appear blurry, but the images clearly indicate a difference in size of the colonies formed by H1650 and H1650-ER1 cells.

5. For fig 6C, it is not clear if the spheroids are derived from the erlotinib treated subline cell SP or just the erlotinib treated cell line. A comparison of these would be interesting. Statistical analysis is needed for fig 6.

The spheroids were derived from H1650-ER1 cells under the continuous presence of erlotinib. We modified the text on p. 12 to clarify this point: “The resistance phenotype of these stem like cells was further confirmed by investigating spheroid forming ability of H1650-ER1 cells under continuous exposure to 10 µM and 50 µM of erlotinib (Fig 6C).”

Statistical analysis is now included; p-value > 0.05 indicates that erlotinib had no significant, detectable effect on spheroid formation (page 12).

6. The terminology should be changed from CSC to SP cells or “putative” cancer stem cells when the authors refer to their SP subline cells as CSCs. The authors have not conclusively demonstrated that the SP subline cells are truly CSCs.

We removed all instances of the CSC terminology and refer to them as SP cells, putative CSCs, cells with CSC properties, or CSC-like cells throughout the manuscript, as discussed in our response to Reviewer 2, comment 1.

Discretionary revisions:
If erlotinib resistance and SP are the markers of a CSC population for NSCLC, then deriving these cell populations from fresh NSCLC tumors and performing serial xenografting will test this hypothesis.

We thank reviewer 2 for this suggestion. We are working with a collaborator to obtain NSCLC tumors for in vitro and subsequent serial xenografting analysis. The current
manuscript motivates this work and suggests the likelihood of identifying CSC populations in these tumors.

Level of interest: An article of importance in its field
Quality of written English: Acceptable
Statistical review: Yes, and I have assessed the statistics in my report.
Declaration of competing interests: I declare that I have no competing interests