Reviewer's report

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

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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.
2. Statistical analysis is missing from large parts of the data and is essential to support some of the authors’ conclusions.

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.
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.
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.

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

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.

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.

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.

**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