Editor's comment

Title: Identification of A Hepatocellular Carcinoma Cell Line Capable of Monitoring The Differentiation of CD13(+)CD166(-) Cancer Stem Cells And Effects of Sorafenib

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Editor's comment:

Dear Rose,

Please find attached my decision form regarding the manuscript MS: 1649917584144627. Should you have any additional questions, do not hesitate to contact me.

Antonio Mazzocca

DECISION FORM

1. COMMENTS

Comments to be passed to the authors:

Dear Dr. Abei

Thank you for submitting your manuscript entitled "Identification of A Hepatocellular Carcinoma Cell Line Capable of Monitoring The Differentiation of CD13(+)CD166(-) Cancer Stem Cells And Effects of Sorafenib." Expert reviewers in the field have evaluated your manuscript and raised concerns, so we cannot accept the manuscript in its present form. However, if you choose to resubmit with responses to the major compulsory revisions, I would be willing to reconsider it, provided it clearly addresses all the concerns of the reviewers, and states how this has been achieved in the rebuttal letter. The review comments are enclosed at the end of this email.

Please note that the revised manuscript may have to be re-reviewed, which might result in additional delays to publication while the original reviewers assess the resubmission. If the reviewers give a low rating, we will not be able to accept the manuscript for publication.
Thank you for submitting your manuscript.
Sincerely,
Antonio Mazzocca, M.D., Ph.D.,
Associate Editor
BMC Cancer

Reviewer no. 1

Reviewer's report: This manuscript describes the identification of a HCC cell line which shows CSC hierarchy and allows monitoring of CSC differentiation. The topic is timely and potentially clinically relevant given the current interest in establishing #in vitro# hierarchical models for screening drugs targeting CSC in order to develop novel therapeutic strategies for HCC. The experiments are in general well controlled and clearly described. Furthermore the conclusions are supported by the experimental evidence presented. However, the #Discussion# section is lengthy and should be shortened.

Specific Comments:

Page 7, paragraph 1: How many times did the authors perform #in vitro# experiments?

Page 9, paragraph 2: The #in vivo# experiments are limited by the low number of mice used.

Minor Comments: This manuscript contains many inaccuracies which need to be addressed. Only some of them are listed in this review.

Page 2, paragraph 3: ##5FU.# should read ##5-FU.# This comment applies to other parts of the manuscript.

Page 5, paragraph 2: ##5-FU## should read ##5-fluorouracil (5-FU)##

Page 6, paragraph 2: ##mIgG1## should read ##mouse IgG1##

Page 7, paragraph 1: ##5-fluorouracil (5-FU;## should read ##5- FU(##

Page 8, paragraph 1: ##1000 cells## should read ##1x103 cells##

Page 17, paragraph 1: ##resuls## should read ##results##

Page 20, paragraph 1: ##in vitro## should read ##in vitro##

References: Reference 25 should be Reference 23
Reference 23 should be Reference 25
Reference 29 should be Reference 28
Reference 28 should be Reference 29

Reviewer no. 2
Reviewer's report: In this manuscript, the authors aimed to identify hepatocellular carcinoma cell lines that demonstrated a cancer stem cell hierarchy in order to identify an in vitro (cell line) model that would more closely reflect the cancer cell hierarchy that is noted in vivo such that the cell line model could be used to screen drugs that have been developed to target stem-like cancer cells. The authors identified one cell line, Li-7, that demonstrated what the authors called a population switch where, over time in culture, the slow-growing stem-like cells (CD13+CD166-) slowly disappeared from the cultured cells as they were outgrown by the rapidly dividing progenitor-like cells (CD13-CD166+). Using sorted Li-7 cell populations, the author demonstrated that CD13+CD166- cell populations make other cell populations (e.g. CD13-CD166+ and CD13-CD166- cells), possess a large proportion of cells that express high ALDH activity, and possess a high expression of certain stem cell genes (Oct4, Sox17, Myc, and KRT19). The authors also demonstrated that CD13+CD166- cells are relatively resistant to 5-FU; however, that they are particularly sensitive to Sorafenib, and that a combination therapy of 5-FU and sorafenib suppressed the growth of bulk Li-7 cells better than either therapy alone.

Major Compulsory Revisions
1. The main purpose of the paper was to present an in vitro model that could be used for drug screening. It is not clear why a population switch is necessary for the effectiveness of an in vitro model. Why would cells undergoing a population switch be any more effective than simply having a heterogeneous cell population with some cells exhibiting stem-like characteristics and some not. Furthermore, I am concerned about using cells that have been left in culture for 2 months or more for any type of experiment, and am concerned about the results obtained from cell populations that have been left in culture for so long.
2. Along these lines, the authors do not clearly state why the population switch is so meaningful. What would the results be if the different populations that existed in the other cell lines were examined? Additionally, why was the Li-7 CD13+CD166+ cell population not investigated in any of the assays performed in the manuscript?
3. If the other cells lines tested contain heterogeneous cell populations, the authors should perform all of the experiments in this manuscript with at least one or two of the other cell lines.
4. It is not clear whether the other cell lines tested were also heterogeneous in nature regardless of whether they demonstrated the population switch (e.g. did the other cell lines contain populations of cells with various CD13/CD166 expression profiles). For all cell lines tested, it would be essential to know what cell populations were present in each cell line (if any), what percentage of the cells made up the different cell populations, and how the cell populations change over time both in culture and in vivo.
5. Experiments that compare cells passages in vivo vs. cells passaged in vitro would be very beneficial.
6. The authors did not define the statistical analyses that were used in this
manuscript, nor did they define the level of significance in the methods section or in the figure legends. This information absolutely needs to be included in the manuscript.

7. Using the cell counting kit-8 does not necessarily reflect true cell death. The authors stated that Sorafenib has been shown to inhibit proliferation (induces G0 cell cycle arrest). A truer test of cell death (that has more clinical relevance) would be to re-plate cells treated with Sorafenib (or 5-FU) and determine the number of cells that are able to grow back and/or are able to truly survive the therapy. What are the characteristics of the cells that survive?

8. The authors assert that CD13-CD166+ cells are rapidly growing progenitor cells. It is not clear why the authors refer to these cells as progenitor cells. What studies were performed in order to ensure that they are indeed some type of progenitor cell?

9. It is unclear why the authors looked at ALDH activity in Li-7 cells as they did not describe previous work that showed that high ALDH activity in HCC cells correlated with stemness nor did they show enough evidence in the manuscript to support the idea that high ALDH activity = CSC properties in HCC cells. The authors should mention whether ALDH activity has been shown to play a role in HCC. The authors should also explain why the relatively slow-growing CSCs had the highest expression of ALDH activity when it has been shown previously that highly proliferative progenitor cells have a higher ALDH activity than their slower-growing counterparts in some systems [see work by Jan Moreb’s or David Hess’s groups].

10. The authors state that CSC populations have been shown to be relatively resistant to cancer therapies because of a higher expression of drug pumps and a higher ability to detoxify ROS. There has also been some important work done that has demonstrated that ALDH activity itself protects cancer cells from both chemotherapy and radiation. Seeing as the CD13+CD166- cells express high ALDH activity, it would be prudent to investigate what Sorafenib does to ALDH activity in Li-7 cells. It would also be important to investigate whether ALDH confers resistance to HCC cells # what happens to the sensitivity of the cells if ALDH activity is blocked or downregulated?

11. Overall, the impact of this paper would be stronger if the authors had a plausible mechanism for why Sorafenib selectively targeted the CD13+CD166- population. What happened to the cells that were treated with Sorafenib? Did the expression of drug pumps decrease? Did the expression of ROS-detoxification enzymes decrease?

12. The authors state that there are differences in CSC marker expression patterns between cells in vivo and in vitro, and that only CD13 expression is consistent. Are the authors concerned that this fact does not support the use of their in vitro model because it shows that the cells are different in vivo vs. in vitro? How does the expression of the markers in vivo change the cell behavior? Why is the marker expression so different in vivo vs. in vitro?

13. The authors stated that CD13+ cells focally accumulated near vessels in a xenograft tumor developed by Li-7 cell injection. If these results are truly based
on a tumour that developed in one mouse, these results are meaningless. What would be more appropriate is to calculate the average expression of CD13 near vessels in tumors formed in multiple mice. Additionally, it is unclear why undifferentiated cells would be located so close to a vessel. Would they not want to seek out a more hypoxic environment?

Minor Essential Revisions

1. The methods section in the Abstract does not reflect much of the work that was done in the study and should be updated to more accurately reflect that

2. The authors need to be more clear what cells are being used in each experiment. What was the passage of the sorted cells that were used? Were 2 month old cultured Li-7 cells used in any of the experiments outside of Figure 1?

3. Why is the CD13+CD166+ cell population not investigated in any of the assays? This should be explained, or the results should be shown in this manuscript.

4. In the Methods section, when describing the ALDH assay, it seems as though ALDH activity and 7-AAD are fluorescing at the same wavelength. Please explain how you can use the same laser to distinguish between ALDH and 7-AAD (would be helpful to also identify the different wavelengths used).

5. The authors need to be consistent with use of terminology throughout the manuscript. It is unclear what is meant when the authors begin by describing the cell populations as CD13-CD166+, and then change the descriptions to CD166+ or CD166-. Are these the same populations, or when the cells were sorted, were they only sorted based on CD166 expression? If this was done, why were the cells sorted solely on CD166 expression?

6. The authors use the terms CSC and non-CSC when referring to the different cell populations. Because the authors did not definitively show that the cell populations were in fact CSCs, and only that they possessed some CSC properties, it would be more appropriate to use the terminology stem-like cells/tumor-initiating cells.

7. When the authors performed the spheroid assays, the number of colonies that were formed was investigated. The average size of the colonies that formed is equally useful information and should be included in this manuscript.

8. The mRNA expression of the various stemness genes (Oct4, Sox17, Myc, KRT19) should be validated at the protein level. Were other markers investigated? If not, the authors should describe the relevance of the markers that were chosen as this was never clearly described.

9. The authors suggest that Sorafenib selectively kills CD166- cells in culture because CD166 expression is increased following Sorafenib treatment. Is it possible that the cells simply upregulated their expression of CD166?

10. What is the efficacy of Sorafenib in vivo? How well do the Li-7 results presented here compare to in vivo studies?

11. It would be helpful if the authors indicated the characteristics of the tumors that the various cell lines examined in this manuscript originated from.
12. The importance of ALDH activity in HCC cells and why it was investigated in this study is unclear. It would be helpful if the authors were more explicit about why they looked at ALDH activity and why their ALDH results were meaningful.

Discretionary Revisions
1. It would be helpful to describe the mechanism of action of Sorafenib in the manuscript to provide readers with that information who are unfamiliar with the specifics of the drug.
2. It is very difficult to differentiate between the 6 cell lines in Figure 5c.
3. The discussion section is not presented in a coherent manner and readers are left unclear as to what the salient points of the study were and why they were important.
4. Overall, the writing is not quite at the quality that it should be for a scientific publication: some typos, misuse of connecting/joining words (e.g. however, although, therefore), and some misuse of words (some examples below)
   Example: On page 15, line 18 # did you mean to say #as long as# instead of #unless#?
   Example: On page 16, line 10 # did you mean to say #clonogenic# instead of #chronologic#?

Confidential comments for the editorial staff which should not be passed to the authors:

2. DECISION.
Recommendation on the suitability of this manuscript for publication:
The present manuscript is potentially an interesting article in its field. However, it cannot be accept in the present form. A revision and additional experiments in response to the significant reviewer comments are necessary to significantly strengthen the value of the manuscript and reach the priority for publication in BMC Cancer. For this reason, the present manuscript needs to undergo major revisions. In this case, the authors should revise their work to improve the writing, the readability and the scientific soundness of their manuscript. If the authors respond to all major compulsory raised by reviewers, I would be willing to reconsider a modified version of their manuscript.

3. STATISTICS.
No, the manuscript does not need to be seen by a statistician.

4. LANGUAGE.
English needs some language corrections before being published

5. INTEREST LEVEL:
An article of limited interest
Declaration of competing interests I declare that I have no competing interests

Best regards

Antonio Mazzocca

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