Author's response to reviews

Title: Cancer stem-like sphere cells induced from de-differentiated hepatocellular carcinoma-derived cell lines possess the resistance to anti-cancer drugs

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Version: 4  Date: 25 July 2014

Author's response to reviews: see over
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Version: 4 Date: 25 July 2014

Author's response to reviews: see over
Dear Dr Amancio Carnero,

Thank you so much for your kind invitation to submit our revised manuscript (MS: 1448508355126326).

We here submit the revised manuscript, together with point-by-point account of how we have responded to each of the reviewer’s comments. You will know that revisions were made satisfactorily in the new manuscript (revised points are blue in the text). We also added Shoichi Hazama as an author of the new manuscript.

We hope that we have answered the reviewers’ questions adequately. We understand that final acceptance depends on satisfactory resolution of these issues.

We appreciate the thoroughness with which the reviewers regarded our paper and hope that it is now suitable for publication in the journal, *BMC cancer*.

Thank you very much for your consideration.

Sincerely yours

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Reviewer's report

Title: Cancer stem-like sphere cells induced from de-differentiated hepatocellular carcinoma-derived cell lines possess the resistance to anti-cancer drugs

Version: 3 Date: 19 May 2014

Reviewer: José Manuel García-Heredia

Reviewer's report:

Minor Essential Revisions:
Although the authors showed clear results about its method for obtaining cancer stem cells from poorly differentiated or undifferentiated HCC cells, they must modify some parts of the manuscript, in order to clarify the results.

Thank you very much for your comments. According to your indications, we revised the some parts of the manuscript in the new manuscript.

Some tables should be included, inside a figure or as a separated table. This is required for figures 4, 5 and (maybe) 7. These tables should be accompanied by the deletion of all percentages referring these figures included in the text. As an example: "CD24 positive: HLE, 88.1%; HLE-sphere, 58.6%; SK, 32.2%; SK-sphere, 11.4%; CD90 positive: HLE, 83.9%; HLE-sphere, 43.6%; SK, 75.7%; SK-sphere, 13.1%; Fig. 5". In a table, or a barchart, these percentages will be clearer to read.

Thanks for your valuable comments; on the basis of your recommendation, we added the bar-charts for flow-cytometric results (Figs 4, 5 and 8), and simplified the descriptions for these results in the text (lines 252 to 261 on page 11). In addition, flow-cytometric analyses were changed to single-staining, in order to facilitate reading comprehension (Figs 4 and 5).

In figure 1 and 2, they should use the same scale for all micrographs. In addition, the morphology of SK cells in micrograph of Figure 2F is not clear. I think that are "spheroids adherents", according to the text, but it should be clarified.

On the basis of your indication, we added or substitute the micrographs with same scale bar in Figure 1 and 2.
Level of interest: An article whose findings are important to those with closely related research interests

Quality of written English: Needs some language corrections before being published

Statistical review: No, the manuscript does not need to be seen by a statistician.

Declaration of competing interests:
I declare that I have no competing interests
Reviewer's report

Title: Cancer stem-like sphere cells induced from de-differentiated hepatocellular carcinoma-derived cell lines possess the resistance to anti-cancer drugs

Version: 3 Date: 9 June 2014

Reviewer: Sandra Muñoz-Galván

Reviewer's report:
In this article, Hashimoto and colleagues provide a novel method for induction of cancer stem-like sphere cells from poorly differentiated hepatocellular carcinoma cell lines. These sphere cells express stemness and cancer stem cell markers and are resistant to a high variety of anti-cancer drugs. Consistently, they show increased expression of ABC transporters, G0/G1 cell cycle arrest and high expression of p21, and reduced levels of ROS together with high HIF1# mRNA levels.

Although the development of this new method is of relative interest, the authors must address some important issues before being suitable for publication.

Thank you very much for your valuable comments, which have allowed us to refine our presentation and data in the previous manuscript. According to your indications, we added several important experiments. On the basis of the new data, we revised the previous manuscript.

a) Major compulsory revisions
- It is important to include the sphere-forming HLE cell line in the RT-qPCR, drug sensitivity, ABC transporter, cell cycle and ROS experiments, as well as the non-sphere-forming HuH-7 and Hep 3B cell lines. Only with these controls the authors will be able to conclude that their sphere induction method works specifically for de-differentiated HCC cell lines.

According to your recommendation, we include the data of sphere-forming HLE cell line (the RT-qPCR, drug sensitivity, ABC transporter, cell cycle and ROS experiments) in the new manuscript (Figs. 3, 7-10, and added related descriptions in the text). As a result, HLE-sphere cells also exerts increased chemoresistance compared to parental HLE cells (new Fig. 7). So, we concluded that the sphere cells induced from de-differentiated HCC cell lines
possess the resistance to anti-cancer drugs. We did not evaluate HuH-7 and Hep 3B cells for above experiments, as background of cells were different among cell lines to comparison. In this study, we screened by sphere formation and then further examined properties of sphere-formed cells by comparison with those parental cells.

- There are a lot of “data not shown”. This is not acceptable and the authors must provide the cited results either in the main figures or as supplementary information.

  On the basis of your mention, we added the data in the Result section in the new manuscript (Supplemental Fig. 1, 2, and main Fig. 5C).

- The reference gene used for RT-qPCR experiments should be always the same. If the authors have reasons for alternatively use GAPDH or PGK1 in different experiments, they must support these reasons and indicate which one was used in each case in the appropriate figure legend. If not, results have to be normalized only to one of them.

  We agree with you that the importance of the reference gene selection for the qPCR experiment. Because normalization against a single reference gene is not suitable in some cases (Vandesompele J. et al., Genome Biol. 2002; Bustin SA. et al., Clin Chem. 2009). In this our study, both GAPDH and PGK1 were measured simultaneously (these two genes were selected by preliminary experiments using 12 considerable reference genes), and each value normalized with both internal measurements were used for statistical analysis. So, qPCR experiments were performed in the same condition. We revised description in the new manuscript (lines 160 to 161 on page 7).

b) Minor essential revisions
- The Student’s t-test and Mann-Whitney U-test are used in the RT-qPCR experiments. Is there any reason to not to use the same test in all cases? The authors should clarify this issue.

  The Student’s t-test and Mann-Whitney U-test were selected according to the examination of normal distribution. To simplify, we applied non-parametric tests in the new manuscript (line 211 on page 9, and related results).

- The percentages referred in the sixth paragraph of the Results section do not
match with those in the referred Figure 8.

Thank you for your indication. The represented histograms were one of typical result among independent experiments. The values described in the text were statistic values of those results. We revised description of new Figure 9.

- The Results section would be highly improved if the authors included partial conclusions in the different paragraphs and link them more fluently.

Thanks for your recommendation. We added a brief summary of the paragraph (lines 236 to 239 on page 10, lines 271 to 272 on page 12, and lines 301 to 302 on page 13).

c) Discretionary revisions

- Panel labeling in Figure 1 should be in the same order as it is cited in the text to facilitate reading comprehension.

As you pointed out, we changed panel labeling in Fig. 1, and revised description in the new manuscript (lines 223, 224, and 228 on page 10).

- Materials and Methods should be avoided in the Figure Legends.

We revised description in the Figure Legends.

**Level of interest:** An article whose findings are important to those with closely related research interests

**Quality of written English:** Acceptable

**Statistical review:** No, the manuscript does not need to be seen by a statistician.

**Declaration of competing interests:**

I declare that I have no competing interests