Author’s response to reviews

Title: Sphere-forming cell subpopulations with cancer stem cell properties in human hepatoma cell lines

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Author’s response to reviews: see over
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Dear Dr. Tim Shipley,

On behalf of co-authors, we thank you very much for giving us the opportunity again to revise our manuscript (MS: 1591875628468550), and appreciate editor and reviewers for their positive and constructive comments and suggestions.

We have made revisions according to the reviewers’ comments and tried our best to answer the reviewers’ questions. All the changes been made have been marked in red in the revised manuscript. Attached please find the letter to the reviewers. Looking forward to hearing from you.

Thank you and best regards.

Sincerely yours,

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Response

Dear Reviewers:

We appreciate the reviewers’ constructive comments. Below is a point by point response to the comments.

1. Reviewer Malcolm Alison:

There is still a big problem with the English language throughout e.g. tenses, 'CSCs is regarded'; starting sentences with 'And... and the use of 'Sphered cells' throughout including the title - replace 'sphered cells' with 'sphere-forming cells'.

Response:

We are very sorry for our incorrect writing. We have replaced ‘sphered cells’ with ‘sphere-forming cells’, and corrected any mistakes we’ve found.

2. Reviewer Nicholas Barker:

1) The inclusion of data demonstrating resistance to a range of therapeutic agents certainly strengthens the manuscript. However, I still think important mechanistic insight would be provided by a simple analysis of candidate CSC marker expression in the spheres following treatment. Is there selection for CSC phenotypes within the spheres following treatment, as has been suggested as a contributing factor in tumor relapse in patients?

Response:

Thank you for your suggestion and we totally agree with your standpoint. A reliable CSC marker is very important for developing targeted therapies.

Liver CSCs have been characterized by a variety of phenotypes including CD133, CD90, OV6, EpCAM, CD44, and new potential markers are continuously being discovered like CD13 (J Clin Invest. 2010,120:3326-3339), CD176 (Int J Exp Pathol. 2011,92:97-105). However, their validity as a liver CSC marker remains obscure. Controversial results have been reported by different research groups. For example,
Kimura O et al. reported CD133$^+$ fraction in Hep3B and Huh7 were 16.8% and 2.7%, respectively (Cancer Sci. 2010,101:2145-55), whereas some other groups reported more than 90% in Hep3B and 60% in Huh7 (Gastroenterology. 2007,132:2542–2556; Int J Cancer. 2010,126:2067-78). In Huh7 the CD13-positive cells typically existed in a CD133$^{\text{strong}}$ fraction, but in PLC/PRF/5 the CD13-positive cells were CD133-negative (J Clin Invest. 2010,120:3326-3339). Some researchers indicated that different culture conditions and differentiated degree of the cells, especially the latter, were important factors. The roles of these markers in defining functionally distinct populations of cells from progenitor to differentiated hepatocytes need to be systemically studied. Therefore, finding a sensitive and specific CSC marker still has a long way to go. We have examined some phenotypes including CD133, CD44 and OV6 in spheres before or after cisplatin treatment. Although positive subpopulations were increased by different extent, it was insufficient to draw any conclusions. Nevertheless, finding potential targets for CSC therapy is what we have been working on, not only cell phenotypes, but also cell signal pathways. The clinical relevance of the in vitro findings should also be demonstrated and validated in primary tumor samples with immunohistochemistry and gene expression analysis.

2) It is also surprising that no enrichment for CD133 was seen using IF in the spheres, despite this being evidenced by Western blot in Figure 4B. Does this mean that CD133 isn't a good CSC marker? This should be discussed.

Response:

Actually, as shown in Figure S2 by IF and Figure 4 by Western blot, CD133 is enriched a little in the spheres compared with the parental cells. But it didn’t mean that CD133 is a good CSC marker. CD133 had less cross-expression with other potential CSC markers in HCC as referred above. Besides, there has been other evidence supporting this view (Cancer Letters. 2009,275:185-193; J Surg Res. 2011 Apr 24 Epub ahead of print; J Hepatol. 2011 Feb 17 Epub ahead of print; Front Biosci. 2011,3:701-10; Int J Cancer. 2011,128:501-10; Radiother Oncol. 2010,94:375-83). The transmembrane protein CD133 has been widely used to isolate putative CSC populations in several
cancer types including liver cancer, colorectal cancer and glioma, especially in the earlier years. It may play a role in the stem-like characteristic of CSCs, but its validity as a CSC marker and its clinical usefulness are controversial and questioned recently. The following is some evidence.

CSCs are radioresistant and chemoresistant. But Hongo K et al. reported that colorectal cancer CD133$^+$ cells, although showing some features of CSCs, were less resistant to 5-FU than CD133$^-$ cells (J Surg Res. 2011 Apr 24 Epub ahead of print). Dittfeld C et al. reported that CD133 expression was not selective for sphere forming, tumor-initiating or radioresistant subpopulations in the HCT-116 colorectal cancer cell line (Radiother Oncol. 2010;94:375-83).

Chen X et al. reported that well-differentiated cell lines were positive for CD133$^+$/ALDH$^{\text{high}}$ and CD133$^+$/EpCAM$^+$ at 1.5-15% and 2.3-8.3%; whereas, poorly-differentiated cells were almost all negative for these markers. CD133$^+$/ALDH$^{\text{low}}$ HLE cells were more resistant to cisplatin, doxorubicin or sorafenib than their positive counterparts. CD133$^+$/EpCAM$^-$ Huh-7 cells or CD133$^+$/ALDH$^-$ HLE cells exhibited a higher invasion rate than their positive counterparts (J Hepatol. 2011 Feb 17 Epub ahead of print).

Those imply that at least CD133 cannot be regarded as a CSC marker in all cell lines. The validity of a CSC marker could not be judged easily by proliferation, metastasis, tumor-initiation ability or drug-resistance. It needs to be functionally accompanied with CSC-directed mechanism and cancer cell differentiation. For this purpose, we have been doing our further research.

3) Please quantify the Western blots in Figure 5 - this is much easier for the reader to interpret.

Response:

We have made corrections according to the Reviewer’s comments as shown in Figure 5.

Again, special thanks to the reviewers’ good comments.
Respectfully!

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