Author's response to reviews

Title: Endothelial cells do not arise from tumor-initiating cells in human hepatocellular carcinoma

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Author's response to reviews: see over
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Editorial Board, BMC Cancer

Dear Editors,

Thank you for your favorable review of our manuscript entitled “Endothelial cells do not arise from tumor-initiating cells in human hepatocellular carcinoma” and for inviting us to submit a revised version (submitted herewith). We have addressed each of the concerns raised by the reviewers in the point-by-point response below:

Reviewer: Matias Avila

1. The number of experiments performed in the xenograft mouse model need to be clearly mentioned in the Materials and Methods section. To be more conclusive this experiment should be repeated at least three times in duplicates using different HCC cell isolates.

As pointed out by the reviewer, we omitted to specify that the results described in this manuscript represent data generated from multiple experiments. We utilized xenografts derived from the HCC tumors of three different patients as well as two different human HCC cell lines. Xenografts were generated in duplicate in different mice and also analyzed separately and independently. We have added the following text to the Xenografts paragraph of the Methods section: “The xenografts analyzed in this study were generated from two different human HCC cell lines (HepG2 and Huh7) as well as from primary cells isolated from three different HCC specimens originating from three distinct patients. Xenografts were generated in duplicate in different mice from each cell line or patient sample and analyzed independently as described below.”

2. The individual panels (A, B, C, etc) presented in Figures 2, 3 and 4 should be better described in the main text. There is not a direct reference for the information presented in each panel, rather the authors make a general reference to the figure in the description of the results.

As requested by the reviewer, we have included direct and detailed references to the data presented in each Figure and Panel within the main body of the manuscript text.
**Reviewer: Kwan Man**

1. *Despite that the authors thought that their primary HCC xenograft model is more biologically relevant to human HCC than the cell lines, I would still like to see some results from the cell lines.*

As explained in our manuscript, we strongly believe that studying patient-derived material is the “gold-standard” in providing the most accurate biological data pertaining to human HCC. However, we previously made use of human HCC cell lines to develop our xenograft models in the laboratory. As requested by the reviewer, we have thus included data from cell lines in the revised version of the manuscript, and have made corresponding changes to the Methods (inserted new paragraph entitled “Human HCC cell lines”, reallocated description of preparation of tumor cell suspension from patient samples into the “Patient samples” paragraph), Results and Discussion, and Figures to reflect the inclusion of this new data. We utilized the widely used HepG2 and Huh7 cell lines to generate intrahepatic xenografts in immunodeficient mice. The histopathology of these xenografts, which resemble human HCC, is shown in a revised Figure 1 (panels E and F). As shown in a revised Figure 2 (panels C through F), staining of these xenografts with antibodies against human and murine CD31 corresponds with findings in the patient-derived xenografts (panels A and B), in that the antibody that recognizes murine CD31 decorates endothelial cells in vascular structures within the tumors while staining with the anti-human CD31 is completely negative. These observations suggest that intratumoral ECs in xenografts generated from human HCC cells are of murine origin.

2. *The authors demonstrated their clinical results in the moderately differentiated recurrent HCC. I would like to see some extra results from other differentiated recurrent HCC, especially in poorly differentiated recurrent HCC.*

In order to clearly distinguish whether endothelial cells in primary human HCC tissues originated from the tumor or from the surrounding normal liver, we sought out an extremely unique subset of HCC patients: those who received a liver transplant from a sex-mismatched donor, went on to develop recurrent disease within the liver, and were candidates for surgical resection of the recurrent HCC from the liver allograft. Only under these rare circumstances could we successfully apply fluorescence in situ hybridization for X and Y chromosomes in combination with immunohistochemistry for endothelial cell markers to address the issue of tumor vs. host origin of intratumoral endothelial cells in human HCC.

Unfortunately, the very small number of patients that met these criteria at our institution all had recurrent HCCs that were moderately differentiated. This may reflect the fact that the majority of patients who qualify for liver transplantation as treatment for HCC have moderately differentiated tumors prior to transplantation (because those with poorly differentiated tumors are often excluded from transplantation due to advanced disease or concerns about poor post-transplant outcomes), or the fact that patients with poorly differentiated HCC recurrences post-transplant tend to present with locally advanced or widely metastatic disease rather than isolated intrahepatic lesions that are...
amenable to surgical resection. Thus, we cannot provide the data requested by the
reviewer. Given that we were unable to identify such patients at our institution despite
many years of performing large numbers of liver transplants for patients with HCC as
well as taking an aggressive approach to the surgical management of post-transplant
recurrences, we suspect that investigators at other centres would also find it very
challenging to identify the type of patients necessary to generate the requested data.
We hope that dissemination of our results may stimulate other centres to identify such
patients and perform similar analyses to further our collective understanding of
endothelial cell biology in HCC.

Although we feel that our analysis of the most common type of HCC (moderately
differentiated) provide a valuable contribution to the literature, we acknowledge the
possibility that the endothelial biology of well or poorly differentiated tumors may be
different. We have thus included the following statement in the Discussion section of
the text to clearly highlight this limitation of our study: “...all of the resected HCC
recurrences that we studied by FISH were moderately differentiated tumors; due to the
extremely unique characteristics of the patients from whom these samples originated,
we were unable to study specimens that were either well differentiated or poorly
differentiated. Although it is possible that endothelial cell origins are different in such
tumors, we feel that the observations presented in this study provide valuable insights
into the pathobiology of human HCC.”

In summary, we feel that we have clearly and thoroughly responded to each of the
concerns raised by the reviewers regarding our original submission, and have made
appropriate changes in the revised version of the manuscript to reflect these responses.

Thank you for considering our resubmission. We sincerely hope that that our
manuscript will now be deemed acceptable for publication in BMC Cancer.

Please do not hesitate to contact me if you require further clarification of our responses
or any other information.

Sincerely,

Anand Ghanekar, MD PhD