Author's response to reviews

Title: Impact of Stem Cell Marker Expression on Recurrence of TACE-Treated Hepatocellular Carcinoma Post Liver Transplantation

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Author's response to reviews:

Dear Dr. Monga,

We are resubmitting a revised version of our manuscript, now titled, “Impact of Stem Cell Marker Expression on Recurrence of TACE-Treated Hepatocellular Carcinoma Post Liver Transplantation” for consideration for publication in BMC Cancer. We wish to thank the reviewers for their careful reading of our paper and the many helpful and constructive comments. The major issue raised was the need to more carefully describe the characteristics of the individual patients included in the study, particularly with regard to why some patients received TACE and others did not. We have therefore obtained additional clinical data on all subjects, reanalyzed all the clinical data, and added an additional table with individual patient information. The TACE group did have larger tumors and the patients were more frequently outside of the Milan criteria. We have extensively revised the paper to address and discuss this issue as well as to better address the other issues raised by each of the reviewers. We wish to note that in obtaining more detailed clinical information from the transplant team records we added several of our surgical colleagues as new co-authors and made a change to the patients included in the groups. This change was necessitated because one of the TACE patients had received therapy with other modalities as well as TACE and thus had to be excluded, and one patient had been scheduled for TACE but did not receive it prior to transplant and thus had been misclassified. These facts were not reflected in the electronic medical record and only came to our attention when we conducted the more detailed review requested by the reviewers. We greatly apologize for these inaccuracies in the original data set and greatly thank the reviewers for their comments that have allowed us to correct this error. All statistics have been recalculated with this new data set (16 TACE patients, 23 non-TACE patients) and the statistical validity of our original conclusions was confirmed.
We believe that these changes have significantly improved the paper and hope that it is now acceptable for publication. We have indicated significant changes in red to make it easier for the reviewers to assess the changes. Detailed point-by-point responses to the reviewers comments are below.

Sincerely,
Steven Weinman

Reviewer 1

1. The HCCs from TACE-treated tumors were higher stage and had more microscopic necrosis and fibrosis. Can the authors comment on whether high EpCAM and CD 133 staining could result from more necrosis and fibrosis. The reviewer brings up a very good point and we now address this in detail in the discussion. After TACE we are looking at tumor regrowth. It is clear that the necrosis and fibrosis was directly induced by TACE but the subsequent appearance of more EpCAM and CD133 could be either because the treatment induced de-differentiation and expression or because a subpopulation of positive cells survived the TACE and repopulated the tumor. A recent study by Zen et al (Liver Transplantation 17:943, 2011) suggests that the dedifferentiation process may be the more likely explanation. Our data cannot distinguish between these possibilities and we now discuss this fact.

2. The authors use the term “downstage” to allow patients to qualify for transplantation. However, all the patients appear to be either stage I or II (within Milan criteria). Is there any staining performed on patients with more advanced stage which were initially outside Milan but downstaged? We apologize for any confusion due to our previous incomplete data presentation and now show individual data for each patient in the new Table 2 as well as summary data in Table 1. Fifty percent of the TACE patients initially fell outside of the Milan criteria and TACE was performed for downstaging. Only 7% of the non-TACE patients were outside of Milan but due to high MELD scores and short wait times at the University of Kansas they were still able to receive transplants. These facts are now presented and discussed in both results and discussion.

3. The title makes no reference to TACE, but that is a key aim of the study, looking at progenitor cell expression in TACE vs non-TACE treated tumors. I would alter the title to discuss TACE. Thanks for making this good point. We have now changed the title.

4. The current guidelines, Milan and others, are based on pre-transplant criteria (size, number of lesions, vascular invasion) that can be assessed pre-transplant to predict recurrence. Staining explanted livers may help predict recurrence (if
EpCAM staining is validated as a marker), but it can only be determined post-transplant. Have the authors examined EpCAM expression in pre-transplant biopsy of tumors to show similar findings?

This is an extremely important point but unfortunately, our patients were not biopsied prior to transplant and we were not able to assess this. This is one of the limitations of the study and we now discuss this. We believe that a better understanding of tumor biology associated with recurrence may be helpful in future studies trying to determine serum biomarkers that correlate with EpCAM expression.

5. There are some minor issues in the tables. Not all the numbers add up in Table 1 and Table 3.

Thank you for alerting us to these errors in the tables. We apologize for this and have corrected them. The old Table 3 is now Table 4.

Reviewer 2:

1. The authors showed evidence that high EpCAM expression in HCC specimens is associated with higher tumor recurrence in patients with TACE treatment prior to liver transplantation. The weakness of the present study is that the results were derived from a relatively small patient group. These data have to be confirmed in large-scale studies.

We agree completely and have now stressed this point in the discussion. We do feel that this cohort of patients, however, does provide important information that could be useful to other investigators while these larger studies are in progress.

3. Till now, several markers were used to identify or characterize stem cells or cancer stem cells. The major problem is that there are no unique markers for these specific cell types. The suggested markers for cancer stem cells (e.g., EpCAM) are also expressed on hepatic progenitor cells (which are known to be activated in cirrhotic liver) or differentiated epithelial cells. Therefore, it will be very interesting to further determine the expression profile of the EpCAM-positive cells in these tissues, using simultaneous immunohistochemical techniques. For instance, was there any immunohistochemical analyses for cytokeratin 19 performed?

We did not perform CK19 staining but anticipate doing this in the larger cohort. We are working now on a follow up study of 130 patients. We now acknowledge this in the discussion.

4. The authors mentioned in the Results section, that there was more fibrosis
observed in the TACE-treated tumors vs. the non-treated group. How was that determined?

The presence of fibrosis in the TACE-treated tissues was dramatic and readily observed on H&E sections. These tumors routinely consisted of broad areas of bands of fibrosis with scattered bile duct elements entrapped. Such structures were never seen in the non-TACE treated tumors. We did not formally quantify this phenomenon as it has been reported by others.

5. The reviewer pointed out some numerical errors and transposed numbers in the abstract.

Thank you for calling our attention to these errors. We apologize and have corrected them.

Reviewer 3:

1. The reviewer raised the issue of whether the increased CSC staining present in TACE treatment tumors was caused by the TACE or if it simply reflected the fact that the decision to use TACE was reserved for patients who had biologically more aggressive tumors to start. The reviewer asked us to raise this point earlier in the paper and address this concern by providing additional information on the rationale for TACE vs no-TACE.

We agree completely with the reviewer that this is an essential point and have now provided the requested information. The new Table 2 summarizes each case individually. The patients selected for TACE did tend to have larger tumors and a significantly larger percentage (50% vs 7%) were outside Milan criteria than the non-TACE patients. We could not confirm that TACE’d tumors had an initially higher growth rate and for those patients in whom multiple imaging studies were done, the non-TACE tumors had a greater mean growth rate. We now present this data in the new Table 2 and discuss it explicitly in results and discussion. We do not believe that our data can be used to answer the question of whether or not TACE caused the increase in marker staining, and therefore have restricted our conclusions regarding tumor recurrence to the TACE population. A recent study by Zen et al (Liver Transplantation 17:943, 2011), however, suggests that dedifferentiation may be induced in the tumor elements that recur after TACE and we now discuss the implications of our study in this context.

2. Can the authors explain why these are called stem cells since they don’t appear to be pleuripotent?

The reviewer brings up an issue that is debated in the field. The concept of “cancer stem cells” is different than pluipotent stem cells. There is strong evidence for the existence of a subset of cells within certain tumors that have some stem cell-like properties including ability to repopulate all the cell types of
the tumor, resistance to chemotherapy, and ability to re-seed tumors at very low inoculum levels. These have been variously called “tumor initiating cells,” “cancer progenitor cells,” or more commonly “cancer stem cells.” We have chosen this latter designation since it is the most common in the literature. We now discuss this issue.

3. The reviewer asked for clarification on a number of issues related to the ability to score the tumors for marker expression. The specific questions are answered below and we have tried to clarify these issues by giving more detail in the methods section.

a. What did they do in cases where little viable tissue was available for examination? Could they always find 5 hpfs?

Most of the sections examined had viable tumor, even after TACE. There were two cases in which no viable tumor could be identified and we did not include these in the study. For the included patients, we were able to identify at least 5 hpfs of viable tumor cells in all samples. In the case of the TACE-treated tumors, these were sometimes nests of HCC that arose either at the periphery or in the center of large fibrotic areas. There was clearly less abundant viable tumor after TACE, but enough to perform the analysis.

b. Did tumors that recurred after TACE have more viable cells than tumors that did not recur?

The reviewer raises an interesting point but we are not able to answer it with the specimens available. We did not quantitate total viable hepatocellular tumor cell mass because the entire tumors were not saved as paraffin blocks and the specimens available to us in this retrospective study frequently were samples only and did not necessarily capture all tumor present. We did not examine any tissues after the post-transplant tumor recurrences.

c. Embolized tumors give the viewer limited fields to examine, whereas viable tumors leave one with a lot of flexibility. By examining only 5 hpfs, one might select high staining or low staining areas within a tumor unless staining is homogeneous. How did they choose areas to examine?

We made as best an attempt as possible to examine 5 representative areas and cover the entire sample geographically. We always had sufficient areas of viable tumor in both untreated and TACE tissue.

d. Did the three observers view the same five fields, or were they free to choose any five fields?

Each observer chose her/his own fields but each was instructed to try to obtain a representative sample of the whole tumor present in the slide.

e. Since many post-TACE livers had multiple tumors, did they compare treated vs. untreated tumors? This might be a better internal control.
f. Can they compare synchronous tumors in a subanalysis.

These tumors were examined retrospectively from paraffin blocks. In the patients with multiple tumors, it was frequent that more than one tumor had been treated. There were relatively few cases in which we could identify both treated and untreated tumors from the same patient and therefore we did not perform this subanalysis. We agree that the suggestion is a good one and would like to approach it in a prospective study where careful matching of the specimens to the radiological data is performed at the time of tissue collection.

g. How did they identify tumor cells in embolized tissue?

Evidence of residual carcinoma present within embolized/treated tissue was identified on routine H&E stained paraffin sections by the same criteria used to diagnose hepatocellular carcinoma within untreated tissue. Hepatocellular carcinoma is characterized by neoplastic cells with increased nuclear to cytoplasmic ratios which are arranged in irregularly expanded trabeculae and pseudoacinacinar structures.

h. Was the scoring system (0-3) based on number of stained cells or density/cell or a combination?

We apologize for the confusion and have now provided more detail on the scoring system in the Methods. Essentially a combination system was used that was agreed upon by all observers after a preliminary assessment of the observed staining patterns. For CD90 and CD44, staining varied little in intensity and therefore a criterion of number of positive cells was used. For EpCAM and CD133, there was variability in staining intensity as well as in number of positive cells and thus a composite scale taking into account both number and intensity was used. We have now explained this in detail in the methods and provide examples in Figure 1.

i. What was the concordance among the investigators?

There was good agreement among the observers. In approximately 80% of the samples all scores were within 1 on the 4 point scale. In approximately 20% there was a variance of 2 points on the 4-pt scale. There were no cases of 3 point variance. The mean value was used to classify samples as low (0-1.5) or high (1.6-3).

j. Please give complete information for the primary antibodies – were all antibodies from Dako?

Full details are in Methods. Most were from Abcam.

4. The reviewer asked us to clarify several issues and include some additional references in the introduction. We have now done so.

a. The rationale for downstaging is quite clear – it is a non-invasive observation
that has proven useful. Size usually correlates with tumor behavior, and has made OLT reasonably successful.

We have now discussed downstaging and its outcomes in more detail, citing the studies by Yao and others. We did not mean to imply that downstaging did not have value, only that the biological basis of it was not established.

b. They fail to acknowledge that tumor staining requires a resected liver – certainly not a practical solution to tumor recurrence.

We certainly agree with this point and did not mean to imply that pre-transplant biopsy should be part of the transplant decision. The goal of this study is to learn more about the biology of tumors that recur in the hope that this knowledge might ultimately allow us to identify more practical biomarkers. We have now discussed this issue in detail.

5. Why did they choose four tumor markers out of a possible six – why not CK19 and CD13?

These other markers are reasonable but they were not in our panel as earlier studies had not used them and recognition is more recent.

6. The most puzzling thing about this paper is the suggestion that TACE causes expression of a stem cell marker that correlates with HCC recurrence. This is certainly not the universal experience. More discussion about this controversial finding is essential to making this paper meaningful and broadly applicable.

Although we are not aware of any studies that have prospectively compared histology of tumors before and after TACE, there have been previous observations that TACE-treated tumors display a more undifferentiated phenotype with mixed cholangiocellular and hepatocellular characteristics when compared to non-TACE tumors (Zen et al Liver Transplantation 17:943, 2011). We now discuss this issue in detail. We are now careful not to conclude that these cells were caused by TACE, but we cannot yet exclude this possibility.

7. Table 1 needs some changes:
   a. TNM classification – all patients were within Milan criteria, so T stage is the same as size – consider using tumor size instead of T stage.
   b. Median size would be helpful.
   c. Number of tumors rather than single/multiple.
   d. Since they have only 40 patients, a table of all patient data should be considered (could be provided as a supplement if space is limited).

We have revised the tables and provided more information as the reviewer has
requested. The reviewer is correct in that none of these patients had known vascular invasion so the TMN classification just indicates size and number. We now give this information more precisely in an expanded table 1 and detailed individual patient information in Table 2.

8. Figures
a. What is the significance of cytoplasmic staining with EpCAM – are CAMs normally localized to the cell membrane? Figure 1 shows staining limited to the cytoplasm in grade 2, but appears to show cytoplasmic and membrane staining in grade 3.

EpCAM staining has been frequently observed in both cytoplasm and in plasma membrane in many tumors. See Spizzo et al, J. Clin. Pathol. 2011, 64:415 for examples. The significance of this is uncertain.