Author's response to reviews

Title: The occurrence of mesenchymal-epithelial transition and the role of hepatocyte nuclear factor 4alpha in metastatic tumor formation of hepatocellular

Authors:

Dianbo Yao Dr (yaodianbo@163.com)
Songlin Peng Dr (psl514@126.com)
Chaoliu Dai Dr (daichaoliu@163.com)

Version: 2 Date: 9 July 2013

Author's response to reviews:

Dear Editors,

We are very pleased that both of the two reviewers with considerable expertise have found the merit in our manuscript. According to the reviewers' comments, we extend and strength our observations. We found that thanks to the reviewer's good advice, our study has become much better. Thanks again for consideration of our manuscript for publication in your journal.

Reviewer # 1 (Dr Marco Tripodi)

Major concerns

In particular since the EMT master genes Snail and Slug have been shown to directly cause HNF4 and E-Cadherin transcriptional down regulation and, since HNF4 has been shown to act as a direct Slug and Snail repressor, it should be easily feasible to examine (by IHC) their expression in tissue samples (i.e. primary tumor, metastasis and normal tissue).

Thanks a lot for the reviewer’s advise. We examined the expression of Slug and Snail in the tissue samples by IHC, and it is found that expression of Snail and Slug in the metastases was significantly decreased compared with that of primaries. The expression of Snail and Slug in normal tumor-adjacent tissues was mostly weak or negative. Spearman correlation analysis showed that the expression of Snail and Slug in the primary tumors and their corresponding metastases was negatively correlated with the expression of E-cadherin, Fibronectin and N-cadherin, confirming again that Snail and Slug were the EMT master regulatory genes. In addition, Spearman correlation analysis showed that the expression of HNF4alpha in the primary tumors and their corresponding metastases was significantly positively correlated with the expression of E-cadherin, and negatively correlated with the expression of Snail, Slug and Fibronectin, suggesting that HNF4alpha might induce the MET through inhibiting the transcription of EMT master regulatory genes Snail and Slug.

Minor points:

1- In the keywords, substitute “mesenchymal epithelial transformation” with “transition”
2- E-cad staining in primary tumor in D (left, lower panel) is the same of 1C (left, upper panel,: Figure 1C and 1D can be joined.
Thanks for reminding us, Figure 1C and 1D have been joined.

Reviewer # 1 (Dr Udayan Apte)
Major Compulsary Revisions.
1. In this study the authors use the presence of E-cadherin as the primary means of showing MET. In figure 4 they also show staining for Vimentin. It may be a good idea to look at multiple marks of MET (vimentin, fibronectin, N-cadherin, etc.). This would help strengthen one of the main themes of this manuscript. It would be nice to see staining for more of these markers throughout the clinical samples.

Thanks a lot for the reviewer's advise. We examined the expression of vimentin, fibronectin, N-cadherin in the tissue samples by IHC, and it is found that the expression of Fibronectin, N-cadherin and Vimentin in the metastases was significantly decreased compared with that of primaries, confirming the occurrence of MET in the metastases. In addition, the spearman correlation analysis was made between the E-cadherin, fibronectin, N-cadherin, vimentin, and the HNF4alpha, Slug, Snail, and the close relationship was found.

2. Studies include only 10 human samples. Additional samples are required.

We reviewed our clinical data, and have found another 3 patients suitable for our study. Though there were only 13 primary tumor samples in all now, the sample number of the corresponding metastases from the 13 patients was 31. The examination from the 31 paired metastases and primary tumors is helpful for our study for the mechanism of HCC metastases formation. In addition, as the samples of HCC with primary tumors and their corresponding metastases were limited, it is rather difficult to get too many samples. We hope that the reviewer would be satisfied with what we have done.

Minor Essential Revisions:
1. It is confusing as to what the y-axis in Figure 1B and 2B is. There is no label. Is this referring to the scoring system based upon E-cadherin expression (none, weak, intermediate, strong)? Also, the figure legend needs rewritten. This is also confusing as to what we are looking at in this figure.

We are sorry for the mistake in our manuscript. The label has been added. It is the scoring value of protein expression (0 absent, 1 weak, 2 intermediate, 3 strong staining). In addition, the figure legend has also been rewritten. In the future we will be definitely more careful to avoid this kind of mistakes.

2. In the text describing Figure 1D and 2D it is said that the normal adjacent tissue has increased expression of E-cadherin as compared to distant metastases. It may be a good idea to show the distant metastases as well for a direct comparison.

As we introduced in the manuscript, in the metastases most of E-cadherin
expression were also slightly reduced, though in some cases (19.4%, 6/31) the expression of E-cadherin in the metastases was similar with that in the normal tissues. In the revised manuscript, we selected to join the figure, and the normal adjacent tissue, primary tumor and metastases were showed together. Thanks again for the good advice.

3. In Figure 2C the distant metastases show increased expression of HNF4a, but it looks to be diffuse throughout the cell and not at a high concentration within the nucleus. Is there any explanation for this? Would this alter its activation of E-cadherin expression?

As we introduced in the second part of “The expression of HNF4alpha increased in HCC metastases and was closely related with the expression of MET marks in HCC specimens” in the result, the position of HNF4alpha in tumor would also be changed. It was previously introduced in the Chellappa K et al’s study that approximately 80% of tumor samples showed a P1-HNF4# staining that was not exclusively nuclear; in contrast, in normal tissue HNF4# staining was observed only in the nucleus. They found that cytoplasmic P1-HNF4# staining in human colon cancer correlates with staining for active Src, and proposed that the described interaction between Src kinase and HNF4# may also be relevant in other cancers, such as hepatocellular carcinoma, which has been shown to have high levels of active Src and a loss of P1-driven HNF4#. This suggested that the expression change of cytoplasmic HNF4# is also needed to be studied. Now, we propose that the expression change of HNF4# may be related with that of E-cadherin, and certainly the regulation effect in vivo is still needed for study more masterly in the future.

4. What may be an explanation for the comparable Vimentin expression in SK-Hep-1 cells and Hep3B cells since Vimentin is another marker for MET? Does HNF4a have nothing to do with Vimentin expression? This is where it may be nice to have Vimentin staining done throughout the clinical samples to help with this comparison and make things a little more clear.

As we see in the figure 4, it could be found that the expression of vimentin in SK-hep-1 cells is a little stronger than that in Hep3B cells, but not “comparable”. We are sorry for the incorrect description in our manuscript and the figure legend has been corrected. In addition, we examined the expression of vimentin in the clinical samples. It was found that 61.3% (19/31) of metastases showed decreased Vimentin expression, the decreased expression of Vimentin is significant. However, the Spearman correlation analysis showed that the correlation between expression of HNF4alpha and that of Vimentin in the primary tumors and their corresponding metastases was not significant (correlation coefficient = -0.23, p=0.75), though it was found that the expression of Snail and Slug in the primary tumors and their corresponding metastases was positively correlated with the Vimentin. This is still needed for further research.

In addition, we have also highlighted (with underlines) all changes made in the revised manuscript, to make it easier to review it.

We also review our revised manuscript by ourselves to ensure that our revised
manuscript conforms to the journal style, according to the “Instructions for authors”.

Thank you very much for your attention and consideration,
Looking forward to your decision,
Sincerely yours,
Chaoliu Dai