Author's response to reviews

Title: The role of hepatocyte nuclear factor 4alpha in metastatic tumor formation of hepatocellular carcinoma and its close relationship with the mesenchymal-epithelial transition markers

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Author's response to reviews: see over
Dear Editors,

We are very pleased that both you and the reviewers have found the merit in our manuscript. According to the comments, we revised again our manuscript. Thanks again for consideration of our manuscript for publication in your journal.

The quality of the immunohistopathology has improved significantly in the revised manuscript. In addition, while you have demonstrated, as expected, that a transient expression of HNF4a increases E-Cadherin in SK-Hep-1 cells. However, you have not demonstrated that this drives an MET (ie. there is no evidence provided that junctions form and/or that the mesenchymal cells are epithelialized).

Thanks a lot for the reminding. In our study, the role of HNF4alpha in the regulation of E-cadherin expression in the HCC cells with mesenchymal phenotypes was further confirmed, via transfection. Though in our study, it was found that the transfected cells were not epithelialized (the morphology of cells did not change from a fibroblastic appearance to a cuboid epithelial Shape as supposed, and the data were not showed), but the role of HNF4alpha on the induction of MET could not be easily denied. We introduced the related content in the discussion (in the sixth paragraph in page 14 and 15). In the Späth GF’s study, it was also found that HNF4 could only result in reexpression of cytokeratin proteins and partial reestablishment of E-cadherin production, and the cells were not epithelialized. But they found that only the transfectants with increased HNF4 are competent to respond to the synthetic glucocorticoid dexamethasone, which induces the second step of morphogenesis, including formation of the junctional complex and expression of a polarized cell phenotype. This suggested that HNF4 should be at least an important part of the MET inducers. In addition, it was also introduced that the expression of HNF4alpha could directly induce the MET in fibroblasts (NIH3T3 cells), and Utilizing rat hepatoma cell lines, Natalia L Lazarevich et al also proved that dedifferentiated hepatoma cells can be induced to transform to the epithelial phenotype by re-expression of HNF4alpha. These findings suggest that HNF4alpha should be an important inducer of the MET in
HCC cells, or in some cases, it acts an important part of the MET inducers, possibly by regulating the expression of E-cadherin.

In addition, the immunofluorescence shown in Fig 4 does not confirm, as is claimed, the differences in the levels of HNF4a and there are no discernible differences in the vimentin.

The Fig 4 in the revised manuscript has been modified, and the figures, including the ones before merging, have been all showed. Now, in the figure 4 with the figures before merging, we could easily found that the expression of HNF4alpha in Hep3B cells were stronger that that of SK-Hep-1 cells. As for the expression of vimentin, we judged again, and now we agree that the expression difference of the vimentin in the two HCC cell lines was not obvious. In the revised manuscript, the Figure legend of figure 4 has also been modified. We hope that these modifications would be satisfying.

In addition, the data with, what is presumed to be a single stable clone (given that methods indicate you picked clones), shown in Fig 5b is not at all convincing for either upregulated HNF4a or E-cadherin.

We picked the clones when the cells had been cultured in 900 µg/ml G418 for about 2 weeks. At that time, the rare cells with the G418 resistance would grow and form several cell populations. We picked part of the cells respectively in each cell population, and put them in 96-well plate. The cells were cultured respectively and continuously passaged. When the cells were enough for detection, we examined the expression of E-cadherin and HNF4alpha in each picked clone. Without the help of fluorescent protein, the ideal clone was more difficult to get, and many of the picked clones were not satisfying. In the clone we showed in Fig 5b, the expression of HNF4a and E-cadherin was increased. Utilizing gray level difference analysis method in western blot, it was found that the expression ratio of HNF4a or E-cadherin to β-actin in the clone cells was almost twice as much as that in normal SK-Hep-1 cells.
We think that it could also primarily show the role of HNF4a in the expression regulation of E-cadherin. Certainly, we also think that the clone was still not satisfying, and not suitable for the next animal model experiment in vivo. We plan to construct a new plasmid with fluorescent protein, or adenoviral vector, to help us continue our study in the near future. We hope that what we have done would be satisfying. Certainly, if the editors and the reviewers are still not satisfied, we are willing to delete the result of stable transfection, as the role of HNF4a in the expression regulation of E-cadherin in SK-Hep-1 cells was also well confirmed in the part of transient transfection.

As a result, I am now convinced that you have shown that there is an increase in epithelial markers and a decrease in mesenchymal markers in metastatic HCC lesions compared to primary lesions that are consistent with an MET in the former. You have also demonstrated that this shift in markers in the metastatic lesions is associated with HNF4a. These findings alone are significant and worthy of publication. You have also confirmed that, as shown by a number other groups, that HNF4a can upregulate E-cadherin in a single HCC cell line.

Thanks a lot for your favorable comment. We are very pleased.

What you have not shown however, and what the data presented to do not support, is that HNF4a alone is driving an MET in metastatic lesions or HCC cells in vitro. You have also not demonstrated, in any way, that an MET contributes to HCC metastasis.

Now, in our study, the increased expression of E-cadherin, and decreased expression of vimentin, fibronectin, N-cadherin in the metastases of HCC suggested the possible occurrence of MET in the metastases of HCC. In addition, the detection of the HNF4alpha expression in the specimens of HCC and its metastases suggested that HNF4alpha might also play a crucial role in the metastatic tumor formation of HCC. It was also revealed by spearman correlation analysis 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 Fibronectin, primarily suggesting that HNF4alpha might be related with the MET in metastatic lesions. In addition, the role of HNF4a in the expression regulation of E-cadherin was further confirmed via transfection. As for the role of HNF4a in the induction of MET, we selected to discuss in the discussion part (in the sixth paragraph in page 14 and 15). Our findings, combined with other findings, suggest that HNF4alpha should be an important inducer of the MET in HCC cells, or in some cases, it acts as an important part of the MET inducers, possibly by regulating the expression of E-cadherin. As for the role of MET in the metastasis of HCC, it needs to be demonstrated by the metastasis model in vivo. In the revised manuscript, we followed the editor’s good advice, and removed the claims in the title and results. We added the related speculation in the discussion in page 13: ”our findings in the HCC specimens suggested again the possible role of E-cadherin in the metastases formation of HCC. As the re-expression of E-cadherin is proposed to be the important hallmark of MET, it also suggested that the MET might play an important role in metastatic tumor formation of HCC. In our study, some other marks of MET, including vimentin, fibronectin, N-cadherin, were also examined. The results further suggested the possible occurrence of MET in the metastases of HCC.”, and in page 15:” Now, combining with what we have found in the clinical specimens, it could be speculated that in HCC, MET might also play an important role in metastatic tumor formation, and HNF4alpha might just be the important inducer of the MET, or act as an important part of the MET inducers. Certainly, the exact role of HNF4alpha in the metastatic tumor formation of HCC is still needed for further confirmed, maybe through the animal model of experimental tumor metastasis in vivo.” In the near future, we would try to examine the role of HNF4alpha and E-cadherin in the metastatic tumor formation of HCC, via animal model of experimental tumor metastasis in vivo. We hope that the editors and reviewers would be satisfied with what we have done until now, and your support would help and encourage us a lot to continue our studies.
Therefore, if you wish the manuscript to be considered further for publication in BMC Cancer you must deal definitively with the shortcomings described above regarding your functional data. Specifically, you must remove claims that the apparent MET drives, contributes to, or plays a functional role in the metastatic progression of HCC from the title and the results of the manuscript given that you are not presenting data which supports such conclusions. You may, however, speculate on such a possibility in the discussion.

We are sorry for the mistakes. Thanks a lot for your good advices. The title has been changed to “The role of hepatocyte nuclear factor 4alpha in metastatic tumor formation of hepatocellular carcinoma and its close relationship with the mesenchymal–epithelial transition markers”, and the claims in the results have been removed. In addition, we also increased the related speculation in the discussion.

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