Author’s response to reviews

Title: Downregulation of Toll-like receptor 4 induces suppressive effects on hepatitis B virus-related hepatocellular carcinoma via ERK1/2 signaling

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Author’s response to reviews: see over
Responses to Reviewer 1

1. Is the question posed by the authors well defined? Yes; the question is clearly presented and relevant.
2. Are the data sound? Yes; the data is compelling and well presented.
3. Do the figures appear to be genuine? Yes.
4. Does the manuscript adhere to the relevant standards for reporting and data deposition? To my knowledge, yes.
5. Are the discussion and conclusions well balanced and adequately supported by the data? Yes. The discussion is well written and makes a compelling argument.
6. Are limitations of the work clearly stated? No; this is one major deficiency of the manuscript. The authors do not elaborate on limitations of the data or alternative interpretations. Some impressions in this regard:
   - use of a single cell line that may not be reflective of majority of cases of HCC
   - potential overstatement of the role of TLR4 signalling in HBV associated HCC because of the choice of cell line
   - lack of positive experiments (can tumor growth be induced with TLR4 stimulation?)

Respond:

We appreciated for reviewer’s comments on the limitations which we haven’t addressed in the manuscript. We have added few limitations in our discussion session to address it. We have to admit that NF-κB and MAPKs are limited research pathways. ERK1/2 may be the concerned signaling, and it shall not be the only one. Although HepG2.2.15 cell is just a single HBV-related cell line, its mRNA and protein expression level is highest among all 5 cell lines. Besides that, HepG2.2.15 cell carries HBV. Therefore, HepG2.2.15 cell has sufficient characteristics and representativeness for the research. We are conscious that if more cell lines are investigated, then as a result more sufficient evidence can certainly be provided to our experiement. Additionally, to further improve the accuracy of the experiments, we plan to upregulate TLR4 as positive treatment in the future.

7. Do the authors acknowledge any work upon which they are building? Yes.
8. Do the title and abstract accurately convey what has been found? Yes, with the reservation that the relationship with HBV is only tangentially established here because of the choice of cell line.
9. Is the writing acceptable? The writing is of high quality.
Responses to Reviewer 2

In this manuscript, Wang et al. describe the role of TLR-4 in HBV-related HCC. The authors demonstrate higher TLR-4 mRNA and proteins levels in a cell line (HepG2.2.15) expressing HBV-protein(s) as compared to other HCC cell lines. The authors also demonstrate an interaction between HBx and TLR-4. The authors also link TLR-4 levels to the phosphorylation of ERK1/2. While the association of TLR-4 and HCC has been demonstrated earlier, this manuscript sheds light on the role of TLR-4 in HBV-related HCC. This work provides evidence of interaction between a HBV-encoded protein (HBx) and TLR-4. The manuscript is data rich and provides new mechanistic insights on the role of TLR-4 in HBV-related HCC. The manuscript is written in standard English. Appropriate experimental controls are included.

Major comments:
a) The authors describe the use of TLR-4-miRNA (Le-TLR 4) and negative control miRNA (Le-NC) to study tumorigenicity in nude mice. Do the authors mean si-RNA (and not miRNA)? If so this is major error and can mislead readers; this error should be fixed throughout the manuscript (in both the text and the figures).
If the authors have truly used TLR4-miRNA – what is this miRNA? Does this miRNA have other known targets that can potentially modulate tumorigenicity?

Respond:
We used shRNA, which is corresponding to siRNA in vivo experiment. We are very sorry for not picking up such a major flaw in our manuscript, as well as any misleading we have caused so far. We have corrected our manuscript including figure legends.

b) The authors demonstrate that downregulation of TLR-4 can lead to the inhibition of HepG2.2.15 derived xenografts in athymic nude mice. Since the focus of the manuscript is on HBV-related liver cancer – the authors may consider studying whether or not the downregulation of TLR-4 can lead to the inhibition of HepG cells (without HBV) derived xenografts in athymic nude mice.

Respond:
We appreciated the suggestion in regards of assessing downregulation of TLR4 would inhibit HepG2 cells (without HBV) derived xenografts in athymic nude mice. In the last few months, HepG2 cell line with either Le-TLR4 or Le-NC recombinant lentivirus was transfected. Equal amount of HepG2 cells were administrated s.c into right flank of nude mice to determine if HepG2 cells with downregulated TLR4 inhibits tumour growth. In our data, TLR4 downregulation in HepG2 cells did not inhibit tumour growth, indicating that TLR4 downregulation may specifically target HCC related HepG2.2.15 cells (with HBV) caused tumour in nude mice.

Minor comment:
1) The figure legends should be edited for clarity. Each figure and its legend should be sufficient for the reader to understand without having to go back to the text. The authors may consider revising the figure legends.
All figures and figure legends have been rearranged and fitted at the end of the manuscript.

**Level of interest:** An article of importance in its field  
**Quality of written English:** Acceptable  
**Statistical review:** Yes, but I do not feel adequately qualified to assess the statistics