ROUND 1

World Journal of Gastroenterology

Manuscript NO: 72414

Title: FOXQ1 promotes invasion and metastasis in colorectal cancer by activating the HB-EGF/EGFR pathway

Dear Editors,

Many thanks for your warm work about our manuscript entitled "FOXQ1 promotes invasion and metastasis in colorectal cancer by activating the HB-EGF/EGFR pathway".

Thanks very much for you and the reviewers taking time to deal with the manuscript and giving us lots of useful suggestions. According to the reviewers' comments, we have carefully revised it. The reviewers' comments have helped to greatly improve the quality of the manuscript.

Thank you again for your great efforts and time for our manuscript! The following are the responses to the reviewers' comments point by point. We are looking forward to hearing from you as soon as possible.

Sincerely yours,

Prof. Hui Tang

Dec. 31st, 2021

ROUND 1

Detailed Response to Reviewer 1

Q1 EGFR should be defined in the abstract and in the manuscript text.

Response:

We thank the reviewer for this suggestion. According to your suggestion, we have added the definition of EGFR in the Abstract.

Q2 Sections should be numbered.

Response:

We thank the reviewer for this suggestion. According to your suggestion, we have numbered sections in the revised manuscript.

Q3 In the Introduction, "Forkhead Box Q1 (FOXQ1) is a member of the fork head transcription factor family[4], and it promotes tumor genes by activating cell proliferation, invasion and apoptosis[5]." What do the authors mean by "tumor genes"? Tumorigenesis? Tumor genes transcription?. Also in the Introduction "There is evidence that poor efficacy and survival in CRC are associated with abnormally activated signaling pathways, including the EGFR signaling pathway[17]." The poor efficacy is referred to the treatments? this should be stated.

Response:

We are so sorry to make you confused. According to your suggestion, we have revised in the INTRODUCTION section.

Q4 I observe that MAPKs are evaluated. Which one of them? ERK ½ MAPK? This should be stated in the text and figures. As a suggestion, beta-Catenin modulation by FOXQ1 through HB-EGF/EGFR pathway could be also studied, since this protein is relevant for CRC progression and chemoresistance.

Response:

We analyzed the expression of 84 genes which related to EGF/PDGF signal pathway in DLD1-shFOXQ1 with the Human EGF/PDGF Signaling RT^2 profiler™ PCR Array. Among them, RAS was down-regulated significantly which was described in the RESULT "The knockdown of FOXQ1 suppressed the expression of HB-EGF and blocked EGF/PDGF signaling pathway in vitro" and TABLE 2.

Thanks for your suggestion. We are very interested in your suggestion about beta-Catenin, and we are glad to study beta-Catenin in our future research.

Q5 Cell proliferation should be assessed with more than one test. For example Neutral red uptake, trypan blue, MTT, MTS.

Response：

Thanks for your suggestion. Cell Counting Kit-8 (CCK-8) contains the detection reagent based on WST-8 which widely used in cell proliferation and cytotoxicity. Actually, WST-8 is similar to MTT compound. So we believe that using CCK-8 detection is reliable and effective.

Q6 In "Expression and prognosis of FOXQ1 and HB-EGF in CRC and normal colorectal tissues" Section of the Results. The authors state that "FOXQ1 was also associated with worse overall survival (Figure 1C)". From what is observed in the Figure, it should be clarified that is FOXQ1's increased expression.

Response

We thank the reviewer for this suggestion. According to your suggestion, we have revised this sentence in RESULT section "Expression and prognosis of FOXQ1 and HB-EGF in CRC and normal colorectal tissues".

Q7 In the figures, the statistical comparison between two samples should be indicated with asterisks. Also noted that the authors use two criteria, simultaneously expressing two values of probability of error when only the smallest error value should go. For example, P<0.01.Also, To show consistency throughout the text, they should unify the criteria.

Response

Thanks for your suggestion. According to your suggestion, we have changed the statistical comparison criteria to asterisks in figures.

Q8 Section "FOXQ1 inhibition induces HB-EGF suppression and EGF/PDGF signaling pathway blockade in vitro". I think this section is very interesting, however, these results only suggest what they show, that FOXQ1 is involved in the modulation of the mentioned pathways. It is known that there are many cross signals at the intracellular level. To be able to suggest that the EGFR receptor is involved, tests should be carried out by inhibiting the activity or blocking EGRF (with antibodies) with subsequent treatment of

exogenous FOXQ1 and finally see how the signaling pathways are modulated. Colocalization assays by immunocytochemistry can also be performed. Otherwise, the direct involvement of EGFR can only be argued in the discussion section.

Response

We thank the reviewer for this suggestion. We totally agree with the reviewer's comments. Our present results only suggest that the role of FOXQ1- induced Invasion and metastasis in CRC was related to activate the HB-EGF/EGFR pathway. FOXQ1 in involved in the modulation of the expression of HB-EGF, an important ligand of EGFR, thereby regulating the expression of downstream genes in the EGFR signaling pathway. As reviewer suggested, the experiments by using inhibitors to blocking EGFR should be one of the optimal method to elucidate that the EGFR is involved in FOXQ1/ HB-EGF/ EGF/PDGF axis, alternatively, a rescue experiment, by adding exogenous recombinant human HB-EGF (rhHB-EGF) protein into CRC cells with FOXQ1 knockdown was performed, followed by detecting the expression levels of HB-EGF, as well as of downstream genes related to the EGFR pathway. This rescue experiment can also confirm our result. As your suggestion, we have revised all sentences that related to the direct involvement of EGFR in the FOXQ1/ HB-EGF/ EGF/PDGF axis. In addition, colocalization assays will take into account in our forthcoming work.

Q9 In the final section of the results. Not my expertise, but the analysis of the colorectal tumors tissue looks scant. More details about the selected patients should be included and further analyzed in the results,(age, tumor stage, tumor classification, treatment).

Response

We thank the reviewer for this suggestion. According to your suggestion, we have added a new Table 3 to analyze 65 patients including age; gender; size of tumor; Lymphatic metastasis; Tumor differentiation; TNM stage and AJCC clinical stage according to 7th issue.

Detailed Response to Reviewer 2

Q1 A better Discussion is needed. Most of the Discussion is basically the repeat of results. Comparision of new findings with previous ones are a must. Refernce to figures and p-values should be removed from Discussion unless the result is so significant that it needs to be re-referenced.

Response

We thank the reviewer for this suggestion. According to your suggestion, we have rewritten the DISCUSSION section and removed the references to figures and p-values.

Q2 Did authors applied any p-value correction?

Response:

Yes, we have performed Bonferroni correction in GraphPad Prism 8 and SPSS v.19.

Q3 On figures, use the conventional *//*** signs to represent significance of p < 0.05 / 0.01 / 0.001 instead of a/b/c.**

Response:

We thank the reviewer for this suggestion. According to your suggestion, we have revised all figures in our manuscript.

Q4 Within the main text, exact p-values must be presented with 4 digit decimals.

Response:

We thank the reviewer for this suggestion. According to your suggestion, we have revised all P-values in our manuscript.

Q5 In Discussion authors wrote: "In our previous studies, we found that the mRNA...". Citation must be included!

Response：

We thank the reviewer for this suggestion. According to your suggestion, we have cited our previous study, the corresponding reference [38] was also added in DISCUSSION section.

Q6 First two paragraph of Discussion can be megerged in my oppinion.

Response:

Thanks for your suggestion. According to your suggestion, we have merged first two paragraphs into one in the Discussion.

Q7 Please include the vulcano plots as well if they are mentioned witin the text.

Response：

Thanks for your suggestion. Figure 2C is the volcano plot which we mentioned in our manuscript.

Q8 As per their position within the manuscript, the ordering of Table 2 and 3 should be changed.

Response：

Thanks for your suggestion. According to your suggestion, we have revised in our manuscript.

Q9 A new table about the clinicohystopathological details of the 65 patiens must be included.

Response：

Thanks for your suggestion. According to your suggestion, we have added a new TABLE 3, in which age, gender, size of tumor, lymphatic metastasis, tumor differentiation, TNM stage and AJCC clinical stage of enrolled 65 patients has been included in the manuscript.

Detailed Response to Reviewer 3

Q1 The abstract section can improve—add a focus point in the abstract section.

Response：

We thank the reviewer for this suggestion. According to your suggestion, we have rewritten the abstract in a more straightforward way by adding a focus point.

Q2 Rewrite the methods, results and conclusion (in the abstract) in a more straightforward form.

Response

We thank the reviewer for this suggestion. According to your suggestion, we have rewritten the methods, results and conclusions in the abstract.

Q3 FOXQ1 regulated the expression of HB-EGF, which initiated a cascading effect on multiple important node genes in the EGFR pathway. What does it mean?

Response

We are so sorry to make you confused. The meaning of this sentence is that Knockdown of FOXQ1 suppressed HB-EGF expression, and also lead to the decrease of EGFR and its downstream genes AKT, RAF, KRAS expression levels. In order to describe more clearly, we

have revised in the corresponding paragraph.

Q4 HB-EGF expression levels and EGFR pathway activation states were detected by Western blot before and after recombinant human HB-EGF (rhHB-EGF) protein was added to DLD1-shFOXQ1 cells. What does it mean?

Response

We are sorry to make you confused. Because FOXQ1 knockdown led to decreased expression of HB-EGF. So in this rescue experiment, exogenous recombinant human HB-EGF (rhHB-EGF) protein was added to DLD1-shFOXQ1 and SW480-shFOXQ1 cells, then the expression levels of HB-EGF, as well as of downstream genes related to the EGFR pathway was detected. The aim of this rescue experiment is in order to confirm our result reversely.

We thank the reviewer for this suggestion. According to your suggestion, we have made some revises in the manuscript.

Q5 Authors are suggested to use the full form when used for the first time throughout the manuscript.

Response:

Thanks for your suggestion. According to your suggestion, we have reviewed the manuscript and revised.

Q6 The introduction section looks good. Authors can try to include the existing research limitations also, how the present research unravels those limits.

Response

We thank the reviewer for this suggestion. According to your suggestion, we have revised in the INTRODUCTION section.

Q7 Aim of the study should need to add as the last paragraph in the introduction.

Response:

We thank the reviewer for this suggestion. According to your suggestion, we have rewritten the last paragraph in the INTRODUCTION section by adding the aim of the present study.

Q8 Material and methods also look good. Need a logical flow of the writings with enough references.

Response:

We thank the reviewer for this suggestion. According to your suggestion, we have added references [22] and [23] in Materials and methods in more logical way.

Q9 Check all the symbols.

Response:

Thank you for this suggestion. According to your suggestion, we have double checked all the symbols.

Q10 Cutoff values for the scoring system were assigned as follows: high expression of FOXQ1 and HB-EGF were defined as an IRS of ⩾ 4 (4, 6, 8, 9 and 12); and low expression was defined as an IRS of < 4 (0, 1, 2 and 3). Any references?

Response:

We are so sorry for our mistake. According to your suggestion, we have inserted the corresponding reference [23] in this sentence.

Q11 Exogenous recombinant HB-EGF protein at a final concentration of 50 ng/mL was added to the cell culture medium when the cell density reached 80%. Why is so?

Response:

We are so sorry to make you confused. We performed this experiment according to previous studies. According to your suggestion, the corresponding reference [22] has been cited in the manuscript.

Q12 The results section can improve by adding significant results

Response:

We thank the reviewer for this suggestion. According to your suggestion, we have revised the RESULTS section.

Q13 The writing of results is good. Need to maintain a logical flow of the writings.

Response:

We thank the reviewer for this suggestion. According to your suggestion, we have revised RESULTS section in a more logical way.

Q14 Figures presentation is up to mark.

Response:

We thank the reviewer for this suggestion. According to your suggestion, we have revised all Figures.

Q15 Figure legends are self-explanatory. Need to confirm without the repetition of the results and discussion in the figure legends.

Response:

Thank you for this suggestion. According to your suggestion, we have revised all Figure legends in the manuscript.

Q16 The discussion is good. The discussion section can improve by including the data from other sources about related works.

Response:

Thank you for this suggestion. According to your suggestion, we have rewritten the DISCUSSION section by including more related references, including [37], reference [40], and [47].

Q17 The conclusion needs to address future perspectives.

Response:

Thank you for this suggestion. According to your suggestion, we have rewritten the CONCLUSION section and addressed future perspectives.

Q18 Novelty of the work should be added by the author in the conclusion section.

Response:

Thank you for this suggestion. According to your suggestion, we have rewritten the CONCLUSION section, and highlighted the novelty of present work.

Q19 Many spacing, punctuation marks problem found in the tables.

Response:

We are sorry for our carelessness. According to your suggestion, we have double checked and revised the spacing, punctuation marks problem in all the tables.

Q20 Spacing, punctuation marks, grammar, and spelling errors should be reviewed thoroughly. I found so many typos throughout the manuscript.

Response:

We thank the reviewer for this suggestion. According to your suggestion, we have revised the manuscript and double checked the grammar and spelling errors in the manuscript.

ROUND 2

Detailed Response to the Re-Reviewer

Manuscirpt improved significantly. There are a few typos here and there, which should be checked prior publication. In Table 3, "(mm)" is missing from size of tumor.

Response: Thanks very much for the re-reviewers taking time to deal with the manuscript and giving us lots of useful suggestions. According to the re-reviewers' comments, we have carefully revised it.