

## Responses to the Comments of Editor and Reviewers

Dear Editor and Reviewers,

Thank you so much for your kind advice for our manuscript entitled "Huanglian decoction suppresses the growth of hepatocellular carcinoma cells by reducing CCNB1 expression". We also appreciate the constructive comments from the reviewers. These comments are most valuable for us in preparing a more valuable manuscript. We have revised the manuscript based on the reviewer's suggestions, and retained traces of the revisions. Specific responses to the reviewer's comments are described below:

Reviewer #1:

**Scientific Quality:** Grade D (Fair)

**Language Quality:** Grade B (Minor language polishing)

**Conclusion:** Major revision

**Specific Comments to Authors:** Huanglian Decoction suppresses the growth of hepatocellular carcinoma cells by reducing CCNB1 expression. The authors utilized several databases to find the main DEGs in HCC and Huanglian Decoction inhibits the growth, migration and invasion via CCNB1. There are some questions are not clear.

Majors:

1. The authors only did the *in vitro* experiment to verify that Huanglian Decoction suppressed HCC growth, migration and invasion via CCNB1. As they mentioned in the discussion, the major limitation is the absence of *in vivo* work. There are lot of TCM or single compound worked well *in vitro*, but failed *in vivo*. If they can verify its effect *in vivo* that would be more convincing.

**R:** Thanks for your comments. But I'm sorry, because of COVID-19, animal experiments are not possible in a short time frame. Our main purpose is to study the effect of Huanglian decoction on HCC cells, and to explore its potential functional pathways. We have completed this research purpose, and

*in vivo* research is a future research plan.

2. The authors only used one concentration of Huanglian Decoction to treat each cells, please state why choose this concentration? There was no minimal effective dose to the HCC cell lines and the toxic dose to the normal hepatocyte was absent, which should be clearly detected and essential for the potential therapeutic treatment.

**R:** Thanks for your comments. We initially used different concentrations of Huanglian decoction (0 - 1000 µg/ml) to treat HCC cells, and then we obtained the appropriate Huanglian decoction concentration through cell viability assays. "Here, we performed cell viability assays on liver cancer cells, the outcomes of which are depicted in Figure 6A. These findings demonstrate that Huanglian decoction exhibited time- and dose-dependent effects on HCC cell viability. The IC<sub>50</sub> value of Huanglian decoction after 48 h of treatment was approximately 100 µg/ml for PLC/PRF/5 and 200 µg/ml for HepG2 cells."

3. The authors found 5 bioactive compounds in Huanglian Decoction to target CCNB1. Why didn't do the further investigation on each compound or their synergistic effect additionally? This will be helpful to understand the mechanism of Huanglian Decoction to inhibit the expression of CCNB1.

**R:** Thank you for your professional advice. Because the purpose of this article is to explore the effect of Huanglian decoction on HCC, discover its main target, and verify it, and explore the potential pathways of CCNB1. The study of each compound or its synergy is our future task.

4. For the preparation of Huanglian Decoction aqueous extract, the authors should provide more details, like soaking time, which equipment and temperature were used for the concentration, please provide the gravity not the rpm. Before the *in vitro* and *in vivo* studies, a chromatography and mass

spectrometry should be done to authentic the bioactive compounds are constantly presented in the extract.

**R:** Thank you for your professional advice. We have revised it based on your comments, and the revised position is at method section (*Preparation of Huanglian decoction aqueous extract*).

Minors:

1. Please check the typos, like 3.2 line 1 “Huang Ren Tang” change to “Huang Lian Tang”; 3.7 line 5 “Si-CCNB1” to “si-CCNB1”; discussion paragraph 3 “CCNB2”, the refs 36-42 studied CCNB1 not CCNB2.

**R:** We are very sorry for the mistakes in this manuscript and the inconvenience caused in your reading. We have corrected it.

2. The full name of TRT?

**R:** Thanks for your comments. We have revised it. The full name of TRT is TongRenTang pharmacy.

3. WB method didn't state the gravity of centrifugation nor which membrane was used for transfer, PVDF or NC or others?

**R:** Thanks for your comments. The centrifugation gravity of the WB method and the membrane used have been added to the method section (*Western blotting analysis*).

Reviewer #2:

**Scientific Quality:** Grade B (Very good)

**Language Quality:** Grade A (Priority publishing)

**Conclusion:** Accept (General priority)

**Specific Comments to Authors:**

Major:

1. In the section 3.7, only 3 groups in the experiments shown in Figure

C/D/E/F were insufficient, the effect of Huanglian Decoction on CCNB1 cannot be demonstrated clearly. It is recommended to increase to 4 groups like the experiment shown in Figure A/B (add a Huanglian Decoction without si-CCNB1 group).

**R:** Thank you for your comments. It is an important and valuable suggestion. We have added Huanglian decoction (without si-CCNB1) group. The modified result is shown in Figure 7.

Minor:

1. The “Conclusion” was absent in the ABSTRACT, please add it.

**R:** Thank you for your comments. We have already added.

2. In the INTRODUCTION, the authors did not provide reference about the utility of Huanglian Decoction in the treatment of liver cancer, please complement related reference.

**R:** Thank you for your comments. We have added relevant references. REFERENCES- [15-20].

3. In the Figure1, the meaning of “Patients” and “Samples” is unclear for the readers. Please modified as “tumor samples” and “non-cancerous samples” as in the article.

**R:** Thank you for your comments. We have revised it (Figure1).

4. In the last sentence of the figure legends of Fig2, “Downregulated genes are marked in light blue”. It seems like green according to the picture. Please check again.

**R:** We apologize for the wrong color description. You are right, it is green, and we have modified it (Figure1).

5. In the section3.5 line12, authors wrote that “we noticed that CCNB1

promotes tumor development”. The “promote” was not suitable here, please modified into “associate”.

**R:** Thank you for your comments. We have revised it.

Reviewer #3:

**Scientific Quality:** Grade C (Good)

**Language Quality:** Grade B (Minor language polishing)

**Conclusion:** Major revision

**Specific Comments to Authors:**

1. The quality control of Huanglian Decoction and its drug-containing serum should be provided.

**R:** Thank you for your comments. The description of the quality control of Huanglian Decoction has been added to the method section (*Preparation of Huanglian decoction aqueous extract*). This study did not use drug-containing serum, and further studies will be carried out in animal models in the future.

2. To collecting compounds of Huanglian Decoction, only using TCMSP database is insufficient, it should be multiple databases such as OMIM and GeneCards etc.

**R:** Thanks for your suggestions. We believe that TCMSP (Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform) is a relatively complete database of traditional Chinese medicine. In order to verify whether there are any omissions, we have queried several classic Chinese medicine databases (TCMID-Traditional Chinese Medicines Integrated Database; ETCM-The Encyclopedia of Traditional Chinese Medicine; TCM@Taiwan-Traditional Chinese Medicine Database@Taiwan). We have selected an oral bioavailability (OB) index  $\geq 30\%$  and drug-likeness (DL) index  $\geq 0.18$  to screen compounds. Through comparison, the results show that our compound collection has no omissions (Supplementary Table S1).

3. To identify the DEGs, not only TCGA and GEO datasets, but also more datasets such as HIT, TCMgeneDIT, TCMID, and ETCM etc. could be used.

**R:** Thank you for your professional advice, but the analysis has been completed, we may not be able to add. Next time we will use more datasets to identify DEGs.

4. Authors should provide the experimental evidences of Huanglian Decoction regulated cell cycle through CCNB1.

**R:** Thank you for your comments. We have supplemented the experiment based on your suggestion. "The cell cycle analysis revealed that Huanglian decoction treatment caused a significant accumulation of cells in G2/M phase in both cell lines (Figure 7G; H), which may be regulated by CCNB1."

5. The main apoptosis-related pathways should be validated by the experiments.

**R:** Thank you for your comments. We have revised the manuscript (*Protein expression of CCNB1, Bax, caspase 3, caspase 9, CDK1, p53, and p21 in the different groups by western blot*) and added experiments (Figure 8).

6. Authors should discuss how the compounds in Huanglian Decoction could activate what signal pathways to inhibit cancer cell apoptosis, cell cycle, cell migration and invasion, and descript multi-compounds, multi-tardets and multi-effects feature of Huanglian Decoction in HCC treatments.

**R:** Thank you for your comments. We have added the experiments (Figure 8). "Induction of apoptosis in cancer cells is a key therapeutic strategy for cancer treatment. After Huanglian decoction treatment, we found that apoptosis of HCC cells was increased significantly and that the expression of apoptosis-related proteins was increased. The results of the si-CCNB1 group and the si-CCNB1+Huanglian decoction group showed that Huanglian

decoction mainly affected cell apoptosis through CCNB1. The cell cycle is a complex and complicated process. We found that Huanglian decoction can induce G2/M phase arrest. Cyclin B1 plays an important role in G2/M transition and during M phase [43,44]. The cyclin-dependent kinase inhibitor p21 protein plays an important role in G2 phase arrest [45] and has been shown to contribute to cell cycle arrest through transcriptional repression of cell cycle regulatory genes. Therefore, Huanglian decoction may regulate G2/M cell cycle arrest through p21. Our study showed that CCNB1 silencing suppressed the expression of CCNB1 and CDK1 but increased the expression of Bax, caspase 3, caspase 9, p53 and p21. Bax has been reported to be directly activated by p53 in the absence of other proteins to permeabilize mitochondria and initiate the apoptotic program [46-48]. Moreover, p53-induced apoptosis involves triggering the caspase-9 initiator and its downstream caspase-3 executioner. Previous studies have shown that CCNB1 silencing can inhibit cell proliferation and promote cell senescence by activating the p53 signaling pathway in pancreatic cancer [21]. We found that the same phenomenon exists in HCC. Therefore, we speculate that Huanglian decoction can prevent HCC cell progression by inhibiting CCNB1 expression and activating the p53 signaling pathway.”

7. GOs and pathways enrichment analysis could be changed to a bubble diagram.

**R:** Thank you for your comments. We have modified it.

**Supplementary comments:**

(1) The authors did not provide original pictures. Please provide the original figure documents. Please prepare and arrange the figures using PowerPoint to ensure that all graphs or arrows or text portions can be reprocessed by the editor;

**R:** Thank you for your comments. We have prepared the original figures as

zip file, including PPT and tiff figures.

(2) PMID and DOI numbers are missing in the reference list. Please provide the PubMed numbers and DOI citation numbers to the reference list and list all authors of the references. Please revise throughout;

**R:** Thank you for your comments. We have already added it in the manuscript.

(3) The “Article Highlights” section is missing. Please add the “Article Highlights” section at the end of the main text; and

**R:** Thank you for your comments. We have already added it in the manuscript.

(4) Please don't include any \*, #, †, §, ‡, ¥, @....in your manuscript; Please use superscript numbers for illustration; and for statistical significance, please use superscript letters. Statistical significance is expressed as <sup>a</sup>*P* <0.05, <sup>b</sup>*P* <0.01 (*P* > 0.05 usually does not need to be denoted). If there are other series of *P* values, <sup>c</sup>*P* <0.05 and <sup>d</sup>*P* <0.01 are used, and a third series of *P* values is expressed as <sup>e</sup>*P* <0.05 and <sup>f</sup>*P* <0.01.

**R:** Thank you for your comments. We have revised the problem in the manuscript.