Dear reviewers,

Thank you very much for your letter and for the reviewers' comment concerning out manuscript "Cell culture-adaptive mutations in HCV promote viral production by enhancing viral replication and release" (No. 36386). Those comments are all valuable and very helpful for revising and improving our paper, as well as the important guiding significance to our researchers. We have studied comments carefully and have made correction which we hope meet with approval. The main corrections in the paper and the responds to the reviewers' comments are as flowing:

1. Reviewer No ID 02453015

1) English needs to be improved by native speakers.

   Response: We agree with your suggestion. We have read the full manuscript carefully, and also disputed the special company to polish the English. The professional English language editing company named Medsci, and the certificate may be verified at www.medsciediting.com/certificate/(code: 1508-4985-0DD2-CFD4- 67C6).

2) Novelty should be emphasized.

   Response: Thank you for your thoughtful suggestion. We have written up the research motivation, and research perspectives, et al. to emphasize the novelty, and added the content in the last paragraph of discussion section.

3) The abstract is not in the correct format.

   Response: According to your comment, we have re-written the abstract in the correct format of WJG.

4) How did the authors determine Low and High viral titers? What is the threshold level?

   Response: Thank you very much for your comments. We determine the low and high viral titers dependent on the clinical guidelines and previous studies. (1)The clinical practice considers that the HCVRNA $<10^2$ IU/ml is low viral load, and $>10^5$

IU/ml is high viral load. Although the load and titer are not the same, but we think they could be used as a part of the reference. (2)The previous study entitled “Direct visualization of hepatitis C virus-infected Huh7.5 cells with a high titre of infectious chimeric JFH1-EGFP reporter virus in three-dimensional Matrigel cell cultures. **J Gen Virol,2014**” had reported a “high titer infectious chimeric JFH1-EGFP reporter virus” with “**1×10(6) ffu/ml”**. But that titer was little lower than the titer of our study.

5) Figure 1B. How to show single site mutations with similar length by electrophoresis? It can only be shown by sequencing.

   Response: We thank you for pointing out the important issue. Yes, you are correct. It is our mistake. The mutations only be shown by sequencing, and we have added the comment in the “2.3 Construction of the JFH1 variant” in “2 Materials and Methods” section. The electrophoresis only showed the quality of HCV RNA.

6) The rationale for the combinations of mutations should be described. Why not try other combinations?

   Response: Thank you for your good question. In fact, we also had been puzzled for a long time before the experiments started until we known that the mutation V2440L was reported previously and more and more studies were confirmed that NS5A would be an important target for drug selection. So we designed our experiments using single mutation and combinate mutation at the core of NS5A. And we will try other combination patterns in the next study.

2. Reviewer No ID 01564209

1) English typing and grammar needs attention at places and should be checked carefully best by a native English speaker. Also there are frequently missing space between two words.

   Response: We are so sorry for these mistakes. We have checked and approved the manuscript. The manuscript has also been provided for language certificate by professional English language editing company named Medsci, and the certificate may be verified at www.medsciediting.com/certificate/(code: 1508-

4985-0DD2-CFD4-67C6).

2) Fig. 1B is not convincing. The size of the “HCV RNA” seemed to be much larger than the expected 9.6 kb. In my opinion the denoted HCV RNA bands are artefacts.

   Response: Thank you very much for your comments. We have repeatedly confirmed the samples first, and then we found that the RNA ladders marked shown in the diagram was errors. We have corrected the data and updated the right chart in the manuscript.

3) Fig. 2 How many experiments were performed in order to determine ffu/ml? A statistical calculation should be performed and significance should be annotated best from in minimum three independent experiments.

   Response: Thank you very much for your suggestions. Viral titers were expressed as focus-forming units per milliliter (ffu/ml) assayed in duplicate and performed three times. This aim of the experiment design is to select the best one which could gain the highest titer comparing to the wild type JFH1. We have shown the results using specific value and times value as well to express the relationship of high and low. Considering the reader's easy understanding, we have added the P value between the mJFH1 and JFH1, mJFH1 andJFH1-mE2/p7/NS5A in the manuscript.

4) Fig. 3B. I assume that Fig 3B shows two parallel experiments; however, a stistical diagram summarizing the two to (more convincing) three Western blot experiments should be shown including statistical analysis evaluating the differences between the 6 mutants.

   Response: As you requested, we have performed additional statistical analysis using the grey values of the blots from the three independent experiments. Please kindly find the results in the manuscript. And we have added the P value between the mJFH1 and JFH1, mJFH1 andJFH1-mE2/p7/NS5A.

5) Chapter 3.3. 3rd and 12th line. What are exactly the “ten” HCV RNAs? Please specify.

   Response: Thanks to you for your good comments. We have added the full

names of “Ten HCV RNAs” in Chapter 3.3. This will help the readers to understand the paper better.

6) Fig. 4C. How can you show beta-actin in supernatant?

   Response: Thank you very much for your comments. We know that infectious HCV virions should be released into the supernatants from cells. In order to study whether the mutations could affect the virion release, we collected the supernatants and cell lysates after the ten HCV RNAs electroporated into Huh7.5 cells, and then infected the naïve Huh7.5 cells with them. The final HCV levels which were represented by anti-NS3 Western blot of the total proteins from the infected cells were showed as Fig. 4C. Thus the “supernatants” means that the samples from the top line were infected with “supernatants” not the “lysates”.

7) Chapter 3.3. The virion release should be discussed in more detail. From the Western Blot analysis (Fig. 4C) one can infer dramatic differences in terms of the 6 HCV mutants.

   Response: Thank you very much for your insightful suggestion. Focus on the virion release analysis shown in Fig.4C, we have added the discussion in the “DISCUSSION” section as follow: (Please kindly find this part in the manuscript)

   Previous study demonstrated that HCV p7 promotes a late step ofassembly and release of infectious virions and NS5A plays a major role in regulating the release of infectious virus particles in cell culture. In this study, there three mutants located at NS5A(C2274R, I2340T and V2440L) and one located at p7 (H781Y). Our result showed these mutants obvious promoted the HCV viral particles release(Fig.4). HCV core is located on the cytosolic side of the ER membrane, assembly probably initiates in the cytosol before further maturation, and release occurs by transfer of nascent particles across the ER membrane to enable access to the secretory pathways in hepatocytes. The mutants in NS5A and P7 induced amino acids changes may involve these steps. The specific mechanism needs to be further studied in future.

8) Discussion and Conclusion section. A summary and conclusion of the key results of the experiments are missing leaving the reader alone with the findings of this

well performed experimental study.

Response: Thank you very much for your comments and suggestions. According to your suggestion, we have given a summary and conclusion at the last part of the manuscript, and we think it will help the readers to understand the study.

3. Reviewer No ID 00503405

In this original study the authors assayed the mechanism of in vitro cell culture adaptive viral mutations responsible for enhanced viral production in cell lines. They provided evidence that the adaptive C-virus mutations led to a robust infectious titer via promotion of viral replication and release. The study is well designed and well presented, all the used techniques are adequate for answering the original aims. Some minor language polishing are needed. Also, the format of the manuscript must be changed to the requirement of WJG. After minor revision I suggest to accept the manuscript for publication.

Response: Thank you very much for your positive and useful comments. We have corrected the forms and language mistakes, and revised the manuscript carefully following the editorial advice.

We hope that the revision is acceptable for the publication in your journal.

Looking forward to hearing from you soon.

Yours Sincerely

Jun Cheng