Author's response to reviews

Title: Saikosaponin-d Increases the Radiosensitivity of Hepatocellular Carcinoma Cell line SMMC-7721 by Adjusting the G0/G1 and G2/M Checkpoints of Cell Cycle

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Author's response to reviews: see over
Dear Editors and Reviewers:

Thank you for your letter and for the reviewers’ comments concerning our manuscript entitled “Saikosaponin-d Increases the Radiosensitivity of Hepatocellular Carcinoma Cell line SMMC-7721 by Adjusting the G0/G1 and G2/M Checkpoints of Cell Cycle” (MS: 8842196221023094). Those comments are all valuable and very helpful for revising and improving our paper, as well as the important guiding significance to our researches. We have revised the manuscript, and would like to re-submit it for your consideration. Revised portion are marked in red in the paper. Point by point responses to the reviewers’ comments are listed below this letter.

We would like to express our great appreciation to you and reviewers for comments on our paper.

Looking forward to hearing from you.

With best wishes,

Yours sincerely,
Baofeng Wang
2013.08.18

1. Response to comments: The authors emphasize that "...their recent clinical practice of combining SSD administration with radiation in treating patients with hepatocellular carcinoma revealed that this joint treatment was more effective than either monotherapy alone, indicating a contributory effect of SSD on radiotherapy." Therefore, they would like to investigate the molecular mechanisms of SSD mediated radiosensitive responses. However, the author did not provide the reference or literature for their previous clinical work. Because the in vivo tumor responses and cell line responses to chemo-radiotherapy may be different, the detailed treatment of patients using SSD and radiation should be stated more detail.

Response: First of all, it is a very good and meaningful question. At present, the clinical experience in treating patients with liver cancer comes just out of traditional Chinese folk prescription chai hu tang. We initially observed that this prescription could increase the effectiveness of radiation therapy in patients with hepatocellular carcinoma. Because of the small number of cases, at present, we need more substantial clinical data to support. Just as the reviewer said that the therapeutic effect of a drug in vivo and in vitro may be very different, a lot of effective treatment in vitro may actually have no effect in the body, or the result is not satisfactory. SSD is the main component in this prescription, and therefore, in this experiment, the effect of SSD combined with radiation on SMMC-7721 hepatoma cells in vitro was initially observed, and its possible mechanism of action was explored to provide experimental basis for further future clinical use.

2. Response to comments: Although the author claimed that SSD could increase the radiosensitivity of SMMC-7721 HCC cell ine, no survival curves and dose modifying factor (DMF) were provided in this study. Because the survival curve using colony formation assay (or MTT assay) is the most important parameter to determine the radiosensitivity in treated
cells, this data need to provide before SSd can be claimed as a radiosensitizer.

Response: Many thanks for the question. We have in the relevant articles clearly shown that SSd can significantly increase the inhibition effect of the radiation on hepatoma cell colony formation, currently, the article has been accepted and is being typeset. But because the main purpose of this article is to study the relationship between the radiosensitizing effect of SSd and the cell cycle, in this article we observed the inhibition effect of SSd before and after combined with radiation on the growth of SMMC-7721 cells through MTT assay, and its effect on cell apoptosis and cell cycle through flow cytometry.

3. Response to comments: The author compared the oxia (or Normaxia?) and hypoxia effects when using SSd and radiation treatment on cells. The idea is good, but use of CoCl2 to represent the hypoxia need to be more conserve. Is it possible to know the level of hypoxia using CoCl2? Also, the authors did not show the change of HIF-1a after CoCl2 treatment. This is the most important evidence that CoCl2 can be used for hypoxic mimetic.

Response: Thank you for your question. Our study and previous studies have found [1-4] that in CoCl2-induced hypoxic cells, HIF-1a expression significantly increased, and we also found that the increase of the expression of HIF-1a was closely correlated with cell radiosensitivity. This model can also simulate the malignant biological behavior of tumor cells, such as high rates of invasion and metastasis as well as resistance to radiotherapy and chemotherapy.


4. Response to comments: In Figure 1, 2Gy seemed still cause the growth inhibition of cells in hypoxia, although no significance was pronounced. Please provide the p value of this group.

Response: We apologize for this error. We have made the necessary corrections in Figure 1 according to the Reviewer’s comments. P=0.0461.

5. Response to comments: In figure 2A, the base lines (the crosses) varied in each subfigure of flow cytometric analysis for apoptosis. The cell number also seemed varied a lot in different dataset, please solve these problems or discuss it.

Response: We apologize for this error. After a careful look for reasons, we found that we may have put together the pictures of the same experiment with different repetition numbers. We have now corrected the Fig2A.

6. Response to comments: It is not clear the purpose of using PTX-478 combined with or without radiation. It should be used with SSd+IR under hypoxic condition, based on the authors assumptions.
Response: This is a very good question. Because PTX-478 is now the most accepted inhibitor of the expression of HIF-1α in hypoxic cells, it is here a positive control to indirectly show that in hypoxic conditions, SSd can also increase the radiosensitivity of hepatoma cells, and its effect is similar to that of PTX-478, with no significant difference between the two. Considering the Reviewer's comments, in future experiments, we will observe the effect of the two combined on the radiation therapy of hematoma cells.

7. Response to comments: in figure 3A, what is the blue peak of the cell cycle distribution schemes? The author should address it and define it for discussion, particularly combined with figure 2.
Response: Thank you for your question. SubG1 period is the peak of apoptosis, and it can be detected, using PI single staining and flow cytometry. The appearance of subG1 peak has no effect on cell cycle analysis.

8. Response to comments: In figure 4, the lanes of each blot were not line up, therefore it is difficult to compare the difference between these molecules. Additionally, the HIF-1α level should be added in these data. Is there any relationship between the changes of these molecules to cell cycle redistribution?
Response: Thank you for your suggestion. This problem may be related to the quality of glue, voltage stability and other related factors, and the interference of experimental external factors may have produced a little effect on protein electrophoresis, causing the protein molecules in each channel not completely in a line. But after our repeated test verification, the marked protein is indeed the target protein. As for HIF-1α, P53 and the intrinsic link between apoptosis-related molecules and the cell cycle, we will do further study in our future experiment.

9. Response to comments: Overall, because the authors used PTX-478 for almost all data to compare to SSD, it is unclear that the point of this paper is the radiosensitivity increased by SSD, or the importance of hypoxia when using SSD. The authors need to clarify this point in manuscript title and throughout the manuscript.
Response: Thanks for reviewer’s question. Our preliminary findings showed that either in oxic or hypoxic conditions, SSd showed certain radiosensitizing effect on SMMC-7721 cells. The radiosensitizing effect of SSd in hypoxic condition on SMMC-7721 cells was related to its increasing Go/G1 arrest and its reducing G2/M-arrest; in oxic conditions, the radiosensitizing effect of SSd was only related to its increasing Go/G1 arrest, and not to the decrease of G2/M-phase;

Minor Essential Revisions
1. Response to comments: Is oxia the normaxia? Please be consistent.
Response: We apologize for this error. We have made the necessary corrections in the revised paper according to the Reviewer's comments. We would like to thank you for your helpful comments.
2. Response to comments: In figure 4A, it is suggested that the description of each lane should be put on the top of the blot but not in the middle of two dataset.
Response: Thank you very much for reviewer’s comments. We have made the necessary corrections in figure 4A according to the reviewer’s comments.
3. Response to comments: The topic of each result paragraph need to rewrite to reflect the
importance and significance of the findings. For example: "SSd Inhibited Cell Growth", but the data included radiation treatment, then the authors should clearly state the combined treatment is important in the topic.

Response: We apologize for this error. We have made the necessary corrections in the revised paper according to the reviewer’s comments. We would like to thank you for your helpful comments.

4. Response to comments: How many times of experimental repeats to obtain the statistical analysis results need to provide.

Response: Thank you for your question. We have made the necessary corrections in revised paper according to the reviewer’s comments.

We have revised and improved the contents of this manuscript. These changes will not influence the content and framework of the paper. We did not make a list of the changes but they were marked in red in the revised paper. Thank you very much for your comments and suggestions.