Author’s response to reviews

Title: Relationship Between the Invasion of Lymphocytes and Cytokines in the Tumor Microenvironment and the Interval After Single Brachytherapy Hypofractionated Radiotherapy and Conventional Fractionation Radiotherapy in Non-Small Cell Lung Cancer

Authors:

Lin Li (962553528@qq.com)
Hong Cheng Yue (810658329@qq.com)
Yun Wei Han (530018842@qq.com)
Wei Liu (18715798944@163.com)
Liang Geng Xiong (755745502@qq.com)
jianwen zhang (zhangjianwen66@126.com)

Version: 1 Date: 17 Jun 2020

Author’s response to reviews:

Dear editor:
Thank you very much for your valuable suggestions. We attach great importance to it and seriously answer the questions raised by reviewers. The reply is as follows:

Technical Comments:

Reviewer 2: The main concern with this article, as it is submitted, is that the resolution of the histopathology and flow cytometry images in all figures is bad (the images are quite blurry). Therefore, I am unable to compare the results described in the main text of the manuscript with the figures to verify the findings of this study. I recommend that the authors submit revised high-resolution figures to enable a comprehensive review of this study.

Reply: Thank you for your suggestion. We have modified all figures in detail (Figure 1-8), and added the notes and content description. The notes of tables are further improved according to the requirements.

Reviewer 3:
1. Title - correct the spelling of 'radiotherapy'.
Reply: Thank you for your suggestion. We have modified the spelling.
2. Abstract - background - line 1 - end with a period.
Reply: Thank you for your suggestion. We have modified Abstract - background with a period.
3. Check other such small typos, grammatical, spelling, capitalization, punctuation, spacing errors throughout manuscript, which are plenty
Reply: Thank you for your suggestion. We have checked and modified typos, grammatical, spelling, capitalization, punctuation, spacing errors again.
4. Please include any specific justification (citing refs. if any) why the particular time-points were chosen for the various methods (e.g. days 7 and 14 for micro-PET/CT, max. and min. diameters of tumors measured every 2 days from the 12th day after inoculation until the 14th day after radiation, etc.)? Could there be different findings at intermediate or further time-points?

Reply: Thank you for your suggestion. (1) The relationship between the effects of radiation on invasion of lymphocytes and cytokines in tumor microenvironment and the interval after radiation is not clear at present. In our previous clinical study, we found that tumor cell necrosis was obvious on day 7 after brachytherapy for lung cancer. Therefore, we chose on days 7 and 14 as research time points. Other time points are selected and designed for future research. (2) In order to maintain the consistency of study time, PET/CT time was consistent with the time points of lymphocyte and cytokine examination. (3) Tumor diameter was measured every 2 days to ensure that the tumor size met the research requirements.

5. How many repeat trials/ replicates were performed for each method and for the statistical analyses? Were all results consistent across all replicates? This is important to ensure reproducibility.

Reply: Thank you for your suggestion. We repeated the study two times, and the results were basically the same.

6. The sample sizes used seem to be low. Please provide better sample size or justify the ones used.

Reply: Thank you for your suggestion. (1)The number of tumor-bearing mice in each group was determined by combining literature (reference 20) and statistics (10-15 mice). (2)Although the more animals there are, the more representative they may be, there are corresponding problems: (1) cost problem: the more animals there are, the higher the cost will be. (2) practical operation: the more animals there are, the more operations there are, and the larger the research bias may be.

7. Were any other animal strains tested? Else, how could you predict the generalizability of the data?

Reply: Thank you for your suggestion. Our study was a first basic study of this technique in tumor radiotherapy, and it has not been studied in other animal models and cell lines. In the future, we should carry out multiple animal models and cell lines studies to summarize the possible universality of this technique.

8. Please include any limitations as well as recommend future directions of the study.

Reply: Thank you for your suggestion. Our study was a first basic study of this technique in tumor radiotherapy, which has some limitations and needs further improvement. In the future, we should conduct a variety of animal models and cell lines studies, adjust and shorten the time interval studies, as well as in vivo studies using immunocheckpoint inhibitors on the basis of existing studies.

9. Figures 2, 3 - please use scale bars as appropriate, and need to increase figure resolutions optimally (seems quite blurry even when magnified).

Reply: Thank you for your suggestion. We are so sorry for not adding scale bars to these images when they were originally taken. In order to maintain the authenticity of the original pictures, we did not add extra. The magnifications of the images were shown in legend for readers to read.

10. Fig. 1 legend - please explain a bit more for non-expert readers.

Reply: Thank you for your suggestion. We have further labeled and explained.
11. In background, please provide the basics (definition, fundamental concept, significance, etc.) of the pertinent strategies like radiotherapy, CFRT, SBRT, stereotactic radiosurgery, hypofractionated radiotherapy, SBHFRT, and so on (supported by suitable refs.) to make the work understandable and of interest by non-experts in the field.

Reply: Thank you for your suggestion.

The definitions and meanings of radiotherapy, CFRT, SBRT, stereotactic radiosurgery, low-fractionation radiotherapy and SBHFRT mentioned in the background are limited by the length and the number of words in the article. Further explanation of these concepts is of limited significance and can be further understood by the reader from the basics.

12. Fig. 3 - better to use lines, labels, etc. to distinguish the 3 panels (and 2 rows) for A, and similarly for B, C, D.

Reply: Thank you for your suggestion. It has been modified and added red arrows.

13. Ref. #18 and "abstract" - remove highlights.

Reply: Thank you for your suggestion. It has been modified.

14. Remove vertical lines on left (e.g. on pages 5, 11, 12, 18, 21).

Reply: Thank you for your suggestion. It has been modified.

Reviewer 4: The article from the authors Lin Li MD et. al, titled "Relationship Between The Invasion Of Lymphocytes And Cytokines In The Tumor Microenvironment And The Interval After Single Brachytherapy Hypofractionated Radiotherapy And Conventional Fractionation Radiotherapy In Non-small Cell Lung Cancer", compared the immune cell accumulation and tumor necrosis and apoptosis rates between SBHFRT and CFRT in non-small cell lung cancer mouse models.

Comments:

1. The manuscript was poorly written for instance
   I. Proper references are missing for few sentences in the background section check carefully.
   Reply: Thank you for your suggestion. We have checked and added proper references in the background.
   II. Text formatting need to be done carefully, for instance "tregs"/ T reg cells, "High-Sugar DMEM"/High-Glucose DMEM
   Reply: Thank you for your suggestion. It has been modified.

2. Authors did not explain methodology clearly like IHC, Cell culture, Apoptosis assay and analysis.

   Reply: Thank you for your suggestion. We have explained the experimental methods (cell culture, immunohistochemistry, ELISA, apoptosis) in detail.

3. Figure 1A Author didn't mentioned the significance between the control and other groups.

   Reply: Thank you for your suggestion. Figure 1 is research design and route. The significance between the control group and other groups can not be mentioned. We have modified all figures in detail, and added the notes and content description. The magnifications of the images were shown in legend for readers to read. The notes of tables are further improved according to the requirements.

4. Figure 3 the images are low quality unable draw the conclusions. Author encouraged to add with higher quality images and quantification graphs.

   Reply: Thank you for your suggestion. We have modified and added clear pictures respectively (Figure 1-8), and added the notes and content description. The magnifications of the images were shown in legend for readers to read. The notes of tables are further improved.
Reviewer 5: Thank you for giving me the opportunity to review this article "Relationship Between The Invasion Of Lymphocytes And Cytokines In The Tumor Microenvironment And The Interval After Single Brachytherapy Hypofractionated Radiotherapy And Conventional Fractionation Radiotherapy In Non-small Cell Lung Cancer"
Article is well written by giving the right amount of introduction, need for this study to be conducted. Observed conclusions are well discussed.
Here are a few minor changes I would like to see:
1. In the title, the word radiotherapy has two 'r' by mistake. please fix it.
Reply: Thank you for your suggestion. It has been modified.
2. As per my understanding, interferon-gamma is abbreviated as IFN and not INF.
Reply: Thank you for your suggestion. It has been modified.
3. Figures 2B-E - clarity of the pictures are not good. It's very grainy especially 2E. Please upload higher resolution figures before publication.
Reply: Thank you for your suggestion. We have modified and added clear pictures respectively (Figure 1-8), and added the notes and content description. The magnifications of the images were shown in legend for readers to read. The notes of tables are further improved.
4. Figure 2A - Since clarity of the picture is not good, I was not able to read the x-axis. Is it 'time/day'? If so, it doesn't make sense. Please re-label the axis.
Reply: Thank you for your suggestion. We have modified and added clear pictures respectively (Figure 1-8), and added the notes and content description. The magnifications of the images were shown in legend for readers to read.
5. For all the histology images, I don't see scale bar added. Please add.
Reply: Thank you for your suggestion. We are so sorry for not adding scale bars to these images when they were originally taken. In order to maintain the authenticity of the original pictures, we did not add extra. The magnifications of the images were shown in legend for readers to read.
6. For figure 3: Quantitative bars would provide more info and ease for the readers instead of pointing to the table. Please add bar graph with p value and sample number next to the histology pictures for better understanding.
Reply: Thank you for your suggestion. We are so sorry for not adding scale bars to these images when they were originally taken. In order to maintain the authenticity of the original pictures, we did not add extra. The magnifications of the images were shown in legend for readers to read.
7. Discussion tries to summarize the observations by citing relevant papers. At the end, please add pictorial representation of a possible mechanism (especially when trying to explain all the IL10, IL-12, CD86 activity).
Reply: Thank you for your suggestion. The authors themselves drew a possible mechanism in figure 8 to explain the possible mechanism of tumor necrosis in relation to lymphocyte and cytokine interactions.
Some conceptual questions/possible changes:
1. What is the reasoning behind not checking for tumor infiltration in mice by doing an antibody staining?
Reply: Thank you for your suggestion. Our study was a first basic study of this technique in tumor radiotherapy, which has some limitations and needs further improvement. Antibody staining to detect tumor invasion in mice will be carried out in future studies.
2. In my personal opinion, 6 mice per group is very less especially when there are no other in-vitro and other clinical samples to support with. What is the reasoning behind doing a small scale study? How do you conclude observations with certainty?
Reply: Thank you for your suggestion. The number of tumor-bearing mice in each group was determined by combining literature (reference 20) and statistics (10-15 mice). Although the more animals there are, the more representative they may be, there are corresponding problems: (1) cost problem: the more animals there are, the higher the cost will be. (2) practical operation: the more animals there are, the more operations there are, and the larger the research bias may be.

3. Authors have mentioned that CD86 activity increased on day 7 and possible reasoning behind that is radiation associated with antigenic proteins. It would be ideal to see antibody staining (like MHC-II or IgM) or other APCs staining to support that theory.

Reply: Thank you for your suggestion. We should carry out this kind of research next, because our study was only first step.

4. Did the authors look for any infiltration of tumors cells after radiation since that's a possibility?

Reply: Thank you for your suggestion. About the infiltration of tumor cells after radiotherapy, a very meaningful topic, will be carried out in the future.

5. In your opinion, if you take the SBHRT irradiated tumor cells and grow them in-vitro, would you see less proliferation (Ki67) and less invasion of these cells? It would be nice to see some supporting data at least at in-vitro level since sample size is less.

Reply: Thank you for your suggestion. (1) We radiated Lewis cell lines by means of 0 Gy, 2 Gy, 4 Gy, 6 Gy, 8 Gy and 10Gy in vitro respectively. The results showed that the cell colony number decreased significantly after radiation of 6Gy and 8Gy, and no cell colony formation occurred after radiation of 10Gy. (2) In our study, the one-time radiation dose of SBHRT was 11.3Gy, which was larger than the one-time radiation of 10 Gy in vitro. The Lewis cell line radiated by 11.3Gy in vitro would have no cell colony formation, so cell proliferation and invasion could not be observed. (3) The tumor microenvironment is very complex. Our aim is to observe the changes of lymphocytes and cytokines in tumor microenvironment before and after radiotherapy, which can not only form tumor microenvironment in vitro.