Author's response to reviews

Title: Anti-tumor effect of Liqi, a Traditional Chinese Medicine prescription, in tumor bearing mice

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Version: 4 Date: 21 January 2009

Author's response to reviews: see over
Dear Dr. Sabina Alam,

Thank you very much for your email dated Jan 6, 2009 and the referees’ reports for our manuscript entitled “Anti-tumor effect of Liqi, a Traditional Chinese Medicine prescription, in tumor bearing mice” (manuscript ID 1205479634216499). Enclosed please find the revised version of our manuscript which was completely revised, taking into account all of the Reviewers comments.

The point-by-point responses to each of the Reviewers' comments are detailed below.

We thank the Reviewers for their insightful comments, critiques and suggestions, which were extremely useful to improve the scientific value of the manuscript.

We will be looking forward to hearing from you.

Sincerely,

Jia Ye

Replies to Reviewers

We would like to thank the reviewers for the constructive and positive comments.

Replies to Reviewer #1: Jong-Hwei Pang

**Reviewer's report:**

Additional comments and questions remained unanswered were in red color:

2. The inhibition of tumor growth in nude mice with destructed immune functions by Liqi could reach 38.69% that was similar to two other animal models with normal immune functions (34.54% and 42.54%). Does it mean that the anti-tumor effect of Liqi is not involved very much with the immune responses? The result in Fig. 1 might be simply against what the author claimed in this manuscript.

Response: for Sarcoma 180 tumor and Lewis lung carcinoma implanted mice, the inhibition 34.54% and 42.54% represented the inhibition of tumor weight. For SGC-7901 implanted nude mice, the inhibition of tumor weight was 27.42% which is lower than the other animal models. The inhibition 38.69% represented the inhibition of tumor volume.

Figure 1B is suggested to be removed from the manuscript in order not to confuse readers.
Response: According to your comments, we remove Figure 1B from the manuscript and modify the legend for Figure 1. In section Anti-tumor activity on page 6, we delete the description about tumor size measure and the reference 11. In section Effect of liqi on tumor growth in the tumor-transplanted mice on page 9, we delete the description about figure 1B.

3. As we know that Immune-related anti-tumor reaction involves the induction of apoptosis, however, in Table 3, data showed no subG1 (apoptotic cells). Why? By the way, the data in Table 3 was indeed the opposite to what author discussed in the manuscript.
Response: in this experiment, we did not investigate the effect of Liqi on cell apoptosis. In addition, subG1 phrase commonly appears when apoptotic cells exist, but it does not appear inevitably. In Table 3, we typed the results inversely. We have corrected the error. The authors did not answer the question why cell apoptosis was not observed in this experiment. Is it because that SGC-7901 cells were isolated from nude mice? The authors can discuss more about this point.

Response: thank you for comments. We agree with your opinion.

Immune-related anti-tumor reaction often involves the induction of apoptosis. Cytotoxic T lymphocytes (CTLs) and nature killer (NK) cells are highly effective killers of tumorigenic cells. The lytic activity of CTLs and NK cells is localized in specialized granules in their cytoplasm and through the regulated secretion of these granules, killer cells can selectively induce target cell death. Perforin secretion from the lytic granules caused cell death by disrupting the plasma membrane through pore formation. Killer cell lysis was also found to be accompanied by extensive cellular deterioration beyond damage to the plasma membrane, in particular nuclear disintegration. Other components of the lytic granules were subsequently identified as serine proteases, called granzymes. These proteases trigger rapid apoptosis in the target cells.

Because it lacks a thymus, nude mice cannot generate mature T lymphocytes. Therefore they are unable to mount most types of immune responses, including: killing of virus-infected or malignant cells (requires CD8+ cytotoxic T cells) ; antibody formation that requires CD4+ helper T cells; cell-mediated immune responses, which require CD4+
and/or CD8+ T cells; delayed-type hypersensitivity responses (require CD4+ T cells); graft rejection (requires both CD4+ and CD8+ T cells).

So we think that cell apoptosis was not observed in this experiment due to that SGC-7901 cells were isolated from nude mice.

4. It is better and more meaningful to measure the IL-2 and TNF-alpha activities directly from tissues, not after the simulation by Con A or LPS.
Response: It’s good idea. However, in tumor bearing mice, the time point of IL-2 and TNF- alpha releasing peak was difficult to be determined. After stimulated by ConA and LPS, it was stable and easy to be determined.
The data obtained by stimulating with ConA and LPS can not be used to explain what really happened in the animal models.
**Response:** Thank you for the comments. We propose to perform these researches in our future work.

5. It is better that data from normal control should be added to Figure 4, 5 and 6.
Response: According to your comments, normal control was added to figure 4 and 6, figure 6 shows that effect of Liqi on lung metastasis of tumor cells, no tumor colony was found in the lungs of normal control. In our experiment, we measured TNF-alpha activities after the stimulation by LPS. The healthy animal without tumor xenograft which was stimulated by LPS could not be taken as normal control. It is no meaning to compare the model group/ the treated group with the healthy animal without tumor xenograft and LPS stimulation.
The data obtained by stimulating with ConA and LPS can not be used to explain what really happened in the animal models.
**Response:** Thank you for the comments. We propose to perform these researches in our future work.

 replies to Reviewer #2: Kazumi Yagasaki

**Reviewer's report:**

Major points
The revised version seems to be well improved compared with the previous manuscript. High effective dose of liqi, pointed out by the two reviewers as an important problem, is well explained by authors. Experimental procedures are also sufficiently described. However, this manuscript still has small inadequate points and discrepancies. Some corrections noted below should be done before acceptance for publication.

Minor points
1. Page 1, Line 7 (P1L7): ShangHai; ‘H’ may be ‘h’. See, P3L2 from bottom.
2. P2L10: Cell cycle ‘assay’ may be Cell cycle ‘analysis’.
3. P4L7 from bottom: ‘humidity 60%±5%’ ; ‘relative humidity 60±5%’ seems better.

Response for points 1-3: according to your comment, we have revised the mentioned part.

4. P4L1 from bottom: ‘---food ad libitum.’ ‘Food’ may be a pellet for rodents. If authors used a commercial pellet, then the supplier probably knows the composition. As commercial pellet for rodents usually composed of many kinds of sources derived from plants and animals, there is a possibility that components such as polyphenols and other components in the pellet interfere with the effect of liqi. If possible, authors are requested to address this possibility. If impossible, authors are requested to show its composition briefly in the text. For example, “food composition is as follows: protein?%, carbohydrate ?%, fat %, minerals %, vitamins %, fiber % and moisture %.”

Response: in our experiment, food pellets meet Feed Standard of Medical Laboratory Animal of China. Food composition is as follows: protein18-25%, fat 4-5%, calcium 1.0-1.8%, phosphonium0.6-1.2%, vitamins A 12500-15000IU/kg, vitamins D 1250-1500IU/kg, fiber 4-5% and moisture 8-10%, lysine 0.98-1.42%, cystine0.76-1.10%, tryptophane 0.22-0.34%. We mention this in P4L1-5 from bottom in the manuscript.

5. P5L14: Probably PBS did not contain Ca and Mg to prevent cohesion of cells. In that case, ‘Ca- and Mg-free phosphate-buffered saline [PBS(#)]’ may be better expression than ‘phosphate-buffered saline [PBS]’.

Response: according to your comment, we have changed “phosphate-buffered saline [PBS]” to “Ca\(^2+\)- and Mg\(^2+\)-free phosphate-buffered saline [PBS]”.
6. P5L9-8 from bottom: What time did you give animals liqi everyday? Authors are requested to mention it briefly.

Response: we give animal liqi at about 9 o’clock daily. We mention this in P5L3 from bottom in the manuscript.

7. P6L11: Between ‘cytometer’ and ‘and’, Please insert ‘(FACS Calibur; Becton Dickinson, USA)’. Therefore, ‘(FACS Calibur; Becton Dickinson, USA)’ can be deleted from P6L20-21.

8. P6L2 from bottom: ‘cells/L’. Is ‘L’ correct? mL?


10. P7L6: 0.5 mCi is very high. Probably 0.5 µCi?

11. P7L9: Authors used Ci in P7L6 as described above. Thus, 7.4x1011 Bq/mmol seems better to be replaced 20?Ci/mmol to make the unit of radioactivity the same.

12. P8L12 from bottom and P8L1 from bottom: Change properly 57 of C57BL and 1# of F1#, respectively.

Response for points 7-12: according to your comments, we have revised the mentioned part.

13. P9L4: Did authors use Student’s t-test in Table 3 to compare the difference between the two groups? Authors used Dunnet t-test to compare the differences among three groups. Can Dunnet t-test apply to the comparison of the difference between two groups? If inapplicable, a description ‘Student’s t-test or’ seems better to be inserted just before ‘Dunnet t-test’.

Response: yes, we used Student’s t-test in Table 3 to compare the difference between the two groups. According to your comment, we insert ‘Student’s t-test or’ before ‘Dunnet t-test’.

14. P9L12-8 from bottom and Fig. 1: Description is different from the order of Fig. 1A and B. According to the order of the description in P9L12-8 from bottom, it seems better for readers to replace Fig. 1A (tumor weight) with Fig 1B (tumor volume).
Response: according to the comment of Dr. Jong-Hwei Pang, we have removed Fig 1B from the manuscript. The descriptions about Fig 1B in the manuscript are deleted.

15. P9L6-4 from bottom and Table 3: According to the description in results (P9L6-4 from bottom), *P < 0.05 in the legend of Table 3 may be **P < 0.01.
16. P10L12 from bottom: 'tumorbearing' may be 'tumor bearing'.
17. P11L8 and 15: antitumor may be anti-tumor (see, your submission title, P1L1)
Response: according to your comment, we have corrected the above errors.

18. P12L17: Authors mention that ‘TNF-# has a direct cytotoxic effect and induces apoptosis of tumor cells [26]’. However, some cancer cells are insensitive to TNF-#. Authors should indicate directly from their experiment or indirectly by citing literatures that sarcoma 180 cells are sensitive to TNF-#.
Response: according to your comment, we cite literatures that sarcoma 180 cells are sensitive to TNF-α. In P12L7-12 from bottom, we replace the sentence “TNF-α has a direct cytotoxic effect and induces apoptosis of tumor cells [26]” with the following sentences “TNF-α had an anti-tumor activity against sarcoma 180. In addition to direct cytotoxicity against tumor cells, TNF induced a host-mediated factor which contributed to the anti-tumor effects [25]. Rychly J et al. [26] also demonstrated that TNF-α induced strong necrosis in sarcoma 180 in vivo and showed total regression. The infiltration of inflammatory cells was observed in the sarcoma 180 tumor. Their result suggested that cell infiltration may be of importance for tumor regression.”

19. P15-18: reference section: There is no consistence in writing authors's name. For instance, please compare ref. 9 with 10. Authors should revise the reference section according to “Instruction for authors”.
Response: according to your comment, we revise the reference section following “Instruction for authors”.

Level of interest: An article of importance in its field
Quality of written English: Needs some language corrections before being published
**Response:** We have made language corrections according your comments.

**Statistical review:** Yes, and I have assessed the statistics in my report.

**Declaration of competing interests:**
I declare that I have no competing interests.

**Reviewer:** József Molnár

**Reviewer's report:**
The MS 1205479634216499 on "Antitumor effect of Li.." was read and found to be acceptable for publication.

*We thank all Reviewers for their careful and competent analysis and hope to have properly addressed their queries.*