Author's response to reviews

Title: Effect of low frequency magnetic fields on melanoma: tumor inhibition and immune activation

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Author's response to reviews: see over
Dear Professor,

Thank you very much for your letter in which you send us the reviewers’ comments on our paper (MS: 1791616852830263). In the light of the reviewers’ comments, we have revised our manuscript carefully. The questions raised by the reviewers are responded as follows:

Response to Reviewer Junqing Han,

Reviewer's report:
Comments to Authors
1. The authors report a novel finding of rotary magnetic fields may inhibit the growth of melanoma cancer cells and study the possible mechanism, which may be helpful for the therapy of melanoma. The article is really interesting and well described.
2. In Figure 4 A, P value between sham MF Tumor mice and MF Tumor mice is 0.33, how did you get the result of survival rate of melanoma model mice was elevated after MF exposure?
Response: Thanks for your comments! It’s our fault to make this mistake. The $P$ value between sham MF tumor mice and MF Tumor mice should be 0.03, which is statistically significant. All of our results proved that MF could inhibit the growth of melanoma cancer and promote the survival time. Thank you very much for pointing out this writing mistake in our paper.

Response to Reviewer Boris Pasche,

Reviewer's report:
Overview
The authors challenge mice with the B16-F10 melanoma cell line through tail vein injection to induce lung metastasis. After challenge, the authors treat the mice with “ROTARY Magnetic Fields” to induce inhibition of cancer cells and stimulate the immune system. It appears that the immune system is activated in a positive manner (anti-cancer) but the direct inhibition of melanoma cells in not clear. Also, the
percentage of mouse survival of sham tumor mice vs. treated tumor mice was not statically significant although, they do see inhibition of melanoma cells.

Positives:

- This group is examining the immune system with respect to a magnetic field’s influence. They are definitely do that with their cytokine/chemokine data.
- Exposure for their system is reported in both Tesla and frequency.
- Their FACS / T-reg data is also very interesting (Upon MF exposure, Tregs decreased in mice that were challenged with melanoma cells)
- Their organ size/weight data is very interesting (Enlarged spleen, after challenge with melanoma cells, will shrink in size/weight upon MF exposure)

**Response:** Thanks for your positive comments!

Negatives:

- The article is riddled with grammatical and syntax errors, at certain points throughout the article it hinders the readers ability to understand.

**Response:** Thank you for your comments. We have revised our paper very carefully.

Dwayne G. Stupack, a professor in the Moores UCSD Cancer Center, offered us kind help to polish our manuscript, especially the Introduction and Discussion Section. We hope this version is much improved.

- I would like to see another melanoma cell line and a separate cancer line undergo the same exposure. The authors only have one cell line used for both the in vivo and in vitro experiments. The use of other cancer models may possibly indicate specificity to a cancer type, immune system, or none of the above.

**Response:** Thanks for this good suggestion! We agreed with the reviewer that using one more cell line may confirm whether the inhibitory effect of MF was specific in melanoma cancer. However, we only have one cell line in our lab. We don’t have enough time to buy cell line and treat it with MF since the limitation of revise time. Moreover, in our previous study (see below), we explore the anti-tumor of MF on different cancer cells. We found that MF could inhibit the proliferation of BGC-823,
MKN28, A549 and LOVO cells, while no significant effect was detected on MKN-45 and SPC-A1 cells. Therefore, we think the inhibitory effect of MF is not melanoma-specific.


-I would also like to see more histology slides (even if placed in the supplements) of other tissue types to see if proliferation was altered. Also, some H&E slides would be helpful, especially of the spleen.

Response: According to your suggestion, we add the HE slides of lung and spleen in Figure 4 and Figure S2 in the revised manuscript.

While I understand that the authors are describing the current literature related to Magnetic Fields and its influence on cancer, it could be better written and organized. Their writing, in the introduction, is constantly jumping back and forth from positive to negative effects of magnetic fields. The authors give examples of effects from ELF-EMF to mobile and cordless phones that are clearly not in the same range and do not make it a point to discuss this difference or at least make a mention of it. In addition, the authors make little to no mention or description of ‘Rotary Magnetic Fields’; this is KEY if you want to explain where your system fits i.e. is it similar to RF EMF or ELF-EMF. Why mention ELF-EMF and RF-EMF at all if your system is not first explained in order to be able to make comparisons. Hence, I believe it would help this article to expand its introduction with a little more information on ROTARY MF and Melanoma, which they also give little to no information.

Response: Sorry to make you confused in the introduction section. We rewrite the introduction section focusing on the antitumor effect of MF and practicability of MF. In our MF system, the key point is low frequency, which is non-invasive and non-ionizing and even has non-thermal effects. Therefore, we make it clear in the
revised title and revised introduction section. We also added the current situation of melanoma. Thanks for pointing out our drawbacks.

Response to Reviewer Robert M Lafrenie,

Reviewer's report:
The manuscript, "Effect of rotary magnetic fields on melanoma: tumor inhibition and immune activation", describes in vitro and in vivo experiments that show exposure to a particular rotating magnetic field pattern (0.4T, 7.5 Hz) slows cancer cell growth. The in vitro data shows that treatment of B16-F10 cells with the rotating field causes a small increase in annexin V staining cells, a small shift in cell cycle, and a small decrease in cell proliferation (CFSE dilution and CCK-8 staining). In vivo, mice were transplanted with B16-F10 cells and exposed to the rotating field. EMF-exposed mice were shown to survive longer (although p=0.33), show lower levels of Ki-67 proliferation marker, enhanced anti-tumor IP-10 cytokine and decreased chemokines and cytokines, decreased number of splenic Tregs and increased dendritic cells. In my opinion, additional experiments need to be performed in order to support the conclusions made regarding the relatively small differences in data.

1. The apparatus for generating the 0.4 T, 7.5 Hz needs to be more fully described. The exposure volume for the cells and animals needs to be described. The characteristics of the magnetic field are critical for understanding any biological effects and therefore a clear and reproducible description of the apparatus and field exposures are required.

Response: Thanks for your comments. We agreed with reviewer that the apparatus and MF is critical in our study. It should accurate and reproducible. Therefore, we add a picture and an instruction of our apparatus and make it clear in method section.

2. The effects described are quite small with the changes in proliferation being 15-30% after 5 days (CCK-8 counting and CPSE dilution). These should be supported by other methods such as simple cell counting.
Response: Thanks for your suggestion. We add result of simple cell count in Figure 2. In consist with CCK-8 and CFSE, the inhibitory effect of MF is slight but significant.

3. The data in figure 1 is not very compelling. The changes in flow cytometry show changes from 15% to 21% for annexin staining. In my experience, inter-experiment variation is frequently at least this variable, and the error bars (SEM) shown are much smaller than I usually get for this experiment. Similarly, the cell cycle variability seems to show unexpected lack of variability (SEM). I would also like to know how the samples were gated for the flow cytometry since no sub-G1 cells are seen which seems inconsistent with the relatively large number (15-20%) of annexin stained apoptotic cells. In order to be convincing, additional experiments should be performed to verify this data. For example, caspase-3 cleavage, DNA fragmentation gels, or nuclear morphology, should support the annexin V staining, and cyclin measures, or BrdU labelling should support the proliferation data.

Response: Thanks for your comments! Our data were calculated based on three independent experiments. The apoptotic cells were increased slightly from 15% to 21% after exposure to MF. We also detected the apoptotic associate gene bcl-2 and survivin, and found no change after exposure to MF. Therefore, we thought the inhibitory effect of MF may not mainly through induction of apoptosis, but through other indirect way, such as activation of immune cells. In the revised manuscript, we put the results of apoptosis in the supplemental section.

4. Figure 3 (electron microscopy) needs to be supported by determining the number of affected nuclei in each condition to determine if this effect is significant.

Response: We add the proportion of affected nuclei in different groups in the revised Figure 3. The alteration of ultrastructure is significant. Thanks for your good suggestion.

5. For the in vivo experiments, the statistics underlying the survival curves needs
to be better explained. IF this is a Kaplan-Meier statistic, then p=0.33 is not significant and therefore the magnetic field has no effect.

Response: It’s our fault to make this mistake. The $P$ value between sham MF tumor mice and MF Tumor mice should be 0.03, which is statistically significant. All of our results proved that MF could inhibit the growth of melanoma cancer and promote the survival time. Thank you very much for pointing out this writing mistake in our paper.

6. The relative number of Ki-67 staining cells for each condition also needs to be counted for each condition. What is being sectioned for Ki-67 staining in the mice without tumours?

Response: Thanks for your suggestion. We add the proportion of KI-67 positive cells in different groups in revise Figure 4. The Ki-67 staining was done in lung tissues (explained in question 7).

7. The levels of plasma cytokines and splenic T cells seem reasonable, but it would be nice to see the levels of immune mediators and cells in the tumor itself. Are these cells sequestered in the tumor?

Response: In our study, the mice model was induced by injection of $2 \times 10^5$ B16-F10 melanoma cells into the tail vein in C57BL/6 mice. It has been known that the lung metastases were the primary characteristics after intravenous challenge of C57BL/6 mice with melanoma cells. We confirmed the lung metastatic tumors by HE and immunohistochemistry of lung tissues. However, we cannot seperate the tumor from lung tissues. That’s why we did not detect the cytokine and immune cells in tumors.

I hope this version of the manuscript can be accepted for publication in your journal. Great thanks to you for your time and effort you expend on this paper.

Yours Sincerely

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