Author’s response to reviews

Title: Engineered Measles Virus Edmonston Strain Used as a Novel Oncolytic Viral System Against Human Hepatoblastoma

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Author’s response to reviews: see over
Dear editor:

I am very appreciated for your giving me this chance to revise my manuscript numbered MS3847916167128706, I have carefully read the reviewer’s advices and made the manuscript revised accordingly. Also, the revised manuscript has been kindly edited by Elsevier Webshop as you suggested, the certificate of English editing issued by Elsevier Webshop has been submitted online as a supplementary file. Now, I submit it to you, if there is still any problems, please inform me and I would like to revise it again.

The point to point responds to the reviewers are listed as follows:

**Reviewer 1: Jian-Dong Huang**

Reviewer's report:

Hepatoblastoma is a common liver tumor in children. The authors tested attenuated measles virus against HB cell lines both in vitro and in vivo, and found virus strain MV-CEA could induce apoptosis, delay tumor growth, and prolong survival rate. The manuscript could be improved by addressing the following questions.

1. The manuscript should be revised for its English and structure. The figure number is inconsistent with the number labeled in main text! It showed total 13 figures in figure section, but only 5 in text. In the discussion part, the authors focused too much on other people's work, but little discussion with their own work.

   **Answer:** The figures have been rearranged and are consistent with the number labeled in main text. The discussion was also rewritten, thank you for your suggestions.

2. There are many inconsistent data in this manuscript. In Result part (page 8), the authors stated 'the positive rate of CD46 receptor was 90.82% in Hep2G and 80.03% in HUM6'. However, in Figure legend (page 22) they said 'high levels of CD46 receptor was observed in human HB cell lines with the rate of 86.82% in Hep2G and 92.03% in HUM6 cells'! In page 9, the authors observed MV-CEA induced CPE for 120 hours, but they only showed 96h data in Figure 5,6,7,8,9.

   **Answer:** Sorry, it is a mistake in writing and has been corrected in the revised manuscript. (Page23,Line2)

3. Statistical analysis is missing in Figure 6, 7, 8, 9.

   **Answer:** The statistical analysis has been added in the new version.
4. Authors test the MOIs of 1, 0.1 and 0.01. How about the larger MOIs (10 or more)? Will it show too much damage in normal cells and increase safety issue?
   
   Answer: We did not test the MOIs of 10 or more, because the concentration of MOIs = 1 has been very high and can be rarely encountered in the normal condition. MVs with this MOI level has demonstrated so dramatic CPE on tumor cells but not the normal cells that the concentration is sufficient for demonstrating the oncolytic mechanism.

5. The authors proved MV-CEA cannot infect the normal liver cells L-02. How about other kinds of normal cells? Such as kidney cells? The authors should test the presence of the virus systematically in all major organs. It will be even better if the authors can measure the virus load in different tissues including tumor over a time course.
   
   Answer: The MV3 enters the cells through the interaction of the H-glycoprotein with the MV receptors. Presently the identified MV receptors include CD46, CD150(SLAM) and Nectin4. The wild-type MV enters more efficiently through the SLAM receptor, whereas the MV-Edm enters the cells predominantly through the CD46 receptor. Both the wild-type MV and MV-Edm can use the Nectin4 receptor, but the efficacy of Nectin4 is so lower than that of CD46 that it can be ignored when a high level of CD46 were noted. Only cells that express high levels of the CD46 or nectin4 receptor are infected by MV-Edm and lytically killed. In normal conditions, the normal cells do not express the CD46 or nectin4 receptor, this fact has been well known and has been tested in a proportion of documented literatures such as reference 9-15, 16-22. It is not necessary to detect all of the other normal cells one by one, so we only test the direct-related cells L02. In the other hand, HB is very appropriate for localized treatments because most of the tumor is located within the liver, therefore, verifying of the normal liver cells L-02 is enough for the further explorations.

6. In Figure 11, the serum CEA level on 20d has dropped dramatically compared with 15d, but the tumor size between day 15 and 20 seems not change too much. But authors didn't discuss it at all.
   
   Answer: Sorry, the time point has been mistaken; it has been corrected in the revised manuscript. (Page11,Line1)

7. In discussion part (page 14), the authors discussed MV-Edm therapy is the immune response, which can induce cytotoxic T-lymphocyte response. But they used nude mice and SCID mice to apply this experiment. How did virus work in immunocompromised mice?
   
   Answer: The statement of immune response has been removed from the manuscript. This part was still in studying by another group in my lab (the data were not shown). We would discuss it in a new paper.

8. Images of the tumor in control and treatment groups should be shown.
Answer: We did not take the photos of the experimental animals, because the appearance was not specific and could not be distinguished from animal models erected by other tumors such as lymphoma, ovarian cancer, breast cancer, and renal carcinoma. If the reviewer still strongly recommended the images, we had to re-do the animal models and add them in the next revisions although I think it was not necessary.

Reviewer 2: Pavlos Msaouel
Reviewer's report:
This is an appropriately conducted study showing the in vitro and in vivo efficacy of MV-CEA again hepatoblastoma cell lines and a xenograft model. Below are some issues that I believe the authors should address.

Major revisions:
1) Given the fact that the average age of hepatoblastoma diagnosis is one year and most cases will occur before two years of age this means that a significant number of patients may not be immune to measles prior to MV-CEA treatment (children in the west are vaccinated at 12-15 months of age, although in other areas with higher measles prevalence the target age may be ~9 months). This issue deserves to be addressed in the discussion section.

Answer: In our country, the vaccinated age of MV is 8-9 months after birth, and the MV vaccine has been proven safe in these populations. In the other hand, most of the HB patients who were younger than 8-9months are low-risk or standard-risk, most of them can receive satisfactory results by surgical resection or general chemotherapy, this has been addressed in the discussion. (Page15, Line2-7) High-risk patients were very rare in this group. From the comments, I guess that the reviewer must be a clinical oncologist, who I am much respected. The question he raised is a typical clinical concerning. But the aim of the present study is just to evaluate the efficacy of recombinant MV-Edm against human HB. It is only an experimental investigation, but not a clinical trial. The question will be explored and discussed in the next research when a clinical trial was initiated.

2) In the “materials and methods” section (page 7) there is a “Western blot analysis and ELISA” subsection which does not look like it corresponds to any of the results mentioned in the manuscript.

Answer: Sorry, it was a mistake in writing, the results have been missed. In the revised manuscript, they have been added. (Page10, Line10-12)

3) In the “statistical methods” subsection, the authors should also mention the statistical test (and software) used to obtain the p values in their flow cytometry apoptosis comparisons.
Answer: Thank you for your kindly recommendation. In the revised manuscript, they have been added. (Page8,Line14-17)

4) In the “results” section (page 9) in the “MV-CEA successfully replicated in human HB cells and induces cell lysis” the authors should also provide data (with figures) on the actual intracellular and supernatant viral titers (not only CEA concentration). The title of this subsection should also be modified as this particular experiment may show successful replication of infectious MV-CEA but not cell lysis induction.

Answer: When MV-CEA was replicated in tumor cells, the CEA concentration is completely paralleled with the viral titers, the results have been proven by a lot of work groups such as Russell SJ and Peng KW group. Now an understanding has been reached that the CEA concentration can be used as an alternative against viral titers for detection of viral replication (Please see the reference 14). Thus additional data of viral titers are not necessary. The aim of this subsection is just to demonstrate the cell lysis induced by MV-CEA instead of the replication of infectious MV-CEA. The extracellular MV-CEA has been washed out by PBS for at least 3 times, and they can not replicate in the absence of host. Therefore, the supernatant MV-CEA must come from cell lysis. It just serves as an indirect reflection of MV-CEA replication in tumor cells.

5) It appears that relatively high CEA concentrations (~100ng/ml) were also detected in mice injected with inactivated MV-CEA. Where did that CEA come from? Given the fact that Hep2G is known to intrinsically produce CEA, these cells themselves may be the source of the CEA although I would expect the serum CEA levels to actually increase (at least somewhat) along with the tumor burden over time. On that note, the authors should also mention the percentage of hepatoblastoma patients that are found to have increased CEA levels and how this may affect the potential clinical application of MV-CEA in such patients.

Answer: The CEA concentrations detected in mice with inactivated MV-CEA might be from the injection of MV-CEA themselves. Although Hep2G is known to intrinsically produce CEA which would impact the serum CEA, the impact on the final result would be minor because all of the comparisons were carried out within the Hep2G groups instead of between the Hep2G and other groups. In addition, in the clinical practice, fewer than 10% of patients with HB can produce CEA and the amount is very little; it is likely unnecessary to worry about the confusion of intrinsic CEA. This has been discussed and added in the main text. (Page15,Line18-22)
6) The authors should also discuss the potential role of Nectin 4 (PVRL4), which was recently identified as the epithelial cell receptor for MV, in engineered MV-Edm tumor selectivity.

Answer: The potential role of Nectin 4 has been discussed in the revised manuscript, the discussion has been rewritten and the knowledge of MV infection has been added (Page13, Line10-23)

Minor revisions:

1) In the “introduction” section the authors cite reference#33 when discussing that CD46 is highly expressed in tumor cells. However, that study did not involve tumors. A paper by Jurianz et al. (Complement resistance of tumor cells: basal and induced mechanisms. Molecular immunology 1999, 36, 929-39) may be more relevant in addition to the already appropriately cited reference#32.

Answer: The improper reference has been revised. (Page20, Line16-18)

2) A few typos in the “introduction” section: page 4, beginning of 2nd paragraph:“advantages” should be “advantage”; On the same paragraph, in the sentence “Detection of CEA in the serum is a widely available via clinical assay” the word “via” should be removed.

Answer: The improper writing has been revised as you recommended.

3) In the “Materials and methods” section (page 6) in the “Assessment of MV replication in human HB cells” subsection it is stated that 2.0x10^4 cells well plated per 6-well. This is quite a low density and I suspect that the authors meant to write “2.0x10^5”.

Answer: It is a mistake and has been revised, thank you. (Page6, Line10)

4) In the “Materials and methods” section (page 6) in the “In vivo experiments”subsection female SCID mice are mentioned but, as far as I could gather, only nude mice were used in this paper. Also, please mention in the main text the number of mice used per group (it is only mentioned in the figure legends).

Answer: It is a mistake in writing and has been revised, thank you. (Page6, Line22)

5) The authors mention on a figure legend that MV-CEA was given every other day for a total of 5 doses. On the last paragraph of page 10, they mention that “The CEA concentration reached its maximum on day 15 after the last viral dose. This coincides with peak viral replication in vitro.” I am not sure however that the in vitro and in vivo data do indeed coincide since it appears that in the in vitro experiments the CEA levels peak between 3-4 days after the (single) MV-CEA administration.
whereas in the xenograft model the serum CEA levels peak 15 days after the last (out of 5 total) MV-CEA administration.

Answer: The statement of “This coincides with peak viral replication in vitro.” was impropriated. It could not be concluded from the results, so the sentence has been removed. Thank you for your kindly warning. (Page10, Line23)

6) In the discussion section (page 14) the authors state that “…using a variety of techniques we have demonstrated extensive apoptosis after transfection of MV-Edm in human HB cell lines. These results are in agreement with prior work that has indicated that apoptosis is the main mechanism of death for MV-induced syncytia”. However, I believe that the authors used only one technique (flow cytometry) to demonstrate apoptosis. The other techniques reported in this manuscript demonstrate cell death without distinguishing apoptosis from necrosis. In addition, I would use the word “infection” of the cell lines rather than “transfection” since a replicating oncolytic virus was used.

Answer: In fact, the western blotting was also used, but the results have been missed, they have been added in the revised manuscript. (Page10, Line10-12)

Reviewer3: Kenzaburo Tani

Reviewer's report:

Zhang S-C et al. reported the in vitro and in vivo anti-hepatoblastoma effects of their MV-CEA virus using hepatoblastoma(HB) cell lines of Hep2G and HUM6 cells and MV-CEA. They concluded that the engineered measles virus Edmonston strain MV-CEA has potent therapeutic efficacy against HB lines and xenografts. Trackable measles virus derivatives merit further exploration in HB treatment, too. Although it seems that this is the first report to suggest the possible clinical benefits of MV-CEA for HB, the followings are my comments.

Major comments:

1) The oncolytic superiority of their MV-CEA to other reported MV-CEA strain should be compared and discussed because there have been published many articles reporting the possible clinical effectiveness of MV-CEA for various tumors including breast cancers, ovarian cancers, glioblastoma and prostate cancers. Authors should refer these previous results for comparison.

Answer: The oncolytic superiority of our MV-CEA and its application in HB has been discussed and added in the last paragraph in the discussion, thank you for your advices. (Page15, Line2-7, Line14-17)
2) In vitro experiments using neutralizing antibody should be added and discussed more about this important issue for clinical translation. Because many researchers have been struggling with this important issue for effective clinical translation.

Answer: The blocking antibody as well as the agonist is most used to test the specificity and effectiveness of the target receptor. Till now it has been well known that ONLY two receptors of MV-Edm, CD46 and nectin4, are identified. The specificity and effectiveness of CD46 has been well described in the published literatures, the blocking antibody and the agonist have been both used. There has been a consensus on the fact if a high level of CD46 is expressed in tumor cells, the tumor cells must be infected by MV-Edm. For the nectin4 receptor, it is an epithelial cell receptor for MV found in respiratory tract. Although it can also been used by MV-Edm, its efficacy is much lower than that of CD46, it is used only when CD46 is negative in target cells. The efficacy level of these two receptors is not in one grade, this issue has been well described in published literatures; please see the reference 44-46.

3) We would like to know whether cell lines of Hep2G and HUM6 are appropriate model for high risk HB. Are they drug resistant?

Answer: These two lines have features that are the most characteristic of human HB and have been widely used in HB-related investigations. Although the HB subtype and the risk level (either standard-risk or high-risk) induced by these two lines are still uncertain, it is not critical for the oncolytic virus to be used in the HB biotherapy. Only cells that express high levels of the CD46 receptor are infected by MV-Edm and lytically killed. This has been discussed and added in the main discussion. (Page12, Line2-7)

Minor comments:
1) The numbering of figures is wrong.
Answer: The figures have been rearranged and are consistent with the number labeled in main text.

2) In page 5, line 14, 2x105 should be 2x10^5
Answer: It has been corrected in the revised manuscript.

3) In page 6, line 23, the origin of mice should be clarified.
Answer: It has been added in the revised manuscript.

4) In page 9, line 7, what does more mean?
Answer: The impropriate statement has been removed.

5) In page 9, line 16 and page 10,line1, Human HB Cells should be Human HB Cell Line.
Answer: It has been corrected in the revised manuscript.

6) In page 10, line 8, what is control group?
Answer: It was L02 and has been clarified in the revised manuscript.

7) In page 10, line 16, please clarify the total dose of virus.
Answer: It has been clarified in the revised manuscript.

8) In page 11, lines 1-2, please clarify the usage of UV-inactivated and untreated.
Answer: It has been clarified in the revised manuscript.