Peer Review File

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Reviewer A

Major comments:

1) In introduction section the interest of orthotopic model compared to ectopic model could be discuss.

Reply 1: Thanks for your comment. We have checked the literature and the difference between orthotopic model and ectopic model were discussed in the introduction section. Thank you.

Change in the test: We modified our test as advised, please see Page 3 line 71-80.

2) L220 – 224: Could you explain the sentence « Moreover, in a second 49-day-old model »? Did you perform additional experiment with other mice? Could you also add more information regarding the frequency of mice with metastases? Then, could you explain the sentence « Pathological testing indicated that the heterogeneity of both metastatic and local tumors was consistent. »

Reply 2: Thanks for your comment. We didn't perform additional experiment with other mice. This mouse was from the same batch of experiments and we just want to emphasize it. I am sorry for the confusion and have revised it. The frequency of mice with metastases was summarized in Table 1. The meaning of the sentence « Pathological testing indicated that the heterogeneity of both metastatic and local tumors was consistent. » was to explain the tumor was metastases rather than primary tumor.

Change in the test: We modified our test as advised, please see Page 7 line 230.

3) L258 – 259: Could you develop the idea mention in the sentence « These processes resembled the biological patterns of primary tumor development, progression, and spontaneous metastasis. » and also add more ref?

Reply 3: Thanks for your comment. And we have developed it and added more references. The greatest feature of malignant tumors is the ability to metastasize and spread during their growth and proliferation. We observed the same characteristics in our study by implant the tumor fragments into the orthotopic model. This mimics the in vivo oncology characteristics and has important implications.

Change in the test: We have added more reference, please see Page 8 line 269.

4) L 303–311 : What is the interest to use your surgical procedure in Balb/c with cell line model wherease iv injection (as described in Jarry et al 2022 / ref 16) lead to lung tumor development for 100 % of mice (vs 60.86). You procedure should be use more for PDOX model? Could you discuss that?

Reply 4: Thanks for your comment. This is our first formal experiment, and although the tumor formation rate is not very high, it is still significant. The use of tissue fragments to

build orthotopic models is our pursuit and the tumor formation rate may be improved with the improvement of technology. The ultimate purpose of our study was to establish a PDOX with our modified method and this is a preliminary attempt. The materials and method we provided were essential for practicing model building and may provide reference for novices.
Change in the test: None.

Minor comments:
1) L191–194: The author mention that 2 mice were died during the experimentale procedure and 2 within 24h. Then the author mentions that « pulmonary hemorrhage and pulmonary atelectasis accounted for the majority of the 72-hour surgical mortality ». Could you add more precision in the text? How many mice died during the surgical procedure have had pulmonary hemorrhage and pulmonary atelectasis? Same question for the mice died within the 24h. For these mice how did you observed the pulmonary hemorrhage and pulmonary atelectasis?
Reply 1: Thanks for your comment. We have added more precision information of events during procedure. Autopsy revealed that all dead mice developed hemothorax of varying degrees after implantation. This leads to the development of atelectasis and respiratory failure. We speculate that the cause of death in the four mice was pulmonary hemorrhage. We will closely observe the condition of the mice after the operation. If mice were dead, autopsy was performed immediately. Hemothorax was found in the two mice died during the surgical procedure and the latter two mice were found to have numerous blood clots in the thorax.
Change in the test: We modified our test as advised, please see Page 4 line 112-113.

2) L195 – 200: The author mentions that « The remaining mice were monitored for 2 months daily. They were humanely euthanized by CO2 inhalation when they were moribund due to respiratory failure brought on by a heavy tumor load, prostration, and severe lung infections. » In M&M section it's mentionned that 4 mice were euthanatized every week for 7 weeks. Then the mice were not euthanatized consequently to moribond statut? Could you add precision?
Moreover, table 1 shown tumor incidence and number of mice with metastasis. Were euthanized animals counted in this table? If yes, do you consider that the first euthanatized mice without observed tumor could developp tumor later?
Reply 2: We sincerely appreciate the valuable comment. This question is hard to avoid in our observational study. This is our experimental design. In our study, 4 mice were actively euthanatized and sacrificed every week for 7 weeks to observe the formation of tumors were not consequently to moribund status. The initial plan was to observe for ten weeks, but according to ethical requirements, mice with these conditions must be euthanized even though they don't developed tumor. This resulted in the experiment eventually lasting two months. Tumor incidence and number of mice with metastasis in thr table 1 included the euthanatized mice. In my opinion, with longer observation time, the first euthanatized mice may have tumor formation. The interpretation of our results is subject to some bias.
Change in the test: We have added the footnote in table 1.

3) L205 – 2013: Could you add precision regarding the days where the prelevment have be done? These informations should be indicated on Fig 2.

Reply 3: Thanks for your comment. We have modified our text as advised. The precision time of the gross specimen harvest was added in the legend of Fig 2.
Change in the test: legend of Fig 2. See page 16 line 465-466 and line 468.

4) L216 – 217: No tumor has been observed before day 10. What assay(s) have been done to demonstrate this obsvervation?
Reply 4: Thanks for your comment. The earliest detection of tumor formation was on day 10, included in our pilot experiments. This may be subject to some chance and bias, no assay(s) to demonstrate this observation.
Change in the test: None.

5) L104 – 106: Can you add precision regarding PET/CT assay? How many experiments have be performed? with the same mice?
Reply 5: Thanks for your comment. To better monitor the progression and metastasis of cancer, small- animal PET/CT scans were performed on 6 mice. And the detailed procedures regarding PET-CT examination was exhibited in method section (page5-6/line 166-179). Intense FDG uptake was demonstrated in the tumor. In consideration of cost, all mice were subjected to only one experiment. A total of three mice had tumor formation, and the most representative one is shown in our manuscript.
Change in the test: None.

6) L219 – 220: Could you add precision regarding the frequency of mice with metastases?
Reply 6: Thanks for your comment. The frequency of mice with metastases was summarized in Table 1. 16 mice of the whole observation group were discovered metastases.
Change in the test: None.

7) L227 – 233: Could you add precision regarding the PET/CT assay? See previous point.
Reply 7: Thanks for your comment. To better monitor the progression and metastasis of cancer, small- animal PET/CT scans were performed on 6 mice. And the detailed procedures regarding PET-CT examination was exhibited in method section (page5-6/line 166-179). Intense FDG uptake was demonstrated in the tumor.
Change in the test: None.

8) L238 – 240: Same recomandation, could you add more precision on the sample?
Reply 8: Thanks for your comment. Research funds are limited, and we tentatively plan to procure these mice for experiments.AND the sample size estimates were based on our previous studies, maybe subjected to some limitation.
Change in the test: please see Page 11 line 340.

9) Fig 5: The indication regarding Black Arrow should be reenforced.
Reply 9: Thanks for your suggestion. In order to keep the chart format consistent, we ultimately decided not to modify the model of the arrow. Thank you very much.
Change in the test: None.

10) L321 – 323: You mention that you are going to reproduce these experiments. Can you indicate the usefullness of such experiment and why do not way the generated result before do this manuscript?

Reply 10: Thanks for your comment. Graduation is imminent and time is limited, there was not enough time to verify the effect of the method. The controlled trial was aborted due to the COVID-19.

Change in the test: None.

11) L323 – 324: You mention that you « we anticipate constructing orthotopic lung cancer models using tumor pieces derived from clinical patients. » If you use immunodeficient mice, how do you consider the tumor microenvironment?

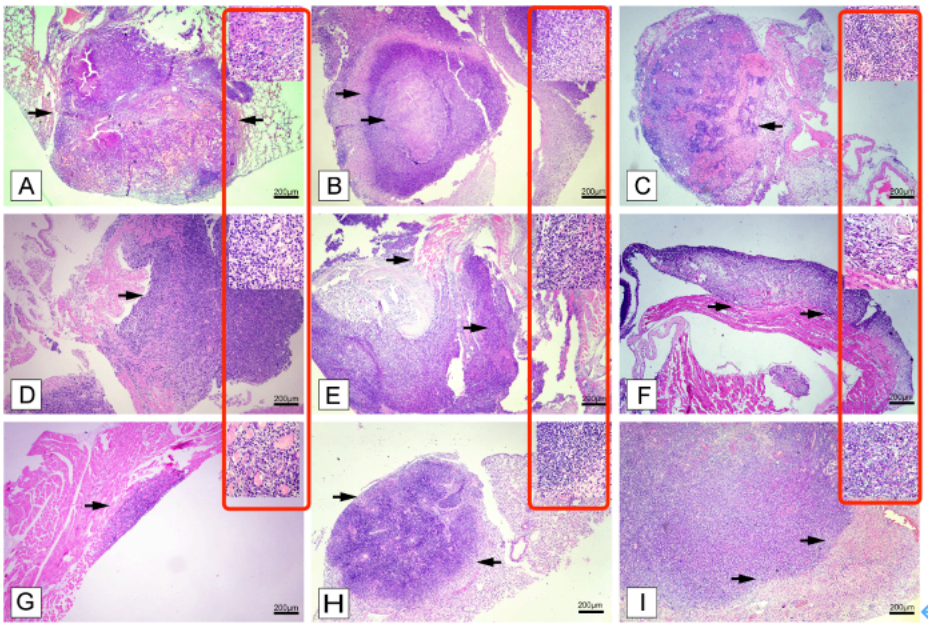
Reply 11: Thanks for your comment. Tumor fragments from patients contain many components in addition to malignant cells, but also stromal, endothelial, and immune cells and an extracellular matrix that work together to sustain the tumor. This is conducive to the formation of transplanted tumors. However, whether the micro-environment of the transplanted tumor is consistent with the primary tumor over time needs to be further studied.

Change in the test: None.

Reviewer B

1. Figuren5

Please also provide the scale bar in the figure or magnification in the legend for these enlarged images.



Reply: Thank you. We have revised this figure as your suggestion and resend it to you.

2. Table 1-2

Please explain BALB in the table footnote.

Reply: Thank you. BALB is the name of mouse lines, no use of abbreviations and don't have to explain that.

3. References/Citations

a) Please add the citation for Hoffman et al. at the end of the sentence.

> 83 Hoffman et al. developed a patient-derived orthotopic xenograft (PDOX) nude mice
> 84 model using orthotopic surgical implantation. A study showed that the amount of

b) Please double-check if more studies should be cited as you mentioned "studies". OR use "study" rather than "studies".

> 290 techniques (21). Recent studies have demonstrated that organ-site-specific implantation
> 291 of tumor cells is crucial for optimum tumor growth and development in vivo (22). An

Reply: Thank you. We have revised the text and added the citation.