Author's response to reviews

Title: Immunohistochemical analysis of cancer stem cell markers in pancreatic adenocarcinoma patients after neoadjuvant chemoradiotherapy

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Author's response to reviews: see over
Dear Editor, Solera

We are pleased to submit the revised version of our manuscript: “Immunohistochemical analysis of cancer stem cells in pancreatic adenocarcinoma patients after neoadjuvant chemoradiotherapy”

We are very grateful to you that I had a peer review of this manuscript.

We have revised the manuscript according to the comments from editors and reviewers.

We now hope that our paper will be suitable for publication in BMC Cancer and look forward to hearing from you concerning your editorial decision.

Sincerely yours,

Tatsuzo Mizukami

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Response to the Reviewer Dr. Deepa Patil

Major revisions:

1. Most of the stem cell markers are expressed by acinar cells. Is there any information about how this relates to ductal adenocarcinoma, which arises from ductal epithelial cells? Perhaps the authors could explain this in the discussion.

Reply 1: We have information about how acinar cells can convert to pancreatic cancer cells from the reported research paper (Reference: Dev Cell 26: 3-7, 2013.). However, we do not have any information about their relationship between acinar cells and CSCs markers. We used acinar cells and so on as a positive control in immunohistochemical staining, referring to the research paper of the past. So, we added these informations in the methods sections in Table 1.

2. How was the response to neoadjuvant therapy graded? What was the percentage of residual cancer in each of these cases/groups?

Reply 2: We used the grading system of Evans et al (Reference: Arch Surg 127: 1335-1339, 1992.) to determine the pathological effects of neoadjuvant therapy.

The detail of the grading system we used is explained in Table 2.

3. Please explain Evan’s criteria in the methods section in Table 2.

Reply 3: According to your suggestion, we added to the explanation of Evan’s criteria in the methods section in Table 2.

4. Discussion: EpCAM, CD24, CD44, and CXCR4 results are different from published literature. The discussion should include the reasons why their data is different from literature. Is it related to the antibodies or number of cases?

Reply 4: As you have mentioned, the reasons why our data is different from published literature mainly in prognostic factor may be related to the
antibodies we used. However, considering that the results are consistent regardless of the type of antibodies in published literature (Reference No.12, 13), we assume that the difference in results would rather be due to the number of cases. Moreover, the major difference between our study and previous studies is the resected specimens used. In our study, we used the surgical specimens influenced by NACRT. So, there is a possibility that the result is different from the previous reports using the surgical specimens not influenced by NACRT. We added these explanations in Discussion.

Discretionary revisions:
5. Was there any staining observed in pancreatic intraepithelial neoplasia? If so, was there any change in the percentage or intensity of staining with any of the markers?

Reply 5: No. When we choose the fields for the evaluation of the immunoreacting score, we selected only the area of invasive ductal carcinoma at random excluding that of intraepithelial neoplasia on H-E staining slide. So, we added this explanation in Methods (P7 lines 5-13).
Response to the Reviewer Dr. Aatur Sighi

Major Compulsory Revisions:
1. The manuscript states the patients selected within their study have localized advanced pancreatic adenocarcinoma, however locally advanced disease is considered to be at least stage III disease. Per AJCC, stage III disease is clinically staged as T4, any N and M0. However, per Table 2, none of the patients had cT4 tumors. Thus, the term “locally advanced” should be omitted from the manuscript.

Reply 1: The definition of the term “locally advanced” pancreatic adenocarcinoma is ambiguous, nothing has been clearly defined. So, we defined “locally advanced” pancreatic cancer as the borderline resectable pancreatic adenocarcinoma that meets the criteria of NCCN unlike your definition. In our study, it is not true in all cases according to your definition, so we omitted the term “locally advanced” from the manuscript, as you have pointed out.

2. The selection criteria for patients enrolled in their study is ambiguous and should be clearly stated. The study enrollment was for a 10 year period and only enrolled 28 patients. It seems unlikely that only 28 patients were considered eligible during this time. If so why?

Reply 2: In our facility, surgery for liver disease, including liver transplantation is the mainstream. On the other hands, surgery for pancreatic disease is only a few in a year. When it comes to locally advanced pancreatic cancer, the number is further limited. Moreover, among the patients who received NACRT before surgery, resectable ratio is finally less than 60%. This is the reason why the number of cases was less. Please understand that it is not because we selected cases as it is convenient.

3. The authors evaluate their immunohistochemical stains by defining a positive
score as an averaged score > median score for a specific antibody. How did the authors arrive at this method? If this is an accepted method for these antibodies, they should reference prior reports using this method. Previous studies the authors cite have used different semiquantitative methodologies and it may be best to refer to those.

Reply 3: The methodology we used was in reference to previous report (Reference No.22), so we added the description of “according to previous reported criteria [22]” in immunohistochemical evaluation (P7 line 18-9).

4. Table 5, the only CSC marker considered significant was CXCR4 and per the authors correlated with liver metastases. The overall number of positive cases was 8, however, the table states 9 with a p-value of 0.498. This error may be significant considering the borderline p-value and will need to be reanalyzed and corrected. Whether this is statistically significant will effect the discussion within the manuscript.

Reply 4: As you pointed out, we carelessly mistook the overall number of positive cases for 9 despite of 8 in fact. So, we have correctly modified Table 5 and p-value in the manuscript (P11 line 4).

5. The Discussion primarily restates the results of the study, rather than relating the data with previously published findings and possible future directions. In addition, the authors assume their immunohistochemical results are directly related to cancer stem cells and do not consider a stem-cell independent correlation. Certainly, cancer stem cells are considered to be a minor population of cells, however the immunohistochemical stains are scored in a fashion where the majority of cells are immunoreactive. How do the authors explain these findings? The Discussion should be revised to reflect how their data adds or conflicts with published literature and alternative possibilities to the function of these proteins.

Reply 5: As you have mentioned, cancer stem cells in a true sense are considered to be a minor population of cells, so it appears to be unable to
find them by the immunohistochemical scoring system we used. However, in our study, we used the term “cancer stem cells marker” in the sense that one of the phenotype caused by differentiation of cancer stem cells rather than cancer stem cells themselves in the true sense as is so often the case with previously published reports. If the term confuses readers, we plan to replace the term “cancer stem cells marker” with more suitable one (i.e, “cancer stem-like cells” or solely “prognosis prediction marker”). Apart from this discussion, we added another supplementary figures and revised the Discussion in a manner as your suggestion (P3 lines 24-29, P13-15).

Minor Essential Revisions:
1. Within the Methods, immunohistochemical evaluation was performed by 2 independent observers. The text notes that discrepancies between observers were resolved using a conference microscope. It may be of interest to also enter how many discrepancies were there within the Methods.

Reply 1: In most cases, there was no disagreement in principle. However, since there were only two minor discrepancies in the evaluation of CD24, I agreed to the opinion of the pathologist.

2. Within Table 2, the histologic classification was considered indistinguishable for 1 case. The methods should reflect how histologic classification was done and why 1 case was consider indistinguishable. Furthermore, instead of indistinguishable, a better term may be ungradeable. Table 5 omits this case, why? This should be entered.

Reply 2: We performed the evaluation of histological classification in accordance with guidelines of the UICC, so we added this in the “Patient characteristics” of “Results” (P8 lines 3-5).

Because the tumor cells were denaturalized with the effect of radiation chemotherapy, we could not exactly evaluate the degree of differentiation in 1 case. According to your suggestion, we entered this case and reanalyzed the results.
Apart from your indication, we noticed description mistakes in the section of the “Histological classification” in Table 2 and Table 5, so we have correctly modified them along with the change of term.
Response to the Reviewer Dr. Wenqing (Wendy) Cao

Major Compulsory Revisions:
1) This is a small sample size retrospective study done only in 17 cases in NACRT group. There are only 1 or 2 cases in many groups listed in Table 4 and 5. It is highly recommended the authors to expand their sample size.

Reply 1): Thank you for your advice and suggestion. As you have mentioned, we also fully understand that the larger the sample size is, the more credible the result obtained by it is. In our facility, surgery for liver disease, including liver transplantation is the mainstream, but surgery for pancreatic disease is only a few in a year. So, it will take a long time to expand the sample size in our facility. In such reason, to extend sample size is very difficult in our facility.

Minor Essential Revisions:
2) When evaluating IHC expression, the authors used two to six visual fields to evaluate staining intensity and estimate the fraction of positive stained tumor cells. How did the authors define the visual fields and how did they apply the visual fields to different markers? Please explain.

Reply 2): Thank you for your advice and suggestion. We selected the visual fields and applied the visual fields to different markers as described below. At first, guided by the microscope, two to six visual fields were selected randomly per section using a ×4 objective and a ×10 ocular lens on each H-E staining slide and marked it by circling each area. And then, we superimposed the slide which was stained with CSCs markers on the HE staining slide, and have marked it by tracing the mark. We added these explanations in Methods (P7 lines 5-13).

3) Most pancreatic cancer patients are resistant to chemoradiation therapy. It would be interesting to see some kind of association between stem cell marker expression and treatment response. Although the authors showed in Table 5 that there are no significant differences between stem cell marker expression
and treatment response, this might be biased by small sample size.

Reply 3): Thank you for your advice. We absolutely agree with your opinion. Unfortunately, however, with respect to the issue of sample size, it is very difficult to be solved by our facility as described Reply 1).

4) Many results in the current manuscript appear to be inconsistent with the published data. Authors did not interpret and discuss them. These discussions may make the study more significance to the audience.

Reply 4): Thank you for your advice and suggestion. The reasons why our data is different from published literature may be related to the antibodies we used or the number of cases. Moreover, the major difference between our study and previous studies is the resected specimens used. So, there is a possibility that the result is different from the previous reports using the surgical specimens not influenced by NACRT. We added these explanations in Discussion (P14 lines 9-11).