Peer Review File

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Reviewer A

The authors provide an excellent introduction into the potential role of EMT and cancer stem cell phenotype in gastric cancer in support of their hypothesis.
The purpose of this study was to evaluate EMT and CSS markers and identify associations with prognosis and peritoneal disease.
In this retrospective review, the authors randomly selected patients whom underwent gastrectomy at a single institution.
The authors met their goal of identifying a strong association between Slug and CD133.
The conclusions are supported by the results and not over-stated.
The figures are blurry and could benefit from ensuring they meet the journal requirements.

Reply: Taking the reviewer's advice, we revised all our figures (Figure 1 and Figure 2). The figures are now in resolution of 600 DPI.

Updated files will be attached in separate files. (Figure_1.tif, Figure_2.tif)

Reviewer B

The authors aim to evaluate the prognostic ability of the transcription factor Slug and the stem cell marker CD133 in gastric cancer after curative therapy. To accomplish this aim, the authors performed histochemistry on 196 gastric cancer specimens for multiple cancer-relevant proteins, and retrospectively correlated expression with clinical variables. The strength of the article is the positive correlation between CD133 and Slug with peritoneal recurrence and overall survival. However, I would recommend to revisions to the manuscript from its current form due to the following concerns:

Major points:
1. Please clarify in your Tables, and manuscript, when you are using Clinical staging and Pathologic staging. For the purposes of this post-hoc analysis it would be best for us to understand that the associations you are making are with pathologic staging variables. For example, in Table 2, the inclusion of (presumably) clinical stage 2 and 3 variables in your analysis of Slug and CD133 expression is not helpful. Especially since you do not seem to be advocating the Slug and CD133 should be used to make pre-operative decisions. Thus, all factors related to cancer staging should be based on pathology.
Reply 1: We agree with the reviewer's suggestion. We have reviewed the manuscript accordingly. We definitely agree that there needs to be a clarification of the variables in the tables of the manuscript. The T stage and N stage variables in Table 1 and Table 2 were referring to pathological/surgical stages.

For clarification, in Table 1 and Table 2 we added "pathological(p-)" to T stage and N stage. In contrast, "Stage" variable was deleted. By these changes we hope our analysis and results are delivered more precisely. Thank you for pointing this out.

Changes in the text:

Table 1, Table 2, and Table 3. (see Page 19 line 354, page 20 line 357, and page 21 line 360)

1) Stage variable was deleted
2) "p-" (pathological) was added to T stage and N stage variables.
   T stage → pT stage
   N stage → pN stage

Table 4 (see Page 20 line 364)

1) "p-" (pathological) was added to T stage and N stage variables.
   T stage → pT stage
   N stage → pN stage

2. To point #1, it would be extremely interesting to know whether the peritoneal recurrences also retained elevated Slug and CD133 expression. Presumably the authors have access to tissue in some patients to evaluate?

Reply 2: We deeply agree with the reviewer's opinion. We have reviewed the medical records in search of the peritoneal samples. Among peritoneal recurrent cases, 10 samples had cytology or tissue. Unfortunately, due to the retrospective aspect of this study, there were limitations to perform further study. First, most of the samples were cytology samples which were not made into cell blocks at the time of acquisition. The rest of the samples remaining after diagnosis were discarded. Secondly, the immunohistochemical staining of Slug and CD133 is not routinely done at out institution. For this reason, we hope you generously understand that additional staining cannot be done at the moment.

Since peritoneal tissues are obtainable initially through surgical staging from more advanced stage of gastric cancer patients, we plan to do an expression study of the peritoneal carcinomatosis environment and the epithelial-mesenchymal transition markers based on RNA-sequencing in a different setting instead in the future.

3. It would be beneficial to look at as many pathologic variables that could be associated with Slug and CD133 expression. Examples include lymphovascular invasion, signet ring cell morphology, lymph node positivity ratio, HER2 over-expression, etc. Were any of these considered?

Reply 3: Yes, the reviewer is correct. This part of the manuscript was not clear. We have already looked at lymphovascular invasion, perineural invasion, signet ring cell morphology, and lymph node positivity by surgical staging for association with Slug, CD133, or both, although we have not presented these in detail in the previous manuscript.

To reflect the reviewer's point, we presented data of "grade/differentiation, histology (signet ring cell type), lymphovascular invasion, perineural invasion" to Table 1, Table 2, and Table 3. Also, Table 4 has also been changed. Univariate analysis results were added (1) to clarify the process of survival analysis and (2) to show pathological variable analysis.

Changes in the text: 4

1. Table 1, Table 2, and Table 3. (see Page 19 line 354, page 20 line 357, and page 21

line 360)

1) Signet ring cell histology, lymphovascular invasion, perineural invasion variables were added.
2) Histology variable was renamed to Grade/differentiation.

2. Table 4 (see Page 20 line 364)
   1) Univariate analysis result (p-value) was added with addition of age/sex/signet ring cell histology/lymphovascular invasion, perineural invasion variables.

4. Representative photomicrographs of the TMA staining should be included as a figure.
Reply 4: We added representative photos of CD133 and Slug staining as Figure 1.

Changes in the text:

1. Figure 1 and explanation were added in Method-Tissue Microarray (TMA) and Immunohistochemistry (IHC) section.
"Representative photographs of variable Slug and CD133 expression is shown in Figure 1." (see Page 7, line 142)

2. Figure legend was added.
"Figure 1. Immunohistochemical expression levels of Slug and CD133. Slug staining showed (A) negative (0), (B) weak (1+), (C) intermediate (2+), and (D) strong (3+) intensities. CD133 staining showed (E) negative (0), (F) weak (1+), (G) intermediate (2+), and (H) strong (3+) intensities." (see Page 18, line 399)

3. Numbering of Figures were changed due to this.
   1) Figure 1 → Figure 2
      Figure 1A, 1B → Figure 2A, 2B (see Page 9, line 194)
      Figure 1C → Figure 2C (see Page 9, line 197)
      Figure Legends; Figure 1 → Figure 2 (see Page 18, line 403)
   2) Figure 2 → Figure 3
      Figure 2 → Figure 3 (see Page 10, line 207)
      Figure 2E, 2F → Figure 3E, 3F (see Page 10, line 214)
      Figure Legends; Figure 2 → Figure 3 (see Page 18, line 406)

Minor points:
1. Is there a reference for the semi-quantitative system for scoring IHC?

Reply 1: Yes, there is a reference for this. It is added in the method text and the reference list.

Changes in the text:

1. Reference citation is added in the main text.
   ➔ (17) (see Page 7, line 17)
2. Citation is added in the reference list.
   ➔ Yuji Toiyama, Hiromi Yasuda, Susumu Saigusa, et al. Increased expression of Slug and Vimentin as novel predictive biomarkers for lymph node metastasis and poor prognosis in colorectal cancer, Carcinogenesis. 2013;34(11):2548–2557. (see Page

17, line 362-364)

2. Would recommend including how documentation of peritoneal recurrence was done – I assume this was radiographic evidence of recurrence.

Reply 2: PC documentation according to CT finding were additionally described in main text.

Changes in the text:

"The peritoneal recurrence was documented radiologically by computed tomography (CT) findings. Nodules or masses in the peritoneal cavity, omental haziness, ascites, and peritoneal thickening or enhancement were key CT findings of PC."

(see Page 6, line 115-118)

3. Recommend being more explicit about the IHC methods as it applies to all antibodies; for instance, the antibody information for Slug is given, but not so for the others listed. This should be done.

Reply 3: Yes, it accordance with the reviewer's suggestion the antibody information for each antibody in the method section of the main text was added.
Changes in the text: The following sentences were added to the main text.

"Immunostaining was performed as follows. Tissue sections (4-㎛-thick) were cut from paraffin-embedded blocks. After deparaffinization and rehydration, antigen retrieval and blocking of endogenous peroxidase were performed. The antibodies and dilutions used were: anti-Slug (ab38551; Abcam, Cambridge, UK) at 1:500 dilution and anti-CD133 (GTX60471; GeneTex, San Antonio, TX, USA) at 1:650 dilution. The sections were incubated at room temperature for 90 min with anti-CD133 antibody and overnight at 4℃ with anti-Slug antibody. Positive controls were used as suggested in the supplier's sheet. The primary antibody was omitted in negative-control experiments."

(see Page 6, line 127-134)

• Table 1: For "Primary location", "Whole" has 2 patients but 0.0% - please correct.
Reply: We have modified the table as pointed.
Changes in the text: 0.0 → 1.0 (see Page 19, line 354)

• Line 172 and 179 refer to Figure 3; there is no Figure 3 – please correct

Reply: We have corrected the text as pointed.

Changes in the text: 1) Figure 3 → Figure 3 (see Page 10, line 207)

2) Figure 3 → Figure 3E, 3F (see Page 10, line 214)

• The authors should be more explicit about limiting their conclusions to the highly selected group of patients chosen for the study, i.e. gastric adenocarcinoma of clinical stage 2 or 3 who underwent a curative gastrectomy with adjuvant chemotherapy.

Reply: An explanation to this point was added in the method section of the main text.

Changes in the text:

> "This inclusion criterion was applied due to our specific interests in those who are at higher risk of recurrence." (see Page 5, line 100-102)

• The TCGA database is an easily accessible resource to characterize exome data and directly correlate it to survival and could provide a source of external validation for your study.
• Would also consider adding that your data has yet to be tested in other populations; unless you consider using TCGA data as suggested above.

Reply: Thank you for providing these insights. In response to the reviewer's recommendations, we searched for any available data on TCGA and GEO database whether there is a dataset that contains expression data, peritoneal recurrence, interval to recurrence, and survival. Unfortunately, the TCGA-STAD data did not include recurrence patterns (type of involved organs). There was an array data at GEO database (GSE15081) which included genes of Slug and CD133 for analysis and peritoneal relapse data, but no overall or peritoneal specific survival was publicly available. Some other GEO datasets did not include Slug/CD133 genes (GSE8657) or did not include peritoneal involvement data (GSE66222, GSE62254). Please understand that comparing our results with publicly available data was difficult to apply in our research.