Peer Review File

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Reviewer A

Your manuscript, "The prognostic value and potential immunotherapeutic efficacy of ACVR1 in treating gastric cancer", presents the results of a bioinformatic characterization of patients with gastric cancer that overexpress the ACVR1 gene. According to your results, the ACVR1 gene has the potential to be a therapeutic target and can evaluate the prognosis of gastric patients. Among differential features of this study in comparison with previous literature, this manuscript presents predictions about the immune state of patients overexpressing the ACVR1 gene as well as putative therapeutic strategies to be further studied. Though the potential contribution of this study to their field, I would like to comment on some concerns:

Major comments

1. Are TCGA tumor and normal samples balanced to run this comparative analysis? Once you retrieved this data from the Xena repository, you could use the TCGA-GTEx dataset instead. GTEx is a dataset including normal samples of different tissues. Then, you could increase the number of participating normal tissues. Otherwise, is there any specific reason to limit this analysis to TCGA samples?
Reply 1: This paper mainly analyzes TCGA database and Xena database respectively, and does not use TCGA-GTEx dataset. Moreover, we performed the GEPIA database (TCGA tumor and GTEx normal samples included) to verify this comparative analysis and obtained the same results.
Changes in the text: None.

2. Regarding your independent cohort of over 200 patients. Is this cohort representative of the Chinese population with gastric cancer? Please describe the statistical power of your sample.
Reply 2: Yes. The data of 200 patients were obtained from postoperative patients diagnosed with gastric cancer in Nantong University Hospital from 2010 to 2011. According to the staining intensity of IHC from these patients, the immunohistological microarray data were classified into high and low-expression groups.
Changes in the text: None.

3. Regarding Figure 4C, it seems that you elaborate a nomogram with all variables with $p<0.05$ in the univariate analysis. Should not it be performed only with relevant variables in the multivariate analysis?
Reply 3: First, we finish the univariate analysis, then the meaningful factors in the univariate analysis are selected for multivariate analysis. Then, the selected multivariate analysis (including gender, TMN stage, tumor stage, age, and pathological grading) was used to predict clinical outcomes in gastric cancer patients.
Changes in the text: None.

4. In addition to the previous comment, these findings (accuracy and relevance of the AVCR1 gene for prognosis in gastric patients) must be confirmed by external or independent validation. Could you apply the nomogram to data from other sources? I include a brief list of available datasets for your evaluation:
Asian Cancer Research Group (ACRG, GSE66229 dataset, n = 297) (Cristescu et al., 2015), Yonsei University Severance Hospital (YUSH, GSE84437 dataset, n = 433) (Kim et al., 2019), Korea University Guro Hospital (KUGH, GSE26899 data set, n = 93) (Oh et al., 2018), the National Cancer Centre of Singapore (NCCS, GSE15459 data set, n = 192), the Kosin University College of Medicine (KUCM, GSE26901 dataset, n = 109) and The University of Texas MD Anderson Cancer Center (MDACC, GSE28541 data set, n = 40). Please consider evaluating your nomogram in some of these cohorts.
Reply 4: In the next study, we will conduct the illustrated external or independent validation to verify the accuracy and relevance of the AVCR1 gene in gastric cancer patients.

5. The discussion is very interesting regarding the potential participation of AVCR1 in immune-related pathways and microenvironment. However, there are no descriptions of other molecular scores for gastric cancer patients. Are there differences in the localization of the tumor (Siewert classification)?
Reply 5: This article mainly studies the relationship between ACVR1 and gastric cancer, and other molecules will be studied in the future. The classification proposed by Siewert et al. is based on the anatomical characteristics of the esophagogastric junction. In our study, Siewert classification of patient tissues has not been analyzed. In the future research, we can use this classification for further research.

6. How do you envisage the inclusion of this gene in the clinical routine? Many papers are approaching this niche, some with more genes in the score. To put this in context, a recent report has compared many of these molecular scores using independent cohorts (https://doi.org/10.3389/fgene.2023.1206609). How the sole evaluation of the ACVR1 gene will impact this context? Please discuss it.
Reply 6: In our next study, molecular biology experiments will be performed to further verify the biological function of this ACVR1 gene and further evaluate the role of ACVR1 in human tissue specimens. In our research, in addition to research the prognostic survival role of ACVR1 gene, we also investigated that ACVR1 expression was significantly correlated with immune infiltration, which may provide a potential therapeutic target for gastric cancer immunotherapy. However, the context (https://doi.org/10.3389/fgene.2023.1206609) merely found that the GES7 is a reliable gene-expression-based signature to improve the prognosis estimation in gastric cancer. Hence, our evaluation of the ACVR1 gene will not affect that context.

Minor comments
7. Please separate data collection from public data and your independent cohort selection in different items of the methods section.
Changes in the text: We have modified our text as advised(see page 6, line 174).

8. Please describe all abbreviations in their first mention. For example, TPM (Transcripts per million), TME (tumor microenvironment), TMB (tumor mutation burden), and ddH2O (laboratory-grade water).
Changes in the text: We have modified our text as advised(TPM: see page 6, lines 184-185. TMB: Have explained. See page 2, line 52. ddH2O: see page 6, line 192.).

9. Please describe supplier and catalog IDs for all used products in the elaboration of results for this study.
Reply 9: I will add it in Word form to the zip package.

10. Please follow the HUGO guidelines for formatting gene and protein names. All human genes must be written in caps and italics.
Reply 10: We have modified our text as follow the HUGO guidelines in revised manuscripts.

11. Please check the heading of the Table in Figure 4B, though you describe a multivariate analysis, there is written "p-value univariate analysis". Similar to Figure 4A.
Reply 11: It was originally submitted as a multivariate analysis, I didn't notice it become univariate analysis. Thanks for reminding.
Changes in the text: We have modified our text as advised(see page 30, line 916).

Reviewer B

The article shows a very interesting strategy to use open and published data for in silico analysis. However, the authors do not provide evidence or statistical analysis to demonstrate that all the cohorts used, considering all the different sources and human populations, are representative or at least balanced with each other. Most in silico analyzes were performed well as independent approaches, so each data set may not be related to another and disconnected from the biological perspective of the biomarker, which is ACVR1.
Reply: Although the data of different platforms did not indicate the source and population of samples, the results obtained from different platforms all indicated that high expression of ACVR1 was associated with poor prognosis and immune infiltration of gastric cancer. The data used by R software are same.
Changes in the text: None.

Regarding immunostaining, there is no information on the validation of antibodies and protocols. The quality of the staining and images shown do not allow further interpretation or analysis. Several statistical analyzes mention the segregation of cases based on ACVR1 levels (High and Low), which was established by the staining intensity.
Changes in the text: We have modified our text as advised(see page 8, lines 244-246):the IHC data were processed to classify the expression of ACVR1 into high- and low-expression groups based on the median value and cutoff value of the semiquantitative immunohistochemical score.

In the material and methods section (lines 232-240), the authors mentioned that ACVR1 IHC was rated in at least 5 categories, and they qualitatively separated them as negative or positive, but nothing explains the high and low.
Reply: The expression of ACVR1 into high- and low-expression groups based on the median value and cutoff value of the semiquantitative immunohistochemical score, not negative or positive. So we delete it.
Changes in the text: We have modified our text as advised(see page 8, lines 244-246).

TMB, MSI and methylation (materials and methods section) are unnecessary, as are the results related to the correlation between ACVR1 and TME, ICI, TMB, MSI/dMMR, methylation status, which are very far from being verifiable at the biological level. Therefore, most of the conclusions are overestimated, they may not be representative of a specific population, and the possibility of clinically validating these findings will require, beforehand, a long process of basic and translational research.
Reply: TME, ICI, TMB, and MSI/dMMR which are closely related to immunity, are widely used in clinical practice, providing directions for follow-up clinical research. In the future. Further research will be carried out to verify the molecular biology experiment
Changes in the text: We delete the methylation part in the text.

Reviewer C

It has been correctly addressed that a correlation between ACVR1 expression and cancer progression has been studied and established for several cancer types and in this respect, the only novelty is the focus on gastric cancer. Overall, this manuscript represents a great effort to collect and analyze data from various databases as well as raw hospital data. However, I would like to mention, it's not always "the more, the better". I feel there is redundancy in the information given by different figures and overall the text as well as the figures can be optimized a great deal. Correlations have been drawn between ACVR1 expression in GC and numerous clinical and biological phenotypes. However, I fail to see an obvious clinical relevance of all these correlations.
Several points I would like to highlight:
• The flow of text needs to be improved substantially. Remove redundancies and repetitions.
• Text is written too lengthy and can be significantly shortened.
• There is too much Materials and Methods information in the Results chapter.
Changes in the text: We have modified our text as advised.

• It sounds like the author concludes a causality btw TME and ACVR1 expression, however, correlation does not equal causality. Please explain further.
• Figures: please explain all acronyms and highlight gastric cancer tissue data. Make figures clear and readable and explain every axis in detail.

Reply: This article summarizes the immune pathways that promote or inhibit the occurrence and development of gastric cancer. A detailed introduction of the pathways will be carried out in subsequent studies.

Changes in the text: None.

• Correlations are not always obvious from the graphs. Often enough I fail to see any significance.

• Only include data and graphs that underline your message. Ambiguous or insignificant but relevant data can be added to supplementary figures.

• Make more effort in writing a clear, understandable, and meaningful storyline. The current manuscript reads like a long and hastily written lab report, not a well-thought-through manuscript.

Reply: These results such as TME, ICI, TMB, and so on were studied based on public platform data and IHC data. The correlation between ACVR1 and gastric cancer and immunity was obtained, which provided the direction for further research in clinical trials.

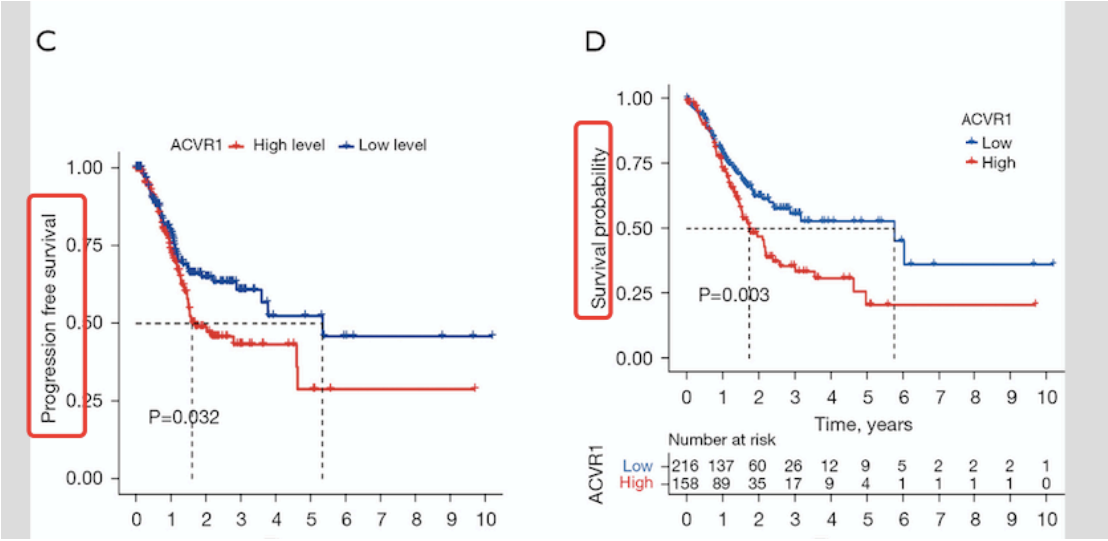
Changes in the text: None.

Reviewer D

1. Figure 2

Please check whether the legends (C) and (D) match the figure 2C and 2D.

979 Hospital cohort; (C) OS of ACVR1 in TCGA divided into high- and low-expression
980 groups by cutoff value; (D) PFS of ACVR1 in TCGA cohort. ACVR1, activin A

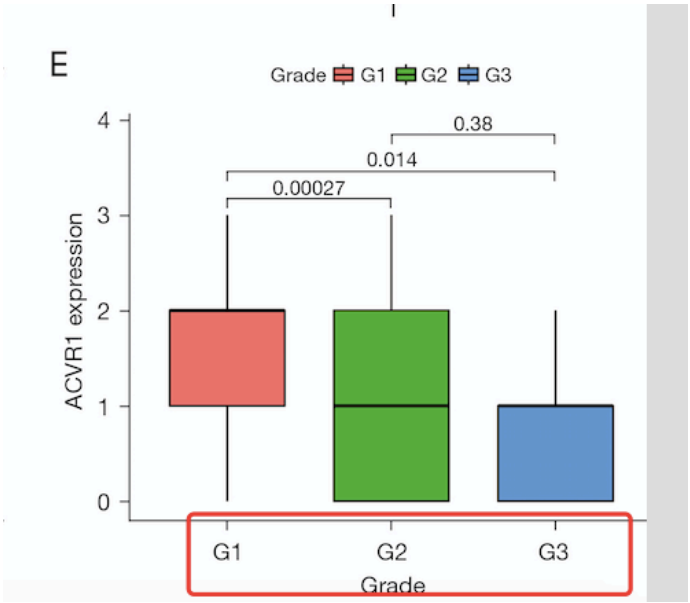


Changes in the text: We have modified our text as advised(see page 28, lines 898-899)..

2. Figure 3

Please check whether the legend (E) match the figure 3E.

992 Hospital cohort; (E) correlation of ACVR1 expression with T staging in the Nantong
993 University Hospital cohort. ACVR1, activin A receptor type-1; TCGA, The Cancer
994 Genome Atlas. (* represent <0.05,**represent <0.01,***represent <0.001)



Changes in the text: We have modified our text as advised(see page 29, lines 911-912).

3. Figure 4

a. Please indicate the meaning of *** in figure legend.

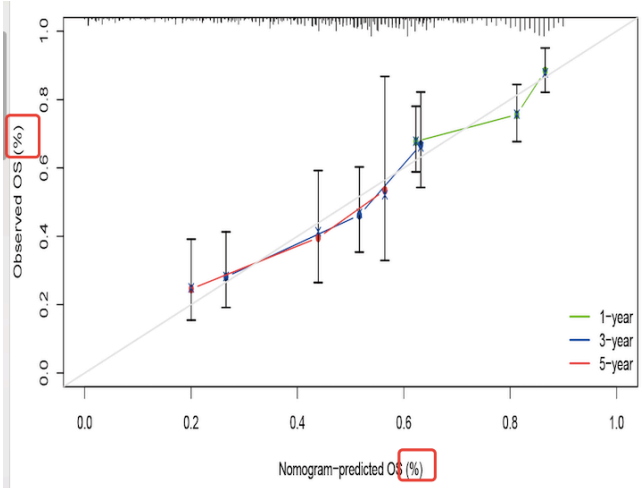
Changes in the text: We have modified our text as advised(see page 31, lines 927-928).

b. Since **Figure 4** is a (A-E) combined image, please also mark the capital letters (A, B, C, D, E) in image.

416 analysis showed that a high expression of ACVR1, $T_{2\text{-}4}N_{1,3}M_1$ stage, stage III,IV and
417 old age(>65) were all associated with OS (P<0.05) (Figure 4A). Multifactorial Cox
418 analysis showed that the high expression of ACVR1 and old age(>65) operated as
419 independent prognostic factors (P<0.05) (Figure 4B). Subsequently, column line plots
420 were constructed for predicting the 1-, 3-, and 5-year survival rates of patients with
421 gastric cancer based on TNM stage, pathologic grade, gender, stage, age, and ACVR1
422 expression, with the results being 0.894, 0.699, and 0.642, respectively (Figure 4C).
423 The calibration curve showed that the actual OS values were more consistent with the
424 predicted OS consequences (Figure 4D). The area under the ROC curve for 1-, 3-, and
425 5-year survival rates of the forecast OS column line graphs were 0.527, 0.583, and 0.627,
426 respectively, which, taken together, indicated a more satisfactory prediction (Figure 4E).

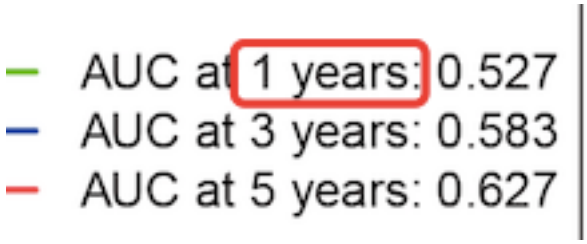
Changes in the text: We have modified our text as advised(see page 30, line 916).

c. figure 4D: please delete the (%), since the numbers are range from 0-1.



Changes in the text: We have modified our text as advised(see page 30, line 916).

c. Please revise '1 years' to 1 year' in figure 4E.



Changes in the text: We have modified our text as advised(see page 30, line 916).

d. Please check if '$N_{1,3}$' in line 416 should be "$N_{1,2,3}$'

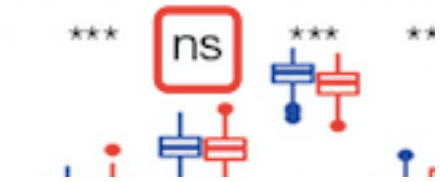
416 analysis showed that a high expression of ACVR1, $T_{2\text{-}4}N_{1,3}M_1$ stage, stage III,IV and
417 old age(>65) were all associated with OS (P<0.05) (Figure 4A). Multifactorial Cox

Pathologic N stage	352	
N0	107	Reference
N1	97	1.629 (1.001 2.649)
N2	74	1.655 (0.979 2.797)
N3	74	2.709 (1.669 4.396)

Changes in the text: We have modified our text as advised(see page 12, line 388).

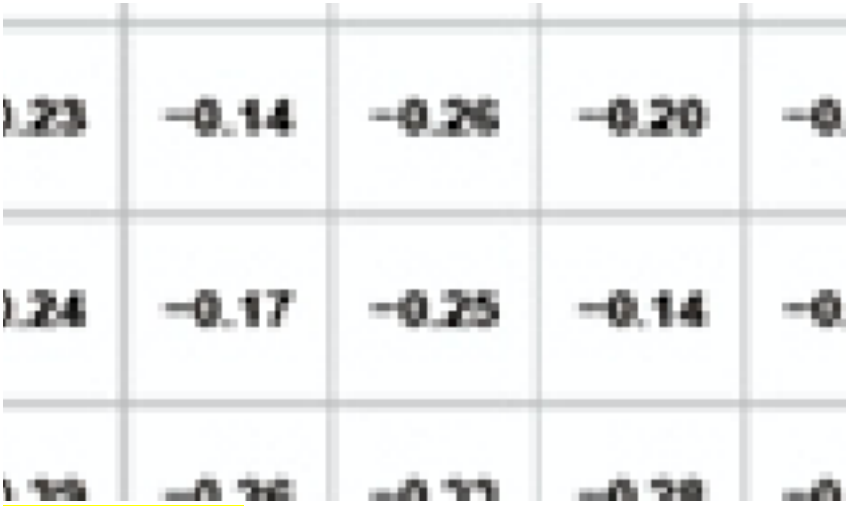
4. Figure 5

a. Please indicate the meaning of 'ns' in figure legend.



Changes in the text: We have modified our text as advised(see page 33, line 940).

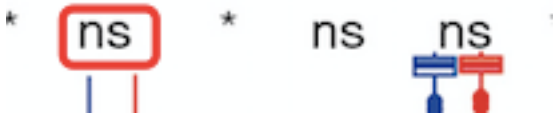
b. Please send us Figure 5B with higher resolution in JPG/TIFF, as the current one is not clear enough.



Changes in the text: We have modified our text as advised(see page 32, line 930).

5. Figure 6

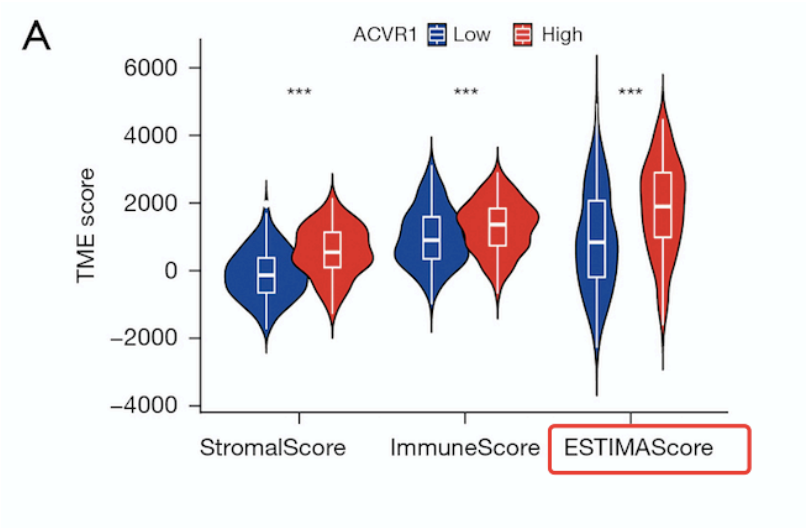
Please indicate the meaning of 'ns' in figure legend.



Changes in the text: We have modified our text as advised(see page 34, line 959).

b. Please confirm whether the legend (A) is correct.

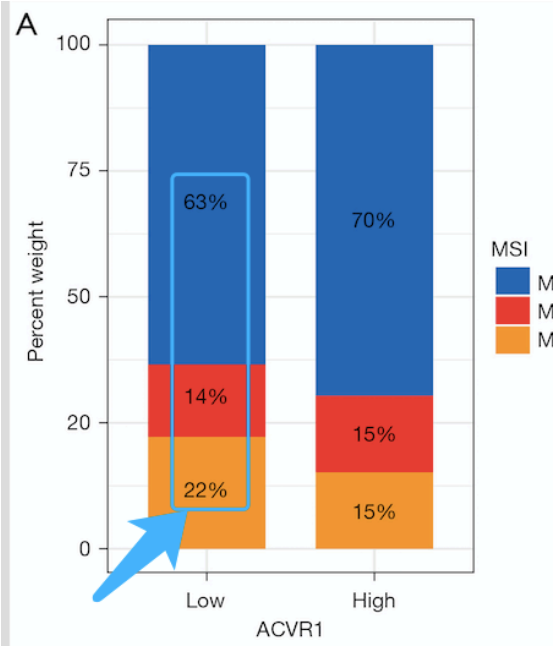
.022 **Figure 6 Correlation of ACVR1 with immune infiltration.** (A) Relationship between
.023 stromal score and immune score with high and low ACVR1 expression; (B) the
024 CIBERSORT algorithm assessed the proportion of immune cell infiltration in the high
481 immune cells, stromal cells, and the availability of immune and stromal cells in th
482 TME. The results showed that the stromal score, immune score, and estimate score o
483 the high ACVR1 expression group were higher than those of the low-expression grou
484 (Figure 6A).



Changes in the text: We have modified our text as advised(see page 33, line 944.see page 14,lines 443-444).

6. Figure 10A

The total percent of low bar is not equal to 100. Please check and revise.



Changes in the text:We have modified our text as advised(see page 39, line 991).

7. **Figure 11** is (A-G) combined picture. Please check if legends (C) and (D) should be legends (F) and (G).

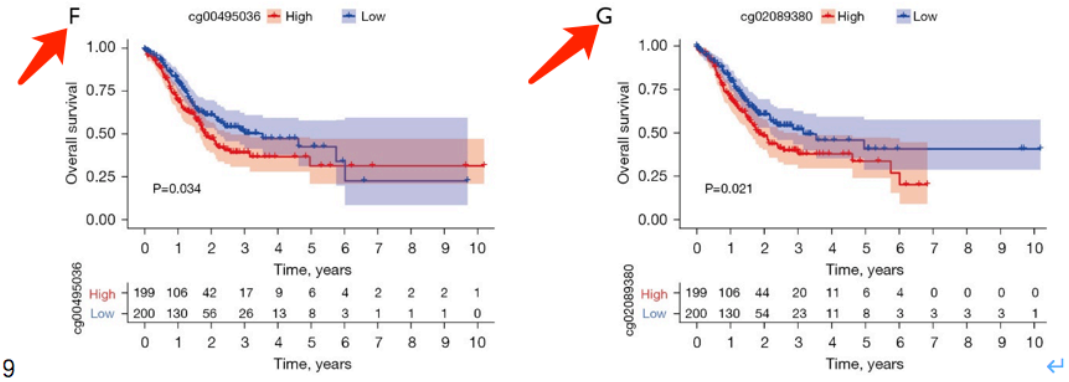


Figure 11 Correlation analysis between ACVR1 and methylation. (A) Relationship between ACVR1 and the degree of methylation occurring in methylated sites; (B) positive and negative correlations between ACVR1 and methylation sites; (C) correlation analysis of the cg00495036 locus with prognosis; (D) correlation analysis of the cg02089380 loci with prognosis. ACVR1, activin A receptor type-1.

Changes in the text:We deleted methylation part.

8. **References**

a. The authors mentioned "studies...", while only one reference was cited. Please revise.

- *Studies have verified that the clinical features of gastric cancer are associated with infiltrating tumor microenvironment (TME) cells, in which the suppression of T cells causes a reduction in the ability to mediate tumor killing and may lead to a poor prognosis of gastric cancer (6).*

Changes in the text:We have modified our text as advised(see page 4, line 111).

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- *however, recent studies have shown that IL-2 can induce CD8$^+$ T cell depletion, suppressing antitumor immune responses, thus making IL-2α double-edged sword (49).*

Changes in the text: We have modified our text as advised(see page 19, line 642).

b. Please confirm if citations are needed for this sentence, as you mentioned "studies". Please revise.

- *Studies have demonstrated that both aberrant upregulation of PD-L1 expression and deletion of PD-L1 expression may lead to the ineffectiveness of PD-1/PD-L1 target inhibitors.*

Changes in the text: We have modified our text as advised(see page 20, lines 664-669).