Peer Review File

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Reviewer A

The paper titled "Mining TCGA database for genes of prognostic value in HNSCC microenvironment" is interesting. This study confirmed that CD8+ T cells were significantly associated with better survival in HNSCC and verified that the top five significantly mutated genes (SMGs) in the TCGA HNSCC cohort were TP53, TTN, FAT1, CDKN2A, and MUC16. A high level of CD8+ T cells and high immune and stroma scores corresponded to a better survival probability in HNSCC. However, there are several minor issues that if addressed would significantly improve the manuscript.

1) What are the cell types and expression characteristics in the immune microenvironment of HNSCC? How are the dynamic changes and connections between cells in different tissues? It is recommended to add relevant content.

Reply: Your comments are greatly appreciated. The TME in HNSCC is comprised of heterogeneous non-malignant cells that integrated in a complex ECM. TME of different types of tumors have their own characteristics and also commonalities. However, the TME of HNSCC is characterized by some unique features compared to other cancer types, such as decreased absolute T cells count in tumor and circulation, decreased number of natural killer cells et al. Moreover, the interaction of various cellular components in the TME along with the production of cytokines and chemokines profoundly impact the function of T cells. In addition, HNSCC is characterized by desmoplastic stromal fibroblasts that promote tumor invasion and progression through autocrine and paracrine factors. We have added relevant content in the revised Introduction (see Page 4, Line 117-134).

2) Figure S1 is missing, please add on.

Reply: We are really sorry for our careless mistakes. Previous Figure S1 and Figure 5B were repeated. We have deleted the previous Figure S1 legend, and added new Figure S1 and its description in the revised manuscript (see Page 29, Line 747-750).

3) This study is based on bioinformatics analysis. It is recommended to increase in vivo and in vitro experimental studies, which may be more meaningful.

Reply: Thank you for your useful suggestion. We must acknowledge this limitation in our study, we will try to perform in vivo and in vitro experiments in further studies.

4) Based on the results of this study, how to develop prognostic markers of immune-related genes to stratify patients with HNSCC? It is recommended to add relevant content.

Reply: Thanks for your valuable comments. In this study, we confirmed that the expression

level of CD8+ T cells and CD4+ T cells, the immune score and the overall classification of immune cell infiltration were significantly correlated with the prognosis of HNSCC patients, therefore the expression level of CD8+/CD4+ T cells could be used to evaluate the prognosis of patients in clinical applications. The expression levels of characteristic genes that used by CIBERSORT and ESTIMATE algorithms, or the expression levels of characteristic lncRNAs of LASSO Cox regression model in this study can be detected through high-throughput sequencing methods, and then the relative T cells proportion, immune score or stromal score can be calculated based on the algorithm models. Hence, using the comprehensive immune measures at the transcriptome level can stratify patient outcomes. We have added relevant content in the Discussion (see Page 13-14, Line 430-448).

5) The introduction part of this paper is not comprehensive enough, and the similar papers have not been cited, such as "CD8+ T-cell exhaustion in the tumor microenvironment of head and neck squamous cell carcinoma determines poor prognosis, PMID: 35434003", "Upregulated YTHDF1 associates with tumor immune microenvironment in head and neck squamous cell carcinomas, PMID: 36523307". It is recommended to quote this article.

Reply: We agree with the reviewer's comments. We have cited the articles that you mentioned and further revised the Introduction (see Page 4, Line 106-108; Line 131-132).

6) It may be more meaningful to add functional research on key genes.

Reply: Thanks for your insightful suggestion. In our study, we verified top five mutation significantly mutated genes in TCGA HNSCC cohort were *TP53*, *TTN*, *FAT1*, *CDKN2A* and *MUC16*, we have searched for functional researches on these five key genes and added detailed discussion in the revised manuscript (see Page 12-13, Line 397-409).

7) It is recommended to increase the weighted gene co-expression network analysis.

Reply: As suggested by the reviewer, we further added the analysis of the weighted gene co-expression network in the text. We downloaded the transcripts per million (TPM) values of tumor and normal samples from the TCGA-HNSCC dataset and performed weighted gene co-expression network analysis (WGCNA) using the WGCNA R package. In the WGCNA analysis, a co-expression network was constructed according to the co-expression of all the genes in the samples, and 130 modules were identified, among which the MEroyalblue module was the most relevant to the normal samples, and the MElightyellow module was the most relevant to the tumor samples (see Page 8-9, Line 269-276).

8) How to judge the prognostic characteristics of HNSCC based on the results of this study? How to provide candidate targets for the treatment of HNSCC? It is recommended to include relevant descriptions in the discussion.

Reply: We agree with the reviewer's comments. Based on the results of this study, high level of CD8+ T cells was significantly corresponding to a preferable survival and CD4+ T cells was significantly associated with poor survival in HNSCC. Therefore, the expression level of CD8+ T cells and CD4+ T cells could be used as prognostic biomarkers for HSCNN patients in clinical applications. Furthermore, high immune score and stroma score corresponded to a better survival probability in HNSCC. Hence, the prognosis of patients can be stratified using the comprehensive immune approaches at the transcriptome level. We have added relevant descriptions in the Discussion (See Page 12, Line 375-380; Page 13-14, Line 430-448).

Reviewer B

1. Original articles should organize the main text in Introduction, Methods, Results, Discussion, and **Conclusions**. Please provide.

Reply: Thanks for kindly reminding us. We have provided the heading "Conclusions" in the revised manuscript (Page 13, Line 435).

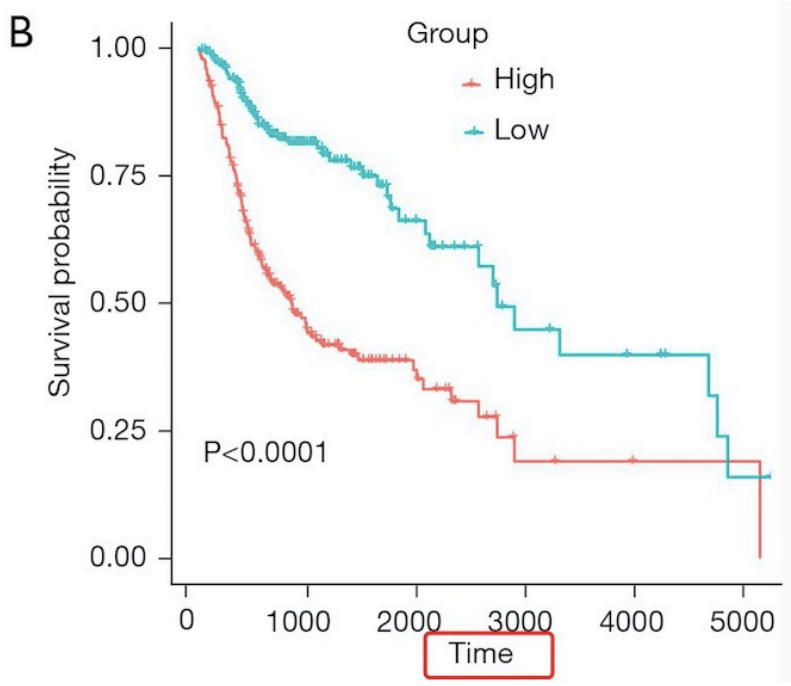
2. **Ref. 36 and 37 are not cited in the main text**. Please check and revise.

Reply: We are sorry for our careless mistakes. We have added the citations in Page 5, Line 144.

3. **Abbreviated terms** used in the figures/tables/legends should be defined in their legends. Please check all figures/tables and revise.

Reply: Thanks for your useful suggestions. We have checked and made further revisions.

4. Please provide the **unit of time** in Figure 2B. Please also check **other survival** plots.



Reply: We are sorry for our careless mistakes. We have added the unit of time in the revised figures (Figure 2-revised, Figure 3-revised and Figure 6-revised).

5. There is **no part C in figure 4**. Please also check the **legends and the citations of Figure 4 in the main text** to ensure they are correct.

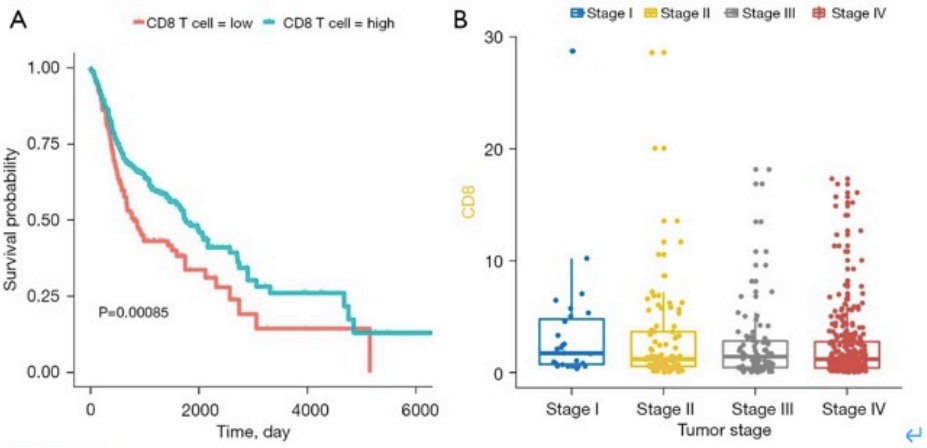


Figure 4 Immune therapy analysis. (A) TIDE predict ICB benefits show little effects on the survival of patients. (B) The expression level of CD8 is significantly related the survival of patients. (C) Expression level of CD8 in different HNSCC stages.

Reply: Thanks for your comments. We have further revised the legends of Figure 4 and also checked the citations in the main text.

6. The **P value in Figure 6D** is 0.00088. Please check and revise.

stages (Figure 6A,6B). A roughly increased stromal and immune scores with the advancing of tumor stage could be seen. There was a significant association between stromal (P=0.015) and immune scores (P=0.0088) and HNSCC patients survival probability (Figure 6C,6D).

Reply: We are sorry for our careless mistakes. We have further revised the relevant content in Page 11, Line 344.

7. Please check and confirm which one is the **title of Table S1**.

heatmap of HNSCC samples. (C) Heatmap plot of genes differentially expressed in tumor and normal tissues.

Table S1 Clinicopathological information for the TCGA data sets.

Supplementary Table 1. Baseline patient characteristics of the 528 HNSCC patients.

Reply: Thanks for kindly pointing this out. We have confirmed and made relevant revisions.

8. Ethical Statement

For research involving human, authors should also state that the study conformed to the provisions of the Declaration of Helsinki (as revised in 2013), available at: https://www.wma.net/wp-content/uploads/2016/11/DoH-Oct2013-JAMA.pdf

Reply: Thank for your suggestion. We have added relevant statements in the revised manuscript (see Page 6, Line 182-183; Page 14, Line 471-472).