Peer Review File

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Review Comments

Comment 1: What is the expression of PD-L1 in patients with surgically removed esophageal cancer? How can patients be further classified based on the status of tumor infiltrating lymphocytes? Can drugs targeting PD-1/PD-L1 pathway be used for treatment?

Reply 1: Thank you for your interest in immunotherapy for esophageal cancer! Programmed cell death 1 ligand 1 (PD-L1), also known as cluster of differentiation 274 (CD274), is a protein in the human body and is encoded by the CD274 gene. The expression of PD-L1 in patients with surgically resected esophageal cancer refers to the genetic testing of the resected tissue after esophageal cancer surgery to assess whether there are PD-L1 related targets for further immunotherapy. In recent years, immune checkpoint inhibitors targeting PD-L1 and PD-1 have shown encouraging clinical effects in the treatment of advanced non-small cell lung cancer, kidney cancer, melanoma and other malignant tumors. Related immunotherapy involving esophageal cancer is still in the preliminary research stage. At present, some related inhibitors have entered clinical trials and have shown long-lasting anti-tumor activity and controllable adverse reactions[1-3]. In this study, we evaluated the tumor infiltrating immune cells (TIICs) of patients with esophageal cancer based on bioinformatics technology, but we have not yet classified them further. In order to answer your question, we retrieved a report combining TIICs and clinical results to classify patients. The study calculated the correlation between lymphocytes and patients' clinical results based on some statistical methods to guide clinical treatment[4]. At the same time, we will actively explore the classification method of tumor infiltrating

lymphocytes in the follow-up research to better guide clinical diagnosis and treatment.

Comment 2: Cancer immunotherapy has completely changed the way of cancer treatment. Immunotherapy largely relies on a comprehensive understanding of the immune landscape of the tumor microenvironment. How to use single-cell transcriptome analysis to study all aspects of immune cells in these tumor microenvironments?

Reply 2: To assess the stromal cells and immune cells in tumor samples for their infiltration, Yoshihara et al. designed an algorithm known as ESTIMATE (Estimation of Stromal and Immune cells in Malignant Tumor tissues using Expression data) to predict the number of stromal cells and immune cells in tumor samples based on the unique characteristics of the transcriptional profiles of cancer samples. And to predict the infiltration of non-tumor cells, single-sample gene set enrichment analysis (ssGSEA) was conducted for calculating the stromal and immune scores. The 20th reference in the article details the method used in this study.

Comment 3: What is the relationship between the integrative stemness characteristics and the prognosis and immune microenvironment in esophageal cancer?

Reply 3: In this study, we investigated genes and immune factors in the tumor microenvironment (TME) of ESCC and EAC that can serve as prognostic biomarkers. To determine the correlations between TIIC infiltration and prognosis in EC, ESCC and EAC patients were divided into the high- and low-infiltration groups according to the median proportion of TIICs. High levels of infiltrating resting DCs and CD8+ T cells predicted shorter OS in ESCC ($P<0.05$, Figure 6A,B), while high levels of infiltrating naïve B cells, activated mast cells, and resting memory CD4+ T cells

predicted shorter OS in EAC (P<0.05, Figure 6C-D). **You can quickly find it in the highlighted part of the results section**.

Comment 4: Current research on esophageal cancer focuses on deciphering the characteristics of immune cells in the tumor microenvironment. However, what is the spectrum of immune cells caused by disease in the para-tumor compartment?

Reply 4: This is a question worth thinking about. **Our research aims to explore the differences in the immune microenvironment between esophageal squamous cell carcinoma and esophageal adenocarcinoma tissues, and to find factors with prognostic value.** In this study, we extracted the public data of esophageal cancer patients from the TCGA database, and used the ESTIMATE algorithm to calculate the scores of stromal cells and immune cells in the patient's tumor tissues to find differentially expressed genes with prognostic value. In addition, the CIBERSORT algorithm was used to analyze 22 tumor-infiltrating immune cell subgroups to determine the immune cell types related to the survival time of patients with esophageal cancer. **There is no doubt that a more detailed study of paracancerous tissue is an interesting perspective**. Thank you for your constructive comments. We will discuss the para-tumor tissues, and the related research will be solved in future publications.

Comment 5: The data in this study all come from genetic and molecular information, which lacks clinical research verification.

Reply 5: Thank you for your constructive comments on this study. It is critical to use human tissue samples to verify the expression and prognostic value of prognostic factors. Due to the limited length of the article and considering the time spent in the experiment, we aim to introduce the application of the ESTIMATE algorithm and a

variety of bioinformatics tools in screening biomarkers. However, we have actively started the exploration of these key genes and immune cells in human tissues, and these experiments will be resolved in time in our future publications. **At the end of the article, we pointed out the limitations of the study based on your suggestions. You can quickly find it in the highlighted part of the discussion section.**

Comment 6: Cancer stem cells induce tumor metastasis and recurrence. But what is the role of cancer stem cells in shaping the tumor immune microenvironment?

Reply 6: In recent years, the interaction between cancer stem cells and their immune microenvironment has gradually been clarified, and it has been discovered that targeting cancer stem cells and their immune microenvironment is expected to become a new strategy for cancer treatment. Studies have shown that cancer stem cells are resistant to the toxic effects of Cytotoxic T lymphocytes (CTL) cells in the immune microenvironment. Many cancer stem cells can evade immune recognition and CTL-mediated killing by low expression of MHC-I[5]. In addition, many studies have shown that tumor stem cells can have synergistic or antagonistic effects with natural killer cells, regulatory T cells, macrophages, B lymphocytes and other immune cells in the tumor immune microenvironment, thereby affecting the formation of the tumor immune microenvironment. For example, regulatory T cells in the immune microenvironment can secrete VEGFA, promote the formation of blood vessels in the hypoxic microenvironment, and provide rich nutrients for cancer stem cells. At the same time, VEGFA binds to the VEGFR on the surface of cancer stem cells to quickly activate VEGFR/JAK2 /STAT3 signaling pathway induces the expression of stem pluripotent transcription factors MYC and Sox2, thereby promoting the self-renewal of cancer stem cells[6]. Other relevant documents are as follows[7-10].

Comment 7: The samples of this study are relatively small, and it is easy to miss important information.

Reply 7: Indeed, a small sample size may bias the results. **At the end of the article, we pointed out the limitations caused by the small sample size.** Overall, the RNA-Seq and clinical data of patients with esophageal cancer are less than those of other tumors. **We collected all available data on esophageal cancer patients in the TCGA database**, including 95 cases of esophageal squamous cell carcinoma and 87 cases of esophageal adenocarcinoma, in order to minimize the bias of the results. With the popularization of second-generation sequencing technology and the diversification of esophageal cancer diagnosis and treatment measures, it is believed that more and more data are available for esophageal cancer in the future, and we will continue to follow up related research.

Comment 8: How to study the mechanism of esophageal cancer metastasis and epithelial mesenchymal transition through methylation-induced gene expression changes?

Reply 8: Thank you for your comments to expand my horizons. Through searching the literature, we believe that methylation-induced genes affect certain key biological processes of esophageal cancer cells by regulating different upstream and downstream signaling pathways [11]. The regulation of cellular processes may lead to esophageal cancer metastasis and epithelial-mesenchymal transition. We will continue to learn and research knowledge in related fields to provide a richer theoretical basis for guiding the diagnosis and treatment of esophageal cancer.

We appreciate for the Editors' and Reviewer's time earnestly, and hope that the revision will meet with approval.

References

[1] Hamid O, Robert C, Daud A, et al. Safety and tumor responses with lambrolizumab (anti-PD-1) in melanoma [published correction appears in N Engl J Med. 2018 Nov 29;379(22):2185]. *N Engl J Med*. 2013;369(2):134-144. doi:10.1056/NEJMoa1305133.

[2] Doi T, Piha-Paul SA, Jalal SI, et al. Safety and Antitumor Activity of the Anti-Programmed Death-1 Antibody Pembrolizumab in Patients With Advanced Esophageal Carcinoma. *J Clin Oncol*. 2018;36(1):61-67. doi:10.1200/JCO.2017.74.9846

[3] Shah MA, Kojima T, Hochhauser D, et al. Efficacy and Safety of Pembrolizumab for Heavily Pretreated Patients With Advanced, Metastatic Adenocarcinoma or Squamous Cell Carcinoma of the Esophagus: The Phase 2 KEYNOTE-180 Study. *JAMA Oncol*. 2019;5(4):546-550. doi:10.1001/jamaoncol.2018.5441

[4] Yagi T, Baba Y, Ishimoto T, et al. PD-L1 Expression, Tumor-infiltrating Lymphocytes, and Clinical Outcome in Patients With Surgically Resected Esophageal Cancer. *Ann Surg*. 2019;269(3):471-478. doi:10.1097/SLA.0000000000002616

[5] Morrison BJ, Steel JC, Morris JC. Reduction of MHC-I expression limits T-lymphocyte-mediated killing of Cancer-initiating cells. *BMC Cancer*. 2018;18(1):469. Published 2018 Apr 26. doi:10.1186/s12885-018-4389-3

[6] Zhao D, Pan C, Sun J, et al. VEGF drives cancer-initiating stem cells through VEGFR-2/Stat3 signaling to upregulate Myc and Sox2. *Oncogene*. 2015;34(24):3107-3119. doi:10.1038/onc.2014.257.

[7] Liu Y, Liang X, Yin X, et al. Blockade of IDO-kynurenine-AhR metabolic circuitry abrogates IFN-γ-induced immunologic dormancy of tumor-repopulating

cells. *Nat Commun*. 2017;8:15207. Published 2017 May 10. doi:10.1038/ncomms15207.

[8] Avril T, Vauleon E, Hamlat A, et al. Human glioblastoma stem-like cells are more sensitive to allogeneic NK and T cell-mediated killing compared with serum-cultured glioblastoma cells. *Brain Pathol*. 2012;22(2):159-174. doi:10.1111/j.1750-3639.2011.00515.x.

[9] Hou YC, Chao YJ, Tung HL, Wang HC, Shan YS. Coexpression of CD44-positive/CD133-positive cancer stem cells and CD204-positive tumor-associated macrophages is a predictor of survival in pancreatic ductal adenocarcinoma. *Cancer*. 2014;120(17):2766-2777. doi:10.1002/cncr.28774.

[10] Zhang X, Zhang Y, Xu J, et al. Antigen presentation of the Oct4 and Sox2 peptides by CD154-activated B lymphocytes enhances the killing effect of cytotoxic T lymphocytes on tumor stem-like cells derived from cisplatin-resistant lung cancer cells. *J Cancer*. 2018;9(2):367-374. Published 2018 Jan 1. doi:10.7150/jca.20821.

[11] Wei H, Liu Z, She H, et al. Promoter methylation and expression of Raf kinase inhibitory protein in esophageal squamous cell carcinoma. *Oncol Lett*. 2017;13(3):1866-