Peer Review File

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Review comments

Comment 1: The authors found that cluster 2, which has increased immune cell infiltration, is associated with poorer survival than cluster 1. This result is a little bit controversial. In most solid tumors, higher infiltration of immune cells is associated with better survival. The deconvolution analysis indicated C2 is associated with higher macrophages and Tregs gene signatures; however, what about CTLs? Also, if the authors perform a classification of MIBC solely based on the immune cell signature, what result will it be? Will CD8 gene signature be associated with better survival?

Reply 1:

In most solid tumors, higher infiltration of immune cells is associated with better survival. However, recent studies targeting the TME have found that several immune-infiltrating cells exhibit important immunosuppressive functions, including myeloid suppressor cells (MDSCs)(1), tumor-associated macrophages (TAMs), regulatory T cells (Tregs)(2), and tumors associated neutrophils (TANs)(3). Some TAMs show M1-like macrophage phenotype, especially in the early stage of tumorigenesis, expressing anti-tumor effect. By reprogramming TAMs, the vast majority of TAMs show an M2-like macrophage phenotype, which plays an important role in promoting tumorigenesis, progression and metastasis. In this study, all four types of cells were highly infiltrated in C2 cluster, which may affect its prognosis(4). In the study of Chaozhi Tang et al.(5), although the degree of immune cell infiltration was high, the prognosis was different from the traditional view due to the different types of infiltrating cells.

As a new favorite in the field of tumor immunity, CTLs are the first-choice immune cells for targeted cancer therapy. CTLs play a major role in destroying virus-infected cells and cancer cells. Naive CD8+ T cells proliferate and activate into CTLs through a series of chemokines and antigen presentation. CTLs then migrate to the TME through chemotaxis, resulting in tumor cell apoptosis(6). However, the ssGSEA used in this paper and other commonly used algorithms such as CIBERSORT cannot directly calculate the degree of infiltration, which is worth further exploration.

One of the aims of this paper is to explore the prognostic factors of MIBC. The starting point of the study is to classify MIBC from the perspective of ferroptosis, to describe the degree of infiltration of immune cells in different classifications, and to further explore the correlation between ferroptosis and immune cells in MIBC.

Of course, it is a good idea to use the immune cell signature alone to classify MIBC, and related scholars have already conducted research in bladder cancer(7).

In bladder cancer, Hualin Chen et al. also performed related analysis for prognostic analysis using CD8 signature(7). In this paper, using CD8 signature for univariate

COX prognostic analysis, we can get that its HR value is 0.85, and the P value is less than 0.05, indicating that CD8 gene signature is associated with better survival.
We have modified our text as advised (see Page 15, line 315).

Comment 2: Ferroptosis is one of the mechanisms of anti-tumor T cells-induced cell death. What is the relationship between ferroptosis-related prognostic genes with the fraction of T cell infiltration?

Reply2:
We used correlation analysis to study the relationship between ferroptosis prognosis-related genes and T cells in this study, and expressed it in the form of heatmap (Supplementary Figure 1). We found that IFNG, ZEB, SLC39A14, and AGPS were all positively correlated with the infiltration degree of activated CD8 T cells, and all were ferroptosis-promoting genes. The two genes, PLA2G6 and SRC, showed a negative correlation with activated CD8 cells, and they were both ferroptosis suppressor genes.
We have modified our text as advised (see Page 15, line 322).

Comment 3: Are these ferroptosis genes expressed most in tumor cells or immune cells? Any correlation between ferroptosis-related genes and other immune cells fraction data?

Reply 3:
In this study, the sequencing results of the TCGA-BLCA dataset were all obtained from solid tumors, not single-cell sequencing. Therefore, we cannot exactly determine the expression of ferroptosis genes in tumor cells or certain immune cells. However, the ferroptosis-related genes used in our study are all from The Molecular Signatures Database and FerrDb , which integrated relevant data from various studies. We consulted its sources and found that the expression of ferroptosis-related genes was mostly on solid tumor cells. In addition, the correlation between ferroptosis prognosis-related genes and other immune cells is shown in the Supplementary Figure 1.

Comment 4: What about the expression levels of ferroptosis-related genes? Are these genes expressed at a higher level, or are they expressed at pretty low levels and therefore subject to the significant variabilities between samples.

Reply 4:
We performed a differential analysis of tumor and normal samples for 41 ferroptosis prognostic genes, and the results were shown in the Supplementary Table 1. We found that 19 genes were differentially expressed. In tumor samples, SCD, TFRC, FADS1, SRC, and GCLM were highly expressed, while ZEB1, WWTR1, FZD7, CAV1, and TXNRD1 were lowly expressed.

Comment 5: There is the minimal discussion about the biological function of the top 5 pivotal genes. How will these genes contribute to the potential poor survival status associated with cluster 2 patients?

Reply 5:
Among 5 pivotal genes , SLC1A6, UPK3A, and SLC19A3 were risk factors for the prognosis of MIBC in this study, while CCL17 and UGT2B4 were protective factors. Through the difference analysis between C1 and C2, CCL17 and UGT2B4 were relatively low expressed in C2. Yuxin Li et al found that bladder cancer patients with high expression levels of CCL17 were associated with a signifcantly better prognosis (8), which is consistent with our study. Xu et al found that high expression of UGT2B4 is associated with low-grade prostate cancer, and UGT2B4 upregulation in tumors is associated with upregulation of metabolic pathways, such as novel IMP biosynthetic processes, glutamine and monocarboxylic acid metabolism(9). UP3KA is not only a prognostic risk factor, but Yongqing Lai et al. found that the measurement of UPK3A in urine is a sensitive new marker with good performance for detecting bladder cancer(10). SLC1A6, a member of the SLC1A family, encodes a transmembrane transporter that mediates L-glutamate and L/D-aspartate uptake, and its overexpression reduces the response of nasopharyngeal carcinoma radioresistant cells to cisplatin and radiation sensitivity. Xuan Zou et al. concluded that the expression level of SLC1A6 was an independent predictor of poor prognosis in bladder cancer patients through multivariate COX regression analysis(11). SLC19A3, which was relatively high expression in C2, encodes hTHTR2 and is mainly expressed in the intestine, liver, kidney and placenta. Mutations in it cause alkaloid-responsive basal ganglia disease and thiamine-responsive encephalopathy(12). However, research on UGT2B4 and SLC19A3 in bladder cancer is still lacking.
We have modified our text as advised (see Page 15, line 322).

Once again, we thank you for the time you put in reviewing our paper and look forward to meeting your expectations. Since your inputs have been precious, in the eventuality of a publication, we would like to acknowledge your contribution explicitly.

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