Peer Review File

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Reviewer A

Comment 1. The authors identified prognostic genes significantly correlated with OS. TCGA cohorts and external validation cohorts consists mainly patients with stage 1-2 RCC. Recent study performed single-cell RNA seq using samples from advanced RCC patients before immune checkpoint inhibitor therapy (K.Bi, et al. Cancer Cell. 2021; 39: 649-661). Results of this study have less meaningful.

Reply 1: Thanks for pointing out the limitation of our study, which is that the number of ccRCC patients with advanced stage was relatively small. While our signature demonstrated promising predictive performance in subgroups of ccRCC patients with advanced stages, as shown in Fig S2h and S2p, further studies are needed to validate its predictive capacity. K. Bi et al concentrated on metastatic RCC patients and revealed how ICB remodels the RCC microenvironment and modifies the interplay between cancer and immune cell populations. In our study, we attempted to build a model which can predict the prognosis of ccRCC patients. However, it's also our limitation that the underlying mechanisms are not clearly revealed and need further research.

Changes in the text: Part 4, paragraph 5, page 23, line 2-3.

Comment 2. Immune inhibitory molecules of several types of immune cells were higher in the NKMS high group than in the NKMS low group. The NKMS high group also showed decreased expression angiogenesis genes. In the ccRCC tumor microenvironment, NK cells are minority (S.Chevrier, et al. Cell. 2017; 169: 736-749). The author should give some references and/or convincing evidence to explain NK cells showed broad relationship with other immune cells and angiogenesis.

Reply 2: Thanks for the scientific concern of our study. Nature killer (NK) cells are cytotoxic lymphocytes which can kill tumor cells without prior sensitization. Regarding ccRCC, though the absolute amount of NK cells is relatively small among all of the tumor-infiltrating immune cells, several studies have addressed the critical role of NK cells in anti-tumor immunity in TME [1-4]. NK cells have also been considered as a potential target for immunotherapy [5]. Recent review has discussed the close relationship between NK cells and TME, including T cells, Tregs, DCs, neutrophils and macrophages [6]. The bridge between NK cells and other immune cells is mainly the

soluble mediators [6]. NK cells can either enhance or impair T cell responses in a direct or indirect way regulating the expression of activating or inhibitory receptors, cytokines, and chemokines [7]. Meanwhile, the antitumor function of NK cells could be inhibited by Tregs by inhibitory molecules secreted or expressed by Tregs [8]. Cazzetta et al reported that DCs release cytokines and chemokines to promote NK cell activation and recruitment to sites of inflammation, and activated NK cells produce IFN-γ, tumor necrosis factor α (TNF-α), and XCL1 to promote DC maturation and recruitment [9]. The cytokines mediated by macrophages, like IL-12, IL-15, IL-18 and TNF-α can promote antitumor cytotoxicity of NK cells while molecules secreted by NK cells, like IFN-γ can induce the differentiation of macrophages in the TME [10-11]. Taken together, we consider that NK cells are closely correlated with immune cells in the TME.

In aspects of angiogenesis, recent study have implied both positive and negative effects of NK cells on angiogenesis in RCC. Guan et al reported that NK cells tend to convert to a proangiogenic phenotype with higher expression of VEGF and PDGF, which may support early tumor growth and promote metastasis [12]. Hofmann et al found that NK cells could inhibit angiogenesis by producing interferon-gamma (IFN-g) and TNF-a, which suppressed HIFa and VEGF expression [13]. Meanwhile, antiangiogenic agents, like sunitinib, axitinib and sorafenib, were found able to increase the susceptibility of cancer cells to NK cell-mediated cytotoxicity [14-15].

I hope the evidence can show the broad correlation between NK cells and other immune cells and angiogenesis.

References:

[1] Schleypen J.S., et al. Renal cell carcinoma-infiltrating natural killer cells express differential repertoires of activating and inhibitory receptors and are inhibited by specific HLA class I allotypes. Int. J. Cancer. 2003;106:905–912. doi: 10.1002/ijc.11321.

[2] Schleypen J.S. Cytotoxic Markers and Frequency Predict Functional Capacity of Natural Killer Cells Infiltrating Renal Cell Carcinoma. Clin. Cancer Res. 2006;12:718–725. doi: 10.1158/1078-0432.CCR-05-0857.

[3] Murphy K.A., et al. Exploiting natural anti-tumor immunity for metastatic renal cell carcinoma. Hum. Vaccin. Immunother. 2015;11:1612–1620. doi: 10.1080/21645515.2015.1035849.

[4] Prinz P.U., et al. NK-cell dysfunction in human renal carcinoma reveals diacylglycerol kinase as key regulator and target for therapeutic intervention. Int. J. Cancer. 2014;135:1832–1841. doi: 10.1002/ijc.28837.

[5] Terrén I, et al. NK Cell-Based Immunotherapy in Renal Cell Carcinoma. Cancers (Basel). 2020 Jan 29;12(2):316. doi: 10.3390/cancers12020316.

[6] Zhou Y, et al. NK cells are never alone: crosstalk and communication in tumour microenvironments. Mol Cancer. 2023 Feb 16;22(1):34. doi: 10.1186/s12943-023-01737-7.

[7] Crouse J, et al. NK cells regulating T cell responses: mechanisms and outcome. Trends Immunol. 2015 Jan;36(1):49-58. doi: 10.1016/j.it.2014.11.001.

[8] Liu W, et al. CCR4 mediated chemotaxis of regulatory T cells suppress the activation of T cells and NK cells via TGF-β pathway in human non-small cell lung cancer. Biochem Biophys Res Commun. 2017;488:196–203. doi: 10.1016/j.bbrc.2017.05.034.

[9] Cazzetta V, et al. Natural Killer-Dendritic Cell Interactions in Liver Cancer: Implications for Immunotherapy. Cancers (Basel) 2021;13:2184. doi: 10.3390/cancers13092184.

[10] Gaggero S, Witt K, et al. Cytokines Orchestrating the Natural Killer-Myeloid Cell Crosstalk in the Tumor Microenvironment: Implications for Natural Killer Cell-Based Cancer Immunotherapy. Front Immunol. 2020;11:621225. doi: 10.3389/fimmu.2020.621225.

[11] O'Sullivan T, et al. Cancer immunoediting by the innate immune system in the absence of adaptive immunity. J Exp Med. 2012;209:1869–82.

[12] Guan Y, et al. Renal cell tumors convert natural killer cells to a proangiogenic phenotype. Oncotarget. 2020 Jun 30;11(26):2571-2585. doi: 10.18632/oncotarget.27654. PMID: 32655841;

[13] Hofmann R, et al. Natural killer cells in patients with renal cell cancer. Urol Int. 1985;40(5):251-6. doi: 10.1159/000281093.

[14] Morelli M.B., et al. Axitinib induces DNA damage response leading to senescence, mitotic catastrophe, and increased NK cell recognition in human renal carcinoma cells. Oncotarget. 2015;6:36245–36259. doi: 10.18632/oncotarget.5768.

[15] Moeckel J., et al, Ullrich E. Sunitinib does not impair natural killer cell function in patients with renal cell carcinoma. Oncol. Lett. 2017;14:1089–1096. doi: 10.3892/ol.2017.6187.

Changes in the text: We have added correlation between NK cells and immune cells in introduction. As seen in part1, paragraph 2, page 4, line 20-22 and page 5, line 3-5.

Reviewer B

Comment 1: Only two ccRCC cases were included in the published scRNAseq dataset used in the study. The sample number is too small to draw meaningful conclusions. There are more of scRNAseq studies on ccRCC recently published that the author group may consider being included.

Reply 1: We thank the reviewer for pointing this out. We have included GSE159115, which contained 7 ccRCC samples, in the revised version of our research to enhance the robustness of our findings. As a result, the number of qualified single-cell samples for scRNA-seq analysis has increased from 11,018 to 20,074. Furthermore, the number of NK cell marker genes identified by scRNA-seq analysis for signature construction increased from 44 to 52, and the signature itself has been refined. Finally, we have removed HCST and added SH3BGRL3 to our signature, and all results based on our signature have been reanalyzed.

Changes in the text: Part 2.1, all the results in part 3, paragraph 2 in discussion, all the figures.

Comment 2: The 7 gene signature mixes risk and protective genes. It would be better to evaluate the risk genes and protective genes separately to give better prognostic scores and more clear interpretations.

Reply 2: We appreciate your input regarding our signature construction. To screen for potential prognostic genes, we first used univariate Cox regression analysis on 52 NK cell markers. Subsequently, the least absolute shrinkage and selection operator (LASSO) Cox proportional hazards regression and the stepwise multivariate Cox regression analyses were used to determine the most predictive genes. At last, our signature comprised 7 genes out of the initial 52 markers. We integrated the risks and protective effects of these genes into our signature to improve the prognostic accuracy of ccRCC patients. The coefficients of protective genes were negative, and those of risky genes were positive. We also explored the construction of models using either risk genes or protective genes exclusively, but observed no improvement in predictive ability compared to the mixed model. If needed, we can provide detailed information on these separate models.

Changes in the text: None.

Comment 3: PLAC8 is upregulated in the groups with higher risk factors in one of the validation studies Fig4a. Is there any interpretation of this result?

Reply 3: We are so grateful for your kind question. As a part of our constructed signature, PLAC8 serves as a risk gene and is upregulated in the high-risk group of TCGA-KIRC, the training set. Meanwhile, in both univariate and multivariate Cox regression analyses, hazard ratio (HR) of PLAC8 was significantly larger than 1, which means higher PLAC8 expression was correlated with poor prognosis. In both E-MTAB-1980 and RECA-EU validation cohorts, significantly higher expression of PLAC8 was enriched in high-risk group, and this was in accordance with the survival analysis that patients with high-risk scores suffered poor overall survival. In addition, recent study reported that overexpression of PLAC8 was correlated with advanced tumor progression and impaired prognosis of ccRCC, which is consistent with our findings [1].

Reference: [1] Shi, L. et al. Overexpression of placenta specific 8 is associated with malignant progression and poor prognosis of clear cell renal cell carcinoma. Int Urol Nephrol 49, 1165-1176, doi:10.1007/s11255-017-1578-y (2017).

Changes in the text: None.

Comment 4: The nomogram is hard to explain in Fig5e. What are the points and total points? How does the 1-3 years expectation fit into the plot?

Reply 4: We appreciate the reviewer for bringing to our attention the issue with the readability of the nomogram in Fig 5e. We acknowledge that we did not put sufficient effort into improving its clarity. We conducted multivariate analysis and incorporated the independent risk factors in the nomogram. The nomogram is a graphical tool that maps predicted probabilities, based on age, grade, stage, and risk score in our study, onto a scale from 0 to 100. The total points accumulated by various covariates correspond to the predicted probability for a patient. [1]

For instance, consider a patient with clear cell renal cell carcinoma (ccRCC) who is 35 years old, at stage 3, grade 3, and has a risk score of -1.0. This patient would receive 10 points for their age, 30 points for their stage, 15 points for their grade, and 32 points for their risk score. The total score would be 87, which corresponds to an estimated 1-year, 3-year, and 5-year survival probability of approximately 95%, 86%, and 80%,

respectively. This nomogram was also validated in E-MATB-1980 with a concordance index of 0.831, which indicates the capacity of clinical use.

Reference: [1] Iasonos A, Schrag D, Raj GV, Panageas KS. How to build and interpret a nomogram for cancer prognosis. J Clin Oncol. 2008 Mar 10;26(8):1364-70. doi: 10.1200/JCO.2007.12.9791. PMID: 18323559.

Changes in the text: Part 2.4, page8, line 24 and page9, line 1-3.

Comment 5: Fig7 g-h these plots have been established before and are not directly related to the NKMS analysis. It would be great to move them to the supplementary figures.

Reply 5: Thanks for the reviewer's suggestion. We have moved the figures to Fig S3 e-g.

Changes in the text: The figures mentioned was moved to Fig S3 e-g.

PS: We apologize for the misspell and misuse of colors in our figures. The misspell was corrected in Fig 3h and the colors of high and low risk groups in Fig 5 were flipped.