Peer Review File

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Reviewer A

In this study the authors aimed to evaluated the gene expression patterns and potential functional enrichment analysis of cuproptosis regulators in hepatocellular carcinoma (HCC) using two very large databases (TCGA and ICGC). According to cuproptosis-related genes differentially expressed in normal and HCC tissues, two subtypes were identified and HCC patients with these two subtypes were found associated to different clinical prognoses and immune characteristics, as well as different degrees of response to immunotherapy. LIPT1, DLAT, and CDKN2A were selected to construct a prognostic signature, which significantly distinguished HCC patients with different survivals. The risk score of the prognostic signature was confirmed to be an independent prognostic factor, and nomograms were generated to effectively predict the probability of HCC patient survival. Interestingly, qRT-PCR, Western blotting and IHC results, revealed a significant imbalance in the expression of these cuproptosis-related genes in HCC. They concluded that the classification and prognostic signature based on cuproptosis-related regulatory genes helps to explain the heterogeneity of HCC, which may contribute to the individualized treatment of patients with the disease. The study is of interest, since the identification of potential signatures and markers able to predict treatment response is a topic of current major relevance. I have only a few comments:

Comment 1: "*Since it is well-known that the etiology and severity of underlying liver disease may affect treatment response to HCC treatments, it would be of potential interest adding the characteristics of enrolled patients.*"

Reply 1: We appreciate your suggestion. According to your suggestion, we have added the characteristics of enrolled patients in Table 2 and Table S2.

Changes in the text: Table 2 and Table S2.

Comment 2: *"To further improve the clinical/therapeutic impact of this study, the authors should recall and discuss potential clinical settings where prognostic signature based on cuproptosis-related regulatory genes might have a role in treatment allocation. For instance, it has been previously suggested the role and pharmacology of cuproptosis in gastric cancer were combination treatment with capecitabine is effective (doi: 10.3389/fonc.2023.1145446). Of interest, it has been previously demonstrated that metronomic capecitabine is also effective and safe in patients with advanced HCC after first-line sorafenib failure, as previously demonstrated (Dig Liver Dis. 2015 Jun;47(6):518-22. doi: 10.1016/j.dld.2015.03.010; J Cancer Res Clin Oncol. 2018 Feb;144(2):403-414. doi: 10.1007/s00432-017-2556-6.), thus suggesting that the cuproptosis-related signature should be explored as a potential predictor of response to metronomic capecitabine."*

Reply 2: We appreciate your suggestion. According to your suggestion, we have

added relevant discussion contents and references in the discussion section. (see Page 19 and 20, line 421-428)

Changes in the text: We calculated the IC50 values for multiple targeted agents to assess the sensitivity of patients and found that both molecular subtypes had their respective sensitive targeted agents, which may indicate that cuproptosis-related regulatory genes play a potential role in it. Clinical trials have reported that Axitinib, ATRA and Veliparib are effective or well tolerated in the treatment of patients with HCC (47-49). In addition, Luminespib, Ponatinib, Akt inhibitor VIII, AICAR and Saracatinib have also been proved to inhibit the malignant phenotype of HCC cells in vitro or in vivo experiments (50-54). Collectively, these findings may provide more suitable personalized treatment options for HCC patients. However, the role of cuproptosis-related regulatory genes in these treatment allocations needs to be further clarified.

Comment 3: *"Regarding the response to immunotherapy, it would be of relevance to report which immunotherapy was investigated (immune checkpoint inhibitors, CTLA-4, PD-1, PD-L1?). The discussion could further improve recalling the emerging role of combination treatment strategy based on immune checkpoint inhibitors plus tyrosine kinase inhibitors to obtain a higher rate of objective tunmor response and longer overall survivale as well described in a recent comprehensive review (Expert Rev Anticancer Ther. 2023 Mar;23(3):279-291. doi: 10.1080/14737140.2023.2181162.)."*

Reply 3: We appreciate your comments. Our analysis of the response of different clusters of HCC patients to immunotherapy is based on the differences in the expression of immune checkpoints in tumor tissues of these patients and the score of TIDE database. In the analysis of the differential expression of immune checkpoints, we have included 47 related immune checkpoints (IDO1, LAG3, CTLA4, TNFRSF9, ICOS, CD80, PDCD1LG2, TIGIT, CD70, TNFSF9, ICOSLG, KIR3DL1, CD86, PD-1, LAIR1, TNFRSF8, TNFSF15, TNFRSF14, IDO2, CD276, CD40, TNFRSF4, TNFSF14, HHLA2, CD244, PD-L1, HAVCR2, CD27, BTLA, LGALS9, TMIGD2, CD28, CD48, TNFRSF25, CD40LG, ADORA2A, VTCN1, CD160, CD44, TNFSF18, TNFRSF18, BTNL2, C10orf54, CD200R1, TNFSF4, CD200 and NRP1), including CTLA-4, PD-1 and PD-L1 you mentioned. In addition, according to your suggestion, we further reviewed the emerging role of the combined therapy strategy based on immune checkpoint inhibitors and tyrosine kinase inhibitors in the discussion, in order to obtain a higher objective tumor response rate and a longer overall survival period. (see Page 19, line 411-417).

Changes in the text: Notably, the recent clinical trials have found that the combination of atezolizumab and bevacizumab can simultaneously target the two key pathogenic hallmarks of HCC immune escape and angiogenesis to achieve a higher objective tumor response rate and a longer overall survival (42-44). This combined treatment strategy has gradually become the first-line treatment therapy for advanced HCC (45). Therefore, we speculate that HCC patients in cluster 2 may also obtain better prognosis by combining tyrosine kinase inhibitors

targeting angiogenesis such as bevacizumab on the basis of immunotherapy.

Reviewer B

Comment 1: "*You refer to "studies" with only one literature citation couple times. Please check and revise.*"

Reply: We appreciate you pointing this out. We have carefully checked across the whole paper about "studies" with only one literature citation and the corresponding references were supplemented.

Comment 2: "*Subfigures should be cited consecutively. For example, Figure 5D should be cited before 5H, unless Figure 5 is cited first.*"

Reply: We appreciate your suggestion. According to your suggestion, we have changed the picture arrangement order to ensure the subfigures cited consecutively.

Comment 3: "*Add the scale bar or magnification of Figure 2H*"

Reply: We appreciate your suggestion. According to your suggestion, we have added the scale bar of Figure 2H.

Comment 4: "*Provide a figure caption for subfigure 5L*"

Reply: We appreciate your suggestion. According to your suggestion, we have provided a figure caption for subfigure 5L.

Comment 5: "*Add the age unit in Table 2, Table S2, Figure 3, and Figure S3.*"

Reply: We appreciate your suggestion. According to your suggestion, we have added the age unit "years" in Table 2, Table S2, Figure 3, and Figure S3.

Comment 6: "*Words are not showing completed in some figures. Please check and revise.*"

Reply: We appreciate you pointing this out. We have checked all the figures and corrected figures with incomplete word display such as Figure S2, Figure 3, Figure 5 and Figure 6.

Comment 7: "*Abbreviations should be spelled out on first occurrence in Abstract/ Main Text/ Highlight Box/ Figure/ Table/ Supplementary.*"

Reply: We appreciate your suggestion. According to your suggestion, we have checked across the whole paper to ensure the abbreviations spelled out on first occurrence in Abstract/ Main Text/ Highlight Box/ Figure/ Table/ Supplementary.

Comment 8: "*There are no blue dots in Figure 2B*"

751 column represents the frequency of alteration. Deletion frequency, blue dots;
752 magnification frequency, red dots. (C) The CNV alteration locations of the

Reply: We appreciate you pointing out this important issue. We have re-written the relevant description in the figure legend of Figure 2B (line 776).

Comment 9: "*Add the title/unit of the Y-axis in each subfigure of Figure 2G.*"
Reply: We appreciate your suggestion. According to your suggestion, we have added the title of the Y-axis in each subfigure of Figure 2G.

Comment 10: "*Indicate "normal liver" and "HCC tissues" in Figure 2H.*"
Reply: We appreciate your suggestion. According to your suggestion, we have indicated "Normal liver" and "HCC tissues" in Figure 2H.

Comment 11: "*Figure 3E is about PFS while 3F is OS. Please check and revise.*"
Reply: We appreciate you pointing out this important issue. According to your suggestion, we have checked and revised the relevant descriptions of Figure 3E and Figure 3F in the main text (line 297-300) and figure legend (line 796).

Comment 12: "*It should be ≤65 and >65 according to Figure 3G and S3.*"

284 expression profiles and clinicopathological parameters including age (<65 or ≥65
285 years), sex, tumor grade (G1–G4), tumor stage (I–IV), and Tumor Node Metastasis

Age (years) (%)	< 65
	≥ 65

Reply: We appreciate you pointing out this important issue. According to your suggestion, we have changed "≤65 and >65" to "< 65 and ≥65" in Table 2, Table S2 and main text (line 304).

Comment 13: "*The value does not match Figure 4I.*"

346 (ATRA) (p = 0.0016), Ponatinib (p = 0.037), AKT.inhibitor.VIII (p < 0.001), Acadesine

Reply: We appreciate you pointing out this important issue. We have checked and corrected the relevant values in the main text (line 366-367).

Comment 14: "*Check the spelling "score" in Figure 6.*"
Reply: We appreciate you pointing out these typo errors. We carefully checked all contents of the manuscript and corrected these errors.

Comment 15: "*Check whether (%) should be removed from Figure 7G.*"
Reply: We appreciate your suggestion. According to your suggestion, we have removed (%) from Figure 7G.

Comment 16: "*Add the observation method of Figure 8B.*"
Reply: We appreciate your suggestion. According to your suggestion, we have added statistical charts to the results of Figure 8B and relevant descriptions in the

material method section of main text (line 230-244).

Comment 17: "*Rewrite characteristics in Table 2 and S2, indicate how the data is presented, and add the unit {e.g., Albumin_result_specified_value [mean (SD)] -> Albumin (g/dL) [mean (SD)]; Child_pugh_classification_grade (%) -> Child Pugh Classification Grade, N (%)}.*"

Reply: We appreciate your suggestion. According to your suggestion, we have rewritten the characteristics in Table 2 and S2.

Comment 18: "*Numbers do not add up in Table 2.*"

Histological grade (%)	G1	45 (15.85)	10 (11.63)
	G2	137 (48.24)	40 (46.51)
	G3	91 (32.04)	30 (34.88)
	G4	8 (2.82)	4 (4.65)
	NA	3 (1.06)	1 (1.16)

Reply: We appreciate you pointing out this important issue. We have checked and corrected the numbers in Table 2 and Table S2.

Comment 19: "*Add the title/unit of heatmap legend (Figure 2D, 3G, 5G, 5H, 6E, 6F, S1, and S3).*"

Reply: We appreciate your suggestion. According to your suggestion, we have added the description of Figure 2D, 3G, 5G, 5H, 6E, 6F, S1, and S3 heatmap in corresponding Figure legend.

Comment 20: "*Red usually represents "dead". Please revise Figure S3.*"

Reply: We appreciate your suggestion. According to your suggestion, we have revised the colors of "dead" and "alive" in Figure 3 and Figure S3.