Peer Review File

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Reviewer A

The development of high-throughput sequencing technology combined with bioinformatics analysis, multiple molecular markers have been identified for the construction of prediction models and the development of therapeutic targets to predict HCC outcome in terms of hypoxia-related genes, The data was validated with TCGA and GEO cohorts

However, there were at least three similar papers from the literature bases: An Inflammatory Response-Related Gene Signature Can Impact the Immune Status and Predict the Prognosis of Hepatocellular Carcinoma.

Front Oncol. 2021 Mar 22;11:644416.

Identification of Seven-Gene Hypoxia Signature for Predicting Overall Survival of Hepatocellular Carcinoma.

Front Genet. 2021 Apr 9;12:637418

Identification of a six-gene signature predicting overall survival for hepatocellular carcinoma. Cancer Cell Int. 2019 May 21;19:138.

Please answer the questions:

Q1: Page 2 , line 54-57. The cited reference was too old to reflect the current HCC status.

Q2: The data related to hypoxia was similar to other papers. Is there possible using another tool to validate the hypothesis, such as cell lines experiment, proteomics, or treatment model.

Q3 Nomogram was a good presentation model, but another cohort will be better for validation.

Comment 1. Page 2, line 54-57. The cited reference was too old to reflect the current HCC status.

Reply: Thank you very much for your valuable comments. Here, we apologize for not being able to cite more recent literature to reflect the current HCC status. As suggested by the reviewer, we have cited the following relatively new literature to reflect the current state of HCC. (see Page 4, line 72-75) Thank you again for your time and effort reviewing our manuscript.

[4] Omata M, Cheng AL, Kokudo N, et al. Asia-Pacific clinical practice guidelines on the management of hepatocellular carcinoma: a 2017 update. Hepatol Int. 2017;11(4):317-370.

[5] Jin C, Li Y, Su Y, et al. Novel copper complex CTB regulates methionine cycle induced TERT hypomethylation to promote HCC cells senescence via mitochondrial SLC25A26. Cell Death Dis. 2020;11(10):844.

[6] Chen Q, Li F, Gao Y, Xu G, Liang L, Xu J. Identification of Energy Metabolism Genes for the Prediction of Survival in Hepatocellular Carcinoma. Front Oncol. 2020;10:1210.

[7] Fu J, Wang H. Precision diagnosis and treatment of liver cancer in China. Cancer Lett. 2018;412:283-288.

Changes in the text: see Page 4, line 72-75

Comment 2. The data related to hypoxia was similar to other papers. Is there possible using another tool to validate the hypothesis, such as cell lines experiment, proteomics, or treatment model.

Reply: Thank you very much for your valuable comments. Your comments have provided us with an important suggestion for a future study. Here, we apologize for not being able to conduct experiments to validate the findings.

It has been documented hypoxia is associated with poor prognosis and therapeutic resistance in HCC patients. Moreover, high-throughput sequencing and data analysis have gradually become more significant tools for biomedical research, which can identify biomarkers for prognosis predicting, recurrence monitoring as well as clinical stratification. Therefore, there is an increasing number of analogue studies which used bioinformatics analyses.

The present study is the first part of an extensive research conducted by our team. The reason we conducted this study is that a systematic study on the relationship between currently known hypoxia-related genes and the prognosis of HCC patients has rarely been reported. Therefore, we collected a total of 200 hypoxia-related genes based on previous literature to construct a prognostic risk model for early diagnosis and accurate prognostic prediction of HCC. To validate the accuracy of the risk model stem from the TCGA database, we analyzed the model in the GEO cohort. However, relying solely on bioinformatics analysis had limited predictive ability. Therefore, further experiments are necessary to test this hypothesis as proposed by reviewer.

Here, we sincerely thank you for giving us such a detailed experimental protocol for verification. In the second component of an extensive research, we will collect more experimental and clinical data to validate these findings, including hypoxia-related genes expression and function. In addition, due to the large number of targeted genes and relatively short follow up period, we need more time to confirm the prognostic value of hypoxia-related genes in HCC. It is hoped that we may aggregate more reliable and meaningful conclusions to submit to ***Translation Cancer Research*** after entire study completion. In the revised manuscript, we have added a description of the defect that this study failed to be verified by experiments. (see Page 20, line 433-436) Thanks again for your review and suggestions sincerely.

Changes in the text: see Page 20, line 433-436

Comment 3. Nomogram was a good presentation model, but another cohort will be better for validation.

Reply: Thank you very much for your valuable comments. Your comments have provided us with a very good inspiration for the further research. Here, we apologize for not being able to plot nomogram for validation using GEO dataset (validation cohort).

In this study, the reason why our nomogram constructed based on the TCGA database failed to be verified by an external database is the lack of clinical data of HCC samples in the GSE54236 dataset. In future research, we will strive to find a more

comprehensive dataset of clinical data to verify our nomogram. In the revised manuscript, we have added a description of the defect that these findings failed to be verified by a larger cohort. (see Page 20, line 430-433) Thanks again for your constructive comments sincerely.

Changes in the text: see Page 20, line 430-433

Reviewer B

The manuscript entitled "Construction and Validation of a Hypoxia-related Gene Signature for

Predicting Prognosis and Evaluating the Immune Microenvironment in Patients with Hepatocellular Carcinoma" by Wang et al try to construct a prediction model for predicting HCC prognosis using six hypoxia-related genes, facilitating the diagnosis and treatment of HCC.

In general, the experimental design is not logically serious. The rationale of this study is flawed: they try to find markers for early diagnosis by using hypoxia genes --- as we knew, the liver has sophisticated blood vascular network and the arterial blood supply in HCC significantly decreases as the stage and histologic grade progressing advanced --- although that does not mean no-hypoxia in early HCC. Therefore, it may not an effective way for HCC diagnosis using hypoxia related genes. Some results are over-interpreted. Not experimental approaches to support the conclusion made in this study. Although this study leads to some interesting finding, the prognostic value of the 6 hypoxia genes needs to be evaluated more carefully.

Comment 1. As said, the rational of this study is flawed.

Reply: Thank you very much for your valuable comments. Your constructive criticisms have provided us with an important suggestion for a future study. Here, we are sorry for the irrationality of this study.

By reviewing the literature, we learned that hypoxia (insufficient oxygen) is always present in tumor areas due to the excessive proliferation of tumor cells and the increasing oxygen consumption. Tumor hypoxia is strongly associated with malignant

progression, poor prognosis, and resistance to chemotherapy and radiation. The biological features of hypoxic tumor cells are closely related to altered gene expression under an oxygen-deficient condition. For example, hypoxia modulates the malignant phenotype of tumor cells through HIF-1α, which regulates the expression of numerous target genes. Moreover, HIF-1α regulates angiogenesis-related genes against hypoxia. Therefore, many investigators used bioinformatic methods to explore the relationship between the expression levels of hypoxia-related genes and prognosis of solid cancer patients.

Based on this background, we conducted the present study to assess the prognostic value of hypoxia-related genes and constructed a gene-based prognosis prediction model for patients with HCC. However, owing to our insufficient understanding of the pathogenesis of HCC, this study has some limitations in the design and some too far-fetched explanations. Therefore, in a future study, we will collect more experimental and clinical data to investigate the association between the expression levels of hypoxia-related genes and the development of HCC, especially the six hypoxia-related genes used for the construction of the prognostic model. Besides, we will learn more knowledge on the pathological mechanism of HCC. In the revised manuscript, we have added a summary to describe the main flaws of this research. (see Page 20, line 427)

Once again, thank you sincerely for your useful critiques and constructive guidance.

Changes in the text: see Page 20, line 427

Comment 2. All the studies are in silico-based analyses. As we knew, both hypoxia and immune checking points are associated with prognosis of cancer patient --- it is very normal that genes related with these two bioprocesses are also correlated --- that does not mean there is a causal relationship between these two groups of genes. Experiments are needed to illustrate this hypothesis.

Reply: Thank you very much for your valuable comments. We are grateful for the helpful criticism on logical confusion of the manuscript. Here, we sincerely apologize for the imprecise statement.

In this study, our prognostic model based on six hypoxia-related genes was used to

calculate the risk score of each patient in order to identify high- and low-risk groups. Furthermore, Kaplan-Meier survival analysis indicated that patients in the high-risk group had worse prognosis than those in the low-risk group. We found that high scores positively correlated with the expression of immune checkpoint molecules, which suggested that poor prognosis in patients with high-risk score were associated with tumor immune escape induced by upregulation of immune checkpoint molecules. Here, we sincerely apologize for the logical confusion of causal relationship between hypoxia-related genes and immune checkpoint molecules. As suggested by reviewer, we have revised the manuscript to state clearly the relationship between risk score and the expression levels of immune checkpoint molecules, which was used to interpret the upregulated immune checkpoint molecules were involved in poor prognosis for patients with high-risk score. (see Page 16, line 338-345)

In a further study, we will implement further experimental studies to investigate whether there is potential relationship between the hypoxia-related genes and immune checkpoint molecules. Thank you again for your time and effort reviewing our manuscript.

Changes in the text: see Page 16, line 338-345

Comment 3. In fig3, panels in left part and fig 4 make nonsense, as the six genes were picked based on TCGA data, the signature score definitely will fit well with TCGA data. But the GEO data is much less impressive, especially fig 3D and 3J.

Reply: Thank you very much for your valuable comments. Your comments will have a constructive guiding effect on our future study. Here, we apologize for not being able to provide strong validation for the results.

In this study, the main reason why we used the TCGA database to evaluate the predictive ability of the prognostic model was that most of the current bioinformatics-based articles followed this analysis method, that is, the TCGA database was used as a training cohort and the GEO database as a verification cohort. However, the sample size of the validated data obtained from the GEO database was small, and we were unable to provide strong evidence for the predictive ability of this model. Indeed, we

have tried to obtain large-sample cohort data like the TCGA database for verification, but due to limited data resources, we can only find GEO data with a relatively small sample size. In our study, the follow-up endpoint of the samples in the GSE54236 dataset (validation cohort) was death, which may be the reason why the predictive power of the prediction model cannot be clearly evaluated in Figure 3D. Additionally, we think that the small sample size of GSE54236 (including only 81 samples) is the main reason why Figure 3D and Figure 3J are not well displayed. The relatively small sample size was a limitation to this study. In the revised manuscript, we have added a description of the defect that the sample size of the validation cohort was relatively small. (see Page 20, line 430-433) Currently, we have begun collecting more clinical data for further validation study on these findings.

In this study, Figure 4A-B was plotted to assess the independent prognostic value of the hypoxia-related risk signature compared to conventional clinicopathological features. Then we built a predictive nomogram which may be helpful to accurately predict a certain clinical outcome (Figure 4C). Each level of independent factors was assigned one score and a total score was calculated by summing up the scores in each patient. The survival probability for the individuals at 1-, 3-, and 5-year was obtained through the function conversion relationship of total scores. Besides, the ROC curve analysis and calibration plots were generated to estimate the accuracy of the nomogram (Figure 4D-G). Thank you for your time and effort reviewing our manuscript.

Changes in the text: see Page 20, line 430-433

Comment 4. Some figure legends are duplicated in the text.

Reply: Thank you very much for your valuable comments. Here, we apologize for the confusion caused by the duplicated legends. In the submitted manuscript, the contents of Figure 2A were presented in first paragraph of the "Results" section, and the contents of the Figure 2B-D were presented in the second paragraph of the "Results" section. In order to make it easier for reviewers and editors to review the legends, we repeated the legend content in Figure 2 in the order of paragraphs. Similarly, the legends of both the Figure 3 and Figure 7 were also repeated. We apologize for any inconvenience this has

caused. In the revised manuscript, we have deleted duplicated figure legends. (see Page 9, line 177-182; see Page 10, line 212-223; see Page 15, line 324-335) Thank you again for your time reviewing our manuscript.

Changes in the text: (see Page 9, line 177-182; see Page 10, line 212-223; see Page 15, line 324-335)