Peer Review File

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**Reviewer A**

The authors Jin-Feng Liu et al. of the submitted manuscript "Disulfidptosis and Ferroptosis Related Genes Predict Prognosis and Personalised Treatment for Hepatocellular Carcinoma" studied the expression pattern of genes related to disulfidptosis and ferroptosis (DRG-FRG) for their prognostic and therapeutic potency in cases of hepatocellular carcinoma.
In summary, based on their intensive and deep in-silico investigations the authors could find eleven DRG-FRG genes (ABCC1, AURKA, DNAJB6, FANCD2, FTL, MYB, NCF2, NQO1, SLC38A1, SLC7A11, and YY1AP1). Consecutively, this eleven DRG-FRGS could be used to stratify HCC patients into low and high-risk subgroups, (ii) to validate their robust predictive capacity by univariable and multivariable Cox analyses in relation to clinico-pathological characteristics and (iii) finally to integrate in the development of a normogram, too. Furthermore, the authors were able to identify a specific biological, immune and drug sensitivity signature for these eleven DRG-FRGs. Therefore, the authors concluded that this signature based on 11-DRG-FRG genes could be used as a promising prognostic and predictive biomarker for HCC.

Overall, the submitted manuscript gives some interesting information and insights of disulfidptosis and ferroptosis related genes signatures for prediction and therapeutic strategies for HCC on theoretical level. The manuscript (including presentation) is comprehensible and convincing. The methods are mostly well described. Although the results and discussion are clearly presented, the authors (see specific comments) must perform some minor changes to improve the manuscript. In conclusion, the presented data are interesting. After incorporating the mentioned specific comments (see below) the manuscript has the potency to be accepted.

Specific comments
**Material and Methods: Please add for all applied online databases the link into the material and methods section.**
**Reply 1:** We have added links to all the online databases that we used in this study (see Page 7, line 208 to 220)
**Changes in the text:**
1) In light of this, leveraging the B-cell specific lncRNA signature, we employed a suite of algorithms, namely QUANTISEQ (http://icbi.at/quantiseq), CIBERSORT (https://cibersortx.stanford.edu), XCELL (https://comphealth.ucsf.edu/app/xcell), CIBERSORT abs.mode (https://cibersortx.stanford.edu), EPIC (http://epic.gfellerlab.org), TIMER (https://cistrome.shinyapps.io/timer), and MCP-counter (https://github.com/ebecht/MCPcounter), to gauge the extent of immune cell infiltration across high-risk and low-risk groups.

2) The TIMER2 database (http://timer.cistrome.org) was instrumental in shedding light on the interplay between immune cells and 11 specific DRG-FRG, thereby enriching our comprehension of the functional significance of DRG-FRG in HCC.

Results:

**# Figure 2: It is not clear why the coefficient of MYB is higher (Figure 2B) than all others in comparison to hazard ratio of Figure 2. Please explain.**

**Reply 2:** We initiated a univariate Cox regression analysis to screen genes related to prognosis in HCC patients (Figure 2A), and found that MYB is one of the genes associated with poor prognosis, with a hazard ratio of 2.4839 (1.3844-4.4567), indicating that patients with high expression of this gene have a 2.4839 times higher risk of death compared to patients with normal expression. Building on this, lasso penalized Cox regression analysis was utilized to further screen genes with high prognostic value, and found that all 11 selected genes were positively correlated with poor prognosis in patients, with the coefficient of MYB being higher (Figure 2B) than all others. This suggests that MYB has a more significant impact on the prognosis of HCC patients compared to other genes, which is consistent with the results in Figure 2A. There are reports in the literature that MYB mRNA expression is higher in HCC tissues compared to matched non-tumor tissues (refer to the following references 1). Additionally, survival analysis revealed that patients with high MYB expression had lower overall survival (refer to the following references 1) and disease-specific survival rates (refer to the following references 2 to 4) compared to patients with negative MYB expression. Therefore, the findings of Figure 2 are consistent with previous reports in the literature. we added some explanation in the Discussion section. (see Page 14, line 406 to 410)

(1). Chang YS, Lee YT, Yen JC, Chang YC, Lin LL, Chan WL, Chang WC, Lin SY, Chang JG. Long Noncoding RNA NTT Context-Dependently Regulates MYB by Interacting With Activated Complex in Hepatocellular Carcinoma Cells. Front Oncol (2021) 11:592045. doi:10.3389/fonc.2021.592045.

(2). Nakajima T, Yasui K, Zen K, Inagaki Y, Fujii H, Minami M, et al. Activation of B-Myb by E2F1 in Hepatocellular Carcinoma. Hepatol Res (2008) 38(9):886–95. doi:10.1111/j.1872-034X.2008.00324. x.

(3). Chen RX, Xia YH, Xue TC, Ye SL. Transcription Factor C-Myb Promotes the Invasion of Hepatocellular Carcinoma Cells via Increasing Osteopontin Expression. J Exp Clin Cancer Res (2010) 29:172. doi:10.1186/1756-9966-29-172.

(4). Yang H, Huang ZZ, Wang J, Lu SC. The Role of C-Myb and Sp1 in the Up-Regulation of Methionine Adenosyl transferase 2A Gene Expression in Human Hepatocellular Carcinoma. FASEB J (2001) 15(9):1507–16. doi: 10.1096/fj.01-0040com.

**Changes in the text:** MYB is a gene associated with poor prognosis in HCC patients, its' HR (derived from univariate Cox regression analysis) and coef (derived from lasso Cox regression analysis) are significantly higher than all others, which suggests that MYB has a more significant impact on the prognosis of HCC patients compared to other genes. The findings from our study are in line with previous literature.

**# Figure 3: The p-values of the survival analysis are surprising since the survical curves**

**for low and high risk crossed and switched after about 7 years. Please explain.**

**Reply 3:** As is well known, HCC is the most common type of liver cancer and is a leading cause of illness and death worldwide. The 5-year survival rate for HCC is only around 10%. Based on the survival model constructed from Figure 3A, we can observe that the 5-year survival rate for high-risk group is 8.24% (15 out of 182), while the 5-year survival rate for low-risk group is 13.67% (25 out of 183). The overall 5-year survival rate is 10.96% (40 out of 365), which is consistent with previous literature reports. Therefore, the 5-year survival rate of HCC patients remains an important focus of research. From Figure 3A and D, we can observe that in the first 5 years, the survival rate of the low-risk group patients (blue curve) is significantly higher than that of the high-risk group patients (red curve). If the cutoff time for the prediction model is adjusted to 5 years, the result of the model in Figure 3A has a P-value of 0.001, and the result of the model in Figure 3D has a P-value of 0.003. These results are consistent with previous findings. Therefore, whether or not all data, including survival data beyond 5 years, are included, the conclusion can still be drawn that the high-risk group registered a markedly elevated mortality rate in contrast to the low-risk group. Only a small proportion of HCC patients achieve a survival benefit of 7 years or longer. In the 7th year, the survival rate of the high-risk group is 1.65% (3 out of 182), while the survival rate of the low-risk group is 2.73% (5 out of 183). The overall survival rate is 2.19% (8 out of 365). By the 10th year, only one person in the high-risk group is predicted to survive, while all patients in the low-risk group have died. After 7 years, the number of survivors and the survival rate for both groups become very small, causing the survival curves for both groups to cross or even reverse. However, this does not affect the final conclusion of our study. we added some explanation in the Discussion section. (see Page 14, line 410 to 417)

**Changes in the text:** Furthermore, the survival curves for low and high-risk groups intersected and reversed after about 7 years according to Figures 3A and D. A possible explanation for this result is that the number of survivors and the survival rate for both groups become very low after 7 years. However, the 5-year survival rate for HCC is only around 10% worldwide, and the rate is 10.96% (40 out of 365) in our study. Therefore, the 5-year survival rate of HCC patients remains a crucial research topic. From Figures 3A and D, we can see that in the first 5 years, the survival rate of the low-risk group patients (blue curve) is significantly higher than that of the high-risk group patients (red curve). Therefore, our conclusion is reliable.

**# Figure 4: Regarding the univariate and multivariate cox-regression analysis, the authors should keep in mind that the pathological stage is still the "best" predictor for HCC, which is "easy" evaluated in the daily clinical setting.**

**Reply 4:** Thank you for the valuable input from the reviewers. We acknowledge that pathological stage is an important prognostic factor for HCC. However, in certain cases, pathological stage may lack sensitivity or be subject to controversy. Therefore, we aimed to explore other prognostic factors such as the expression of DRG-FRG-based signature genes. We incorporated pathological stage as a covariate in both univariate and multivariate Cox regression analyses to control for its influence. Surprisingly, even after considering pathological stage, risk score remained an independent predictor of survival (Figure 4). We also included a comparison with pathological stage in the discussion section to highlight the superiority of risk score (see Page 14, line 389 to 391)

**Changes in the text:** This model demonstrated significant efficacy in predicting overall survival among HCC patients, while also serving as an independent predictor, apart from other clinical variables, such as pathological stage.

**# Figure 5: The findings of Figure 5b are interesting and partially surprising. The authors could show that high risk signature is linked to T-stage, but inverse with grading and M-stage, which is contradictory. Please explain.**
**Reply 5:** The pathologic stage includes T, M, and N stage. The findings of Figure 5b show that high risk signature is linked to pathologic stage ($P < 0.001$). Furthermore, subgroup analysis revealed that that high risk signature is linked to T-stage ($P = 0.003$), but not M-stage ($P = 0.295$) and N-stage ($P = 0.383$). Therefore, the correlation between the DRG-FRG-based signature and pathological stage, including T, M, and N stages, can be interpreted reasonably.
**Changes in the text:** No modifications have been made to the text.

**# Figure 6: Please highlight to significant findings for better reading.**
**Reply 6:** In order to enhance readability, we highlight the clinical covariates labels that have prognostic merit for overall survival along with their corresponding p-values in red color. Changes in the text: we added the explanation in Figure 6 Legends and Figure 6 (see Page 22, line 670 to 671, and Figure 6).
**Changes in the text:** Red labels indicate clinical covariates that have prognostic merit for overall survival along with their corresponding p-values.

**# Figure 7: Please explain the red color of figure 7D.**
**Reply 7:** Red color indicates a significant association between genes and small molecule drugs.
**Changes in the text:** we added the explanation in Figure 7 Legend (see Page 22, line 674 to 675)

**# Figure 8: It is not clear, why the differentially 59 DRG-FRG genes are analyzed and not only the eleven preliminary detected genes. Please explain.**
**Reply 8:** We conducted a more comprehensive analysis by examining not only the eleven preliminary detected genes, but also an additional 48 genes that showed significant differential expression using a rigorous statistical method. This allowed us to study a larger set of genes and better understand the underlying biological functions and pathways involved in the DRG-FRG interaction. The eleven preliminary detected genes may have overlooked important gene expression changes that are crucial in elucidating the complex nature of the DRG-FRG interaction. Therefore, by analyzing all 59 genes, researchers can identify any additional genes that may be playing a role in the observed differences, providing a more complete picture of the genetic factors at play. Additionally, this approach allows for a more robust statistical analysis, as it takes into account a larger number of genes and potential interactions between them. Overall, the analysis of all 59 genes provides a more comprehensive and informative assessment of the genetic differences between the groups, and we have explicitly addressed this in the revised manuscript. (see Page 16 to 17, line 454 to 456, and line 490 to 493)

Changes in the text:
(1). In order to enhance our comprehension of the underlying biological functions and pathways associated with HCC, we conducted GO and GSEA analyses to explore the interactions among the 59 DRG-FRG genes.
(2). This underscores the importance of GO and GSEA analysis in identifying disease-relevant biological functions and signaling pathways, providing a foundation for researchers to explore the mechanisms of HCC formation and progression.

**Discussion: The discussion is largely narrative and repeat most of the findings. Therefore, the authors should shorten the findings and discuss the real important and novel findings of the presented findings in more detail. How could the interesting findings transferred from a theoretical to a practical view (like clinical setting (drug-development, drug-combination))? Please discuss in short.**
**Reply:** We have shortened the findings and discuss the real important and novel findings of the presented findings in more detail. (see Discussion section)
**Changes in the text:** Please refer to the Discussion section.

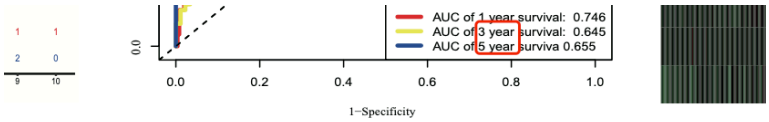
**Reviewer B**

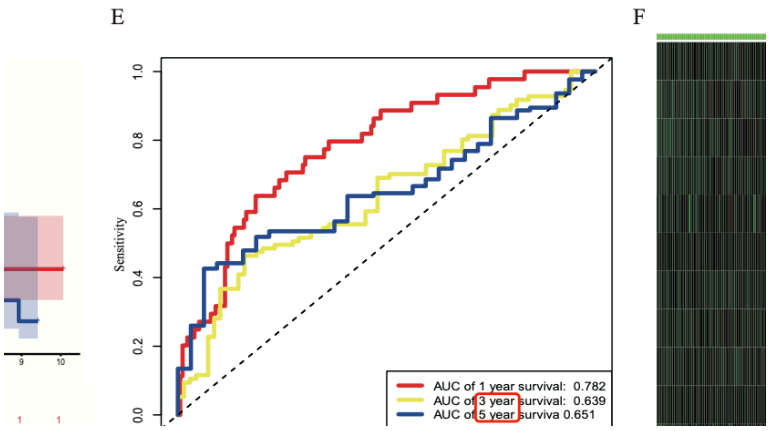
1. Please define ROC in Abstract.
**Reply:** We have added the definition of ROC in Abstract (see Page 2, line 56).
**Changes in the text:** The prognostic performance of this model was evaluated by Kaplan-Meier survival analysis and time-dependent receiver operating characteristic analysis.

2. Ref.23 was not cited in your paper, please cite it in order in text.
**Reply:** Ref. 23 was originally a reference in the discussion section. We had deleted the relevant content and ref. 23 from the discussion section in the previous revision. After updating the references, the reference is no longer cited in this study.

3. Figures should be cited consecutively in text; you should cite Figure 7C-7D between Figure 7A-7B and Figure 8A-8C. Please revise.
**Reply:** Figure 7C and 7D were split out from Figure 7 and renamed as Figure 9A and 9B, respectively. We have revised the figure citations to ensure they are in consecutive order in the text and also added figure legends for the new figures.

4. Figure 3B, 3E: Please revise them to “3/5 years” and resend us updated Figure 3.

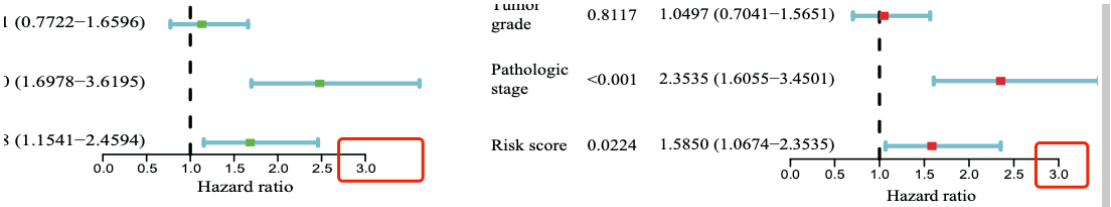



**Reply:** We have revised all instances of "3/5 year" to "3/5 years" and sent back the updated Figure 3.

**Changes in the text:** Please refer to the updated Figure 3.

5. Figure 4: It's better to extend the X-axis for this figure since the CI values go beyond it.




**Reply:** We have extended the X-axis for Figure 4 and sent back the updated figure.

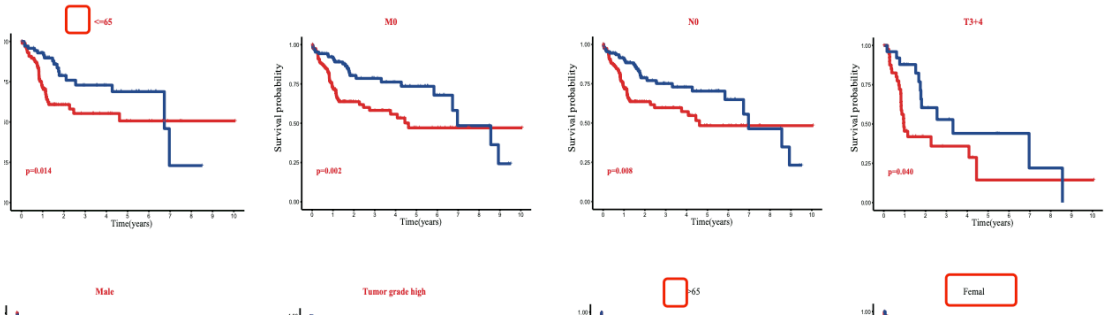
**Changes in the text:** Please refer to the updated Figure 4.

6. Figure 5: Please define ", **" in figure legends.

**Reply:** After the Chi-squared test, it was determined that the composition ratio of various stages in the three subgroups of pathologic stage, T stage, and tumor grade are associated with Riskscore. However, the statistical method performed by R software did not provide a specific P value, and the number of * symbols indicating the level of significance. In order to facilitate the interpretation of the content of Figure 5A, we use * to indicate subgroups with statistical significance.

**Changes in the text:** Please refer to the updated Figure 5 and Figure 5 legends.

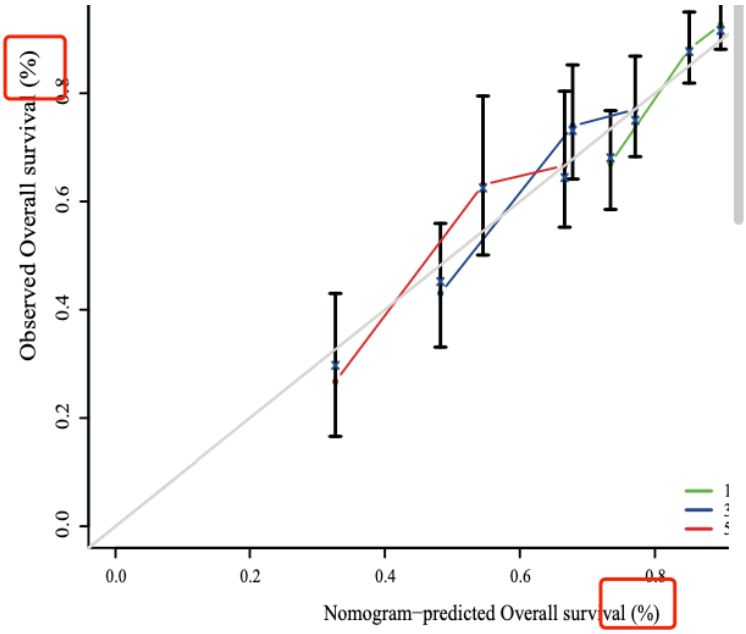
7. Figure 6: Please indicate "Age" for below two images and correct the typo to "Female".




**Reply:** We added "Age" to the two images above and fixed the "Female" typo.
**Changes in the text:** Please refer to the updated Figure 6.

8. Figure 7
a. Figure 7A: Please define "*, ***" in figure legends.
**Reply:** The nomogram model results suggest that patient prognosis is significantly correlated with pathologic stage and risk score. However, the correlation strength is only represented by the number of asterisks used, and specific statistical results such as P value are not provided. In order to facilitate the interpretation of the content of Figure 7A, we use * to signify indicators with statistical significance.
**Changes in the text:** Please refer to the updated Figure 7 and Figure 7 legends.

b. Figure 7B: Please remove "(%)" from X- and Y-axis since numbers on the axis are from 0-1.0.




**Reply:** We have removed "(%)" from the X- and Y-axis in Figure 7.
**Changes in the text:** Please refer to the updated Figure 7.

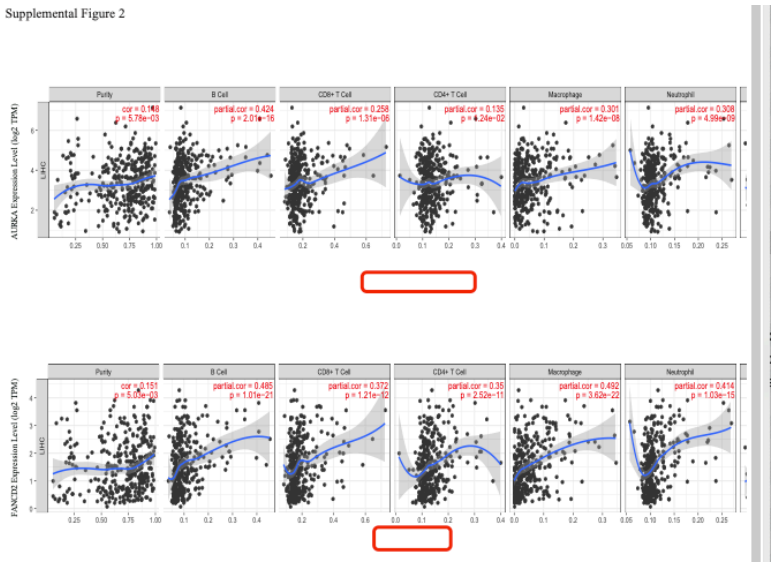
9. Please check if ALL abbreviations have been defined in the figure legends. Like "TCGA" in Figure 3-4, "OS" in Figure 7, "DRG-FRG, KEGG" in Figure 8…

**Reply:** We carefully reviewed the figure legends to ensure that all abbreviations have been defined (see Page 21 to 23, line 634 to 683).
**Changes in the text:** Please refer to the figure legends.

10. Please define "****" in Figure 10 legends.
**Reply:** We've included the definitions for **** in the legends of Figure 10 legends.
**Changes in the text:** Please refer to the figure 10 legends.

11. Please define TPM and TIMER in Supplemental Figure 1 legends.
**Reply:** We've included the definitions for TPM and TIMER in the legends of Supplemental Figure 1 (see Page 23, line 688 to 689).
**Changes in the text:** Please refer to the Supplemental Figure 1 legends.

12. Supplemental Figure 2
a. Please also supplement the description for X-axis in the above two graphs.




**Reply:** The description for X-axis have been added to Supplemental Figure 2.
**Changes in the text:** Please refer to the updated Supplemental Figure 2.

b. Please define TPM and TIMER in Supplemental Figure 2 legends.
**Reply:** We've included the definitions for TPM and TIMER in the legends of Supplemental Figure 2 (see Page 23, line 691 to 692).
**Changes in the text:** Please refer to the Supplemental Figure 2 legends.

13. Please indicate formal **table titles** for Supplemental Table 1-3 **respectively**.

739 DRG-FRG genes using the TIMER database. DRG-FRG, disulfidptosis-ferroptosis.

740 Supplemental Table 1

741 Supplemental Table 2

742 Supplemental Table 3

743 Supplemental Table 4 The expression of 23 disulfidptosis genes in normal and HCC groups

**Reply:** Supplemental Table 1-3's table titles have been updated (see Page 23, line 693 to 695).

**Changes in the text:**

(1) Supplemental Table 1 Information of Ferroptosis-related driver gene;

(2) Supplemental Table 2 Information of Ferroptosis-related suppressor gene;

(3) Supplemental Table 3 Information of Ferroptosis-related marker gene