

## Peer Review File

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### Reviewer A

The paper titled “Investigation of mitochondrial DNA methylation-related prognostic biomarkers in hepatocellular carcinoma using The Cancer Genome Atlas (TCGA) database” is interesting. This study constructed a risk model for HCC based on two identified prognostic genes (ADH4 and DNASE1L3). It also elucidated the pathogenesis of MTDM-associated HCC. Our findings provide novel insights that could lead to the development of future therapeutic strategies. However, there are several minor issues that if addressed would significantly improve the manuscript.

**1) The number and sites of control regions, involved enzymes, and regulators during mtDNA methylation may vary among cell types and diseases. Specific regulatory and functional networks associated with mtDNA methylation mainly include mtDNA, regulatory factors, methyltransferases, nucleotides, mt-rRNAs, and other epigenetic modifications. How to breakthrough mitochondrial DNA methylation-associated networks? Suggest adding relevant content.**

**Response 1) :** Thank you for your kindly suggestion. We have made correction according to your comments. We have added relevant content in the discussion (see Page 15 to 16, line 492 - 517). Specifically, we have expanded on the enzymatic and regulatory heterogeneity. Furthermore, we have included text on multi-omics integration, therapeutic targeting, and clinical biomarkers. This content integrates mechanistic depth, technological innovation, and clinical relevance.

**2) What is the role of mtDNA methylation in promoting mitochondrial dysfunction and lipid metabolism impairment in HCC? Suggest adding relevant content.**

**Response 2) :** Thank you for your kindly suggestion. We have made corrections based on your comments and added relevant content in the second paragraph of the discussion (see Page 16, line 523-534).

**3) Suggest adding various methods to evaluate the biological functions of MTDM, such as copynumber karyotyping analysis and quantitative analysis of the cell proliferation rate.**

**Response 3) :** Thank you for your kindly suggestion. We sincerely appreciate the reviewer’s valuable suggestions to strengthen the functional validation of MTDM. While we acknowledge the importance of copy number karyotyping and cell proliferation rate analysis, we regret that these experiments could not be performed in the current study due to limitations in experimental resources (e.g., access to high-resolution karyotyping platforms) and time constraints during the revision period. We have made corrections based on your comments and added relevant content in the last paragraph of the discussion (see Page 20, line 647-656).

**4) Some of the fonts in all figures are not clear, please modify and upload again.**

**Response 4) :** Thank you for your valuable feedback. We have made the necessary adjustments to the fonts in all the charts to enhance their clarity. The updated charts have been re-uploaded

for your review. If there are any additional issues or further modifications needed, please feel free to let us know. Thank you for your patience and guidance.

**5) Suggest adding weighted co expression network analysis (WGCNA) to determine the different modules involved in MTDM related genes in HCC, which may make the entire study more complete.**

**Response 5) :** Thank you for your valuable suggestion. We have incorporated WGCNA analysis into our study. The analysis results show that the MEturquoise module has the strongest correlation with MTDM scores and is a potential functional module. Thank you again for your suggestion, and we look forward to your further guidance.

**Changes in the text:** A new section titled "##Weighted Gene Co-expression Network Analysis (WGCNA)" has been added to the Methods section (**see Page 7, line 207-224**). A new section titled "##Identification of Key Modules and Genes Associated with Mitochondrial DNA Methylation" has been added to the Results section (**see Page 11-12, line 366-378**). Figure S3 and its caption have been included at the end of the reference list.

**6) Suggest adding cell experiments to verify the role of MTDM related prognostic genes in HCC.**

**Response 6) :** We sincerely appreciate the reviewer’s critical suggestion to validate the roles of MTDM-related prognostic genes through cellular experiments, which would undoubtedly strengthen the mechanistic insights of our study. While we fully acknowledge the importance of functional validation (e.g., CRISPR-mediated knockout, proliferation/apoptosis assays) to establish causality between these genes and HCC phenotypes, limitations in laboratory access, time constraints during the revision period, and current funding restrictions precluded us from conducting additional experiments. To address this gap, we have leveraged existing functional evidence from published studies. For instance, prior work demonstrated that ACSL3 drives lipid metabolism dysregulation in HCC, while CPT1A sustains mitochondrial dysfunction—both genes being central to our MTDM-associated network (see Page 16, line 523-532). Our bioinformatics and clinical correlation analyses robustly link MTDM-related genes to HCC progression and survival outcomes. However, we recognize that the lack of direct experimental validation limits causal inference. Moving forward, we propose a prioritized roadmap for future research (see Page 19, line 646-655).

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**Reviewer B**

**1. Please check if here should be “Figure S2A, S2B”.**

363 four GO results were: pattern designation process, embryonic skeletal joint  
364 morphogenesis, regionalization, and cellular response to cadmium ions (Figure  
365 **S1A,S1B**). Additionally, 16 enriched KEGG pathways were identified (Table S2),  
366 including entries on mineral uptake, retinol metabolism, and drug metabolism-  
367 cytochrome P450 (Figure S2C,S2D).↵

**Response:** Thank you for your feedback. We have corrected the error and confirmed that the proper reference should indeed be "Figure S2A, S2B". The corresponding revisions have been made in the manuscript accordingly.

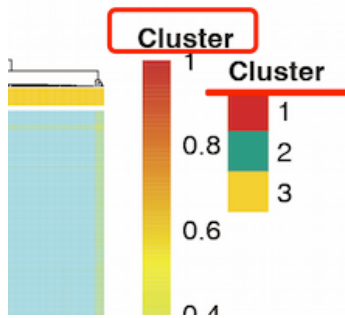
**2. Table S3**

Please provide table head.

**Response:** Thank you for your feedback. As requested, we have added the column header ("Chemical Number") in Table S3 and implemented the corresponding updates in the revised version of the manuscript.

**3. Figure 1**

Here are two “cluster”. Please confirm whether they are correct.



**Response:** Thank you for your feedback. Upon further inspection, we identified that the issue in the figure was caused by the positioning of cluster labels. The figure has been revised accordingly, and the corresponding corrections have been implemented in the revised manuscript version.

b) It is “subtype-specific DEGs” in figure legend, while it is “subtypeDEGs” in figure 1H. Please unify.

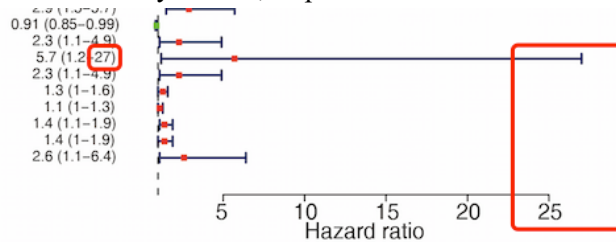
**Response:** Thank you for your correction! The figure legend was labeled as "subtype-specific DEGs", while the label in Figure 1H was marked as "subtypeDEGs". To ensure consistency, we will standardize the label in Figure 1H to "Subtype-specific DEGs" in the revised manuscript version.

#### 4. Figure 2A, C

a. Please revise “Hazard ratio” to “Hazard ratio (95% CI)”.

**Response:** Thank you for your reminder! We have revised "Hazard ratio" to "Hazard ratio (95% CI)" to ensure greater accuracy and clarity.

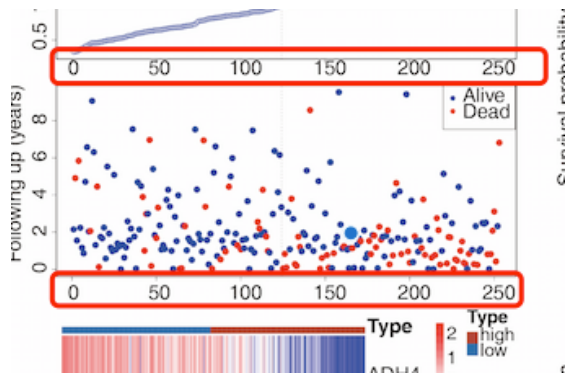
b. **Figure 2A:** To standardize the results, the part that exceeds the horizontal coordinates should be indicated by arrows, or please extend the X-axis.



**Response:** Thank you for your suggestion! We have implemented your recommendation by adding x-axis labels to the figure to ensure clearer data presentation. Should you have any further questions or suggestions, please do not hesitate to contact us.

#### 5. Figure 2D

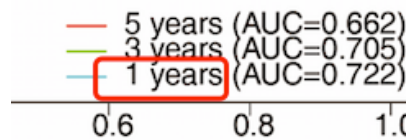
Please add the description of the X-axis.



**Response:** Thank you for your valuable suggestion! We have implemented your recommendation by adding x-axis labels to the figure for improved data clarity. Should you have any further questions or additional recommendations, please feel free to reach out.

### 6. Figure 2F

Please revise “1 years” to “1 year”.

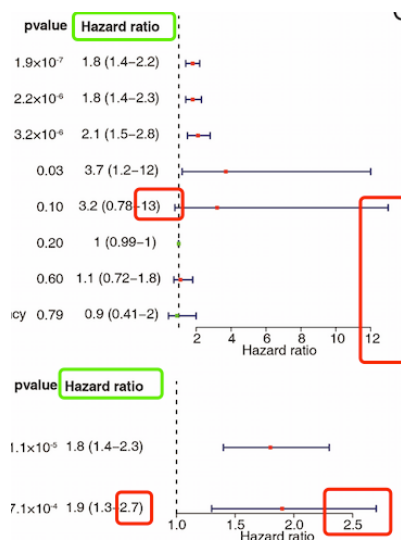


**Response:** Thank you for bringing this to our attention! We have corrected the inconsistency by revising "1 years" to "1 year" in the manuscript. Should you have any further questions or suggestions regarding this or other aspects of the work, please do not hesitate to reach out.

### 7. Figure 3A, B

a. Please revise “Hazard ratio” to “Hazard ratio (95% CI)”.

b. To standardize the results, the part that exceeds the horizontal coordinates should be indicated by arrows, or please extend the X-axis.



**Response:** Thank you for your suggestions! We have implemented the following revisions:

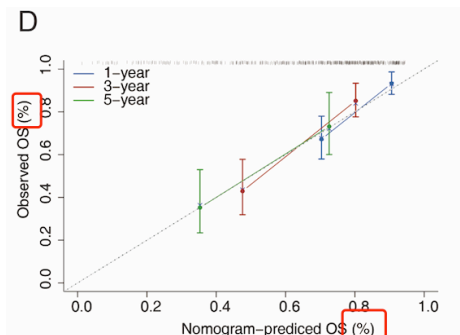
a. "Hazard ratio" has been revised to "Hazard ratio (95% CI)".

b. To standardize the results, we have extended the X-axis to ensure complete data display. Please feel free to contact us if you have any further questions or recommendations.

### 8. Figure 3D

The correct format for the x-axis and y-axis should be one of the following, please revise.

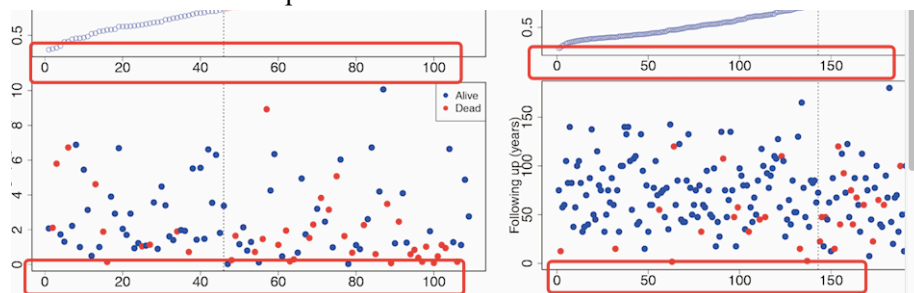
- If the description is with %, the numbers usually should be 0-100.
- If the description is without %, the numbers usually should be 0-1.0.



**Response:** Thank you for your suggestions! We have removed the percentage sign in accordance with your request and performed corresponding formatting adjustments. Please feel free to inform us if you have any further questions or recommendations.

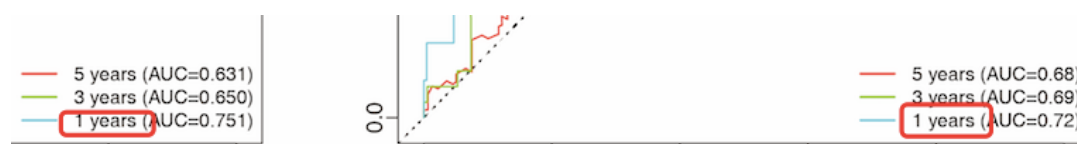
### 9. Figure S4

a. Please add the description of the X-axis.



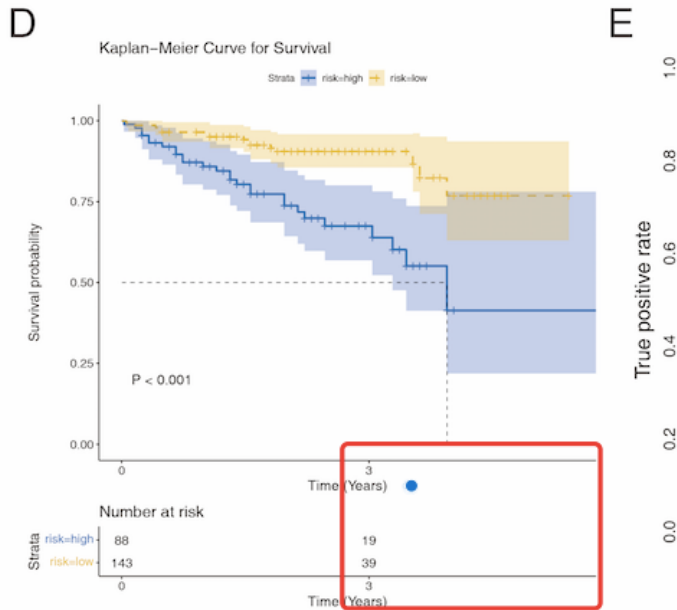
**Response:** Thank you for your suggestion! We have added the x-axis labels in Figure S4 to clarify the data presentation. Should you have any further questions or suggestions, please do not hesitate to contact us.

b. Please revise “1 years” to “1 year”.



**Response:** Thank you for your reminder! We have corrected "1 years" to "1 year" in Figure S4.

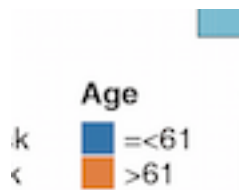
c. Please confirm if any data are missing in the X-axis.



**Response:** Thank you for your feedback! We have verified Figure S4D, supplemented the missing image data, and resubmitted the integrated figure.

## 10. Figure 4

a. Please add the unit of age.



**Response:** Thank you for your suggestion! We have added age units (e.g., "years") to Figure 4.

b. (Figure 4D) should be cited in line 453. Please revise.

450 IV accounted for 50%, 24%, 21%, and 5%, respectively (Figure 4C). Additionally, 246  
 451 samples (low risk =120, high risk =126) had Race clinical profile information, of which  
 452 White (Figure 4D), Asian, Black or African American, and American Indian or Alaska  
 453 native accounted for 50%, 44%, 5%, and 0%, respectively.↵

**Response:** Thank you for your reminder! We have cited Figure 4D in line 464.

## 11. Figure 5

It seems that the description of Figure 5D, 5E in the main text are inconsistent with the Figure 5D, 5E and the figure legend. Please check and revise.

**Response:** Thank you for your feedback! We have reviewed and revised the descriptions of Figure 5D and 5E in the main text to ensure consistency with the figures and legends.

## 12. There are two reference lists. Please delete the unnecessary one.

**Response:** Thank you for your feedback! We have removed duplicate reference lists and

retained only the essential one.

**13. The authors mentioned “studies...”, while only one reference was cited. Change “Studies” to “A study” or add more citations. Please revise.**

*In lung adenocarcinoma, **studies** have found that abnormal DNA methylation in circulating tumor cells is associated with tumor metastasis [12].*

***Studies** have shown that fatty acid metabolism is closely linked to the progression of gastric cancer [59].*

*Previous **studies** have shown that the early activation of CD4+ T cells is crucial for establishing an antitumor immune response [65].*

*In recent years, **studies** have shown that changes in the proportion of eosinophils in peripheral blood may be correlated with the efficacy of immunotherapy [71].*

**Response:** Thank you for your feedback! We have changed "studies" to "a study" to correct the grammatical structure. Please contact us if further revisions are needed.