Peer Review File

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Reviewer A

[General Comment] This manuscript describes a novel method to identify HCC subtypes and predict patient prognosis in HCC patients by performing multivariate cox regression analysis using anoikis-related gene signatures. This paper can be accepted for publication with minor revisions. The following suggestions are intended to improve the quality of this paper and should not be construed otherwise. The authors are recommended to ensure proper English language and grammar editing throughout the manuscript and also expand the abbreviations at their first use.

The overall response to Reviewer: Thank you for your comments and suggestions concerning our manuscript. The comments and suggestions are all valuable and very helpful for revising and improving our manuscript, as well as the essential guiding significance to our research. We have carefully studied the comments and made corrections that we hope meet your high standards.

Our response: Thank you for your advice. As you pointed out, our manuscript had some issues with English grammar and the first occurrence of unexpanded abbreviations. We have made the necessary revisions in the manuscript.

"Line 81-83:

The TCGA-LIHC cohort was combined with the GSE76427 cohort and the batch effect was removed to obtain the new "LIHC-GSE76427" cohort."

"Line 136:

fdr<0.05. The heatmap showed the first 30 DEGs (fig.1A)."

"Line 144:

Copy Number Variation (CNV) data"

"Line 177:

The K-M curves in the high-risk group…"

"Line 217:

… prediction. The article…"

[Comment 1] Please expand on the introduction section. Here are a few suggestions:

a. Which anoikis-related genes are common to HCC?

b. Why is the identification of ANRG important and how does it impact clinical outcomes?

c. Why is immune infiltration of tumors relevant to clinical outcomes? What is the relationship between ANRG and tumor immune microenvironment?

d. Please provide a detailed overview of your research goals

Our response: Thank you for providing valuable feedback on our research. We have carefully considered each of your suggestions and made corresponding adjustments in the revised manuscript. We have expanded on this in the introduction, outlining the roles of some ANRGs in cancer progression. We have underscored the significance of ANRG, particularly its potential value in guiding treatment strategies and directly influencing clinical outcomes. This reinforcement aims to ensure that readers fully grasp the crucial nature of ANRG as a therapeutic target. We further explore the role of the tumor immune microenvironment and the relationship between ANRG and immune cell infiltration. This enhancement aims to provide a more comprehensive explanation of how ANRG may influence the immune response to tumors. Towards the end of the introduction, we have provided a more detailed overview of our research goals. Emphasis is placed on our objective to lay the foundation for developing therapeutic strategies targeting ANRG to improve the clinical prognosis of HCC patients. Thank you once again for your constructive feedback. We believe these revisions will enhance the comprehensiveness and clarity of our study.

"Line 45-47:

Anoikis resistance is believed to contribute to the survival of tumor cells that detach from the primary tumor, thereby facilitating metastasis [10]**"**

"Line 48-53:

Ye et al. discovered that nuclear MYH9 conferred anoikis resistance to gastric cancer cells by identifying the CTNNB1 promoter and, in turn, facilitated the metastasis of gastric cancer [13]. Research by D'Amato indicates that TDO2 promotes anoikis resistance, migration, and invasive capabilities in triple-negative breast cancer, leading to a shorter overall survival [14]. Wang et al. proposed that CPT1A-mediated fatty acid oxidation promotes the metastasis of CRC cells by inhibiting anoikis [15].**"**

"Line 54-63:

The main components of the tumor microenvironment (TME) are immune cells and stromal cells, responsible for tumor dissemination, recurrence, metastasis, immune therapy efficacy, and prognosis [17, 18]. Infiltration of CD4 and CD8 T lymphocytes in tumors exerts anti-tumor effects, and is associated with a favorable prognosis [19]. Tumor-associated macrophages (TAMs) exert a tu-mor-promoting effect by secreting immunosuppressive factors, leading to a poorer prognosis [20]. TME characteristics of HCC include ab-normal angiogenesis, chronic inflammation, and dysregulated ECM remodel-ling, collectively leading to an immunosuppressive environment, thereby promoting the proliferation, invasion, and metastasis of HCC [21]. Therefore, exploring the prognostic value of ANRGs in HCC, differences in the tumor microenvironment, and establishing a new prognostic model for HCC is of significant importance.**"**

"Line 65-67:

In this study, we primarily investigated the prognostic value of ANRGs in HCC. We established a prognostic scoring model based on ANRGs and further explored the differences in the tumor micro-environment among patients stratified by this risk-scoring system.**"**

"Line 329-330:

10. Simpson CD, Anyiwe K, Schimmer AD. Anoikis resistance and tumor metastasis.

Cancer Lett 2008/12/18/;272(2):177-85.”

“Line 335-343:

13. Ye G, Yang Q, Lei X, Zhu X, Li F, He J et al. Nuclear MYH9-induced CTNNB1 transcription, targeted by staurosporin, p romotes gastric cancer cell anoikis resistance and metastasis. Theranostics 2020/6/12/;10(17):7545-60.

14. D'Amato NC, Rogers TJ, Gordon MA, Greene LI, Cochrane DR, Spoelstra NS et al. A TDO2-AhR signaling axis facilitates anoikis resistance and metastasi s in triple-negative breast cancer. Cancer Res 2015/11/1/;75(21):4651-64.

15. Wang Y-N, Zeng Z-L, Lu J, Wang Y, Liu Z-X, He M-M et al. CPT1A-mediated fatty acid oxidation promotes colorectal cancer cell me tastasis by inhibiting anoikis. Oncogene 2018/11//;37(46):6025-40.”

“Line 346-356:

17. Ge Z, Ding S. The Crosstalk Between Tumor-Associated Macrophages (TAMs) and Tumor Ce lls and the Corresponding Targeted Therapy. Front Oncol 2020/11/3/;10:590941.

18. Bagaev A, Kotlov N, Nomie K, Svekolkin V, Gafurov A, Isaeva O et al. Conserved pan-cancer microenvironment subtypes predict response to imm unotherapy. Cancer Cell 2021/6/14/;39(6):845-65.e7.

19. Ostroumov D, Fekete-Drimusz N, Saborowski M, Kühnel F, Woller N. CD4 and CD8 T lymphocyte interplay in controlling tumor growth. Cell Mol Life Sci 2018/2//;75(4):689-713.

20. Xiang X, Wang J, Lu D, Xu X. Targeting tumor-associated macrophages to synergize tumor immunotherap y. Signal Transduct Target Ther 2021/2/23/;6(1):75.

21. Chen C, Wang Z, Ding Y, Qin Y. Tumor microenvironment-mediated immune evasion in hepatocellular carci noma. Front Immunol 2023/2/10/;14:1133308.”

[Comment 2] Please cite these similar articles in your discussion.

Zhong, Z., Xie, F., Yin, J. et al. Development of a prognostic model for anoikis and identifies hub genes in hepatocellular carcinoma. Sci Rep 13, 14723 (2023). https://doi.org/10.1038/s41598-023-41139-9.

...

Our response: Thank you for your suggestions. We have cited these articles in the discussion section and compared them with our study.

“Line 219-221:

In addition, in recent years, there have been articles studying the apoptotic-related genes ANRGs and their association with HCC prognosis [36-39].”

“Line 393-402:

36. Zhong Z, Xie F, Yin J, Zhao H, Zhou Y, Guo K et al. Development of a prognostic model for anoikis and identifies hub genes in hepatocellular carcinoma. Sci Rep 2023/9/7/;13(1):14723.

37. Zhang D, Liu S, Wu Q, Ma Y, Zhou S, Liu Z et al. Prognostic model for hepatocellular carcinoma based on anoikis-related genes: immune landscape analysis and prediction of drug

sensitivity. Front Med (Lausanne) 2023/7/12/;10:1232814.

38. Chen Y, Huang W, Ouyang J, Wang J, Xie Z. Identification of Anoikis-Related Subgroups and Prognosis Model in Liv er Hepatocellular Carcinoma. Int J Mol Sci 2023/2/2/;24(3):2862.

39. Chen Y, Lin Q-X, Xu Y-T, Qian F-J, Lin C-J, Zhao W-Y et al. An anoikis-related gene signature predicts prognosis and reveals immun e infiltration in hepatocellular carcinoma. Front Oncol 2023/4/27/;13:1158605.”

[Comment 3] Please expand the discussion section. Here are some suggestions:

a. In the discussion section, please summarize how your results compare with other published analyses identifying anoikis-related gene signatures with prognostic values.

b. Do the seven signature genes identified in your study match with those identified in other published studies?

c. Are your results (identified gene signatures) expected, why or why not?

d. Can you suggest how these seven ANRGs can be validated?

e. Please provide recommendations on how your results can be used for improving clinical outcomes.

Our response: Thank you very much for your suggestions. Following your advice, we have incorporated the relevant content into the discussion. We summarized the comparative analysis of our research results with other published studies identifying the prognostic value of anoikis-related gene signatures. We emphasized the uniqueness of our study and its beneficial contribution to existing literature in the field. We proposed methods for validating these seven ANRGs and provided recommendations on how to use our research findings to improve clinical outcomes. Once again, we appreciate your valuable feedback, and we believe these revisions to the discussion section will further enhance the depth and practicality of our study.

“Line 221-226:

In comparison to previous research, our study is based on the merged LIHC-GSE76427 cohort, de-rived by combining the TCGA and GEO databases after removing batch effects. This approach pro-vides a more comprehensive coverage of information and enhances credibility compared to individual databases. Through internal validation, our model has demonstrated high predictive capability. Moreover, we not only investigated immune cell infiltration under different risk scores but also explored the expression of ANRGs in tumor microenvironment-associated cells.”

“Line 270-273:

Firstly, the construction and validation of our prognostic model are based on a retrospective data-base; therefore, our research findings should be further confirmed through prospective clinical studies. Secondly, the potential mechanisms of these 7 ANRGs associated with HCC prognosis require further experimental investigation.”

[Comment 4] The fonts in the figure panels are too small to review and blurry when zoomed in. It could be due to the pdf conversion. Please ensure that font sizes are legible.

Our response: Thank you for your valuable feedback. We have indeed observed that the text in some of the figures appears relatively blurry when enlarged, impacting the readability of the manuscript. Your suggestion is highly appreciated, and we have re-uploaded the figures in 400 dpi TIFF format to improve the overall quality of the graphics. If there are any areas where clarity remains an issue, please feel free to let us know.

[Comment 5] Lines 156-157: "Immune cell interactions in HCC patients may offer hints to better comprehend the makeup of the immune microenvironment during particular periods (fig.5D)." Can you please specify which particular periods are being referred to here?

Our response: Thank you for your insightful feedback. Regarding your query about the specific periods mentioned in lines 156-157, in our statement, "particular periods" refer to different stages of the immune microenvironment during the progression of hepatocellular carcinoma, these stages may include the early, middle, and late phases of cancer development.

[Comment 6] Please expand the conclusions section to restate the objective of the study and highlight the key results.

Our response: Thank you for your constructive suggestion. Expanding the conclusion section is indeed necessary. In our revised manuscript, we have elaborated on the conclusion to provide a clearer restatement of the study's objectives and to emphasize the key results obtained. We believe this modification will enhance the overall impact of our study. If you have any further recommendations or specific areas you would like us to focus on, please feel free to let us know. We value your input and are committed to improving the manuscript based on your suggestions.

"Line 279-286:

Our study established a novel risk feature based on seven ANRGs, demonstrating significant efficacy in predicting the survival outcomes of HCC patients. Furthermore, our research revealed a close as-sociation between the risk score and the immune microenvironment, exploring the expression of ANRGs in tumor microenvironment-associated cells. In summary, the nomogram based on our model serves as a reliable predictive indicator for the survival of HCC patients, aiding healthcare professionals in formulating personalized treatment plans in clinical practice. Future investigations into the biological basis of this feature and prospective randomized clinical trials may have substantial clinical implications and potentially lay the foundation for precision medicine**"**

Closing comment to Reviewer: We hope the revised manuscript is now acceptable to you. If not, we are glad to receive any further feedback which we shall continue to apply our best effort to address.

Reviewer B

[Editor's comment] The abstract should be structured with Background, Methods, Results, and Conclusions (200-450 words). Please modify the abstract to it.

Our response: Thank you for your feedback. We have carefully revised the abstract. Please let us know if there are any further concerns or issues.

"Line 6-7:

***Background*:** Hepatocellular carcinoma (HCC) is a major health problem with more than 850,000 cases per year worldwide. This cancer is now the third leading cause of cancer-related deaths world-wide, and the number is rising. Cancer cells develop anoikis resistance which is a vital step during cancer progression and metastatic colonization. However, there is not much research that specifically addresses the role of anoikis in HCC, especially in terms of prognosis.

Methods: This study obtained gene expression data and clinical information from 371 HCC patients through The Cancer Genome Atlas Program (TCGA) and The Gene Expression Omnibus (GEO) da-tabases. A total of 516 anoikis-related genes (ANRGs) were retrieved from GeneCard database and Harmonizome portal. Differential expression analysis identified 219 differentially expressed genes (DEGs), and univariate Cox regression analysis was utilized to select 99 ANRGs associated with the prognosis of HCC patients. A risk scoring model with seven genes was established using the Lasso regression model, and internal validation of the model was performed.

Results: The identified 99 ANRGs are closely associated with the prognosis of HCC patients. The risk scoring model based on seven characteristic genes demonstrates excellent predictive performance, further validated by ROC curves and Kaplan-Meier survival curves. The study reveals significant differences in immune cell infiltration, gene expression, and survival status among different risk groups.

Conclusions: The prognosis of HCC patients can be predicted using a unique prognostic model built on anoikis-related genes in HCC."

[Editor's comment] A highlight box is needed to highly summarize the key findings/recommendations, innovation, and potential implications of the study. Please provide a highlight box for your manuscript.

Our response: Thank you for your suggestions. Below is the highlight box for this article.

Key findings

Constructed a novel anoikis-related gene signature to predict the prognosis of hepatocellular carcinoma patients.

What is known and what is new?

Primary liver cancer ranked sixth among all cancers worldwide and stood as the third leading cause of cancer-related deaths. Hepatocellular carcinoma (HCC), the most prevalent type, accounted for 90% of all primary liver cancers. Anoikis, a specific form of apoptosis triggered by the loss of appropriate cell-to-cell or cell-to-extracellular matrix (ECM) adhesion, plays a crucial role. Research indicates that promoting resistance to anoikis is pivotal in the progression of tumor cells, as this resistance is considered an inherent characteristic of aggressive tumor cells.

It facilitates the survival of cells detached from the primary tumor, thereby promoting metastasis. Distant metastasis of HCC is often associated with a poor prognosis.

We constructed a novel anoikis-related gene signature to predict the prognosis of HCC patients. Through internal validation, our model demonstrated high predictive capability. Additionally, we not only investigated immune cell infiltration under different risk scores but also explored the expression of ANRGs in tumor microenvironment-associated cells

What is the implication, and what should change now?

The nomogram based on our model serves as a reliable predictive indicator for the survival of HCC patients, aiding healthcare professionals in formulating personalized treatment plans in clinical prac-tice. Future investigations into the biological basis of this feature and prospective randomized clinical trials may have substantial clinical implications and potentially lay the foundation for precision medicine.

[Editor's comment] Please provide the full name of "CRC", "GSVA", "OS", "Time-C index", "RNA-seq" in the main text. Please also check through your article to make sure all the abbreviated terms have been defined when they FIRST appear in the Abstract and the main text.

Our response: Thank you for your suggestions. All abbreviations and terms have been defined when first mentioned in the abstract and main text.

"Line 69-70:

which in turn encourages Colorectal Cancer (CRC) cells to metastasize**"**

"Line 109-110:

anoikis-related genes was performed using the " Gene Set Variation Analysis (GSVA)" R package**"**

"Line 131-132:

the correlation between Overall Survival (OS) and clinicopathological characteristics**"**

"Line 136:

The Time-dependent Concordance (Time-C) index was used to verify**"**

"Line 140:

single-cell RNA sequencing (RNA-seq) focused on the TME**"**

[Editor's comment] Please double check the full name of "TCGA", "Kegg", "ROCs", "LASSO", "TIPRGPI" in the main text.

Our response: Thank you for your suggestions. We carefully reviewed and revised some of the full name.

"Line 108:

Kyoto Encyclopedia of Genes and Genomes (Kegg)**"**

"Line 119-120:

The receiver operating characteristic curves (ROCs)**"**

"Line 193-194:

least absolute shrinkage and selection operator (LASSO)**"**

"Line 237-238:

tumor immunological phenotype-related gene index (TIPRGPI)**"**

[Editor's comment] Please unify to use "Cox" and "COX", "Kegg" and "KEGG", "K-M" and "KM" in the manuscript.

Our response: Thank you for your feedback. We have made revisions to the manuscript addressing the identified issues.

"Line 108:

The "c2.cp.Kegg.symbols.gmt**"**

"Line 116-117:

and the Cox model was constructed**"**

"Line 174:

Kegg pathways between cluster A and cluster B**"**

"Line 263:

the Cox model**"**

[Editor's comment] Please revise "ANRGS" to "ANRGs", "DEGS" to "DEGs" in the manuscript.

Our response: Thank you for your feedback. We have made revisions to the manuscript addressing the identified issues.

"Line 94:

516 anoikis-related genes (ANRGs) in total**"**

"Line 95:

the expression of 516 ANRGs in tumor tissues**"**

"Line 152:

Identification of ANRGs associated with prognosis**"**

"Line 154:

these ANRGs was compared**"**

"Line 168-169:

utilizing prognosis-associated ANRGs.**"**

"Line 213:

the ANRGscore model.**"**

"Line 219:

the ANRGs risk score in predicting the clinical prognosis."

"Line 155:

219 differentially expressed genes (DEGs)."

"Line 259:

219 DEGs were obtained by comparing the expression"

[Editor's comment] Please check if any reference should be added since you mention "studies".

"Previous studies have shown that anoikis resistance plays an important role in the progression of HCC [16]."

Our response: Thank you for your advice; we have cited relevant literature.

"Line 71-72:

Previous studies have shown that anoikis resistance plays an important role in the progression of HCC (16, 17)"

"Line 359-361:

17. Peng Y-F, Shi Y-H, Ding Z-B, et al. Autophagy inhibition suppresses pulmonary metastasis of HCC in mice vi a impairing anoikis resistance and colonization of HCC cells. Autophagy.9(12):2056-68."

[Editor's comment] Figures.

All abbreviations in figures and legends should be explained. HCC, DEGs, ANRGs and TCGA-LIHC in Figure 1 for example. Please check all abbreviations and provide the full names in the corresponding legends.

Our response: Thank you for your feedback. We have reviewed all abbreviations and provided full names in the corresponding legends.

"Line 444:

Hepatocellular carcinoma (HCC)"

"Line 445:

differentially expressed genes (DEGs)"

"Line 446:

anoikis-related genes (ANRGs)"

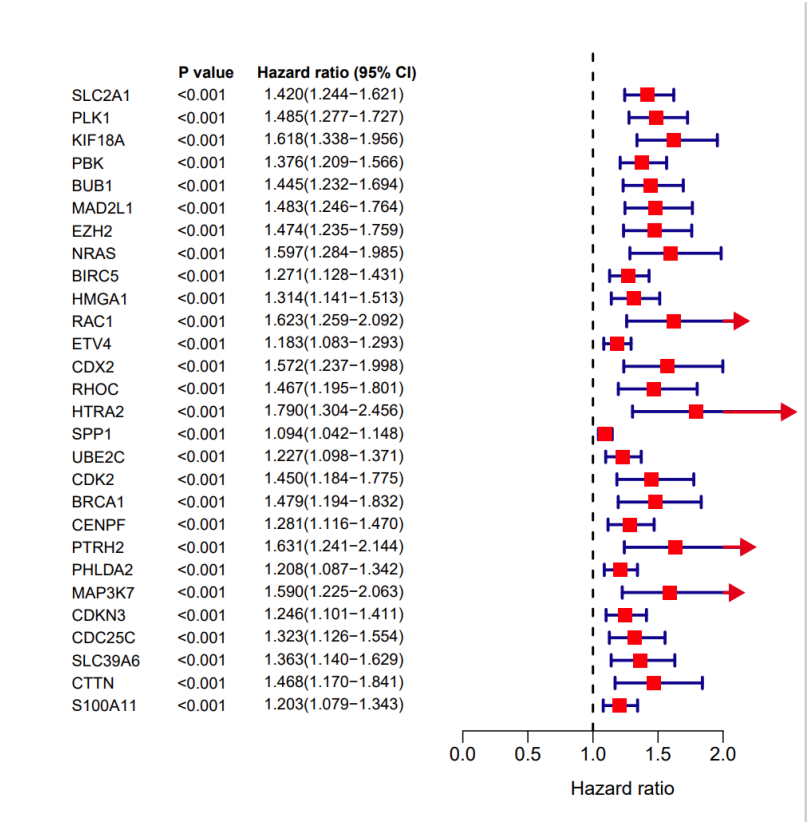
"Line 449-450:

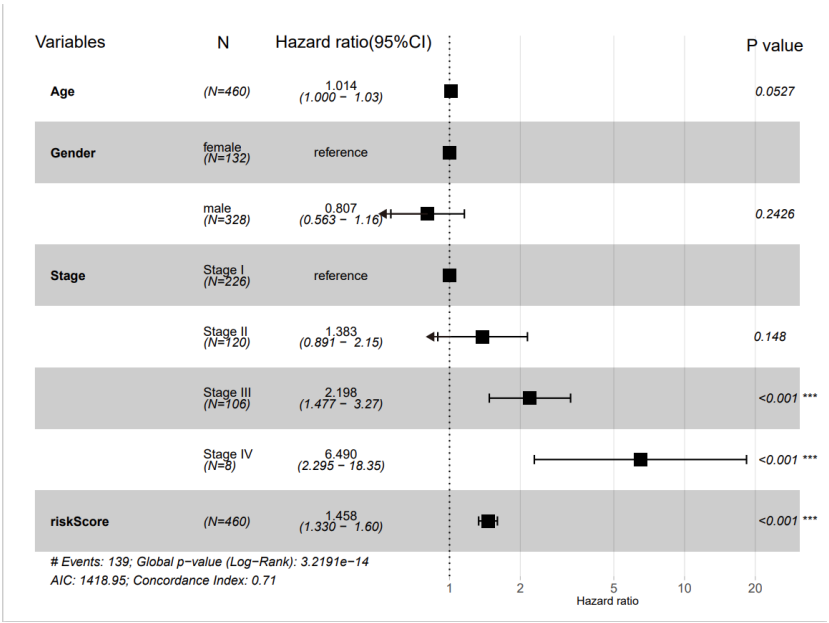
The Cancer Genome Atlas Program—Liver Hepatocellular Carcinoma (TCGA-LIHC)"

Please revise "pvalue" to "P value" and "Hazard ratio" to "Hazard ratio (95% CI)" in Figure 1B.

Figure 1B and Figure 6G: To standardize the results, the part that exceeds the horizontal coordinates should be indicated by arrows.

Our response:





Please double check the P value in Figure 1B legend.

"(B) The forest plot shows the top 28 ANRGs ($p < 0.01$) via the univariate Cox regression analysis."

Our response: Thank you for your feedback. We have made revisions to the manuscript.

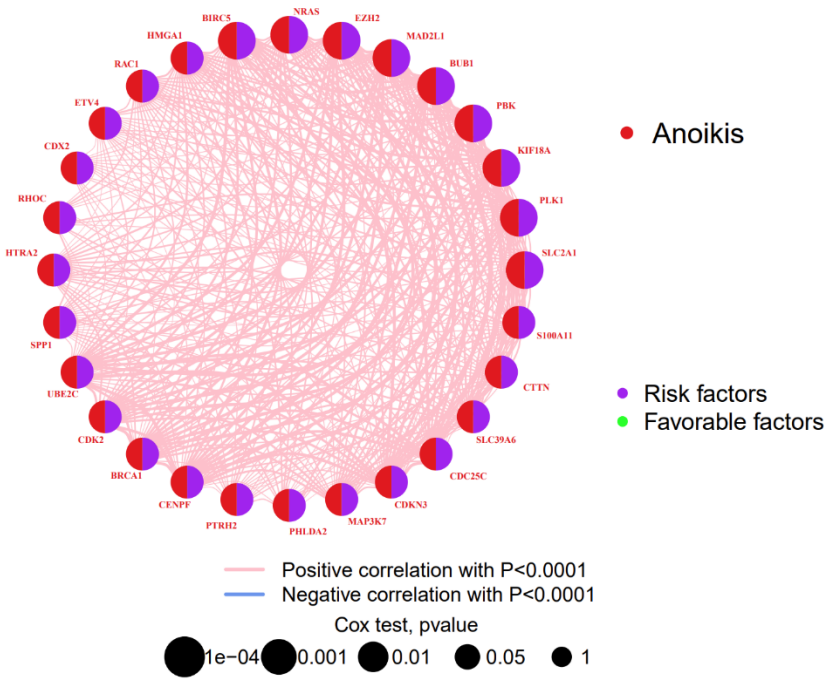
"Line 446:

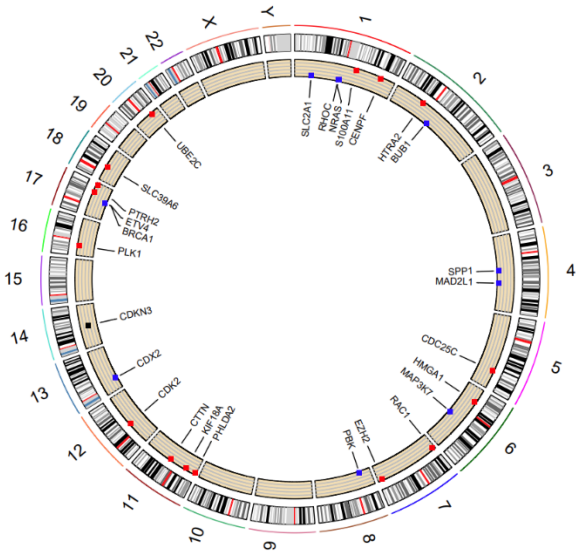
($p < 0.001$)"

There is a spelling mistake in Figure 1C.

Some words are covered and not clear enough in Figure 1C and 1E.

Our response:





Please indicate the meaning of the red, blue and black dots in Figure 1E.

Our response: Thank you for your feedback. We have added the corresponding content in the figure captions.

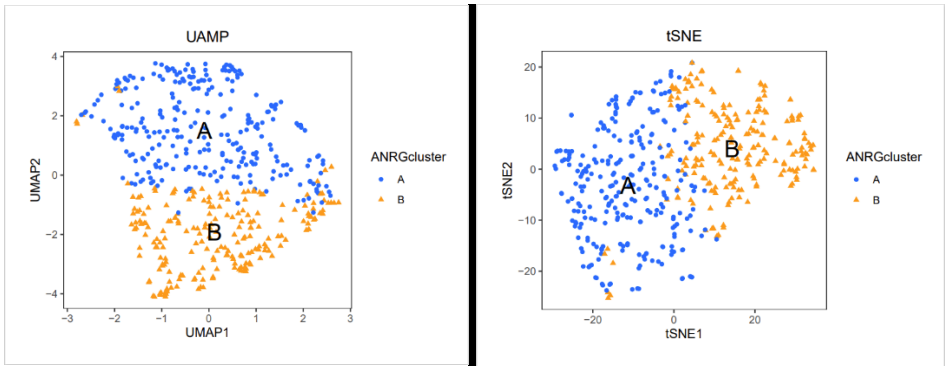
"Line 450-451:

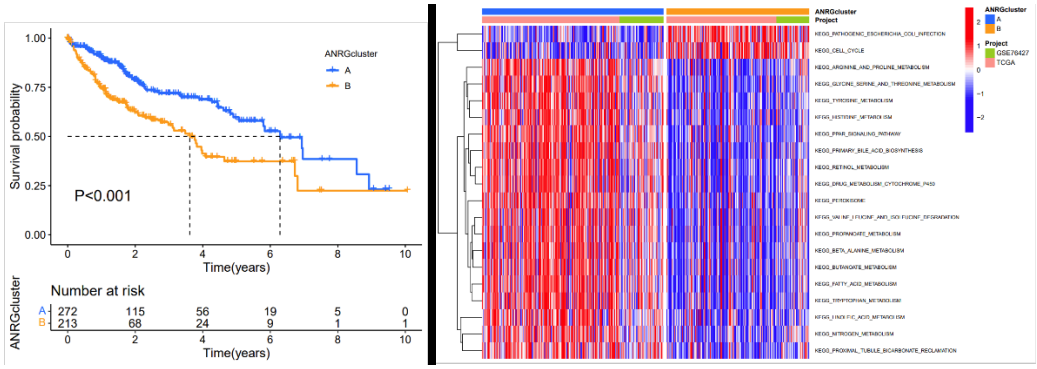
(Red dots: Increase in copy numbers. Blue dots: Missing copy numbers. Black dots: Normal copy numbers.)**"**

Please double check if it is "ARGcluster" or "ANRGcluster" in Figure 2B-2E. Please also

check other figures.

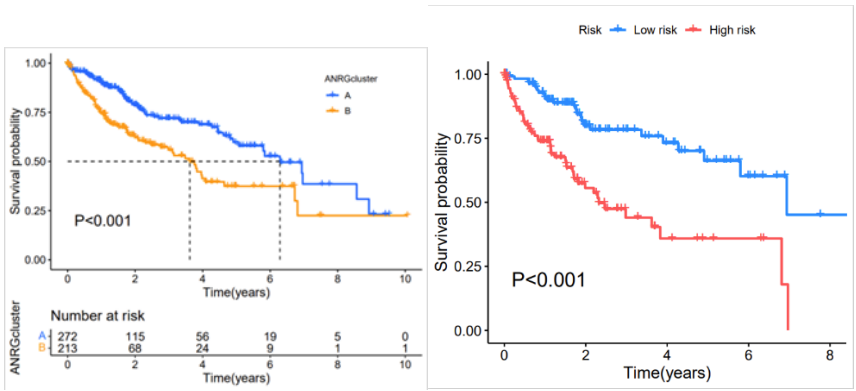
Our response:

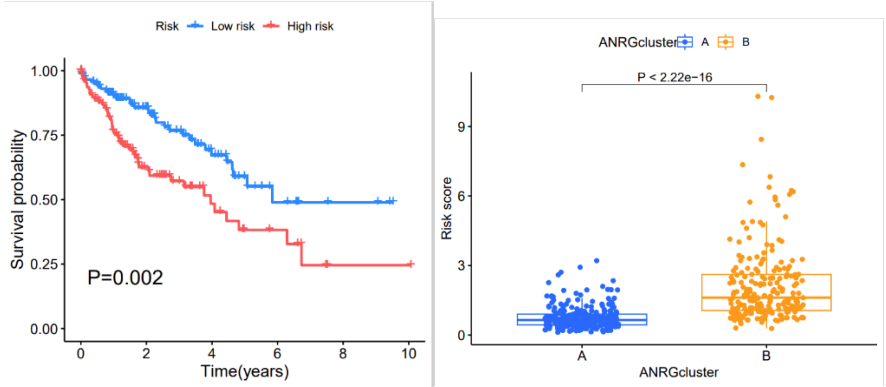


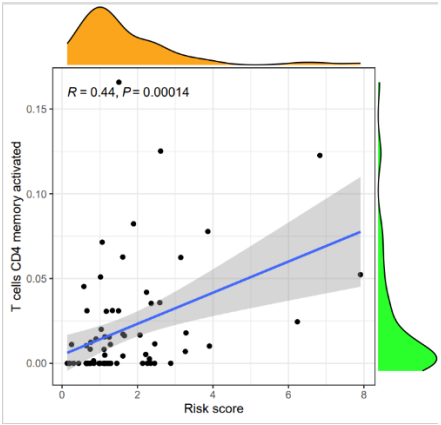


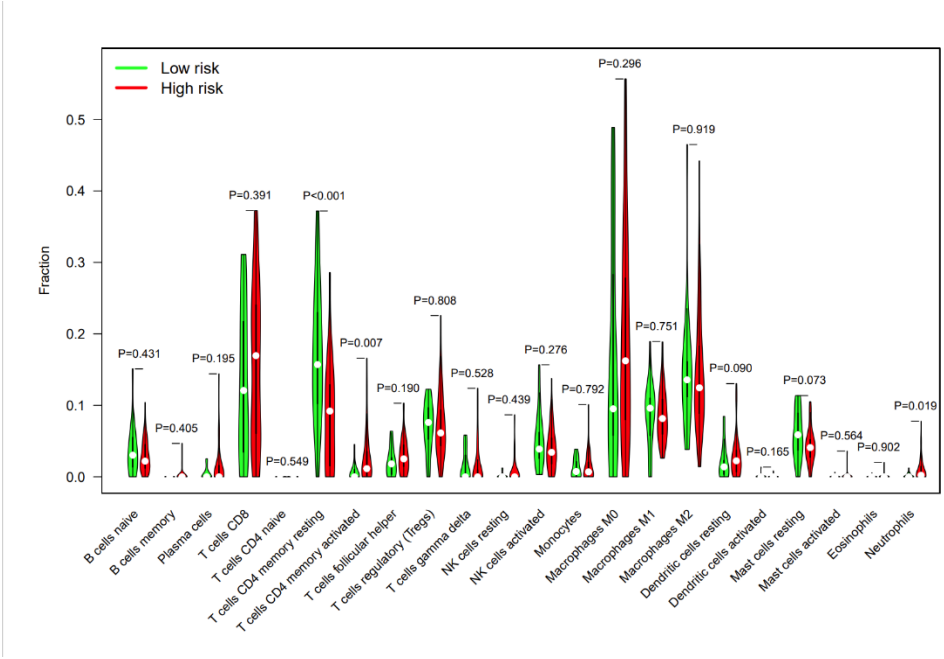
Please revise “p” to “P” in Figure 2D, Figure 4E-4G, Figure 5B, 5C.

Our response:









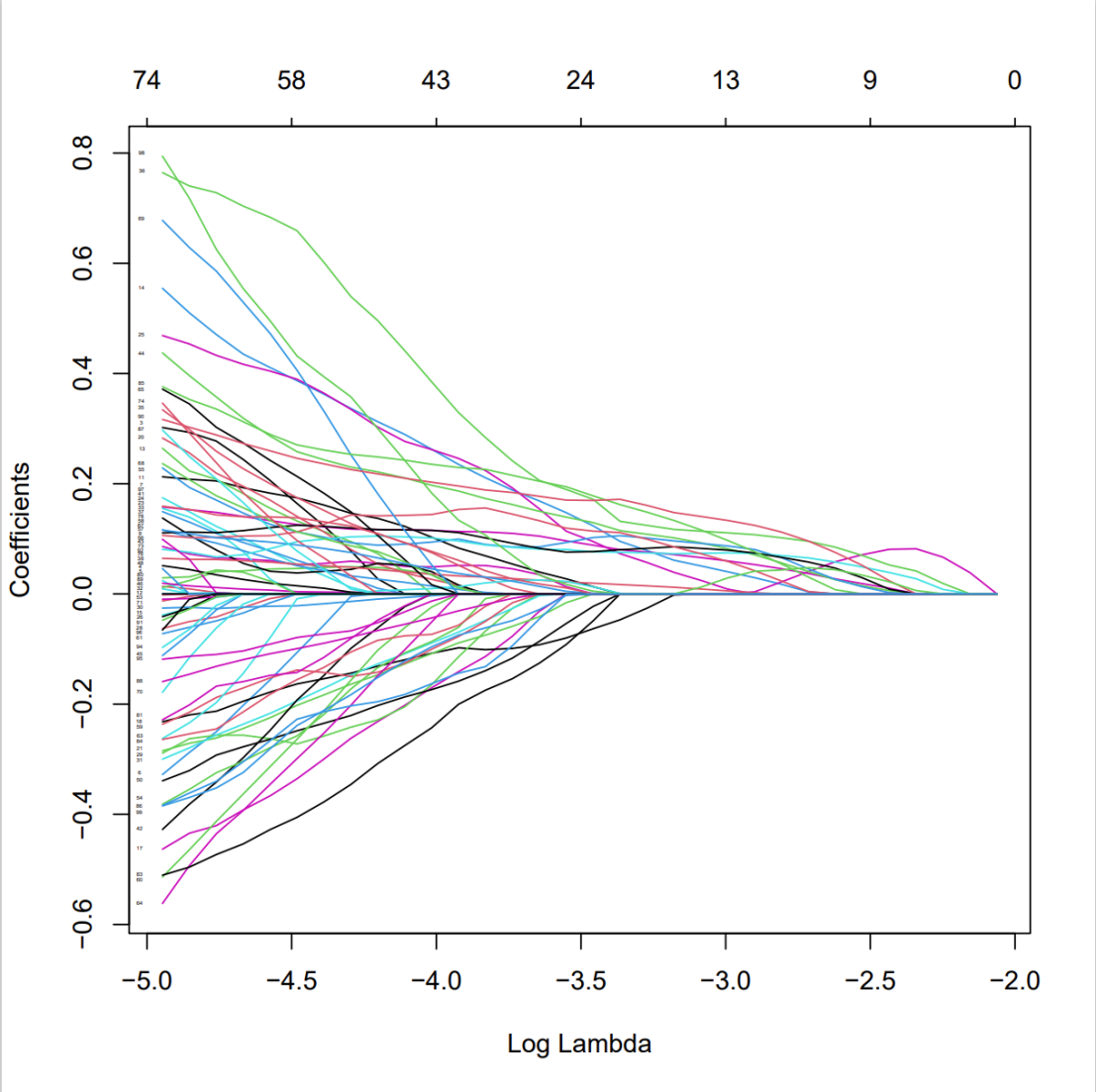
Please indicate the meaning of "*" "***" in Figure 3 legend.

Our response: Thank you for your feedback. We have added the corresponding content in the figure captions.

"Line 462:(*:P<0.05; **:P<0.01; ***:P<0.001)**"**

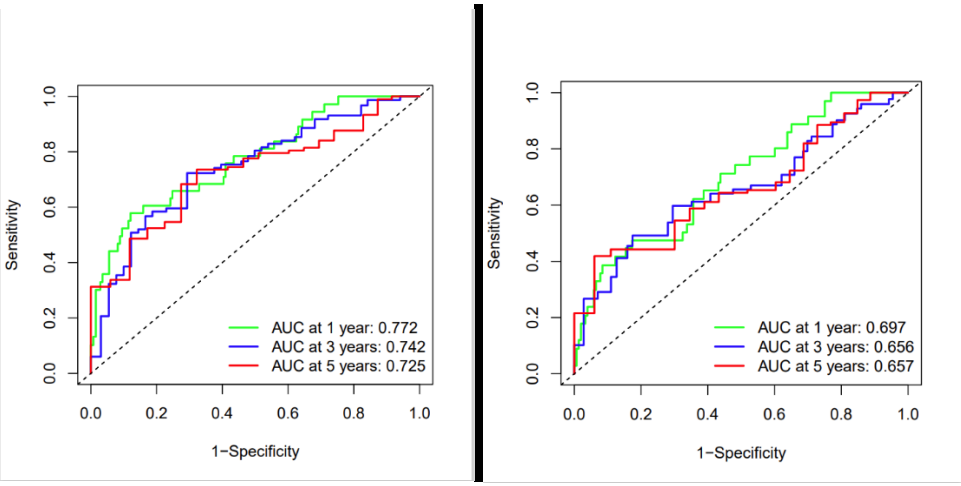
The numbers are not clear in Figure 4B.

Our response:



Please revise “1 years” to “1 year” in Figure 4C, 4D.

Our response:



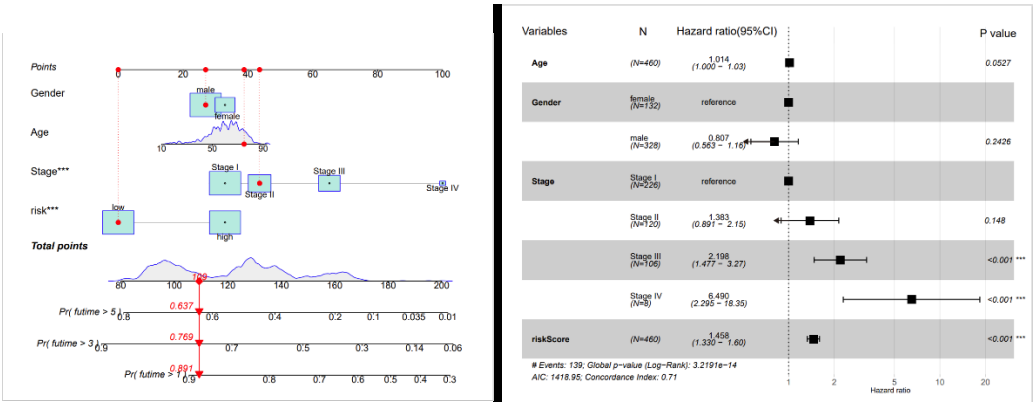
Please indicate the meaning of “***” in Figure 6A legend.

Our response: Thank you for your feedback. We have added the corresponding content in the figure captions.

"Line 485:(*:P<0.05; **:P<0.01; ***:P<0.001)**"**

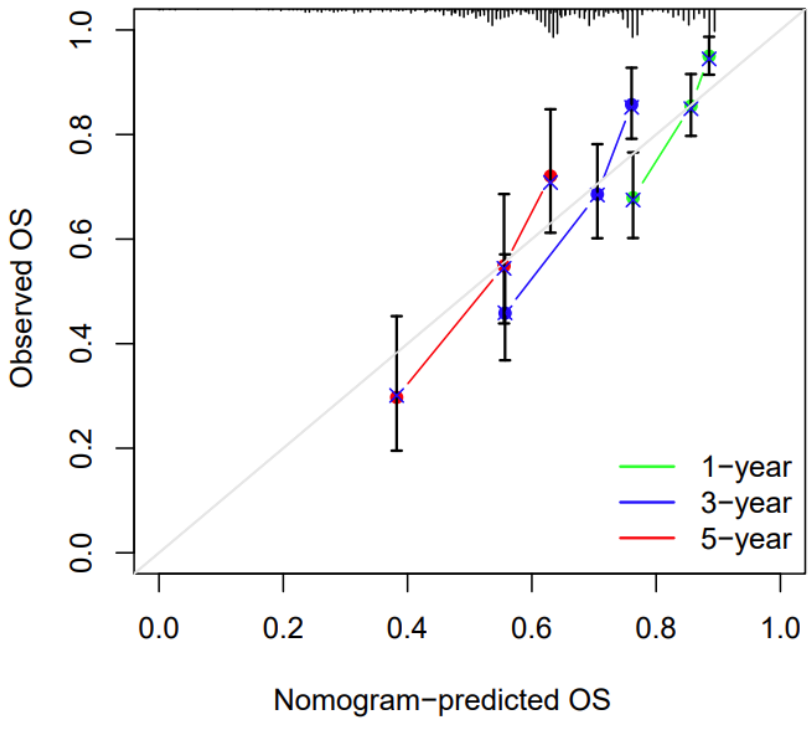
Please revise "femal" to "female" in Figure 6A and 6G.

Our response:



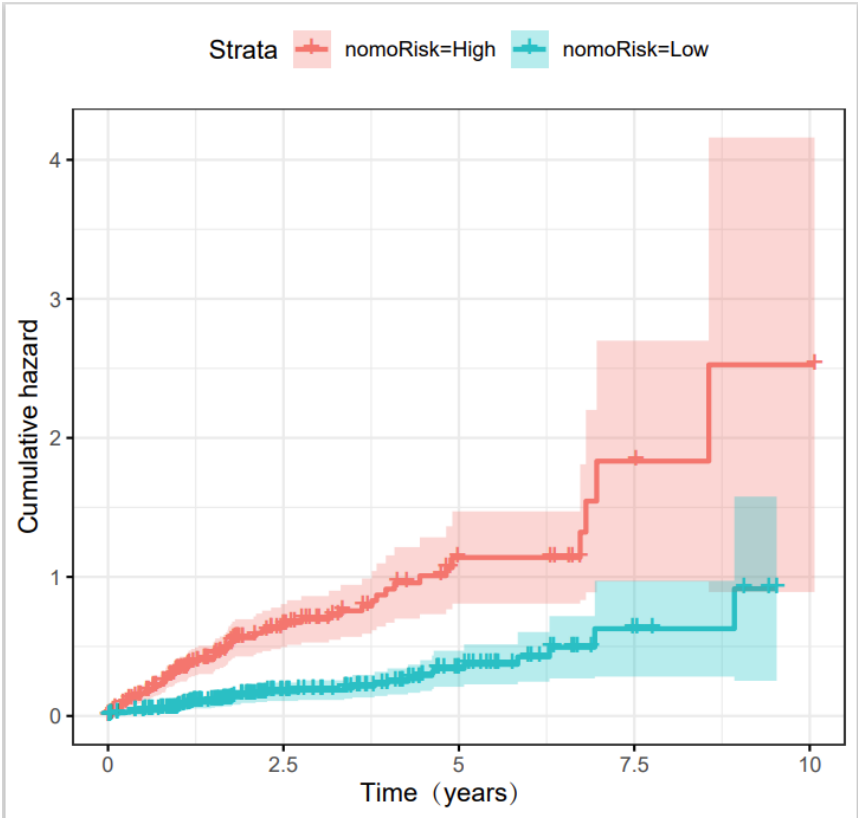
Please check if the unit "%" is correct or not in the x- and y-axis of Figure 6B.

Our response:



Please add unit for Time in the x-axis of Figure 6F.

Our response:



Please indicate the meaning of "***" in Figure 6G legend.

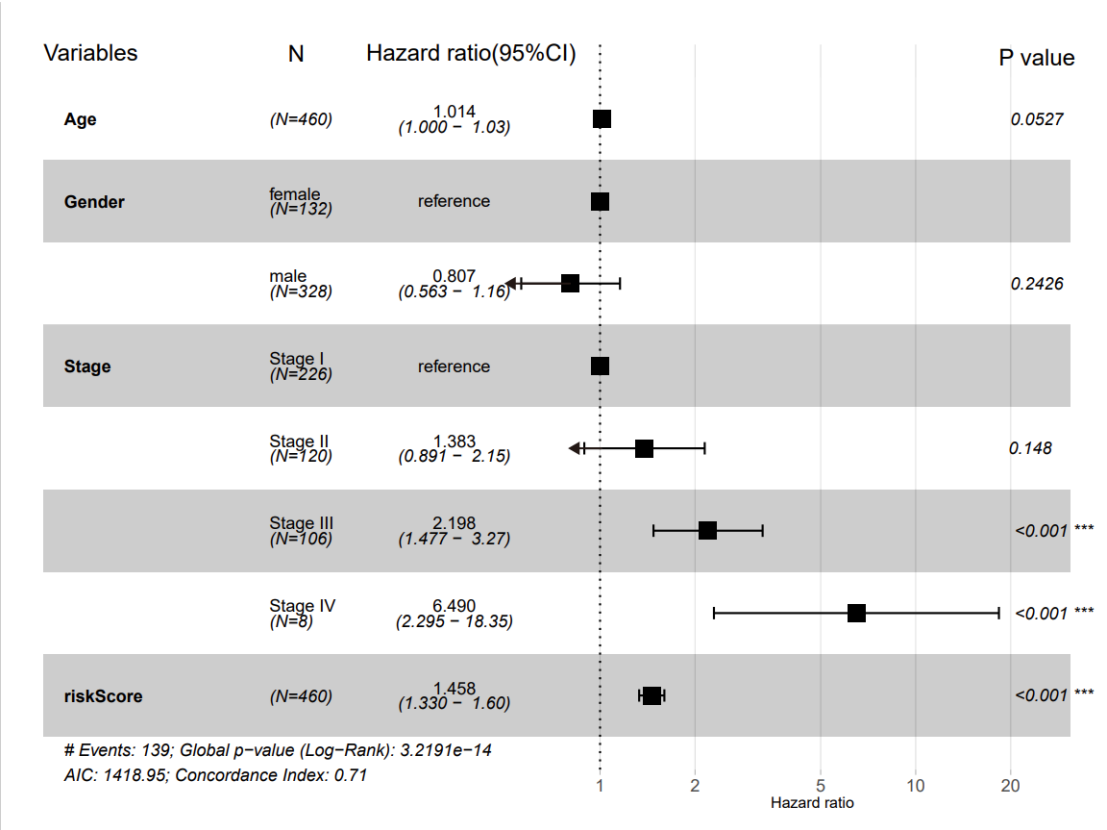
Our response: Thank you for your feedback. We have added the corresponding content in the figure captions.

"Line 485:(*:P<0.05; **:P<0.01; ***:P<0.001)**"**

Please provide headers in Figure 6G.

Please add description for the x-axis in Figure 6G.

Our response:



Please double check Figure 7B legend, whether it should be "GSE166635"."(B) Cell clustering in GSE16635."

Our response: Thank you for your feedback. We have made the necessary revisions to the manuscript.

"Line 488: Cell clustering in GSE166635."

Please double check the data in the following sentence of the main text. There are only 10 cell types in Figure 7C.

"There are 11 different cell types and 20 different cell populations in the GSE166635 dataset (fig.7A,7B,7C)."

Our response: Thank you for your feedback. We have made the necessary revisions to the manuscript.

"Line 224-225: There are 10 different cell types and 20 different cell populations in the GSE166635 dataset (fig.7A,7B,7C)."