Peer Review File

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Reviewer A

In this work, the authors carry out extensive analyses on a number of human tumor mRNA expression sets, focusing on RAC1 mutations, expression levels, and their correlation with the expression of other genes including those that influence the TME and immune cell populations, as well as outcomes data such as tumor stage and survival. Of course, we can't know at this point if there is a causal relationship, but in any case the work presented here could be a valuable reference for others in the field.

Some of the correlations are hard to understand as written. This is especially true for Figure 7. The GSEA for liver cancer simply reports what gene sets are up or down in HCC, does it not? How do we know this is at all related to RAC1? The authors need to give a more detailed explanation of the logic behind this figure. In a lab setting, this question could be addressed by altering RAC1 levels and measuring gene expression.
Reply 1: We did correlation analysis of RACI gene and all genes, and GSEA analysis based on the results of correlation analysis.
Changes in the text: None

Reviewer B

This manuscript investigates the utility of RAC1 as a diagnostic and prognostic marker for cancer using data from public cancer databases. The authors analyzed Rac1 expression, promoter methylation, and their relationship to cancer immunity in detail, and also examined their relationship to survival. Finally, the authors also showed that RAC1-GTP is increased in HCC by immunostaining. The analysis is well done and is expected RAC1 to be useful as a diagnostic and prognostic marker for cancer. However, there are some issues that need to be addressed.

1. Line 219 states that RAC1 expression is increased in RCC, but Line 223 states that RAC1 expression is decreased in RCC, which is correct? To begin with, the data for RCC is missing in Figure 2.
Reply 1: The RCC data is wrong, we have modified it.
Changes in the text: Page 8, line 239-253

2. In Figure S2, the authors stated that methylation was greatly increased in KIRP, but it does not appear to be increased, so it may be better to change the wording.
Reply 2: We have modified our text as advised.
Changes in the text: Page 10, line 292

3. It would be better to include the full notation of some abbreviations in the text (e.g. T size, M sites, etc.).
Reply 3: We have modified our text as advised.
Changes in the text: Page 11, line 336、338、339

4. In Line 358, the authors note that RAC1-GTP is more highly expressed in HCC than in other tumors, but only normal and tumor sites are shown here. Also, the picture in Figure 10C appears to show stronger staining in Normal than in Tumor, so maybe the labeling is incorrect? Furthermore, the authors describe that they scored antibody-positive cells using staining intensity as an indicator, so that data should be presented.
Reply 4: The "the authors note that RAC1-GTP is more highly expressed in HCC than in other tumors" statement is wrong, our study only compared RAC1-GTP expression in LIHC tissues and its adjacent tissues, we have modified our text. At the same time, the icon has been corrected and the data are presented in a bar chart.
Changes in the text: Page 13, line 401; Figure11

Reviewer C

In this manuscript, the authors aim to report a set of alterations associated with cancer progression that could be further studied to be used as prognostic, diagnostic, or therapeutic tools. The authors studied the correlation between RAC1 expression and aspects of different types of cancers. The study was conducted by applying multiple statistical analytical measurements and bioinformatic tools (gene set enrichment analysis and gene set variation analysis). The authors used data from various databases, including The Cancer Genome Atlas, Genotype-Tissue Expression Project, cBioPortal, Tumor Immune Estimation Resource 2, and published articles. In Addition, the authors measured activated RAC1 (RAC1-GTP) levels in hepatocellular carcinoma patients using immunohistochemistry (IHC). The authors showed that high expression of RAC1 was characteristic of most types of cancers and correlated with prognosis and advanced pathological stages. Additionally, the authors determined higher levels of activated RAC1 in liver hepatocellular carcinoma compared to adjacent tissues. The authors utilized these RAC1/tumor correlations and established a prognostic nomogram for hepatocellular carcinoma patients. The study reported significant associations between upregulated expression and poor survival, DNA methylation, immune cell infiltration, immune-related genes, tumor mutational burden, and microsatellite instability in most tumors. To this end, the author concluded that RAC1 can serve as an immunotherapy target and a diagnostic and prognostic biomarker. The study lacks experimental confirmation and clinical data of the patients, such as previous treatment, naïve tumors, etc. However, the authors reported the limitations of the study. Overall, the study is a good reference and assembly of information that may provide researchers with knowledge about RAC1 in different types of tumors. I support its publication at TCR. I listed my suggestions to help produce a better version of the manuscript to make the paper get more attention.
Suggestions:
1. In the method section. The authors should describe the normal tissues used as a reference in

the study. It has been mentioned in the text once that they are adjacent tissues; that needs to be stated in the method's section if this was the case for all the tumors. An example is result section # 206: Tumor and normal tissue expression of RAC1 gene; normal tissue needs to be defined.
Reply 1: We have described normal tissues in the methods section.
Changes in the text: Page 4, line 128-132

2. The subtitles in the method section need to describe the applied method, for example, the type of analysis used (e.g., #168 and #178).
Reply 2: We have modified our text as advised.
Changes in the text: Page 6, line 194-195

3. Method section#186 describes a score of IHC. This score is not reported in the result section.
Reply 3: The data are presented in a bar chart
Changes in the text: Figure11

4. Method section#199 (Statistical analysis) should state the software used for the statistical analyses and the type of statistical tests applied.
Reply 4: We have supplemented the statistical analysis software used and the types of statistical tests used.
Changes in the text: Page 7,8, line 222-228

5. Unify an abbreviation for liver hepatocellular carcinoma LIHC or HCC; the abbreviation in the figure should match the text; for example, it is LIHC in Figure 2 and HCC in section #206).
Reply 5: We have unified the abbreviation of liver hepatocellular carcinoma in the article as LIHC.
Changes in the text: Full text

6. Most of the figure legends could be more informative. The type of applied statistical test, number of tumor samples, etc., should be reported. The authors briefly describe the figures panels. For example, what was the applied analysis in Figure 10 A and 10B? What does the red color refer to?
Reply 6: We have modified our text as advised.
Changes in the text: Page26, line 774-780

7. Figure 3A: Correct the word mutationo. Cancer names can be abbreviated. Figure 3C: It would be helpful if the authors clarify what the positive correlation refers to; is it hypermethylation of DNA, and does a negative correlation mean no change or hypomethylation? What was the reference for this comparison? Normal methylation status in normal tissue?
Reply 7: We have corrected the word mutationot into mutation. Sorry, I don't understand the question raised by the reviewer. What we're doing here is correlation analysis, not difference analysis.
Changes in the text: Figure 3A; Page 9, line 281-282,286-287

8. I highly recommend making a table to highlight the significance of this study. The table can

include a list of cancer types with high RAC1 expression, RAC1 as a prognostic or risk factor, and upregulation/downregulation of immune pathways.
Reply 8: We have made the table according to the suggestion.
Changes in the text: Page 21, table1

9. In #225 (Relationship between RAC1 gene expression and pathological stages, Figure S1), the authors should list the types of cancer where RAC1 was highly expressed in early stages (I and II). This supports part of the rationale of the study and defines RAC1 as an early prognostic marker in some types of cancers.
Reply 9: We looked for cancer types in which RAC1 was highly expressed in the early stages (I and II), but found that both stage III and stage IV had higher RAC1 expression than I and II in the cancers we analyzed.
Changes in the text: None

10. The authors used a huge number of abbreviations. It would be helpful for the reader if all the abbreviations were listed together in one place. Also, in the method and result sections, it would be better to use the full name in the title [e.g., #178; TMB, MSI; #236, CAN]. Abbreviations that are not the type of cancer (e.g., TME, TMB, T stage) are not often used, and it is recommended to write them in the full name.
Reply 10: We have presented most abbreviations in this article in Table 1.Meanwhile, we have modified our text as advised.
Changes in the text: Page20;Full text

11. The phrase “in the absence of” (#247) would be better changed to something else, such as “rather than”. However, if the authors know of patient cases diagnosed with different types of cancers (i.e., one patient with two or more cancers), they should clarify and cite.
Reply 11: We have modified our text as advised.
Changes in the text: Page 9, line 279

12. In the method section and also in result #236, the author should describe the DNA methylation (Which DNA?), and promotor RAC1 methylation should be mentioned in the first place (title and text)
Reply 12: We have changed DNA methylation to methylation of the RAC1 gen promoter.
Changes in the text: Full text

13. In the result section where a parameter is mentioned for the first time, such as DSS, DFI, or PFI (#246), it would be helpful to state the full name.
Reply 13: We have modified our text as advised.
Changes in the text: Page 10, line 307-308

14. Title (269 ##Survival analysis of RAC1 gene expression) can be changed to be more descriptive (example: Survival analysis of patients with altered RAC1 gene expression)
Reply 14: We have modified our text as advised.
Changes in the text: Page 10, line 303

15. In the result section # 307 (Analysis of GSEA and GSVA in HCC for RAC1 gene), the authors reported enrichment of immune-regulated pathways and cell cycle events in HCC, which is RAC1 high. Did the author run this analysis in RAC1 low tumors?
Reply 15: We performed this analysis in tumor KICH with low RAC1 expression, and the results likewise demonstrated enrichment of immunomodulatory pathways and cell cycle events.
Changes in the text: None

16. #309 It would be more realistic to address the question differently; "investigating the biological function of RAC1" is more about the association of altered RAC1 expression and reported changes.
Reply 16: Thank you very much for the reviewer's suggestion. In the future study, we will better explain the biological function of RAC1 through molecular biology experiments and phenotypic experiment.
Changes in the text: None

17. Some subtitles in the result section need to be more specific and informative. For example, # 323: Correlation between RAC1 gene and TME; it can be changed into increased macrophage infiltration into tumor microenvironment of patients with RAC1high tumors.
Reply 17: We have modified our text as advised.
Changes in the text: Page 12, line 361

18. #353 Results: The title should refer to the results but not the methodology, or it can be merged with the previous section (#335).
Reply 18: We have modified title as advised.
Changes in the text: Page 13, line 395

19. #353: The authors mentioned that the expression of RAC1-GTP was higher in HCC tissues than in other tumors and referenced Figure 10C. In contrast, the figure only compares normal and an HCC tumor tissue. The brown staining is higher in the normal tissue; authors should check if the labeling were mixed. Also, check the scale bar in the legend of that figure.
Reply 19: The "the authors note that RAC1-GTP is more highly expressed in HCC than in other tumors" statement is wrong, our study only compared RAC1-GTP expression in LIHC tissues and its adjacent tissues, we have modified our text. At the same time, the icon has been corrected. We have double-checked the scale in the legend for this figure to make sure it is correct.
Changes in the text: Page 13, line 401

20. Legend of Figure 10: authors should start with a sentence to describe the figure, then list the description of each panel.
Reply 20: We have modified our text as advised.
Changes in the text: Page 26, line 774-778

21. Did the author measure the expression of RAC1 downstream effectors?
Reply 21: Unfortunately, we did not measure the expression of RAC1 downstream effectors. However, we will analyze the downstream expression of RAC1 in the future.
Changes in the text: None

22. #395 Discussion: The authors stated, "M2-like TAMs, in particular, induce chronic inflammatory carcinogenesis by releasing proinflammatory mediators." M1 macrophages are the subtypes defined as inflammatory immune cells, but not M2. If the statement is correct, please cite it.
Reply 22: We have modified this sentence.
Changes in the text: Page 14, line 441-444

23. #379 Discussion: "RAC1 plays a role in promoting or suppressing tumors." The presented study cannot lead to the conclusion that RAC1 is a tumor suppressor. The upregulation of RAC1, significant correlation with metastasis, and upregulation of immune suppressor genes support the promoting role. If there is evidence for the suppressor role, it needs to be discussed more and/or supported by citing previous studies.
Reply 23: We have modified the statement to make it more appropriate.
Changes in the text: Page 14, line 424

Reviewer D

In this paper, the authors use multiple databases to analyze Rac1 gene expression across a wide range of cancer types. They find the majority of cancers they look at have an upregulation of Rac1 expression compared to normal tissues. They then characterize Rac1 gene modifications with copy number alteration, mutational analysis and methylation in different cancers and find many cancers have Rac1 amplification. Further, they analyze survival and prognosis relative to Rac1 expression and find in many cancers higher Rac1 expression is associated with a worse progression-free interval while a few cancer types show the opposite trend. The authors then look at gene set enrichment analysis for HCC, hepatocellular carcinoma, which has been previously reported to be associated with higher Rac1 expression, and find an association of Rac1 and immune-related genes. Next, they assess Rac1 expression relative to tumor associated macrophage (TAM) infiltration and immune related genes in other cancers and find a positive association with TAM infiltration and Rac1 expression in most cancer types and find a subset of immunosuppressive genes are correlated with Rac1 expression in most cancers. They also look at microsatellite instability (MSI) and tumor mutational burden (TMB) and find Rac1 expression correlates varyingly (positively, negatively) with immune markers. The authors propose Rac1 could serve as an important biomarker for cancer disease progression and immunotherapy efficacy.
Overall, this paper has an extensive analysis of Rac1 expression, features a wide range of cancers and identifies shared trends among cancer types and Rac1 expression. The association between Rac1 and immune-related genes is intriguing. However, the immune-related genes are

not extensively explored or validated with other approaches in the current version of this manuscript. Further, there are some concerns with the analysis of the datasets. Lastly, the terse text makes this paper difficult to follow and avoiding excessive use of acronyms would greatly improve the readability.

Major Concerns:

1. One concern with the comparison between datasets (tumor versus normal) is that they are from different studies and the counts of transcripts per million could be different based on tissue collection and analysis methods. An explanation of how the authors can control for this and differences in the collection/analysis methods would be useful to have in the main text.
Reply 1: Although the data sets were from different studies, the relevant bias factors were excluded when we performed the meta-statistical analysis, and the statistical results clearly indicated the difference between tumor tissue and normal tissue.
Changes in the text: None

2. In the discussion the authors mention there has not been a Rac1 analysis looking a wide range of cancers but I believe this study should be referenced here as one that has by Lou et al., 2018. Journal of Cancer. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6036885/
a. Related to this, it is not clear why they focus on HCC and Rac1 because there are other studies reporting an association between higher Rac1 expression in other cancers
Reply 2: Lou et al. 's study focused on the relationship between RAC1 expression and prognosis, while our study explored the relationship between RAC1 expression and immunity in addition to discussing prognosis. The primary focus of our research group was on the study of liver cancer. Additionally, subsequent studies were conducted to investigate the relationship between radiotherapy efficacy and rac1 expression.
Changes in the text: None

3. Parts of the manuscript were difficult to follow due to a) the many acronyms used, b) lack of definition of databases within the text, and c) missing explanation of the rational for analysis or conclusions from a result.
Reply 3: 1. We have changed most of the abbreviations in the article to full names for better understanding. 2. We have added to the database definition. 3. We modified the analysis of the results to make them more reasonable.
Changes in the text: Page 14, line 424; Page 14, line 441-444

4. The methods that describe the cutoff for Rac high and low expression for PFI are vaguely defined and need to be reported as the numerical values set for the threshold and how these were determined as “optimal”.
Reply 4: We have modified our text as advised.
Changes in the text: Page 5, line 156-158

5. It is not clear why the authors only look for all gene associations for HCC and then narrow their focus to only immune-related genes for other cancers. Further, the conclusions would be

more supported if the authors validate the expression of some of these immune-related genes relative to Rac1 expression in a few cancer types.
Reply 5: Our research group mainly studies liver cancer. He is particularly interested in radiotherapy for liver cancer.
Changes in the text: None

6. The authors look for an association between immune-related genes and Rac1 and find a correlation between some immune-suppressive genes in Rac1 as well as increased TAM infiltration and Rac1- from this they conclude that Rac1 could be a good immunotherapy target, however it is unclear how Rac1 could be targeted with existing immunotherapies. Rather it seems it may serve predictive marker of immunotherapy efficacy as the authors also conclude, however there are some caveats to this conclusion as well as the authors point out because they do not have datasets to look at immunotherapy efficacy versus Rac1 gene expression directly. I would suggest changing this conclusion or providing a clearer explanation of how Rac1 could be an immunotherapy target
Reply 6: We speculated from the existing results that Rac1 may participate in the immune microenvironment of liver cancer, so can Rac1 be a target for liver cancer immunotherapy? It needs to be further studied in animal experiments and organoid models.
Changes in the text: None

7. For the staining in 10C, can the authors provide some quantification to see how consistent this is? Why did the authors only look at one cancer type?
Reply 7: We have presented it by drawing a bar chart. We also think it is necessary to conduct immunohistochemical analysis in other tissues, but considering the limited time in the early stage, we only conducted immunohistochemical analysis in LIHC tissues, and we will continue to conduct immunohistochemical analysis in other tumor tissues in the future.
Changes in the text: Figure 11C

8. Some of the data is not presented in a clear manner and need to be further explained in the legend or main figure. For instance in Figure 1B-C what do the numbers mean on the plot- what do the different color dashed lines represent?
Reply 8: We have modified our text as advised.
Changes in the text: Page 22, line 648

Minor:
1. Are datasets used only looking at the gene expression tumor cells of other cells (e.g. TAMs) within the tumor sample?
Reply 1: We also analyzed other cells, but selected important results for discussion.
Changes in the text: None

2. Some figures have very small text
Reply 2: Because there's a lot of content in the figure, we can only make the text smaller.
Changes in the text: None

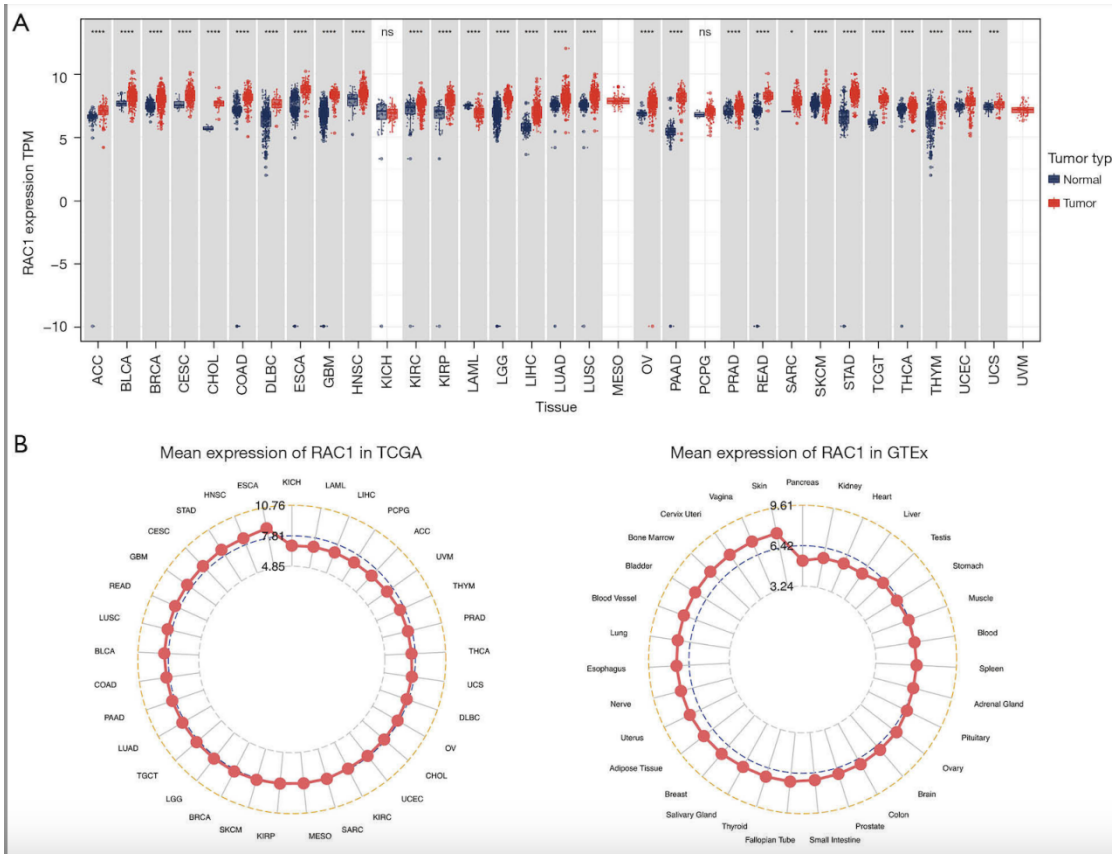
3. Can you please provide p-values for Figure S2?
Reply 3: We have provided p-values for Figure S2.
Changes in the text: Figure S2

4. Would recommend splitting Figure 8 into two figures
Reply 4: We have modified Figure 8 as advised.
Changes in the text: Figure 8 Figure 9

Reviewer E

1. The authors mentioned "studies...", while only one reference was cited. Please revise.
Currently, several studies have demonstrated that tumor mutational burden and microsatellite instability are useful biomarkers that are helpful in predicting the effectiveness of immunotherapy in cancers (21).
Reply: We have modified our text as advised.

2. "C" is missing in Figure 1. Please revise.



Reply: We have revised.

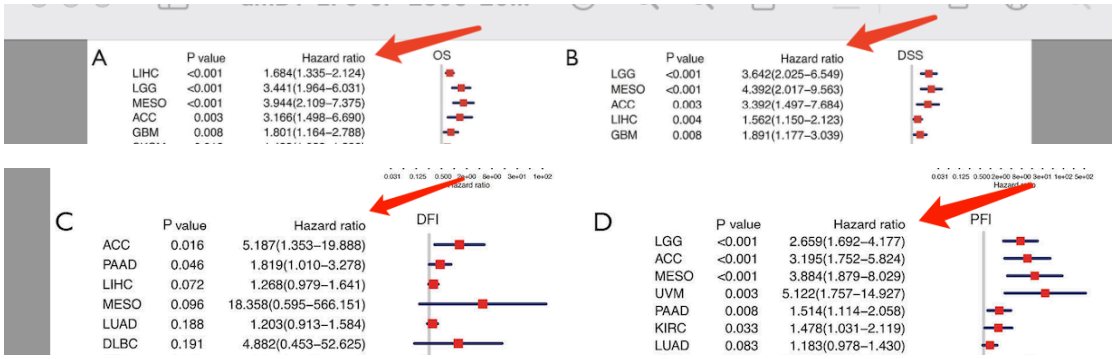
3. Figure 1

3.1 ****: please explain its meaning in the legend.
Reply: We have explained.

3.2 There seems to be no "**" in Figure 1, while it was explained in the legend. Please check and revise.
Reply: We have revised.

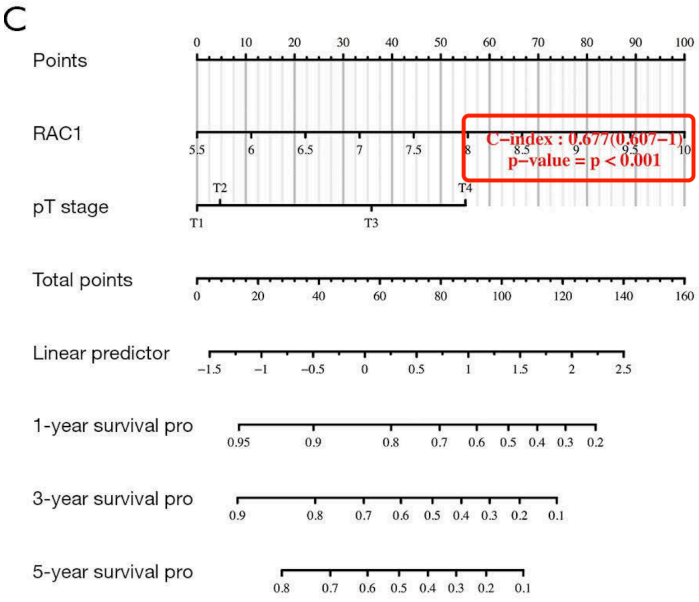
4. Figure 2
*, **, ****: please explain their meanings in the legend
Reply: We have explained.

5. Figure 4
Please add "(95% CI)" accordingly.



Reply: We have added.

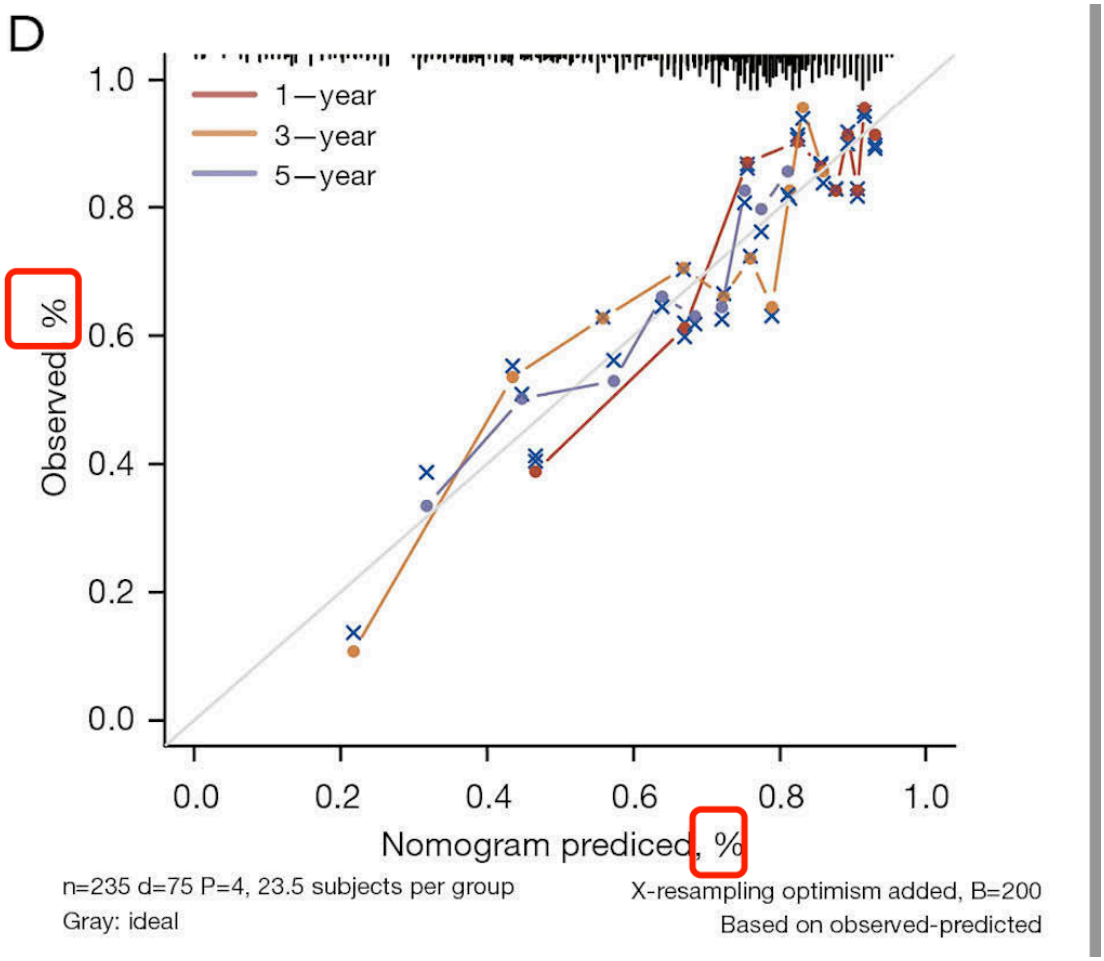
6. Figure 6C
Should the below pointed information (red box) be placed here, since this information covers the data for RAC1. Please check and revise.



Reply: We have revised.

18. Figure 6D

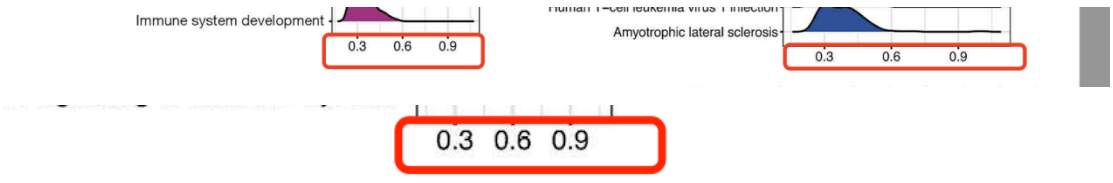
"%" should be deleted. Please revise.



Reply: We have revised.

7. Figure 7A-7C

Please provide the descriptions of the x-axis



Reply: We have explained the x-axis in the Figure 7A-7C

8. Figure 7D

GSCV or GSVA? Please check and revise.

771 Figure 7 Results of GSEA and GSVA for *RAC1* in LIHC. (A-C) The GSEA results of
772 *RAC1* in LIHC, including GO, KEGG, and Reactome. (D) Analysis of the 50
773 hallmark pathways in LIHC according to GSVA.
774

Reply: We have revised.

9. Figure 9

*, **, ***, ****: please explain their meanings in the legend

Reply: We have explained.

10. Figure 10

11.1There seems to be no "***" in Figure 1, while it was explained in the legend. Please check and revise.

Reply: We have revised.

11.2 ****: please explain its meaning in the legend.

Reply: We have explained.

11. Figure S1

, *: please explain their meanings in the legend

Reply: We have explained.

12. Figure S3F

DSS or DFS? Please check and revise.

956 Figure S3 Correlation between *RAC1* promoter methylation and the survival of
957 patients with tumor. Correlation between RAC1 promoter methylation and OS (A-E),
958 DFS (F), DFI (G), and PFI (H,I).
959

Reply: We have revised.