Peer Review File

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Reviewer A

Q: In the present manuscript, authors have utilized a publically available database to explore the markers related to portal vein tumor thrombus (PVTT) by utilizing the different bioinformatics approaches. They identified 110 genes that
were closely associated with PVTT. Among these genes, TMEM165 was the most prominent marker related to PVTT. The manuscript is well-written and the findings have significant potential applications. The authors need to improve the presentation of Figures 1 and 2. The discussion needs to be more concise. A summary figure needs to be added, concluding their findings.

R: Thank you very much for taking the time to review our manuscript amid your busy schedule and providing such constructive feedback. Our images were not sufficiently clear, and the layout was too chaotic, making it challenging for readers to grasp our research rationale and findings effectively. Therefore, based on your suggestions, we have redesigned all the images. In the new figures, we have summarized the single-cell sequencing results into Figures 1-3 and Figure 10. The remaining images present the analysis results of bulk sequencing data. In the previous figures, survival analysis and COX regression curves appeared excessively repetitive, so we have streamlined them and presented the results in alternative forms. With numerous adjustments to our images, we believe the new figures can more clearly convey our research rationale and results.

For the discussion section, we have reduced redundant statements. Through our modifications, the word count of the discussion section has been reduced from 1629 to 1232 words. We have made the discussion of the research more concise and to the point (Pages 31-36, lines 606-726).

Additionally, based on your feedback, we have created a flowchart illustrating the workflow of this study. The flowchart provides a summary of the study's process and the results in the images. Through this, you can more conveniently review the core aspects of our research. For a comprehensive overview and description of the images, we have summarized each figure at the end of the article. You can refer to them in the final pages of the document (Supplementary Figure.docx, pages 42-49, lines 920-1059).

Reviewer B

The authors utilized state-of-the-art single-cell sequencing technology to analyze molecular markers associated with the formation of portal vein tumor thrombus in liver cancer. They described its clinical implications, emphasizing that the presence of portal vein tumor thrombus in liver cancer patients indicates a poor prognosis. Inhibiting the invasion of liver cancer cells into the portal vein could significantly improve the survival period of liver cancer patients. The findings of the study provide new insights for the diagnosis and treatment of portal vein tumor thrombus.

Furthermore, I would like to provide some suggestions for corrections regarding certain aspects of this study:

Q1: Image Annotations: I recommend that the authors include more annotations in the figures to enhance the understanding of the information presented. For instance, in the survival analysis of Figure 7, it would be beneficial to label each lncRNA and miRNA for better clarity.
R1: Thank you very much for your feedback. We have redesigned Figure 7 and, as per your request, added corresponding annotations to it. With these modifications, Figure 7 can now convey its meaning more clearly (Figure 7, pages 46, lines 996-1000).

Q2: Supplementary Files: There are numerous supplementary files attached, and some may not be essential for the understanding of the article. I suggest that the authors organize the supplementary files and exclude unnecessary data, focusing on presenting key research findings.
R2: Thank you very much for your feedback. All supplementary files were created to facilitate the reviewer's understanding of our research workflow and to make it easier for them to check the data in our analysis process. Therefore, following your suggestion, we have removed all supplementary files to streamline the article.

Q3: Statistical Methods: Given the extensive use of statistical methods, the authors could consider consolidating all statistical methods into a dedicated section within the methodology. Significance levels (P-values) for statistically significant differences should be clearly stated.
R3: Thank you very much for your feedback. We have organized all the statistical testing methods used throughout the entire manuscript. They have been consolidated and presented in the Methodology section under the subheading "Statistical Methods," along with the inclusion of significance criteria for statistical tests. These modifications enhance the rigor of the article (Page 22, lines421-426).

Q4: Language Clarity: While the language used in the study is generally coherent, there are instances where expressions may be unclear. It is recommended to use language software or seek professional editing services to improve the overall clarity of the manuscript.
R4: Thank you very much for your feedback. We consulted with professionals and used specialized software to make corrections to linguistic errors in the article. The language of the manuscript is now more fluent.

Q5: Queries and Clarifications: There are some personal inquiries regarding the study. For instance, the use of Mfuzz clustering, which is typically applied in samples with temporal order, may need clarification regarding the relevance of gene expression order in the analysis. Additionally, details about the source of data for immune checkpoint analysis and the support for the settings in single-cell sequencing quality control annotations should be provided.

R5: Thank you very much for your feedback. Mfuzz clustering analysis is typically employed initially for time-ordered expression matrix data. However, in subsequent practice, it has been found to demonstrate good analytical efficiency for grouped data with certain trends. As indicated in articles with PMIDs 36325528, 36418670, etc., in our study, we performed grouping and analysis based on the expression trends of genes, meeting the mentioned application conditions.
The selection of immune checkpoints was based on various databases (such as GSEA) and multiple literature sources (such as PMID: 30546008, PMID: 24655299, etc.). Therefore, we ensure the accuracy of the source of immune checkpoints.

Addressing these suggestions and providing clarifications for the queries will enhance the overall quality and clarity of the manuscript.

Reviewer C

Q: The conclusion of this paper is only based on the database data analysis, lack of clinical sample verification and intensive mechanism experiments, can not draw effective conclusions. In addition, the research is not innovative.
R: We would like to express our gratitude for your careful and thorough reading of this manuscript, as well as your thoughtful comments and constructive suggestions. Here are our responses to your feedback:
Regarding the lack of experimental validation, we regret to inform you that, over the course of the six months from the initiation to the completion of our study, we were unable to collect tissue samples of portal vein tumor thrombosis (PVTT) from our institution and other hospitals. Typically, once PVTT is identified in patients, clinicians often opt for non-surgical treatments such as chemotherapy or interventional therapy, making sample collection exceedingly challenging. Nonetheless, we are actively working on collecting potential samples, recognizing that this is an ongoing process. In the future, if we can amass a sufficient number of samples, we will further validate our research findings. Additionally, we have discussed this limitation in the corresponding section of the manuscript, providing insights for future research.
Regarding the novelty of the article, we utilized the latest single-cell transcriptomic algorithm, hdWGCNA, in our study and were the first to propose the role of TMEM165 in portal vein tumor thrombosis. Furthermore, research on PVTT has been relatively understudied in the field of hepatocellular carcinoma, with previous studies mostly based on clinical cohorts. Therefore, we believe our analysis of PVTT adds a certain level of novelty to the existing body of research.