Peer Review File

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Reviewer A

This original article very thoroughly assesses ubiquitination and deubiquitination, both equally important post translational protein modifications involved in the regulation of metabolic reprogramming in cancer. The authors have used stringent statistacal tools to verify their apporach.

Overall the paper is well written and an impressive compilation of the characterisation of the ubiquitination modification differences in the TME of LUAD and should be a very helpful for future research exploring the matter.

Response: Thank you for your insightful review and encouraging comments on our work. We hope our study will indeed serve as a valuable resource for future research in this area, as you mentioned. We are committed to contribute more to this exciting field.

Thank you once again for your time and positive feedback.

Changes in the text: None

Reviewer B

1) First, the authors need to describe the research design in the title, i.e., a bioinformatics analysis.

Response: Thank you for your constructive feedback and valuable suggestion regarding the potential emphasis on bioinformatics in our study's title. The revised title now reads, "Bioinformatics Analysis and Single-cell RNA Sequencing: Elucidating the Ubiquitination Pathways and Key Enzymes in Lung Adenocarcinoma."

Thanks again for your kind suggestion.

2) Second, the abstract needs some revisions. The background did not describe the knowledge gap on this research focus and did not have comment on the potential clinical significance of this research focus. The methods need to be shortened and provide more details for the development of the nomogram, as well as the assessment of the predictive accuracy. The results need to quantify the findings by reporting statistics such as expression levels, AUC values, and accurate P values. The conclusion is vague and should have more detailed comments for the clinical implications of the findings.

Response: We truly appreciate the time you've spent reviewing our work and your suggestions on improving the abstract. Your keen insights into our research focus, methodology, results presentation, and conclusion are invaluable. Upon careful consideration, we have decided to maintain the current structure and content of our abstract. We believe it suitably encapsulates the essence of our research while leaving room for readers to delve into the main text for the rich details and discussions. The background aims to set the stage for our research without detailing all gaps in knowledge, to preserve conciseness. The clinical significance, while not explicitly mentioned in the abstract, is discussed extensively in the main text. Regarding the methods and results, we intended to strike a balance between detail and brevity. As such, detailed methods, quantified results, and precise P values are available in the body of the paper and supplementary materials. Lastly, our conclusion was drafted to provide an overview of the findings without preempting the detailed discussion in the paper. We understand that opinions on these matters may vary, and we truly appreciate your understanding as we choose to maintain our current abstract.

Your thoughtful review has spurred constructive discussions within our team, and we look forward to any further comments you may have.

Changes in the text: None

3) Third, in the introduction of the main text, the authors need to clearly indicate the clinical significance of this research focus and its knowledge gap in the literature; for example, how the findings from the current study can facilitate the clinical management of LUAD and why the key molecules involved in ubiquitination modification are clinically important.

Response: Thank you for your insightful suggestions on improving our introduction. We appreciate your emphasis on clearly indicating the clinical significance of our research and acknowledging the knowledge gap in the existing literature.
However, we would like to clarify that our introduction was intentionally structured to provide a broad overview of the subject, without delving too deeply into the clinical aspects. We believe that a more detailed exploration of the clinical significance and specific knowledge gaps would be more appropriately discussed within the body of the paper, where we can offer an in-depth exploration without the constraints of an introduction's brevity. Regarding the importance of the key molecules involved in ubiquitination modification, we agree that this is a crucial point and, as such, it has been extensively discussed in our results and discussion sections.
We believe that our findings can potentially facilitate the clinical management of LUAD by offering a deeper understanding of the role of ubiquitination modifications, which could, in turn, inform the development of novel therapeutic strategies.
Your comments have certainly given us food for thought, and we appreciate your efforts in helping to improve the quality of our work. We look forward to any further suggestions or comments you may have.

Changes in the text: None

4) Fourth, in the methodology of the main text please have a brief overview of the research procedures and the questions to be answered by these procedures. Please also indicate the research methodology, the purpose of the nomogram and the indicators for assessing its accuracy, i.e., validation sample and threshold AUC values. In modern statistics, there is no need to have three levels of statistical significance. P<0.05 is adequate.

Response: Thank you so much for your careful review. In the section titled "Multivariate Cox Regression Analysis and Construction of Clinical Prediction Model," we detail the process of constructing a nomogram using multivariate Cox regression. The efficacy and accuracy of this nomogram are evaluated using calibration curves, a robust statistical tool often employed to assess the accuracy of predictive models. Calibration curves provide a quantitative measure of the agreement between the predicted probability derived from the nomogram and the actual observed probability. When it comes to the validation of our model, we depart from the traditional approach of merely partitioning the data into a training set and a test set. Instead, we utilize a resampling method. This technique involves repeated sampling from our dataset, with the cycle being repeated a thousand times. This ensures the stability of our model by reducing the potential influence of sampling variability on our findings. By adhering to these rigorous methodologies and statistical standards, we aim to ensure the robustness and reliability of our findings.

Thanks again for your kind suggestion.

Changes in the text: None

Reviewer C

The paper titled “Deciphering the ubiquitination landscape and identifying key enzymes in lung adenocarcinoma through single-cell RNA sequencing analysis” is interesting. This study examined ubiquitination modifications in LUAD using sequencing data, identifying PSMD14’s critical role in malignancy regulation and its potential as a prognostic and therapeutic biomarker. These insights enhance understanding of LUAD mechanisms and treatment. However, there are several minor issues that if addressed would significantly improve the manuscript.

1) This study mentioned some cell subpopulations and suggested analyzing the heterogeneity and functional changes of different cell subpopulations in LUAD patients.

Response: Thank you for your thoughtful suggestion. In the present study, our primary focus has been to analyze ubiquitination within tumor cells, with the overarching aim of observing alterations in ubiquitination levels within these cells. As you rightly pointed out, the role of ubiquitination in other cell subpopulations bears significant importance, and it is indeed a key area that our future research endeavors will explore. However, within the context of this specific study, our predominant research target remained the tumor cells. We appreciate your

understanding of the specific scope of this current work as we continue to delve deeper into this intriguing field of study.

Changes in the text: None

2) It is necessary to clearly indicate the relationship between PSMD14 and tumor-infiltrating immune cells and the role of PSMD14 play in prognosis in LUAD in the manuscript.

Response: We appreciate your thoughtful suggestions and the time you've dedicated to reviewing our work. We'd like to clarify that, in the context of this particular study, we didn't find it necessary to include immune-related analysis. Our investigations and subsequent validations focused on the role of PSMD14 in promoting the proliferation, invasion, and migration of lung adenocarcinoma cells, and we demonstrated that this is facilitated through the stabilization of the AGR2 protein. This mechanistic pathway, as explored in our work, is not directly associated with immunity. While we understand the relevance of immune-related analyses in the broader cancer research context, we believed that incorporating such aspects into this study might potentially disrupt the central focus of our manuscript. However, we recognize the significance of the interplay between immune cell infiltration and ubiquitination in lung adenocarcinoma, and we plan to investigate these connections in our future research endeavors.

Thank you for your understanding and valuable insights.

Changes in the text: None

3) What is the value of single-cell RNA sequencing technology in exploring ubiquitination and tumor heterogeneity? What is the biggest challenge facing? It is suggested to add relevant contents.

Response: The point you've raised has been duly noted in our discussion section, wherein we elucidate that "Single-cell sequencing provides a means of assessing the risk of tumor susceptibility...".

Thank you for your insightful commentary.

Changes in the text: None

4) It is recommended to increase the relationship between protein ubiquitination, deubiquitination and tumorigenesis in the discussion.

Response: Thank you for your suggestions, which were mentioned in the first paragraph of the discussion.

Changes in the text: None

5) The introduction part of this paper is not comprehensive enough, and the similar papers have not been cited, such as “Systemic immune microenvironment and regulatory network analysis in patients with lung adenocarcinoma, PMID: 35116596”. It is recommended to quote this article.

Response: We appreciate the suggested reference, we don't think this article is necessary.

Changes in the text: None

6) How to use single-cell RNA sequencing technology to screen new diagnostic and prognostic markers for LUAD? It is suggested to add relevant contents.

Response: We greatly appreciate your insightful suggestions. We would like to point out that these points have already been acknowledged and addressed in the discussion section of our manuscript.

Changes in the text: None

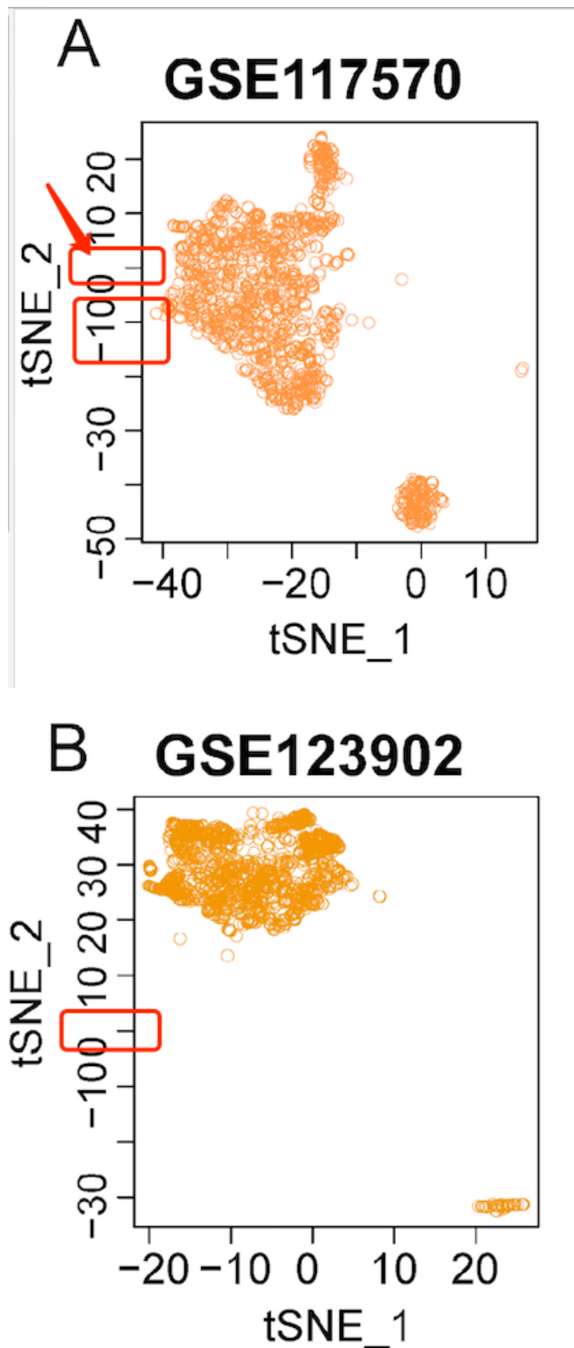
Reviewer D

1. For any experiments involving animals, the authors must indicate the nature of the ethical review permissions, relevant licenses (e.g. Animal [Scientific Procedures] Act 1986), and national or institutional guidelines for the care and use of animals by which the research was conducted. Please supplement the ethical approve number.
- *Suggested wording: “Animal experiments were performed under a project license (No: the license number) granted by The Animal Care and Use Committee of Harbin Medical University, in compliance with ******* national or institutional guidelines for the care and use of animals.”*

Response: We completed them.

2. Figure 3A, Figure 3B, Figure 3C and Figure 3D
Please put the “0” at the right place.

Response: I revised them.



3. Figure 7

HEK293T or HEK-293T? Which one is right? Please check and revise.

Figure 7 AGR2 protein stability is regulated by PSMD14 via deubiquitination. (A) The HA-*PSMD14* and Myc-*AGR2* plasmids were cotransfected into HEK293T cells,

Response: HEK-297T is correct. We revised this in the manuscript.

4. Figure 8

Please explain the meaning of **** in the legend.

Response: "****" means P<0.0001. We revised this in the manuscript.

5. Figure S2C

GSE123902 or GSE123904? Which one is right? Please check and revise.

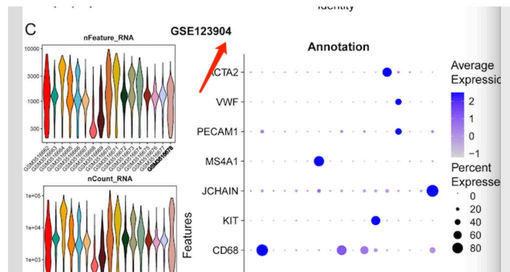


Figure S1 Assessment of data distribution and marker gene annotation in a quality-controlled single-cell public datasets. Assessment of the quality control measures including feature, count, mitochondrial genes, and annotation genes of all samples in the GSE117570 (A), GSE131907 (B), GSE123902 (C), and GSE149655 (D) datasets was conducted.

Figure S2 InferCNV heatmaps. InferCNV was utilized to generate GSE117570 (A), GSE123902 (B), GSE131907 (C), and GSE149655 (D) heatmaps for comparison of copy number differences in all cells, with amplification and deletion represented by red and blue colors, respectively. The intensity of the color, with darker red indicating

Response:

Thank you very much for your careful correction. It has come to our attention that GSE123902 is the appropriate dataset for our study. Notably, both GSE123902 and GSE123904 were contributed by the same group of uploaders but were uploaded at different time intervals. Hence, for our investigation, we exclusively utilized the data extracted from GSE123902. Additionally, we have made necessary modifications to the accompanying figure.

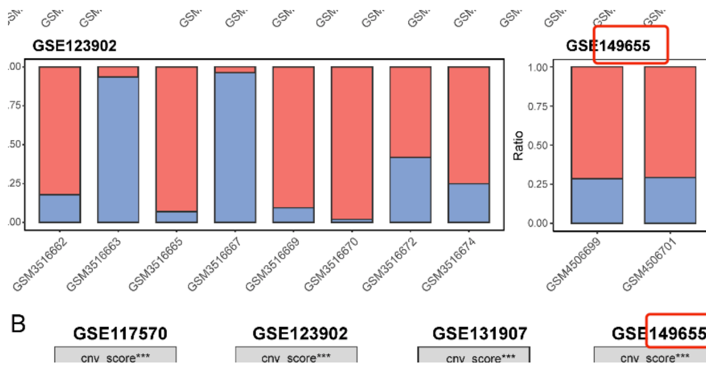
6. Table 3

Please add the unit.

Age
≤65
>65

Response: We added the unit in the revised manuscript.

7. Figure 2: Is “GSE146955” or “GSE149655”? Please check both the legends and the figure.

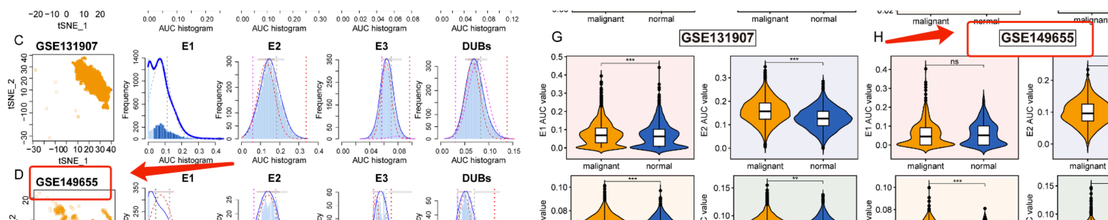


914 **Figure 2 Analysis of malignant and normal cell proportions and epithelial cell**
915 **CNV scores using InferCNV.** (A) The proportion of malignant and normal epithelial
916 cells across diverse samples in the GSE117570, GSE131907, GSE123902, and
917 GSE146955 datasets. (B) Differences in CNV scores between malignant and normal
918 epithelial cells in the GSE117570, GSE123902, GSE131907 and GSE146955 datasets.

Response:
Thank you very much for your careful correction. GSE149655 is correct, and we revised these mistakes in the revised manuscript.

8. Please also check and revise Figure 3.

922 **Figure 3 Evaluation of epithelial cells’ AUC scores using AUCell.** The left panel
923 shows the subpopulation of epithelial cells, while the right panel displays the
924 frequency range of AUC values for E1, E2, E3, and DUBs in epithelial cells in the
925 GSE117570 (A), GSE123902 (B), GSE131907 (C), and GSE146955 (D) datasets. The
926 disparity in AUC values of E1, E2, E3, and DUBs between normal and malignant
927 epithelial cells in GSE117570 (E), GSE123902 (F), GSE131907 (G), and GSE146955



Response:
Thank you very much for your careful correction. GSE149655 is correct, and we revised these mistakes in the revised manuscript.

9. Figure 6: Please indicate the magnification/scale bar of Figure 6H-I in the figure legends.

975 group according to crystal violet staining. Scale bar = 1.0 cm. (H,I) The cell scratch
976 assay demonstrated a significant decrease in cell migration ability in the *PSMD14*
977 knockdown group compared to the control group in A549 (H) and H1299 (I) cells. (J)

Response: We added the scale bar of Figure 6H-I in the revised manuscript.

10. Please also indicate the staining methods of Figure 8E-8F in figure legends.
Response: We added the staining methods of Figure 8E-8F in the revised manuscript.