Peer Review File
Article Information: https://dx.doi.org/10.21037/tcr-24-581

Reviewer A

In the manuscript entitled “RPN1: a pan-cancer biomarker and disulfidptosis regulator”, the authors investigated the role of RPN1 in promoting disulfidptosis. The article is generally clear and follows a logical structure, beginning with an introduction, followed by methods, results, and conclusion. Also, it is an interesting topic regarding cancer research area. However, the following major revisions required for the manuscript:

General Comments

What is the rational to select the two-associated cancer cell lines, breast and lung? Why the researches did not evaluate other cancers experimentally?

Also, it seems that the experimental part should be placed at the end of the article.

We appreciate the reviewer’s thoughtful feedback on our manuscript. MDA-MB-231 and A549 cells were chosen as the two cell lines because we had tested other cell lines, such as intestinal cancer cell lines SW480 and HCT116, but neither glucose starvation nor glucose starvation combined with additional cysteine could induce disulfidptosis in these cell lines. We speculate that disulfidptosis may be limited to certain conditions.

Additionally, this study was initiated based on experimental observations rather than a hypothesis verified through experiments. In the original research article "Actin cytoskeleton vulnerability to disulfide stress mediates disulfidptosis," we found that the researchers only investigated the ability of renal cell carcinoma (RCC) cells (786-O) under glucose deprivation to induce disulfidptosis. Therefore, our initial research interest was sparked by the question of whether other cell lines could also induce disulfidptosis under glucose deprivation. After confirming this phenomenon, we embarked on further research. Therefore, we placed the experimental section at the beginning of the article.

Specific comments

1. Abstract: The aim of the study should present clearly at the end of the introduction part. Hence, it is better to put lines 43-45 at the end of the background.

We greatly appreciate the valuable comments from the reviewer. We have placed the sentence "We demonstrate the inhibition of disulfidptosis upon RPN1 gene knockout in cell lines not previously validated, irrespective of SLC7A11 expression levels, providing additional evidence for RPN1 as a potential universal target for cancer therapy." at the end of the Introduction section.

2. Introduction: this part is too long. It should be summarized. Further, it is not required to mentioned the database name at the end of the introduction where the authors mentioned the aim of the study and the overall study design.

We would like to express our gratitude for the invaluable suggestions made by the reviewers. In the original text, the three paragraphs in the Introduction Section were 231 words, 105 words, and 150 words, respectively. After rewriting, they were reduced to 134 words, 89 words, and 84 words, respectively, meeting the requirement for concise text summarization by reviewers. Additionally, in the conclusion of the introduction, we excluded the TCGA database.

3. Materials and Methods:

- Draw a flowchart of the work and put it at the first part of this section.
- All the used database (i.e. TCGA, GEO, etc.) should be referenced. Furthered, the link assigned to them should be mentioned.
- While the gene expression alteration was significant in 16 cancer, what is the rational to select 12 microarray datasets? Please clearly explain it and add this to materials and methods section. The pipeline that yield to the datasets selection should explained better in all related parts such as abstract and method sections. Also, define the clinical characteristic of the patients.

We sincerely appreciate the constructive feedback provided by the reviewers. We placed the workflow diagram at the beginning of the "Methods" section as follows: *Figure 1* depicts the workflow employed in this study.

Additionally, in the sixth and seventh paragraphs of the Methods section, we cited articles from the TCGA, GEO, and HPA databases, along with providing the corresponding links.

The reason we only validated in 12 microarray datasets is because validation was not successful in the other four types of cancer, possibly due to insufficient available datasets for these four types of cancer. We clearly explained this and placed these statements in the Materials and Methods section, as detailed in the sixth paragraph.

In the Materials and Methods section, we have reintegrated the process of selecting GEO datasets as follows: The process of selecting datasets begins with identifying cancer types that match those in TCGA, such as "squamous cell carcinoma of the lung," rather than broadly categorizing them as "lung cancer." Next, we choose the largest available dataset for this specific cancer type. We clearly explained this and placed these statements in the Materials and Methods section, as detailed in the sixth paragraph. Furthermore, the clinical characteristics of patients involved in each selected dataset can be found in Supplementary Table 2.

4. Results:

- Summarize lines 328-338. It is better to mentione the overall results and pointed to the associated figure for more details.
- In all parts of the article, particularly the “results section”, the abbreviated form of cancer named is adequate. So, revise the manuscript regarding this issue (i.e. Lines 341-343, 374 and 416; There are just examples).
- AUC >0.65 is not considered as strong criteria. Hence, it seems that the candidate gene doesn’t have a good diagnostic value. Please clarify this issue.

We express our gratitude for the insightful suggestions from the reviewers.

In lines 328-338, we summarized the overall results and referred to the corresponding Figure 2C for readers to obtain more detailed information.

In the Results section, we have replaced the full names of cancers with their abbreviated forms in paragraphs 2, 5, 7, 8, and 9.

To enhance the diagnostic value of candidate genes, we set AUC>0.8 as the criterion and accordingly revised Figure 5A-5J. The corresponding modifications can be found in paragraphs 4 to 5 of the Results section.

5. Discussion:

- This part should revise comprehensively.

Thank you for the valuable suggestions from the reviewers. Since Reviewer 1 only mentioned the need for revisions in the Discussion section without specifying the details, we revised the manuscript based on the revision requests made by Reviewer 2.

Reviewer B

In this study, the role of RPN1 was clarified in the disulfidptosis process in both breast and lung cancer cell lines. In addition, the expression level and activity of RPN1, its correlation with prognosis, its diagnostic ability, and its association with immune cells and immune therapy efficacy were analyzed using several bioinformatics tools for various cancer types. These overall outcomes emphasize the significance of this gene as a potential pan-cancer biomarker. Still, more functional studies should be done to validate their relevance in each cancer type and to illuminate its precise molecular mechanism. Thus, this study is the first to integrate data from multiple databases to understand the involvement of RPN1 in diverse cancers and its association with immune response in the tumor microenvironment. However, several amendments were suggested below to improve the manuscript mentioned.

1. The introduction section was found to be too constricted. The information about RPN1 and its relevance to cancer development is insufficient. It is already known that there are recently published data that illuminate the involvement of RPN1 in breast cancer, glioma, or hepatocellular carcinoma. It would be valuable to mention these studies at least shortly to understand perfectly what is already known and what is novel in this study.

We appreciate the reviewer' s thoughtful feedback on our manuscript. In the third paragraph of the Introduction, we incorporated known research findings regarding RPN1 in breast cancer and hepatocellular carcinoma. Detailed discussion on the information about RPN1 and its relevance to cancer development is presented in the third paragraph of the Discussion section. The third paragraph of the Introduction reads as follows:

Several studies have investigated the relationship between RPN1 and cancer. For example, it may promote the progression of breast cancer (8) and be associated with poor prognosis in

hepatocellular carcinoma (HCC) (9). However, the biomarker value of RPN1 across pan-cancers has yet to be investigated.

2. Please see lines between 91-93 and 97-97 in the introduction and insert the related references.
We greatly appreciate the valuable comments from the reviewer. We meticulously examined the first paragraph of the Introduction and inserted relevant references after each sentence and known result.

3. Although the plasmids used to knockout RPN1 were given in the methods, no explanation was given about which technique was used to knockout the gene in human cancer lines.
We would like to express our gratitude for the invaluable suggestions made by the reviewers. We revised this paragraph, as detailed in the second paragraph of the Methods section, which has been modified to the following:

##Gene knockout in cell line

1.Plasmid: The single guide RNA (sgRNA) expression constructs were cloned into the LentiCrispr-V2-puro backbone. The sequences of the sgRNAs used in this study were as follows: RPN1-sg1 (5'-TGTAGGCAACAATCACAGGG-3'), RPN1-sg2 (5'-TGAGGACGTGAAGCGCACAG-3').
2. Lentivirus package: The 293T cell line was seeded into a 6-well plate and cultured until a confluency of 70% was achieved. For the transfection procedure, tube A contained a mixture of the target gene plasmid, psPAX2, and pMD2.G, suspended in 150μl of Opti-MEM medium at a ratio of 3μg:2μg:1μg. Concurrently, tube B was prepared with 24μl of PEI added to an equal volume of Opti-MEM medium. After a 30-minute incubation period to allow for complex formation, the mixture was added to the cells. The culture medium was replaced with complete medium (with 10% fetal bovine serum (FBS), 100 U/mL of penicillin and 100 μg/mL of streptomycin). At 48 hours post-transfection, the supernatant was collected and cleared of cellular debris using a 0.45μm filter membrane. The lentivirus present in the supernatant was then concentrated using the Lenti-Pac Lentivirus Concentration Solution (GeneCopoeia, LT007), in preparation for subsequent gene knockout experiments.
3. Lentiviral Infection: Cell lines designated for infection were plated in 6-well plates and allowed to reach 70% confluency prior to the initiation of the lentiviral infection assay. Prepared lentiviral solution was then added to the culture dishes. After 24 hours, the medium was replaced, and at 72 hours post-infection, selection with puromycin commenced. Following two weeks of selection, stable transfectants were successfully established.

4. No data demonstrate the lack of RPN1 expression at the mRNA and protein level in MDA-MB-231 and A549 cell lines after the knockout procedure. The data should be given in Figure 2 or as supplementary material.

We sincerely appreciate the constructive feedback provided by the reviewers. We added a figure, Figure S1, which supplements the loss of RPN1 expression at the protein level upon knockout in MDA-MB-231 and A549 cell lines. Figure S1 and its legend are provided below and can be found at the end of the figures：

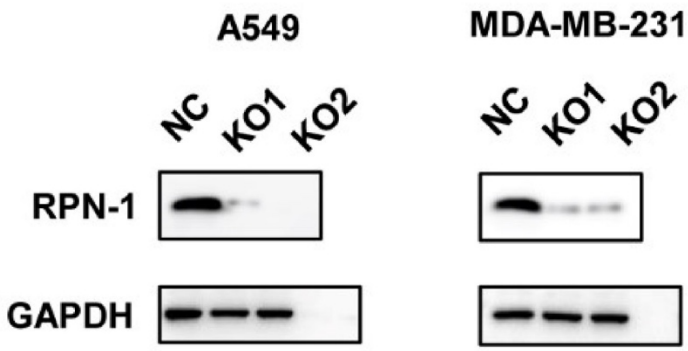

Figure S1 Analysis the expression of A549 and MDA-MB-231 with knockout RPN-1. Western blot analysis was conducted to assess the expression levels of RPN-1 in the A549 and MDA-MB-231 cell lines following gene knockout. The negative control (NC) group received no gene editing, while knockout groups KO1 and KO2 represent two distinct gene ablation variants. GAPDH was employed as a loading control to ensure equal protein quantification across samples.

5. Please check lines 389-390; enrichment in the antigen processing and presentation pathway was shown in CESC and LUSC, not in LUAD as written. Please make the related amendment.

We express our gratitude for the insightful suggestions from the reviewers. We made a modification by replacing "LUAD" with "LUSC". Please refer to the sixth paragraph of the Results section for details.

6. In the Results, entitled “KEGG pathway and tumor microenvironment……..,” lines between 382 and 383, the KEGG pathway enrichment analysis results should also be given in the figure legend. Otherwise, it is hard to follow the statistical values, etc.

We express our gratitude for the insightful suggestions from the reviewers. In the KEGG pathway analysis section, we supplemented the statistical values in the form of an additional table, Table S4. This approach was chosen to prevent excessive lengthening of the figure legend.

7. The Results section presents the study's general results. All obtained results should be presented precisely.

Many thanks for the insightful comments from the reviewers. We believe that all the results have been accurately presented. For instance, in sections 1, 2, 4, 5, and 6 of the Results section, we not only provided statistical values but also explained the significance of the results. Additionally, with the inclusion of figure legends and supplementary tables, such as Table S4, we are confident that readers can obtain the necessary information.

8. There is no considerable explanation from "A" to "O" in the legend of Figure 7.

We are deeply grateful for the insightful feedback provided by the reviewer. In the legend of Figure 7, we have included explanations for the subfigures, which read as follows: (A-F) The correlation between RPN1 expression and ESTIMATE score in LGG, PAAD, PCPG, and SARC tissues. (G-O) The correlation between RPN1 expression and immune cell infiltration in ACC, BRCA, ESCA, LGG, SARC, and THCA tissues.

9. The Discussion section was found to be too constricted. What is already known? What are the main findings that make this study novel? This should definitely be discussed. That kind of lack of information makes it difficult to determine the novelty and significance of the study.

We express our gratitude for the insightful suggestions from the reviewers. We have rewritten the discussion section, incorporating known information about RPN1 along with the findings of our study, to clearly emphasize the novelty and significance of our research. These discussions can be found in the first and second paragraphs of the discussion section, which read as follows:

The RPN1 gene encodes a type I integral membrane protein found exclusively in the rough endoplasmic reticulum (17). It plays a crucial role as part of an N-oligosaccharyl transferase complex, which links high mannose oligosaccharides to asparagine residues within the Asn-X-Ser/Thr consensus motif of nascent polypeptide chains (17). This process is essential for protein N-glycosylation. The UMRC6 cells' increased resistance to disulfidptosis upon RPN1 knockdown suggests that RPN1 may be involved in regulating cell survival pathways (3). However, the precise underlying mechanism remains unclear. In this study, we elucidated the previously unreported mechanistic role of RPN1 in inducing disulfidptosis. Through an investigation into cell death under glucose starvation in breast and lung cancer cell lines, we clarified the role of RPN1 in this process, primarily by inducing cell skeleton breakdown to promote disulfidptosis. To our knowledge, this study represents the first exploration of RPN1's specific mechanism in disulfidptosis.

The relationship between RPN1 and cancer has been partially elucidated. For instance, RPN1 promotes proliferation, migration, and invasion of breast cancer cells through the PI3K/AKT/mTOR signaling pathway (8), possibly by inhibiting apoptosis triggered by

endoplasmic reticulum stress (18). In HCC, survival analysis shows that high RPN1 expression is associated with adverse overall survival in HCC patients (9). Zheng et al. found that the Circ-SNX27 sponging miR-375/RPN1 axis contributes to HCC progression (19). Disulfidptosis-associated genes containing RPN1 have been used in risk models related to prognosis and immune characteristics of brain glioma (20) and HCC (21) patients, serving as independent prognostic factors for glioma. However, the comprehensive expression profile of RPN1 across pan-cancers, its clinical relevance, and its relationship with the tumor microenvironment remain unclear. In this study, we further examined the differential expression and activity of RPN1 across various cancer types. The results revealed strong mRNA expression of RPN1 in 16 solid tumors, with significant differences between tumor tissues of specific cancer types and adjacent non-tumor tissues. Additionally, RPN1 demonstrated robust activity in all analyzed tumor tissues. Validation from the GEO database confirmed consistent mRNA expression trends across 12 cancer types. The HPA database indicated that RPN1's protein expression levels aligned with the observed mRNA expression patterns in TCGA and GEO datasets, covering eight cancer types [UCC, invasive lobular carcinoma (ILC), CHOL, COAD, RCC, HCC, LUAD, and LUSC]. RPN1 exhibited significant diagnostic potential across 12 cancer types, particularly with high accuracy in GBM. Elevated RPN1 expression in tumor tissues correlated with improved OS in DLBC and THYM, whereas it was associated with poorer prognosis in ACC, KICH, LGG, LIHC, and PAAD. Moreover, RPN1 was linked to immune response, being enriched in immune-related pathways, correlating with immune scores in tumor tissues, and associated with various immune cells. Notably, in UCC, RPN1 showed potential in predicting the efficacy of anti-PD-L1 immune therapy. In summary, these findings emphasized RPN1's significance in pan-cancer scenarios and its implications for diagnosis, prognosis, and immune checkpoint inhibitor therapy. To our knowledge, this is the first study of RPN1 in pan-cancers integrating data from multiple public gene databases.

10. The study limitations should also be mentioned in the Discussion section.

We are deeply grateful for the insightful feedback provided by the reviewer. In the final paragraph of the revised manuscript, we added a discussion on the limitations of this study, which reads as follows:

This study has several limitations. Firstly, we did not further validate the differential expression of RPN1 in tumor and adjacent tissues obtained from our own tissue samples, which were used to verify the bioinformatics analysis. Specifically, investigating the expression of RPN1 in cohorts undergoing anti-PD-L1 therapy to validate its biomarker value in the real world was not performed. Secondly, some tumor types did not receive consistent validation across multiple databases, which may be related to systematic sampling biases. Finally, the detailed mechanism by which RPN1 promotes disulfidptosis through inducing cell skeleton breakdown requires further elucidation.

11. There are some typos which should be corrected throughout the manuscript.

Thank you for the valuable suggestions from the reviewers. We carefully proofread the revised manuscript to ensure there are no typographical errors.