

# Peer Review File

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## Reviewer A

Comment 1: The authors checked the correlations between USO1 expression levels and NSCLC prognosis. Although some of the results are interesting, this research is quite descriptive and does not provide enough valuable information. Thus, I think it may be more suitable for other journals.

The authors did some bioinformatics analysis to assess the prognostic values of USO1, a Golgi gene. However, this work is a little superficial and does not provide much important information for the readers. I would say this manuscript is not worth recommending.

Reply 1: we thank the reviewer for their forth-rightness. Indeed, under normal circumstances if there had been no validation of any *in silico* analyses, the authors would not have considered submitting this article to TLCR. Because there is strong validation presented of the various results we believe that the manuscript is still suitable for publication in TLCR and hope that the reviewer understands this position.

Changes in the text: None.

## Reviewer B

Comment 1: This is a very comprehensive study introducing a promising biomarker in NSCLC. The study included an exploratory cohort, and all the data were validated using *in silico* analysis. The results are clearly described, illustrated by tables and figures consistent with an interpretation at the end of the description. However, there are some issues that authors should address.

Reply 1: We thank the reviewer for their kind and constructive comments and have endeavoured to address them all.

Changes in the text: None.

Comment 2. The authors provide the mechanophysical insights into the USO1 in Discussion section but did not discuss why high expression of USO1 confers a good OS for NSCLC in early stage, while in gastric cancer, multiple myeloma is the opposite.

Reply 2: We thank the reviewer for raising this interesting point. To our knowledge the data from the studies in survival for gastric cancer and multiple myeloma is based on the premise that overexpression of USO1 leads to increased invasiveness/metastasis. Having carefully re-read the extant literature we can find no overt OS survival published for gastric cancer or multiple myeloma. The only data currently available relates to Breast cancer (Howley BV et al., 2018). In this manuscript high expression of USO1 mRNA showed no significant change in OS, but did demonstrate that high mRNA was observed to have worse relapse free survival. We therefore used KM-Plot to assess USO1 expression on OS in gastric cancer. Our results show that similar to the data presented in our manuscript for NSCLC, high expression of USO1

is in fact also associated with a better OS. We have included this in our discussion of this and included a Figure for the gastric cancer OS in the Appendix – Supplementary Figure S9, and hope that the additional text/data will be acceptable to Reviewer 2.

**Changes in the text:**

Pages 23-24, Lines 488 – 514.

USO1 has been implicated in tumorigenesis in multiple cancers including gastric (10), colon (11), breast (12), liver cancer (13) as well as multiple myeloma (14) and leukaemia (37). Overexpression of USO1 has been shown to play a role in cell proliferation and cell cycle transition in these studies, and as such in general, one would expect high expression of USO1 to be associated with worse OS. However, survival analysis in tumours with USO1 overexpression has not generally been investigated. For example, in gastric cancer overexpression of USO1 promotes cell proliferation and G0-G1 to S phase transition however, overall survival was not assessed. Using KM-plotter, we have identified that high USO1 mRNA expression in gastric cancer is associated with a significantly better overall survival ( $p=5.6 \times 10^{-7}$ ; Appendix – Supplementary Figure S9) similar to our observed results in LUAD. Our study used a H-scoring system which assessed both staining intensity and the proportion of tumour cells staining for USO1 assigning a score of 0 – 300 in each case. This gives a dynamic range to quantify USO1 abundance. Using this method, we were able to categorize LUAD patients into high and low protein expressers based on the median value. Our study found that USO1 is overexpressed in LUAD as well as LUSC, however, upregulation is associated with superior overall survival in LUAD and not LUSC. We also found many of USO1's first neighbours were also prognostic in LUAD (Appendix - Supplementary Figure S5) but not in LUSC using KM plotter (Appendix -Supplementary Figure S6). As these proteins are all involved in Golgi transport system, it suggests that Golgi transport has a lesser role in LUSC tumorigenesis. Our findings and previously reported studies suggest that USO1, although involved in tumorigenesis, is most likely due to involve regulation of Erk (10,14,15), and is associated with a superior overall survival in LUAD and gastric cancer. The exact reason for this remains to be elucidated and warrants further investigation. It may be that a tumour specific transcript of USO1 is a key element as recently described for hepatocellular carcinoma, where a specific mRNA variant USO1-T (RefSeq NM\_003715.4, transcript variant 2) is associated with worse prognostic outcomes and an aggressive phenotype in HCC (13).

We hope that this re-analysis and subsequent discussion will prove acceptable to Reviewer 2.

3. Examining the Table 1, there is a similar frequency of high and low USO1 expression in tumours with and without lymph node metastasis. A similar frequency of USO1 high and low expression was found in tumour stage I to III. In addition, it is not clear in the manuscript how the authors found a better OS in early-stage NSCLC. Therefore, to identify the independent value of USO1 in survival or the risk of death it is important to perform Multivariate Cox Analysis, controlling for significant variables found in univariate analysis or known variables with impact on prognosis such as TNM stage.

Reply 3: We thank Reviewer 2 for bringing this issue to our attention. We have re-analyzed the

data using multivariate analysis and the results have been incorporated into the manuscript as Tables 2-4 with appropriate text as follows:

**Changes in the text:**

Pages 11-12: Lines 230-243.

Statistical analysis was performed using either the SPSS 25.0. statistical software package (SPSS Inc., Chicago, IL, USA), or Graphpad Prism 5.01 (Graphpad Software, San Diego, CA USA). Correlations between USO1 expression and given categorised parameters were evaluated using the nonparametric Mann-Whitney U-test (for two categories) or Kruskal-Wallis test (for multiple categories). Kaplan-Meier curves were performed for survival curves, and statistical analysis was assessed using the log-rank test. Overall survival was defined as the time from the date of surgery to death. Patients who were still alive or lost to follow-up, were treated as censored data in the survival analysis. Univariate analysis of overall survival was performed using Kaplan-Meier method. Multivariate Cox proportional hazard regression analysis was used to assess the prognostic significance of USO1 and other clinicopathological characteristics on survival.

The correlation of gene expression was evaluated by Spearman's correlation. Overall, 95% confidence intervals (Cis) were used throughout the analysis. Statistical significance was defined as  $p < 0.05$ .

Pages 13-15: Lines 267-296.

Relationship between USO1 expression and clinical outcome in NSCLC: univariate and multivariate survival analysis

At the time of analysis, the number of deaths that occurred was 164. Univariate survival analysis (log-rank test) for all histologies demonstrated significant association between overall survival and age  $>65$ , smoking status, tumour size  $>5\text{cm}$ , stage, and nodal status (Table 2).

\*\*\*\* Insert Table 2 Here \*\*\*\*

Overall USO1 expression did not reach significance ( $p=0.202$ ) (Figure 3A). Univariate analysis in the LUAD group ( $n=82$ ) demonstrated significant association between overall status and the status of USO1 ( $p=0.0283$ ) (Table 3).

\*\*\*\* Insert Table 3 Here \*\*\*\*

Patients with high level expression of USO1 had a better prognosis than those with low-level expression. Figure 3C shows a Kaplan-Meier survival curve in relation to USO1 expression in patients with LUAD. Nodal status and stage reached statistical significance also in the LUAD group ( $p=0.021$  and  $p=0.003$ , respectively) (Table 3).

There was no significant difference in OS and USO1 expression when looking at the LUSC group ( $n=108$ ) on univariate analysis (Figure 3B & Table 3) ( $p=0.8275$ ).

To evaluate if USO1 protein expression is an independent prognostic factor in LUAD, a multivariate analysis using the Cox proportional hazard model was performed and included variables with  $p < 0.05$  in the univariate analysis, which were nodal status and stage. Multivariate

analysis proved USO1 expression as an independent prognostic factor of overall survival in the LUAD group, with high expression associated with better prognosis ( $p=0.048$ , 95% CI = 0.351 – 0.995; Table 4).

\*\*\*\* Insert Table 4 Here \*\*\*\*

4. Please discuss why squamous cell carcinoma, although also presenting high expression USO1, had no impact on OS.

Reply 4: We thank Reviewer 2 for raising this point. We believe that Point 5 also falls into this area and have now included new discussions as follows:

**Changes in the text:**

Pages 23-24, Lines 488-514.

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Pages 24-25, Lines 524-541.

Reanalysis of existing proteomic datasets demonstrated that USO1 and phosphorylated S953 on USO1 are significantly elevated in both LUAD and LUSC (Appendix – Supplementary Figure S8), indicating that USO1 is functionally active in both histological subsets. However,

previous ultrastructural studies on the endoplasmic reticulum and Golgi complex in adenocarcinomas and squamous cell carcinomas have identified clear differences between the LUAD and LUSC subtypes. Electron Microscopy (EM) of LUADs is very heterogeneous and reflects the histological heterogeneity of LUADs. They can be composed of cells resembling those of embryological derivation of the lower respiratory tract, type II pneumocytes, or clara cells (40). Many, however, are composed of cells rich in cytoplasmic organelles and include a very well-developed Golgi complex, rough endoplasmic reticulum and several mitochondria (40-42). In contrast, on EM squamous cell carcinomas show abundant tonofibrils converging on desmosomes and extending into the intercellular bridges. Keratinization is marked with increased number of tonofibrils in a perinuclear arrangement (where the Golgi is usually located). They present a reduced rough endoplasmic reticulum (RER) with only a few ER tubules and abundant intermediate filaments, and the cytoplasm contains relatively few organelles (40-42). As such, the differences in the ER-Golgi may explain to some degree the differences in OS benefit observed between these two subtypes, but will require further evaluation.

We hope that these amendments will prove acceptable to Reviewer 2.

5. It would be very interesting if the authors had included Transmission Electron Microscopy images illustrating the ultrastructure of the endoplasmic reticulum and Golgi complex in adenocarcinomas and squamous cell carcinomas, justifying the expression of USO1. The cytoplasm of adenocarcinomas exhibits a very well-developed Golgi complex, rough endoplasmic reticulum and several mitochondria compared to squamous cell carcinoma which present abundant intermediate filaments. The authors should discuss about this differ

Reply 5: We thank Reviewer2 for bringing this to our attention. We discussed the possibility of doing TEM on samples with our core electron microscopy centre, and their response was that FFPE samples were unsuitable for TEM (*“paraffin embedded samples are not suitable for electron microscopy. Samples for TEM are normally fixed in glutaraldehyde and embedded in vacuum compatible resin.”*). As such we are currently unable to conduct TEM on our samples. We do thank the reviewer for providing the informative comments with respect to the earlier TEM studies in NSCLC. This has formed the basis of a new section in the discussion which may provide an explanation as to why the OS benefit is observable in Adenocarcinomas and not in the Squamous cell carcinoma subtype

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