Peer Review File

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Reviewer A

The authors focused on MRPL19 which is known to have a role in the progression of many cancers, but not yet been appreciated in lung adenocarcinoma (LUAD). Through extensive in silico analyses and some in vitro experiments, the authors concluded that the MRPL19 may have pro-oncogenic effects on LUAD, associating with immune cell infiltration and the poor prognosis of the patients. The study was well organized and written, however I have still some concerns.

Major concerns

1. MRPL19 may be a commonly and inevitably important molecule for cells to keep their fundamental biological activity, which can also be deduced from its ubiquitous expression shown in the Fig.1A of the present study. Although CNV of MRPL19 gene was shown partly in relation to the immune cell infiltration, almost no MRPL19 aberration-originated phenotypes were demonstrated. Because many prognostic or progression-associated markers for LUAD have been reported, the authors may need to provide reasons to focus additionally on MRPL19 in LUAD, for example, superiority to other markers, druggable advantages of MRPL19 or the system involved, etc.

Reply 1: Thanks for your helpful suggestion. Just as what you said, many prognostic or progression-associated markers for LUAD have been obtained and reported by large sample or large multi-center trial research. But the occurrence of lung cancer is a complex biological process and the mechanism of pathogenesis and progression for LUAD has not been recognized completely. In this article, we found that the expression of MRPL19 was upregulated and the high MRPL19 expression was associated with the poor prognosis of LUAD patients, and MRPL19 may have an pro-oncogenic effect on LUAD. We also obtained some potential mechanisms for the functions of MRPL19 in LUAD by bioinformatics analysis. We will investigate these mechanisms in the future research or perform some drug experiments.

2. MRPL19 expressing cells in LUAD tumors: The authors examined MRPL19 gene expression in the TCGA and GEO cohorts and demonstrated the elevated expression in tumor tissues than in normal tissues. Based on the IHC data presented, did what kind of cells in LUAD tumor tissues express MRPL19? Some of normal tissues expressed significant amount of the protein (Fig.1F), and there must be cells expressing the protein other than tumor cells. Because MRPL19 expression showed relation to the infiltrating immune cells into the LUAD tumor, this was caused by tumor cells expressing MRPL19, or by stromal cells including immune cells themselves.

Reply 2: Thanks for your question. Immunohistochemistry (IHC) is an experimental method, which is used to detect the corresponding antigen or antibody of histocyte. In this article, we used IHC to investigate MRPL19 protein in LUAD and normal lung tissues. We interpreted the intensity of the positive staining and the stained regions in tumor cells and normal pulmonary

epithelial cells and compared the expression difference of MRPL19 protein between them. The other infiltrating immune cells can not be differentiated just by IHC staining and are also out of the range of our research. We just want to verify the expression difference of MRPL19 in protein level between tumor and normal tissues.

3. Lentiviral shRNA experiments for MRPL19:

#1: Experiments with more than one shRNA sequence targeting MRPL19 mRNA should be performed to distinguish off-target effects.

Reply #1: Thanks for your suggestion. We had taken into account the occurrence of false positive results when we performed the Lentiviral shRNA experiments for MRPL19. In order to avoid this kind of situation, we used one shRNA sequence targeting MRPL19 mRNA and two cell lines for our experiment by referring a literature published on Cancer Cell International (Xu, G., Bu, S., Wang, X., Zhang, H., & Ge, H. (2020). Suppression of CCT3 inhibits the proliferation and migration in breast cancer cells. Cancer cell international, 20, 218. https://doi.org/10.1186/s12935-020-01314-8). The knockdown efficiency in A549 cells was not as obvious as that in H1299 cells in protein level, after lentiviral vector transduction, but the trend of MRPL19 protein in the two cell lines was consistent, both of which were knockdown. The results may be due to the heterogeneity of the cells. That using more than one shRNA sequence targeting MRPL19 mRNA is quite helpful for our research to avoid false positive results and off-target effects. We will consider using more than one shRNA sequence targeting MRPL19 mRNA and two different cell lines for subsequent experimental investigations in future studies.

#2: Although the shRNA degraded MRPL19 mRNA significantly (Fig.7A), not so much decrease of the protein was observed (Fig.7B). This may partly be due to the stability of the protein. The timepoints of RNA and protein extraction after transfection of the lentiviral shRNA vectors should be presented. Quantification of the levels of protein amount-decrease by densitometry or something would be also appreciated. In addition, at least the protein expression levels examined at the each timepoint for evaluation of migration and invasion assay and apoptosis assay, respectively should be presented.

Reply #2: Thanks for your suggestion. In this research, the level of MRPL19 mRNA declined sharply and the protein level of MRPL19 didn't decreased as obvious as MRPL19 mRNA after MRPL19 knockdown by lentivirus infection, but their trends were consistent. The protein translation is involved from mRNA to protein and this is a complex biological process. Many factors, such as protein translation efficiency, half-life period of mRNA, the stability of the protein etc. would affect the outcome of protein translation. Meanwhile, there are heterogeneity between cells. Thus, not all of the mRNA and the corresponding protein are going to change by the same fold after gene knockdown or overexpression. We have observed this phenomenon during this research. To understand the protein expression at different time points are very important just as what you said, but our article focused on the functions of MRPL19 protein in LUAD. We will explore the reasons and mechanisms about the inconsistent decreasing degree between mRNA and the corresponding protein in our future research.

#3: Images for mitochondrion would be of interests.

Reply #3: Thank you for your helpful suggestion. Our article emphasized on the expression and functions of MRPL19 in LUAD, and their possible mechanism. We would study images for mitochondrion in our future study.

#4: Experiments regarding the findings through the authors' in silico analyses of LUAD should be suitable, but at least discussion on their future plans would be appreciated.
Reply #4: Thanks for your suggestion. We have performed some bioinformatics analysis by GO annotation, KEGG enrichment, Gene set enrichment analysis, PPI network construction and obtained some possible functions of MRPL19 in the process of LUAD and some possible mechanisms involved. We also verified some of these functions by in vitro experiments. We have discussed the limitations of our research in the last paragraph of discussion section (Page 19, from Line 587-592). We would explore some of these mechanisms for MRPL19 in the development of LUAD in the future by experiments in vitro and in vivo.
Minor concerns

4. Fig.6C: please provide the P value for B-cell analysis.
Reply: The P value for B-cell analysis was less than 0.001. We have made the correction accordingly in Figure 6-revised.
Changes in the text: We have modified our text as advised (See Page 29).

Reviewer B

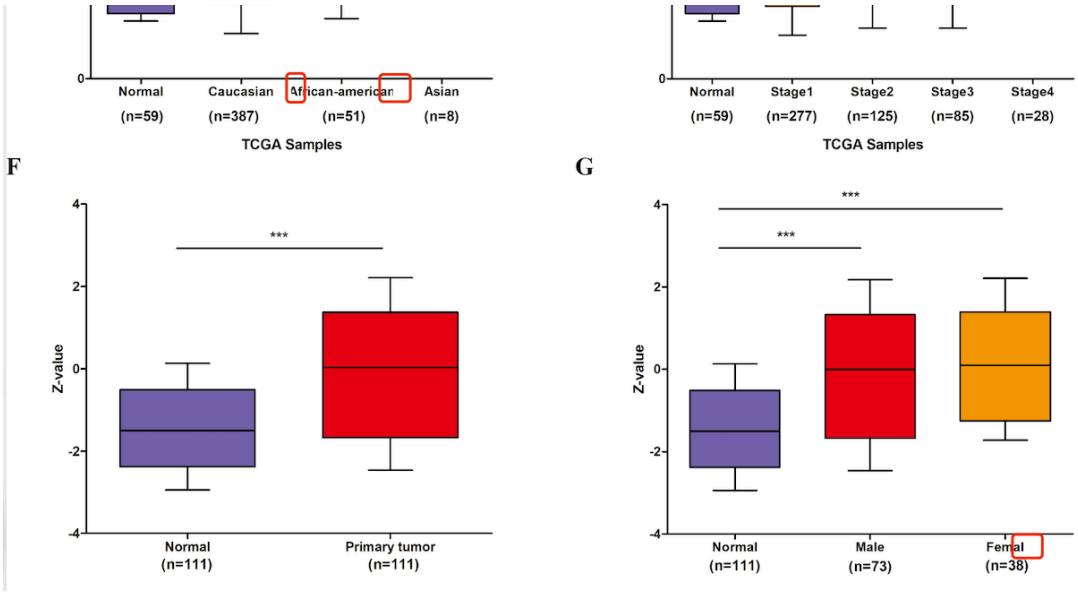
The study is well performed and designed, and the results are well explained and discussed.

My only suggestions are to shorten the first paragraphs of the introduction and the discussion, which perhaps are too general.
Reply: Thanks for your suggestion. We have made the corresponding changes in the first paragraphs of the introduction and the discussion according to your recommendation.
Changes in the text: We have modified our text as advised (See Page 3, Line 79-82; Page 15, Line 474-479; Page16, Line485-490.)

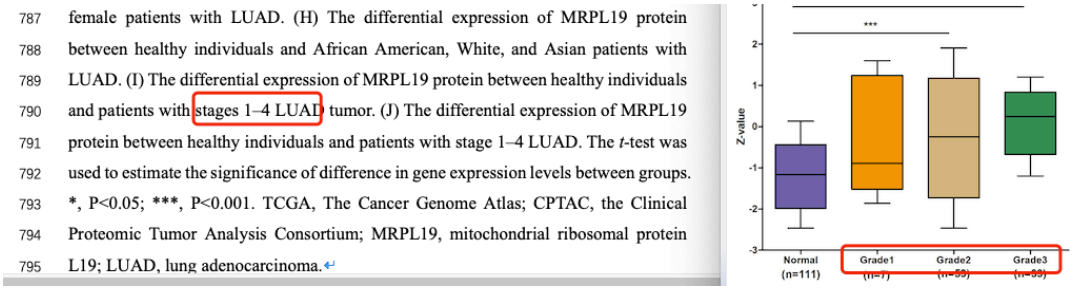
Reviewer C

1. Ref.13 was not cited in your paper, please cite it in order in text.
Reply: We have corrected the mistake and cited Ref.13 in order on Page 4, Line101.

2. Figure 2
a. Please check and complete the figure.



b. The legends and figure do not match to each other, please check and revise.



female patients with LUAD. (H) The differential expression of MRPL19 protein between healthy individuals and African American, White, and Asian patients with LUAD. (I) The differential expression of MRPL19 protein between healthy individuals and patients with stages 1–4 LUAD tumor. (J) The differential expression of MRPL19 protein between healthy individuals and patients with stage 1–4 LUAD. The *t*-test was used to estimate the significance of difference in gene expression levels between groups. *, P<0.05; ***, P<0.001. TCGA, The Cancer Genome Atlas; CPTAC, the Clinical Proteomic Tumor Analysis Consortium; MRPL19, mitochondrial ribosomal protein L19; LUAD, lung adenocarcinoma.

Reply a: We have checked Figure 2 and the mistakes in the red box of Figure 2 have been corrected.

Reply b: We have checked and revised it in the legend of Figure 2I.

3. Figure 5F is a bit vague, please check and resend us a higher resolution version as separate file.

Reply: We have checked Figure 5F.

4. Please provide the observation method/magnification of Figure 7E in the figure legends.

Reply: The pictures of petri dish in Figure 7E was not magnified. These pictures were obtained by photographing directly with a digital camera. The observation method: The inoculated cells were cultured in an incubator, and the number of cells in a single clone was observed by 100 × microscope until the number of cells in most of the single clones in the control group was more than 50. The crystal violet staining was performed. The entire culture plate was photographed. Single clones of 0.3 to 1 mm in size were counted. We have provided the observation method in the figure legends of Figure 7E.