Peer Review File

Article Information: https://dx.doi.org/10.21037/jgo-22-240

Response to Reviewer A

Comment 1: The authors indicated that forced expression of GPC1 in normal esophageal epithelial cell, HET-1A, resulted in them acquiring neoplastic properties, and it can promote tumorigenesis and it may play a role in onset of neoplasia. However, the results from HET-1A shows reproducibility of the results from FLO1 and OE19 cells. There is no investigation of tumorigenesis such as subcutaneous injection of cells. Therefore, I think that the description of tumorigenesis and onset of neoplasia to be overestimated.

Reply 1: Thank you for the comment. We agree with you that the description of tumorigenesis and onset of neoplasia cannot be made based on in vitro cell study results. Therefore, we agree that this was indeed an overstatement on our part. What we intended to show was the effect of GPC1 on viability, migration, invasion, and apoptosis in normal HET-1A esophageal epithelial cell lines. And you are correct in your statement that our results of GPC1 overexpression attained in HET-1A cells were like those in OE19 cells which were overexpressed with GPC1 indicating that GPC1 can drive a normal cell to acquire characteristics of a malignant cell. In the future, we plan to conduct in vivo experiments with subcutaneous implantation of GPC1 knockdown and overexpressing cells to validate in-vitro results.

Changes in the text: The text in the manuscript has been re-written on Page 13, lines 373-380; Page 15, lines 444-447. We have deleted the terms neoplasia, or tumorigenesis in the manuscript and have highlighted the limitations of the in-vitro study and future directions.

Comment 2: What is the mechanism by which GPC1 is overexpressed? The downstream of GPC1 has been well examined, but the upstream of GPC1 expression is much less well described.

Reply 2: Thank you for the question which is a important one. The mechanism of GPC1 activation is not understood clearly. There are reports indicating the role of micro-RNA's especially mi96-5 and miR-149 in colon and pancreatic cancer (Li et.al) which are upstream and target GPC1. These are speculated to activate GPC1 via either promoter hypomethylation of GPC1 (Lu et al), KRAS, and entropic viral integration site 1 (EVI1) (Tanaka et al.) or gene amplification (Witkiewicz et al). To further clarify the downstream mechanism of GPC1 further we have additional data showing GPC1 effect on Wnt/β-catenin using Lithium Chloride. It is hoped that results from this study will lay foundation to explore upstream microRNA pathways in our future studies.

Changes in the text: We have now included the mechanism of GPC1 expression in the revised text and the references (Page 14-15; lines 424-432; readers are directed to references.

Minor comments

1. I think “(Figure 4A)” on page 11, line 319 is “(Figure 4A and B)”. Similarly, “(Figure 4B)” on page 11, line 321 is “(Figure 4C)”; “(Figure 4C)” on page 11, line 326 is “(Figure 4D)”.

Reply 2: thank you and apologies for mislabeling the figures.

Changes in the text: We have corrected the labels in the revised manuscript.

2. I think “CKD4” on page 13, line 362 is “CDK2”. On the same page, lines 362-363, cyclin D1 has no result in Fig. 6C.

Reply 2: thank you and apologies for mislabeling the figures and omitting the western blot figure of Cyclin D1 in Figure. 6C.

Changes in the text: We have corrected the labels in the revised manuscript. We have added a western blot image of cyclin D1 in the revised manuscript.

3. Page 13, line 390, the protein levels of vimentin and SLUG from GPC knocked down cells were not shown in Fig. 9A

Reply 2: Thank you and apologies for omitting vimentin and SLUG levels in figure.

Changes in the text: We have incorporated western blot figures for vimentin and SLUG. We have revised the figure to include a clearer interpretation of western blots for the readers.

4. In Fig. 1D, does it show “notable increase” of the expression? Have you done any statistical analysis?

Reply 2: thank you for your comment. We did not do any statistical analysis. The data shown in Figure 1.D was queried from Oncomine database which unfortunately has been shut down. We have decided to exclude the public data sets from this manuscript until more data is available on esophagogastric cancer.

Changes in the text: Oncomine data base Figure 1 has been omitted in the manuscript.

5. In Fig. 1E, what correlations were shown? I think that this figure should be deleted, and the results should be described based on Table 1. Otherwise, you should state the discrepancy between the RNA and protein levels, because Fig. 1E showed a higher expression of GPC1 from stage II than that from stage III and IV.

Reply 2: thank you for your comment. Unfortunately, we are unable to go back to Oncomine database as it has been shut down. We agree with your suggestion to delete this figure.

Changes in the text: Oncomine data base Figure 1 has been omitted in the manuscript.

Thank you once again for reviewing our manuscript and providing scientific feedback.

Response to Reviewer B

- In the Introduction references #6 and 7 deal with perlecan, not GPC1.

Reply 2: Thank you and we apologize for not being specific with reference to GPC1. Relevant references for GPC1 have been included in the revised manuscript.

Changes in the text: New references related to GPC1 (References 6-9).

- One example of a poorly written sentence can be found in line 92 “it increases expression of tumor growth…”. There are many examples like this in the manuscript.

Reply: Thank you for the feedback. This error has been corrected. We have rewritten the manuscript and all grammar has been checked. We hope the manuscript in its revised form will be more readable.

- Fig.1. The quality of the graphs is poor, difficult to read. In many cases the explanation of the labels is insufficient.

Reply: We apologize for the poor graph quality. We are unable to download the graphs from Oncomine since it shut down in Feb. We have decided not to include the public data sets in this manuscript.

- Fig. 1B seems to include all kinds of esophageal cancers, not just EGACs.

Reply: thank you for the query. Correct this figure shows the expression of GPC1 in Barretina cell line compared to other cancers. As mentioned, in our revision we have omitted the public data set as some data like you have pointed out is confusing in relevance to this work.

- Fig. 1D. What is the difference between the red and black bars?

Reply: Thank you for the query. Red indicates tumor samples and black indicates normal tissue. These figures have been omitted from the revision.

- Fig. 1E. Contrary to what it is stated in the text, the levels of GPC1 don’t correlate with tumor stage.

Reply: Thank you for pointing out this discrepancy. We can only speculate that this discrepancy between stage and GPC1 expression could be explained based on differences between mRNA expression and its protein translation. Furthermore, it is not clear from this public data set as to what percentage of Stage II which were poorly differentiated, in which case a larger proportion of stage II poorly differentiated cases could be responsible for this stage/GPC1 mismatch. Unfortunately, we cannot access Oncomine data since it shut down. We have decided to omit Figure 1 from our manuscript.

- Fig. 1S and 3S are not well explained.

Reply: We apologize for not explaining well; however, we don't have a Fig 1S or 3S in the submitted manuscript. Please let us know if you intended to point out a different figure number and we will be happy to address your concerns.

- Fig. 3. The levels of GPC1 in the cell lines as detected by immunohistochemistry do not correlate with the Western blot results. This should be discussed. Why is the IHC of OE33 missing?

Reply: Thank you for pointing this out to us. We initially performed immunofluorescence with the intention to only localize GPC1 to ascertain if it was cytoplasmic or nuclear in location in the EGAC cell lines. For this purpose, we did not expose the sections to the same exposure time under a confocal microscope. We quantified GPC1 based on qPCR and western blot. However, we now understand from your comments that we should have performed localization with the same exposure time. We have repeated the confocal microscopy with uniform exposure and added images for OE-33, and we hope our new immunofluorescence results correlate with western blot data.

- Line 317: what does it mean that the "GPC1 overexpressing plasmid were labelled with EGFP"?

Reply: Thank you for the comment. This is a typo error. We wanted to clarify for the readers that the Lentivirus plasmids carried an extended-Green fluorescent protein controlled by SV40 as a readout for visual assessment of green fluorescence after transfection.

Changes in the text: We have described the plasmid in detail in material and methods Page 4, lines 102-103.

- Fig. 4. Abbreviations like NC, and EV should be explained in the figure legend.

Reply: Thank you for directing us to this issue. NC is negative scrambled control for GPC1 knockdown comparison, and EV is empty vector to serve as a control for GPC1 overexpression plasmid.

Changes in the text: We have incorporated the changes and explained the terminologies in material and methods as well as in figure legends.

- Fig. 6. The legend should indicate which experiments where repeated three times.

Reply: Thank you for directing us to this issue. We have included the suggested N=3 in relevant experiments.

Changes in the text: Figure legends now include a number of times the experiment was repeated.

- Fig. 7A is very difficult to read.

Reply: We apologize for the poor quality of the image. We have uploaded a higher quality of this image.

Changes in the text: High power and quality of image is now uploaded to the revision.

- In Fig. 7B the beta catenin label is not the right one.

Reply: We apologize for this error. We have corrected the label which should read TRITC.

- In Fig. 10F the changes in beta-catenin localization seem to be the opposite than what it is described in the text. Overexpression of GPC1 in OE19 cells induces membrane localization of beta catenin. This figure puts into question the conclusion that GPC1 is stimulating Wnt signaling.

Reply: Thank you for the critique and pointing out this issue. The Wnt pathway is a critical determinant of cell proliferation during development and regenerative processes, such as stem cell proliferation in adults' Aberrant activation of the pathway has been linked to oncogenesis in multiple systems. Nuclear localization of β-catenin is a hallmark of Wnt activation, yet in many systems it is only incidentally detected in the nucleus. In our IF images the goal was to show the readers differences in nuclear accumulation of b catenin between controls and GPC1 overexpressed cells, and this observation corroborated with western blot data of nuclear and cytoplasmic fraction analysis. Interestingly, Maarten Fornerod and colleagues show that, upon Wnt3a stimulation, unphosphorylated β-catenin is recruited to the plasma membrane (the protein can be detected at the membrane in E-cadherin–/– cells, indicating that it is distinct from E-cadherin-bound, junctional β-catenin). The redistribution of β-catenin to the membrane is an early event in the Wnt response and β-catenin colocalizes with the Wnt co-receptor LRP6, and with axin and APC (another component of the destruction complex). Moreover, the association of β-catenin with LRP6 increases its transcriptional activity. These results imply that β-catenin is activated in a Wnt-receptor complex at the plasma membrane, before translocating to the nucleus.[1]

Changes in the text: We have repeated immunofluorescence staining (Figure 10C) of cells for β catenin and would like to draw attention to the differences in nuclear accumulation as well as membrane staining of β-catenin in GPC1 knockdown and overexpressed cells compared to controls. We have also incorporated these findings in the revised manuscript text Page 12, lines 345-348

1. Hendriksen, J., et al., Plasma membrane recruitment of dephosphorylated beta-catenin upon activation of the Wnt pathway. J Cell Sci, 2008. 121(11): p. 1793-802.

- Fig. 11B and 11C are very confusing. The results regarding the impact of GPC1 on Akt phosphorylation seem contradictory, for example. Why does MK2206 inhibit the total levels of beta catenin, Akt and GSKb? This should be discussed.

Reply: Reply: Thank you for bringing this to our attention. Firstly, we have omitted the triplicate run of western blot previously shown of controls and test groups to avoid any confusion in interpretation of results. Secondly, we repeated the western blot from our lysate library only to get the same results of decreased AKT/GSK/and b catenin. On trouble shooting we found that the stock of MK-2206 was miscalculated with high concentration and we were treating cells with 10mM of MK-2206 instead of 1uM.This high dose could have been responsible for either off target effect of MK2206 and significant apoptosis with resulting low levels of total AKT/GSK and beta catenin. After correcting and redoing the experiment we have now got consistent results which are included in the revised manuscript.

Changes in the text: Repeat western blot has been added (Figure 11).

- Fig. 12C. The changes in pAkt are not obvious.

Reply: We are sorry we could not show obvious changes. Unfortunately, we did not have enough stored lysate from HET1A cells for repeating this experiment and moreover these cells are very slow to grow for us to validate this result in this short time. Therefore, we feel it is best to omit this figure.

Changes in the text: We have omitted this figure (earlier Figure 12C) from the revision.