A point-by-point response to reviewers' comments

Ref: MS #84071, γ-Aminobutyric acid B2 receptor: a potential therapeutic target for cholangiocarcinoma in patients with diabetes mellitus

Reviewer 1:

This study addresses a current topic. The manuscript is quite well written and organized. English should be improved. Figures and tables are comprehensive and clear. The introduction explains in a clear and coherent manner the background of this study.

Authors: Thank you for your time and for thoroughly reviewing our manuscript. In the revised version, the English presentation has been edited by Prof. James A. Will, Emeritus Professor at the University of Wisconsin-Madison, via KKU Publication Clinic. We believe the English presentation of our manuscript now has correct grammar and spelling.

We suggest the following modifications:

1. • Introduction section: although the authors correctly included important papers in this setting, we believe the evolving systemic treatment scenario for BTC should be further discussed and some recently published papers added within the introduction (PMID: 32806956; PMID: 36368251; PMID: 33592561; PMID: 33645367), only for a matter of consistency. We think it might be useful to introduce the topic of this interesting study.

Authors: Thank you for your suggestion. We have added these key papers to the Introduction (Lines: 137-142) and Discussion (Lines: 471-477), accordingly.

2. • Methods and Statistical Analysis: nothing to add.

Authors: Thank you very much.

3.• Discussion section: Very interesting and timely discussion. Of note, the authors should expand the Discussion section, including a more personal perspective to reflect on. For example, they could answer the following questions – in order to facilitate the

understanding of this complex topic to readers:

3.1 what potential does this study hold?

3.2 What are the knowledge gaps and how do researchers tackle them?

3.3 How do you see this area unfolding in the next 5 years?

We think it would be extremely interesting for the readers. However, we think the authors should be acknowledged for their work. In fact, they correctly addressed an important topic in renal cell carcinoma, the methods sound good and their discussion is well balanced.

Authors: Thank you for your suggestion. We have added these points to the Discussion section as highlighted in blue fonts in the revised manuscript (Lines: 471-477, and Lines:524-541).

4. One additional little flaw: the authors could better explain the limitations of their work, in the last part of the Discussion.

Authors: Thank you for your suggestion. We have clarified and explained the limitations of our work, as highlighted in blue fonts in the last part of the Discussion, accordingly.

We believe this article is suitable for publication in the journal although major revisions are needed. The main strengths of this paper are that it addresses an interesting and very timely question and provides a clear answer, with some limitations.

Authors: Thank you very much for your comments. We have revised our manuscript as per your suggestions and believe it improves the scientific integrity and data presentation of the present version.

5. We suggest a linguistic revision and the addition of some references for a matter of consistency. Moreover, the authors should better clarify some points.

Authors: Thank you, we have revised the manuscript as per your suggestion accordingly.

Reviewer 2

I have some concerns about this research:

1. Whether GABBR2 have expression correlation with cyclin D1 or c-Myc in clinical specimen?

Authors: Thank you for raising these interesting points. We have analyzed the correlation of these genes using the transcriptomic data derived from the GSE89749 datasets of CCA tissues from Thai cases. The data showed no correlations between GABBR2, and cyclin D1, and c-Myc at the mRNA level. This information has been added to the Results (Lines: 421-427), and in a supplemental Figure S1 and Discussion (Lines: 528-530).

2. GABBR2 promote b-cat translocation in CCA cells or tumors?

Authors: Thank you very much for raising this important point. To address your question, we have planned to do the additional immunohistochemistry and immunocytofluorescent staining of β-catenin in CCA tissues and CCA cell lines. Unfortunately, after trying our best to find the appropriate β-catenin antibody from our domestic collaborators, it was not available. Thus, the experiments could not be completed within the resubmission deadline. However, we have acknowledged this important point and added it as a limitation in the last paragraph of the Discussion.

2. How to choose the concentration of Glc for cells treatment? How about the long-term treatment?

Authors: A glucose concentration of 5.6 mM is the upper limit of the physiological range of fasting plasma glucose in people without diabetes, whereas 25 mM is within the hyperglycemic range and is similar to the average blood glucose in diabetic rodents used in *in vivo* diabetes and cancer research (Fainsod-Levi et al, 2017). In our model, we cultured the cells in either 5.6 mM or 25 mM for at least 5 passages before use as previously described (Saengboonmee et al, 2020).

Reference

(1) Fainsod-Levi T, Gershkovitz M, Völs S, Kumar S, Khawaled S, Sagiv JY, Sionov RV,

Grunewald M, Keshet E, Granot Z. Hyperglycemia Impairs Neutrophil Mobilization Leading to Enhanced Metastatic Seeding. Cell Rep. 2017; 21(9): 2384-2392. doi: 10.1016/j.celrep.2017.11.010.

(2) Saengboonmee C, Phoomak C, Supabphol S, Covington KR, Hampton O, Wongkham C, Gibbs RA, Umezawa K, Seubwai W, Gingras MC, Wongkham S. NF-κB and STAT3 co-operation enhances high glucose induced aggressiveness of cholangiocarcinoma cells. Life Sci. 2020; 262: 118548. doi: 10.1016/j.lfs.2020.118548.

3. GAPDH is not an ideal loading control for cancer cells, especially after glucose treatment.

Authors: We were also concerned about this point when we set up the NG and HG study in CCA. GAPDH was selected for the loading control in our study as (1) GAPDH expression showed minimal variation in the same type of tissues from different individuals (Barber et al, 2005). (2) GAPDH has been shown as the most appropriate loading control among the other tested housekeeping genes in some cancers, e.g., non-small lung cancer (Liu et al, 2005). (3) In our experience, expression of β-actin varied among CCA cell lines, while GAPDH was not. Moreover, GAPDH expression was not affected by different glucose concentrations as shown in our previous studies (Saengboonmee et al, 2016; Saengboonmee et al, 2020; Saengboonmee et al, 2022).

References

(1) Barber RD, Harmer DW, Coleman RA, Clark BJ. GAPDH as a housekeeping gene: analysis of GAPDH mRNA expression in a panel of 72 human tissues. Physiol Genomics. 2005; 21(3): 389-95. doi: 10.1152/physiolgenomics.00025.2005.

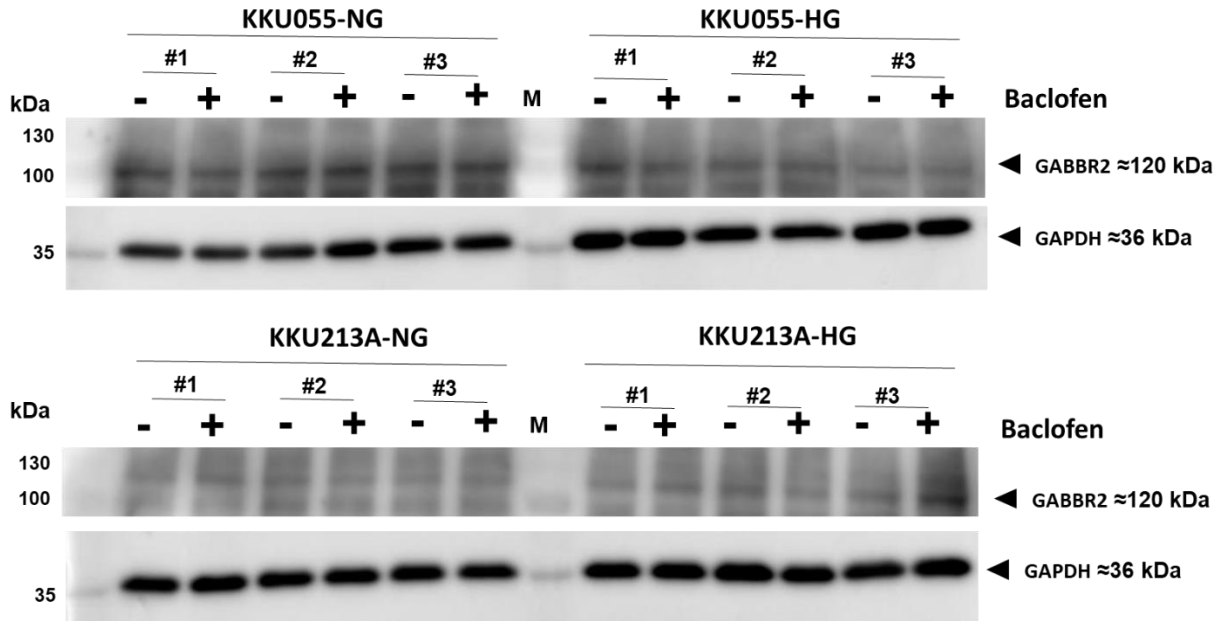
(2) Liu DW, Chen ST, Liu HP. Choice of endogenous control for gene expression in nonsmall cell lung cancer. Eur Respir J. 2005; 26(6): 1002-8. doi: 10.1183/09031936.05.00050205.

(3) Saengboonmee C, Seubwai W, Pairojkul C, Wongkham S. High glucose enhances progression of cholangiocarcinoma cells via STAT3 activation. Sci Rep. 2016; 6: 18995. doi: 10.1038/srep18995.

(4) Saengboonmee C, Phoomak C, Supabphol S, Covington KR, Hampton O, Wongkham C, Gibbs RA, Umezawa K, Seubwai W, Gingras MC, Wongkham S. NF-κB and STAT3 co-operation enhances high glucose induced aggressiveness of cholangiocarcinoma cells. Life Sci. 2020; 262: 118548. doi: 10.1016/j.lfs.2020.118548.
(5) Saengboonmee C, Detarya M, Sangkhamanon S, Sawanyawisuth K, Seubwai W, Wongkham S. High Glucose Induced Upregulation of Cyclin a Associating with a Short Survival of Patients with Cholangiocarcinoma: A Potential Target for Treatment of Patients with Diabetes Mellitus. Nutr Cancer. 2022; 74(5): 1734-1744. doi: 10.1080/01635581.2021.1961830.

4. They authors should prove that Baclofen treatment can not change the expression of GABBR2.

Authors: Thank you for this challenging comment. We proved this point by Western blot using cell lysates from both NG and HG cells of 2 CCA cell lines treated with baclofen for 72 h. As shown in the figure below, baclofen treatment did not affect the expression of GABBR2 proteins in all 3 biological replications. We have mentioned these findings in the Results, Lines: 374-376, of the revised manuscript.



5. ChIP and other solid evidence should be provided to clarify Baclofen and GABBR2

affect the GSK3/β-catenin and STAT3 signaling.

Authors: Thank you very much for your suggestion. We agree that ChIP and additional experiments will strengthen the findings on the effects of baclofen and GABBR2 on GSK3/β-catenin and STAT3 signaling in CCA. The effects of GABBR2 on STAT3 in CCA (Huang et al, 2013), and GABBR2 on β-catenin in lung cancer (Huang et al, 2022), have been reported in previous studies in which our present results confirmed and supported their findings. We have added this point to the discussion section and acknowledged this limitation in the present study. .

References

(1) Huang Q, Zhu CL, Liu CH, Xie F, Zhu K, Hu SY. Gamma-aminobutyric acid binds to GABAb receptor to inhibit cholangiocarcinoma cells growth via the JAK/STAT3 pathway. Dig Dis Sci 2013; 58(3): 734-743. doi: 10.1007/s10620-012-2382-2.

(2) Huang D, Wang Y, Thompson JW, Yin T, Alexander PB, Qin D, Mudgal P, Wu H, Liang Y, Tan L, Pan C, Yuan L, Wan Y, Li QJ, Wang XF. Cancer-cell-derived GABA promotes β-catenin-mediated tumour growth and immunosuppression. Nat Cell Biol 2022; 24(2): 230-241. doi: 10.1038/s41556-021-00820-9.

6. In vivo experiment, such as xenograft are preferred.

Authors: Thank you for your suggestions. We agree that the xenografted animal model may add value to the paper. To answer this, we have set up a preliminary experiment in the xenografted mouse model by submitting the protocol for Animal ethic approval (Approval No. KKU-IACUC 139/64) and asked for an extension of the resubmission deadline.

As there is no report on the appropriate dosage of baclofen for cancer treatment in animals, we used baclofen as a similar anti-tumor dose as natural GABA reported in CCA xenografts by Huang et al. Unfortunately, the dosage of baclofen used has a greater adverse effect, and all mice (n = 12) were not recovered after the first dose. The experiment was then terminated early due to the humane endpoint. Thus, we acknowledge this limitation and added this information in the Discussion instead.

Reference

Huang Q, Zhu CL, Liu CH, Xie F, Zhu K, Hu SY. Gamma-aminobutyric acid binds to GABAb receptor to inhibit cholangiocarcinoma cells growth via the JAK/STAT3 pathway. Dig Dis Sci. 2013; 58(3): 734-43. doi: 10.1007/s10620-012-2382-2.

Reviewer 3

Overall, it is a decent paper. However, in vivo studies should be included.

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Revision reviewer

acceptance.

Authors: Thank you for your suggestions.