Peer Review File

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Reviewer A

In this manuscript, the authors found that Inhibition of BMPR2 suppresses pancreatic ductal adenocarcinoma growth by regulating GRB2/PI3K/AKT axis.

Major critiques:

1. What is the Clinicopathological characteristics of patients with pancreatic ductal adenocarcinoma according to BMPR2 and GRB2 expression.

Reply 1. Thank you for the careful review. We measured the expression of BMPR2 by immunohistochemistry. We have analyzed the clinicopathological characteristics of patients and have added this information in the manuscript (see Page 11, line 4-7, **Table 1**, Page 24).

Changes in the text:

The expression level of BMPR2 and the clinicopathological characteristics were analyzed. As shown in Table 1, high expression of BMPR2 was correlated with TNM stage and tumor size, which indicated that BMPR2 may involve in the growth of pancreatic cancer.

Table 1. Relation between the expression level of BMPR2 and clinical features with PDAC

Clinical features	Classification	High BMPR2 Expression	Low BMPR2 Expression	*P* value
Sex	Male	17(26.15%)	16(24.62%)	0.531
	Female	14(21.53%)	18(27.69c%)	
Age	<60	13(20%)	21(32.31%)	0.279
	≥60	16(24.62%)	15(23.08%)	
Tumor grade	Poor	17(26.15%)	9(13.85%)	0.088
	Moderate	11(16.92%)	6(9.23%)	
	Well	8(12.31%)	14(21.54%)	
TNM stage	I-II	8(12.31%)	19(29.23%)	<0.0001
	III-IV	28(43.08%)	10(15.38%)	
Vascular invasion	Yes	15(23.08%)	13(20.0%)	0.635
	No	22(33.85%)	15(23.08%)	
Tumor size	<3 cm	11(16.92%)	19(29.23%)	<0.0001
	≥3 cm	29(44.62%)	6(9.23%)	
Nerve invasion	Yes	13(20.0%)	18(27.69%)	0.761
	No	13(20.0%)	21(32.31%)	
CA19-9	<39	18(27.69%)	11(16.92%)	0.063
	≥39	14(21.54%)	22(33.85%)	
Tissue type	PDAC tissue	47(72.31%)	18(27.69%)	<0.0001
	Adjacent normal tissue	9(13.85%)	56(86.15)	

2. Antibody information (cas number, corporation) should be provided.

Reply 2. Thank you for your suggestion. We have added the detailed information in our manuscript (**Methods** part, see Page 8, Line 10).

Changes in the text:

Antibody name	Manufacturers	Concentration	Catalog number
BMPR2	R&D Systems	1:2000	AF811
GRB2	Abcam	1:5000	ab32037
β-actin	Proteintech	1:10000	60008-1-Ig
PI3K	Abcam	1:2000	ab140307
p-PI3K	Abcam	1:1000	ab154598
AKT	Abcam	1:500	ab8805
p-AKT^{308}	Abcam	1:1000	ab38449
p-AKT^{474}	Abcam	1:1000	ab38513
Cyclin B1	Cell Signaling Technology	1:1000	12231
CDK1	Proteintech	1:1000	19532-1-AP

3. Both p473 and p308 sites represent full AKT activation, which should be added in WB.

Reply 3. Thank you for your constructive suggestion. The phosphorylation level in Thr308 and Ser473 site of AKT is usually detected to represent AKT activation. We only measured p308 in our previous manuscript. We have further detected the phosphorylation level of p473 site and added the results in **Figure 4C, D** (see Page 15, Line 17).

Changes in the text:

We then detected the level of PI3K, p-PI3K, AKT and p-AKT after down-regulating GRB2, the results showed that p-PI3K, p-AKT308 and p-AKT474 were inhibited after knockdown of BMPR2 in both two pancreatic cancer cell lines. In order to further verify the regulation of GRB2 on PI3K/AKT, we detected the level of PI3K, p-PI3K, AKT and p-AKT, and the results indicated that p-PI3K, p-AKT308 and p-AKT474 were inhibited significantly (Figure 4D).

4. Cell cycle-related proteins (cyclin A/B/C/D/E, p21, p27, CDK2/4/6) should be detected in WB.

Reply 4. Thank you for your suggestion. We revealed the G2/M stage arrest of down-regulation of BMPR2 in pancreatic cancer cells. We further detected the G2/M stage related proteins cyclin B1 and CDK2 by western blot analysis. We have added the results in **Figure 1J** (see Page 13, Line 6).

Changes in the text:

We further detected the expression of two G2/M stage related proteins Cyclin B1 and CDK1, and the results indicated that Cyclin B1 and CDK1 were inhibited significantly after knockdown of BMPR2 (Figure 1J).

5. What is the specific mechanism of BMPR2 regulating GRB2?

Reply 5. Thank you for your review. In our work, we down-regulated the expression of BMPR2 and then performed protein spectrum analysis. By protein-protein interaction (PPI) network analysis, we found that the GRB was the potential target of BMPR2. By enrichment analysis, we found that PI3K/AKT signaling pathway was regulated by BMPR2/GRB2. Therefore, we verified the regulation of BMPR2 on GRB2/ PI3K/AKT axis. However, we didn't further analyze the mechanism of BMPR2 regulating GRB2, and the specific mechanism deserves more study to make the regulation of BMPR2 on GRB2 clearer.

6. Does BMPR2 affect death receptor mediated apoptosis? Additional pro-apoptotic factors (eg. FasL, TNFa, TRAIL) should be added to test the effect of BMPR2 on death receptor mediated apoptosis.

Reply 6. Thank you for your careful review. Our present work mainly focused on the function of BMPR2 on pancreatic cancer proliferation and the potential mechanism that BMPR2 regulating the proliferation. But we didn't detect the effect of BMPR2 on apoptosis of pancreatic cancer cells. BMPR2 has been reported participating in regulation of apoptosis in chondrosarcomas and gastric cancer (Cell Death and Disease. 2014, 5, e1571, Cancer Cell Int. 2019, 19, 354). It really deserves more study to further reveal the effect of BMPR2 on death receptor mediated apoptosis and we thank you for giving the next study direction.

7. Does BMPR2 affect the expression of other apoptosis related mitochondrial proteins, for example, Bcl-2, Bcl-XL, Bax, Bim, Bad, IAPs?

Reply 7. Thank you for your insightful question. As we have replied in Comment 6, we only studied the role of BMPR2 in proliferation of pancreatic cancer, and we didn't detect the apoptosis related proteins. It is very likely that BMPR2 has an effect on apoptosis or other biological behaviors in pancreatic cancer, since BMPR2 is an important receptor involving in multiple signaling pathways such as Smad (Cell Physiol Biochem. 2018;46(1):213-225), TGFβ (PLoS Biol. 2019 Dec 11;17(12): e3000557), Wnt (J Virol. 2018 Mar 14;92(7):e01937-17) and so on. The potential target signaling pathways deserve further study in pancreatic cancer.

8. Does BMPR2 affect cell migration and invasion?

Reply 8. Thank you for your careful review. In our present work, we mainly studied the role of BMPR2 in proliferation of pancreatic cancer. Tumor migration and invasion has totally different mechanism with proliferation. Progress of tumor migration and invasion involves in epithelial-mesenchymal transition, extracellular matrix deregulation and so on, which reflects the mobility of tumor cells. The proliferation of tumor cells is controlled by cell cycle checkpoint proteins, and in our study, we have revealed the effect of BMPR2 on pancreatic cancer proliferation, and the G2/M stage related proteins Cyclin B1 and CDK1 were significantly downregulated after knockdown of BMPR2 and GRB2. Recently, some studies have demonstrated that BMPR2 promotes cell migration and invasion in osteosarcoma Cells (Oncotarget. 2017, 24, 8, 58625-

58641) and Esophageal Squamous Cancer (Int J Oncol. 2017 Jan;50(1):193-202). Therefore, it is possible that BMPR2 may also affect cell migration and invasion in pancreatic cancer.

9. What is the detailed mechanism of BMPR2 and GRB2 regulating the cell proliferation of cancer cells? Does BMPR2 affect Ras, Src and NF-kB signaling?

Reply 9. Thank you for your review. In our present work, we found the upregulated expression of BMPR2 in pancreatic cancer tissues compared with adjacent normal pancreatic tissues. And then we knockdown BMPR2 in pancreatic cancer cells and performed the mass-spectrometric technique combining with bioinformatic analysis, which indicated the potential regulating target GRB2 of BMPR2. By enrichment plot analysis, we focused on the PI3K/AKT signaling pathway, and further studies verified the regulation of BMPR2 on GRB2/ PI3K/AKT signaling pathway. GRB2 is an adaptor protein acting as an intermediate between cell-surface activated receptors and downstream targets, which has been proved in multiple tumor malignancies. The most well known ability of GRB2 is to link the epidermal growth factor receptor tyrosine kinase to the activation of RAS and its downstream kinases, ERK1, 2 (Lancet Haematol. 2018, 5, e128-e129, Protein Pept Lett. 2018, 8, 24:1084-1095, Cell Death Dis. 2019, 18, 10: 546). In our present work, we revealed the regulation of GRB2 on PI3K/AKT signaling pathway in pancreatic cancer proliferation. We have drawn a sketch below to describe the regulating process (Figure R1). In our work, we actually indicated the changes between BMPR2 and GRB2/ PI3K/AKT signaling pathway, but the direct regulating mechanism needs further study.

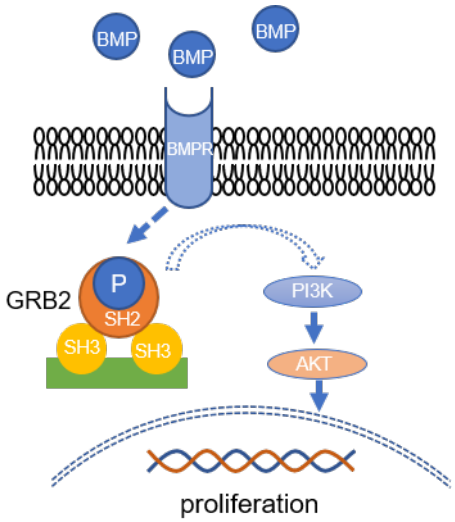

Figure R1. BMPR2/ GRB2/ PI3K/AKT signaling pathway in pancreatic cancer cells.

10. Kaplan–Meier survival analysis of pancreatic ductal adenocarcinoma patients with high or low GRB2 should be supplied.

Reply 10. Thank you for your suggestion. In our previous work, we compared the expression of GRB2 in pancreatic cancer and adjacent normal pancreatic tissues. To analyze the relation of expression of GRB2 and patient overall survival rate, we performed Kaplan–Meier survival analysis according to your suggestion (**Figure 2G**, see Page 13, Line19).

Changes in the text:

The Kaplan–Meier survival analysis was performed according to the expression level of GRB2. As shown in the Figure 2G, higher expression level of GRB2 had shorter overall survival rate.

Reviewer B

This study revealed the clinical significance and biological role of BMPR2 in pancreatic cancer. Moreover, the authors suggested that BMPR2 contributed to tumor growth via regulating GRB2/PI3K/AKT signaling pathway. This is an interesting study. I have some suggestions to further strengthen this study.

1. To further support the conclusions in this study, the authors should confirm that GRB2 knockdown inhibits PI3KA/AKT signaling in cancer cells.

Reply 1. Thank you for your constructive suggestion. To confirm the regulation of BMPR2 on PI3K/AKT by GRB2, we performed western blot analysis to detect the level of p-PI3K and p-AKT after knockdown of GRB2. We have added this result in our manuscript (**Figure 4C, D**, see Page 15, Line 17):

Changes in the text:

We then detected the level of PI3K, p-PI3K, AKT and p-AKT after down-regulating GRB2, the results showed that p-PI3K, p-AKT308 and p-AKT474 were inhibited after knockdown of BMPR2 in both two pancreatic cancer cell lines. In order to further verify the regulation of GRB2 on PI3K/AKT, we detected the level of PI3K, p-PI3K, AKT and p-AKT, and the results indicated that p-PI3K, p-AKT308 and p-AKT474 were inhibited significantly (Figure 4D).

2. According to previous studies, LDN193189 is the BMP signaling inhibitor rather than a specific BMPR2 inhibitor. Thus, the in vivo experiments should be performed using BMPR2 shRNA.

Reply 2. Thank you for your careful review. As you mentioned, LDN193189 is not a specific BMPR2 inhibitor. To further verify the effect of BMPR2 on pancreatic cancer growth, we performed the in vivo experiments using BMPR2-downregulated pancreatic cancer cells (PANC-1-shBMPR2). We have added the results in our manuscript as follows (**Figure 6J, K**, see Page 17, Line18):

Changes in the text:

As we know that, LDN193189 is not a specific inhibitor of BMPR2. In order to verify knockdown of BMPR2 lead to inhibition of pancreatic cancer growth directly, we downregulated BMPR2 using shRNA in PANC-1 cells, and performed in vivo experiments. The results showed that tumors were much smaller in shBMPR2 group compared with the control group (**Figure 6J, K**).

3. How GRB2 regulates PI3K/AKT in pancreatic cancer at least should be discussed.

Reply 3. Thank you for your insightful suggestion. In our work, we didn't reveal the detailed mechanism of GRB2 regulating PI3 K/AKT. We have added the relevant discussion in our manuscript (see Page 17, Line 1).

Changes in the text:

GRB2 was initially discovered as the connect molecule between the epidermal growth factor receptor (EGFR) and the Ras–mitogen activated protein kinase (MAPK) pathway (27, 28). GRB2-mediated receptor dimerization inside cells leads to transphosphorylation of the kinase domains of target proteins(29). Recently, GRB2 has been proved to be involved in PI3K/AKT signaling pathway in colorectal cancer (30). However, this work didn't demonstrate the detailed mechanism that GRB2 regulating PI3K/AKT signaling pathway. In our work, we revealed the level of phosphorylation level of PI3K was significantly decreased. Therefore, GRB2 may involve the phosphorylation process of PI3K, and then affects downstream molecules. Although our present work shows the GRB2 is a mediator in transducing signaling from BMPR2 to PI3K/AKT pathway, the mechanism by which BMPR2 regulates GRB2 remains to be further studied.