Author's response to reviews

Title: Fibroblast activation protein-alpha-expressing fibroblasts promote the progression of pancreatic ductal adenocarcinoma

Authors:

Tomoya Kawase (oneworld_traveller@yahoo.co.jp)
Yumiko Yasui (yumikoy@med.kawasaki-m.ac.jp)
Sohji Nishina (018ep@med.kawasaki-m.ac.jp)
Yuichi Hara (yuich_h777@med.kawasaki-m.ac.jp)
Izumi Yanatori (yanatori@med.kawasaki-m.ac.jp)
Yasuyuki Tomiyama (tomiyama@med.kawasaki-m.ac.jp)
Yoshihiro Nakashima (makazi777@med.kawasaki-m.ac.jp)
Koji Yoshida (kojiyos@med.kawasaki-m.ac.jp)
Fumio Kishi (kishi@med.kawasaki-m.ac.jp)
Masafumi Nakamura (mnakamura@med.kawasaki-m.ac.jp)
Keisuke Hino (khino@med.kawasaki-m.ac.jp)

Version: 2
Date: 28 June 2015

Author's response to reviews: see over
Dear Dr Rooman

We sincerely thank you and reviewers for your constructive and critical comments. We also are grateful for giving us the chance of resubmission of our manuscript.
We have addressed the concerns raised by reviewers.
Response to reviewers is described below. Revised sentences are described in red-color font.
We would be very grateful if you could consider our revised manuscript for the publication in BMC Gastroenterology.

For referee 1:
1. you named a 5-year survival rate for PDAC of 10% in the introduction (page 3,line 21), please specify for which stage.
   Description that a 5-year survival rate is approximately 10% in patients with PDAC was wrong.
   We confirmed that the overall 5-year survival rate was less than 5% in patients with PDAC.
   Therefore, we revised it as described in the line 1 in page 4.

2. how do you explain the untypical results for the factors associated with overall survival in in your study population (uni-/multivariate analysis): no correlation for post-operative chemotherapy, tumor size, lymph node involvement, but for alcohol intake?
   We appreciate the critical comments. We also think it untypical that overall survival was correlated with alcohol intake, but not with post-operative chemotherapy, tumor size and/or lymph node involvement. We defined alcohol intake as one ≥ 3 drinks (37.5 g/day) on the basis of alcohol intake and pancreatic cancer risk deduced from a meta-analysis of the dose-risk relation, as described in the lines 15-17 in page 5. Heavy alcohol intake (≥ 37.5 g/day) and relatively low proportion (18.7%) of T1/T2 stage tumors in our study population might be related to these untypical results, even though the precise explanation remains elusive. Therefore, we revised this issue as described in the lines 2-6 in page 10.

3. Figure 1: survival on the x-axis should be quoted in weeks or months
   According to the referee’s comment, survival on the x-axis in Figure 1 has been quoted in months.

4. please explain: experiments are carried out with only one pancreatic cancer cell line and one embryologic mouse cell line of fibroblasts. How do you ensure that the individual differences of the tumors are sufficiently represented by this set up? I am particularly critical of the point that you used
fibroblasts of another species. Fibroblasts generated from human pancreatic tissue would allow a clearer statement. How can you exclude confounding factors resulting from the use of cells of different species?

We appreciate these critical comments. We also think that the individual differences of the tumors are not sufficiently represented by the use of single pancreatic cancer cell line. Therefore, we have revised Discussion as described in the lines 6-10 in page 15, where we indicated that the critical limitations in this study was the experiments carried out with only one pancreatic cell line and that it remains to be elucidated whether FAP universally activates invasiveness and/or cell progression of cancer cells in patients with PDAC. As for the use of embryologic mouse cell line of fibroblasts, we could not establish the human-originated fibroblasts stably expressing FAP. We agree with the comments that the use of cells of different species may be a confounding factor in terms of evaluation of the effect of CAFs on epithelial tumor cell in human PDAC. However, we wanted to know the effect of FAP on pancreatic cancer cells. In addition, there was no direct contact between FAP-expressing NIH-3T3 cells and MiaPaCa-2 cells in our coculture system. In this respect it should be noted that human FAP, not mouse FAP, was expressed in NIH-3T3cells, and the use of mouse-derived fibroblasts may not have critical influence on the interaction of FAP with pancreatic cancer cells. Accordingly, we have addressed these issues as described in the lines 22-8 in pages 10 and 11, and in the lines 11-17 in page 13.

5. please specify the method of co-culture further: you said you were using six well culture plates and then culture inserts. Did you use the same culture inserts as for the invasion essay? If so, was there any direct contact of the fibroblasts and the pancreatic cancer cells? If there was any direct contact, did you expect any effect of that? If you use the same methodical set up in invasion essay and coculture please revise the corresponding paragraph.

We appreciate these critical comments. We used the same culture plates and culture inserts in invasion assay and coculture system, as described in the line 17 in page 7. There was no direct contact between FAP-expressing NIH-3T3 cells and MiaPaCa-2 cells in our coculture system. Antiplasmin-cleaving enzyme (APCE) has been identified as a soluble form of FAP, resulting from cleavage of the Cys23-Ile24 bond in the transmembrane or extracellular domain [24]. Therefore, it is reasoned that FAP-expressing fibroblasts promoted the invasiveness of MiaPaCa-2 cells even in the absence of direct contact of these two cells, even though we could not measured the APCE level in culture medium. Accordingly, we have added paragraph as described in the lines 22-8 in pages 10 and 11.
6. please add: how often did you repeat the experiments on invasiveness?

The invasion assay was performed 13 times.

7. please explain: why did you choose retinoblastoma (Rb) for your analysis? Did you expect any interaction and when yes, why?

We appreciate these critical comments. As we found that coculture with FAP expressing NIH-3T3 cells activated switching from G0/G1 to S/G2/M in MiaPaCa-2 cells, we then wanted to know the mechanisms underlying this. Rb protein is an inhibitor of cell cycle progression, that is, Rb arrests cells in G1 phase. Rb is phosphorylated and dephosphorylated during the cell cycle; the hyperphosphorylated form predominates in proliferating cells, whereas the hypophosphorylated form is generally more abundant in quiescent or differentiating cells. To be exact, Rb binds to a gene regulatory protein called E2F and blocks the transcription of S-phase genes. Phosphorylated Rb reduces its affinity for E2F, and then dissociates, allowing E2F to activate S-phase gene expression [25]. Therefore, we next examined the phosphorylation of Rb to clarify the mechanisms underlying switching from G0/G1 to S/G2/M in MiaPaCa-2 cells cocultured with FAP-expressing NIH-3T3 cells. We explained the reason why we analyzed Rb phosphorylation, as described in the lines 5-14 in page 12. Although we found that FAP promoted phosphorylation of Rb in MiaPaCa-2 cells, we could not investigate how FAP activates phosphorylation of Rb protein in pancreatic cancer cells in the present study.

For referee 2:
1. Are conditioned media from FAP+ NIH 3T3 sufficient to drive the increased level of P-Rb?
2. Gene expression profile differences in FAP+ NIH 3T3 vs. FAP- NIH 3T3 to identify candidate mediators of the Rb phosphorylation.
3. Will knockdown of Rb abrogate the effects of FAP+ NIH3T3?

We thank referee 2 for these constructive and critical comments. Unfortunately, we could not perform these additional experiments within the limited period for submitting the revised manuscript.