Author's response to reviews

Title: Protein-bound polysaccharide from Phellinus linteus inhibits tumor growth, invasion, and angiogenesis and alters Wnt/beta-catenin in SW480 human colon cancer cells

Authors:

Kyoung-Sub Song (limlab1@gmail.com)
Ge Li (ybhospital@hanmail.net)
Jong-Seok Kim (livingstream@nate.com)
Kaipeng Jing (limlab4@gmail.com)
Tae-Dong Kim (Tae-Dong-Kim@ouhsc.edu)
Jin-Pyo Kim (jinpkim@hanmail.net)
Seung-Bo Seo (winnerhifive@hanmail.com)
Jae-Kuk Yoo (yjk9191@empal.com)
Hae-Duck Park (dsyoonmd@kyuh.co.kr)
Byung-Doo Hwang (bdhwang@cnu.ac.kr)
Kyu Lim (kyulim@cnu.ac.kr)
Wan-Hee Yoon (whyoon@cnu.ac.kr)

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Author's response to reviews: see over
Response to reviewer`s comments

We would like to thank the reviewers for their expert comments on our manuscript (Manuscript ID 8581952545269282). We have revised our manuscript based on the comments and all comments are listed below, followed by individual responses.

Reviewer: Dan Sliva

Specific comments:

1. The title “Protein-bound polysaccharide from Phellinus linteus inhibits tumor growth, invasion, and angiogenesis of SW480 human colon cancer cells by modulating Wnt/beta-catenin signaling.” is not correct. Characterization of “protein-bound polysaccharide” is missing – what is the exact chemical composition of PL from Hankook sin Yak Pharm (page 5, 2nd line from top)? What compound(s) are responsible for the biological activity of PL? If this extract is characterized it is fine but the reference must be provided.

Reply: The protein-bound polysaccharide is extracted from Phellinus linteus (PL) and has a molecular weight of 153 kDa. A reference was added in the revised manuscript as suggested (ref. 2. K.S. Song, S.M. Cho, J.H. Lee, H.M. Kim, S.B. Han, K.S. Ko and I.D. Yoo, B-lymphocyte stimulating polysaccharide from mushroom Phellinus linteus, Chem. Pharm. Bull. 43 (1995), pp. 2105–2108). Additionally, we agree that the title of our original manuscript is not proper and therefore have replaced it with Protein-bound polysaccharide from Phellinus linteus inhibits tumor growth, invasion, and angiogenesis and alters Wnt/beta-catenin signalling in SW480 human colon cancer cells, as suggested by another reviewer, Dr. Calviello.

2. Figure 1: a) quantification of Fig. 1B is missing - in addition, how many times was this experiment repeated? b) TCF/LEF reporter activity (Fig. 1D) – this experiments must be performed also with mutated TCF/LEF reporter gene construct – to show the specificity; the normalization of luciferase activity to the amount of cellular proteins is not correct – an additional reporter gene assay is more suitable.
Reply: a) The experiments were repeated three times separately and as you suggested above, we further evaluated our data. Now, the ratio of cyclin D1/beta-actin and beta-catenin/beta-actin is presented in the revised manuscript. b) When we performed pilot experiment using mutated TCF/LEF reporter gene construct, we did not observe any effects on PL. Therefore, we only employed pcDNA construct as a control in our study. We fully agree with the reviewer that an additional reporter gene assay would be more suitable. However, since normalization of luciferase activity to the amount of cellular proteins is also widely used (Lim et al, Cancer Res., 68:553, 2008), we calculated the relative luciferase activity after normalization of cellular proteins.

3. Figure 2: a) Fig 2A, 2B what is the real amount of invaded/migrated cells, is the incubation time for invasion and invasion the same? b) Fig. 2C – how many times was this experiment repeated (?); statistical analysis is missing.

Reply: a) Figure 2A and B were replaced by two new figures with the indication of real number of invaded/migrated cells, as requested by the reviewer. Same incubation time (72 h after PL treatment) was used in both invasion assay and mobility assay, which was detailed in section Methods of the revised manuscript (page 6, line 6 from bottom). b) We repeated three separate experiments and we have indicated this in the revised manuscript (page 21, line 1-2 from bottom). In addition, Figure 2C is replaced by a new one showing the result of statistical analysis, as suggested by the reviewer.

4. Figure 5C, 5D – how many samples were analyzed, the staining should be quantified and statistical analysis performed (minimally for IHC).

Reply: Eight samples were analyzed in our study. The detailed quantitative description is added in section of Methods of our revised manuscript. Meanwhile, quantification and statistical analysis were performed, and the new data were added in our revised manuscript (Figure 5C, right), as suggested.
Reviewer: Gabriella Calviello

Minor Essential Revisions

1. The article is clear and, on the whole, well written. The findings are interesting, but a title like: “Protein-bound polysaccharide from Phellinus linteus inhibits tumor growth, invasion, and angiogenesis and alters Wnt/beta-catenin signalling in SW480 human colon cancer cells” seems more appropriate.

Reply: Thanks for your comment regarding the title. We agree that the one you provided is much better! Therefore, we have changed the title of our revised manuscript into the one you suggested.

2. Background: Page 3, lines 7-13: “Cancer invasion and metastasis consist of several interdependent.....” This paragraph can be omitted.

Reply: This paragraph was omitted as suggested.


Reply: We agree and have changed it.

4. Page 4, line 4: Change into: “One important signaling pathway involved in the etiology….”

Reply: We have changed it into the one you suggested.

5. Page 4, line 16: Change into: “In the present study, we have investigated the effects of a PL treatment on ....”

Reply: We have done it.

6. Page 4, lines 21-22: Change into: “Our data suggest that the PL-induced down-regulation of Wnt/beta-catenin signaling may contribute to the inhibition of tumor....”
Reply: We have changed it.

7. Results and Discussion: The concentration-dependence of cyclin D1 expression inhibition (fig 1B) is not so clear, the authors should add the cyclin D1/beta-actin ratio value for each blot (obtained at each concentration).

Reply: we have done that as you suggested in the revised manuscript.

8. The different results obtained with the two different conditions intravenous or the intratumoral injections of PL are not explained neither in the Results nor in the Discussion. Moreover, it would be better to specify in Fig 4; “PL intratumoral injection” (in panel A) and “PL intravenous injection” in panel B.

Reply: Figure 4 was specified as you suggested. In addition, thanks for pointing out that we did not discuss the difference of one of our important results. In the Results and Discussion section of our revised manuscript, now we have added the relevant discussion regarding the different results obtained from two different conditions (page 13, line 12-16 from top).

9. Page 9, last line, page 10 lines 1-4: When the author assess the effect of PL on proliferation by using the MTT assay, do they have also evaluated if PL (especially at the high concentrations) is cytotoxic (i.e. by measuring the percentage of died cells in the cell culture, for instance using the trypan blue exclusion assay)? They write: “We previously demonstrated that PL has an anti-proliferative effect for SW480 colon cancer cells and the growth inhibition is mediated by induction of apoptosis and G2/M cell cycle arrest”. But it is important also to evaluate whether PL exert cytotoxic effect (by causing necrotic death).

Reply: We would like to thank for your comment. Although trypan blue exclusion assay is used for cell viability, there are also a number of studies implying that trypan blue can not distinguish between apoptotic cell death and necrotic cell death. Therefore we applied TUNEL assay and detected apoptotic molecules, such as cytochrome C to investigate the inhibitory mechanisms of PL, which has already been published previously (ref. 6. Li G, Kim DH, Kim TD, Park BJ, Park HD, Park JI, Na MK, Kim HC, Hong ND, Lim K et al: Protein-
bound polysaccharide from Phellinus linteus induces G2/M phase arrest and apoptosis in SW480 human colon cancer cells. Cancer Lett 2004, 216(2):175-181.). Since cultured cells that undergo apoptosis eventually die by a secondary necrosis process, we believe that based on our MTT assay data, PL also has cytotoxic effect (by causing necrosis) in SW480 cells.

10. Page 10, lines 9-11 “Depending on the presence of APC mutations and beta-catenin overexpression in SW480 cells, [33] the….”: Change into: “We examined the potential effect of PL on beta-catenin protein level and activity in SW480 cells, since these cells are reported to carry mutations of APC and over-express beta-catenin [33].”

Reply: Thanks for your comment. We have done that as suggested.

11. Discretionary Revisions: The observation that MMP-9 activity is inhibited following the treatment with PL is an interesting finding, but seems not to correlate with the inhibition of beta-catenin overexpression caused by these compounds. Thus, it does not add strength to the main hypothesis (the involvement of the beta-catenin pathway in the anti-cancer effects of PL). On the other hand, it has been previously reported that the expression and activity of MMP-7 is regulated by beta-catenin in human colon cancer cells (Brabletz et al 1999, Am J Path 155: 1033-1038). Thus, it is surprising that the authors did not evaluate the activity of MMP-7 in colon cancer SW480 cells, but, instead, those of MMP-2 and MMP-9.

Reply: Thanks for your suggestion. Yes, we did notice that MMP-7 is one of downstream genes of beta-catenin. However, in the context of invasiveness of colon cancer cells, MMP-2 and MMP-9 have also been reported to play essential roles. Therefore, we first investigated the effect of PL on MMP-2 and MMP-9 activities in the present study. We agree with the reviewer that PL may also regulate the activity of MMP-7 via beta-catenin and this will be investigated in our further study.