Author’s response to reviews

Title: alpha-Mangostin extracted from the pericarp of the mangosteen (Garcinia mangostana Linn) reduces tumor growth and lymph node metastasis in an immunocompetent xenograft model of metastatic mammary cancer carrying a p53 mutation

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Title: $\alpha$-Mangostin extracted from the pericarp of the mangosteen (*Garcinia mangostana* Linn) reduces tumor growth and lymph node metastasis in an immunocompetent xenograft model of metastatic mammary cancer carrying a p53 mutation

Dear Editor:

We thank the reviewers for their constructive criticism and for the opportunity to improve our manuscript, which has been revised and is being resubmitted for further consideration for publication in *BMC Medicine*.

We have made a number of changes (indicated in red in the manuscript) according to the reviewers’ suggestions, as detailed below.

Thank you very much for your efforts on our behalf.

Sincerely,

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Reviewer: Dr. Airo Tsubura

Thank you very much for your excellent advice to improve our research article.

1. Pericarp of mangosteen was obtained from food companies in Thailand. We provided α-Mangostin to Wako Pure Chemical Industries, Osaka, Japan for many requests by researchers. Therefore, α-mangostin is now commercially available from Wako Pure Chemical Industries.

2. Most of analyses (cell viability, caspase, cytochrome c release, Bid and cell cycle) were conducted using mouse mammary carcinoma BJMC3879luc2 cells. However, Akt-phosphorylation analysis only was performed in both MDA-MB231 cells (in vitro) and BJMC3879luc2 cells (in vivo). We are very sorry for complication. To make clear on this, a description has been added in lines 11-14, page 6. In addition, the term “BJMC3879luc cells” has inserted in all in vitro studies using BJMC3879luc cells (page 8) and Figure 2 legend (page 35).

3. MDA-MB231 cells treated with α-mangostin showed similar biological responses as in BJMC3879luc2 cells. This issue is now preparing for submission.
Reviewer: Manabu Kawabata

Thank you very much for your excellent suggestions to improve our manuscript.

1. No any differences in body weights and organ weights were found in a 4-week toxicity study of crude α-mangostin in mice administered 0, 4, 20, 40 and 120 mg/kg by gavage. The 4-week study showed that mice treated 20 mg/kg and more showed significant increase in NK activity (Int. J. Mol. Sci., 9, 355-370, 2008), since 20 mg/kg dose at least does not seem to show harmful effect, we chosen 20 mg/kg as the highest dose in the present study. The description has been made in lines 6 from bottom to end, page 10.

   It was difficult to obtain large amount of pure α-mangostin and extremely expensive. Since a mini-osmotic pump continuously release test agent (0.5µl/hr), it is possible to minimize unwanted loss of valuable test agent. In additional advantages using the mini-osmotic pump, it is possible to ensure accurate exposure to the test agent at predictable levels. Therefore, 20 mg/kg dose and mini-osmotic pump were chosen in the present study. The description has been made in lines 1 to 3, page 11.

2. Phosphoinositide 3-kinase (PI3K) pathway exerts its function through its downstream molecule Akt in regulating various cell functions including angiogenesis, cell proliferation, cell transformation, cell apoptosis, and tumor growth. In fact, expression of Akt dominant negative mutant also inhibited angiogenesis and tumor growth, and decreased the expression of HIF-1α and VEGF in the tumor xenographs (Cellular Signalling, 19, 2487-2497, 2007). Akt has also been reported to phosphorylate and activate endothelial nitric oxide synthase (eNOS), which contributes to angiogenesis through endothelial nitric oxide production. Activation of the protein kinase Akt by VEGF orchestrates several signaling events that contribute to angiogenesis (Cir. Res., 86, 892-896). Involvement of PI3K, Akt and eNOS in endothelial cell biology is apparent under physiological and pathological conditions. In addition, Akt1 null mice showed reduction of size in lymphatic capillary vessels and defection of smooth muscle cell coverage and valve development, suggesting that Akt1 is required isoform in lymphangiogenesis (Am. J. Pathol., 177, 2124-2133, 2010). Thus, Akt-mediated signaling plays an important role in lymphangiogenesis as well as angiogenesis. In the present study, since α-mangostin reduced Akt phosphorylation in vitro and in vivo, this effect may be responsible for reduction of vasculogenesis in tumors. Additional paragraph regarding this has been made in lines 10-24, page 25.

   It was very difficult to quantitate number of lymphatic vessels stained with
podoplanin because of network formation (cobweb-like) in surface of the tumor in the present study. Therefore, number of dilated lymphatic vessels having invading cancer cells was counted. Since another lymphatic endothelial marker, Lyve-1 seems to be much better than podoplanin, we should consider it for future study. However, we realized that previous description (counting for lymphatic vessels containing cancer cells) was not good enough, additional section “Dilated lymphatic vessels with cancer invasion” has been made in page 14. Thank you very much.

In addition, English in overall of the text has been checked again by a scientist who is a native English speaker.

3. Most of analyses (cell viability, caspase, cytochrome c release, Bid and cell cycle) were conducted using mouse mammary carcinoma BJMC3879luc2 cells. However, Akt-phosphorylation analysis only was performed in both MDA-MB231 cells (in vitro) and BJMC3879luc2 cells (in vivo). We are very sorry for complication. Since the automated phosphorylation assay (AlphaScreen SureFire for Akt signaling and GAPDH, Perkin Elmer, Waltham, MA, USA and model EnSpire™ Alpha, Perkin Elmer) used in the present study does not supply Akt-phosphorylation antibody against mouse, MDA-MB231 cells derived from human were used. Furthermore, the results in vitro study were confirmed by western blots. In addition, the data from human cells were compared to the results of mammary cancer tissues from the mouse study (in vivo), demonstrating a similar response of Akt-phosphorylation in both human and mouse cell lines treated with α-mangostin. To make clear on this, a description has been added in lines 11-14, page 6. In addition, the term “BJMC3879luc cells” has inserted in all in vitro studies using BJMC3879luc cells (page 8) and Figure 2 legend (page 35).

Minor points:
1. “Tumor volume increases and the multiplicity…” The word “increases” has been omitted in the Abstract section (page 2).
2. Figure 3A has been re-made. However, since we used Sigma Plot for survival rates, x-axis was automatically aligned. But, since we realized that mice were killed after week 6, time point at week 7 has also made in X-axis. Thank you very much.
3. Although the preset western blotting (Fig.7B) is not so excellent as suggested by the referee, since the results of the western blotting is considered to be supportive for the quantitative analysis of Akt phosphorylation (Fig.7A), we would like to keep the present Figure 7B. Thank you very much. In addition, we realized that another western blot (Figure 2D) required indication of molecular weights because of
cleavedBid (15kDa). We have med indication of molecular weights on Figure 2D.
Reviewer: Yian Wang
Thank you very much for your excellent suggestions to improve our research article.

Specific Comments:
The “Backgroud” and “Methods” in in Abstract section (page 2) have been changed. Thank you very much.

The BALB/c mouse is albino and inbred strain. It is a popular strain and is used in many different research disciplines. This mouse strain is intact immune system. Immunodeficient nude mouse (BALB/c-\textit{nu/nu}) originated from BALB/c mouse. Since we did not describe about BJMC3879 cells originated from BALB/c mice, the sentence has been made “Cell line and animals” section in lines 2-5, page 6.

Repetition of cell culture condition has been deleted in “Cell line and animals” section in page 6.

The full name horseradish peroxidase (HRP) has been added in the Western blotting section, page 9.

Control mice were also treated with vehicle solution (DMSO and 100\% ethanol [1:3, \textit{v/v}]). Additional description has been made in line 7, page 11.

Akt phosphorylation contributes to cell proliferation, apoptotic cell death, cell cycle entry, angiogenesis and metastasis, all important aspects of the oncogenic process. Since both Thr308 and Ser473 are necessary for full activation of Akt, the fact that \textit{\textbullet} -mangostin reduced phospho-Akt-Th308 \textit{in vitro} and \textit{in vivo} suggest inhibition of pathways in Akt downstream. Namely, several modes of Akt pathway dysregulation have been identified in various types of cancer, including breast cancer, and ultimately affect a number of processes including cell growth, survival, proliferation, and motility and/or invasion (Agarwal, 2005 #1304). Therefore, in the present study, the observed anti-tumor growth, apoptotic cell death, cell-cycle alterations, anti-angiogenesis and anti-metastasis may be partially responsible for inhibition of Akt-phosphorylation. These descriptions regarding Akt phosphorylation have been made in lines 7-9 and lines 14-22, page 21 and lines 10-24, page 25 (the Discussion section). Thank you very much for your suggestion.
We did not examine sequence of p53 in BMJ3879 mammary carcinoma, yet. We detected p53 mutation by immunohistochemistry as in human pathology. As you suggested, we should examine the sequences of p53 for future. An additional paragraph regarding p53 have been added (lines 9-20, pages 23).

Most of analyses (cell viability, caspase, cytochrome c release, Bid and cell cycle) were conducted using mouse mammary carcinoma BJMC3879luc2 cells. However, Akt-phosphorylation analysis only was performed in both MDA-MB231 cells (\textit{in vitro}) and BJMC3879luc2 cells (\textit{in vivo}). We are very sorry for complication. To make clear on this, a description has been added in lines 11-14, page 6. In addition, the term “BJMC3879luc cells” has inserted in all \textit{in vitro} studies using BJMC3879luc cells (page 8) and Figure 2 legend (page 35).

Since density of lymphatic vessels with cancer cells in tumors is very low, generally focal photography cannot demonstrate differences between control and treated groups. Only quantitation for whole area of tumors can tell us the difference. Therefore, Figures 6I and J cannot distinguish between control and α-mangostin-treated groups. They showed representative podoplanin-immunohistochemistry (dilated lymphatic vessels containing intraluminal tumors) in the control and the α-mangostin-treated groups. However, we realized that previous description (counting for lymphatic vessels containing cancer cells) was not good enough, an additional section “Dilated lymphatic vessels with cancer invasion” has been made in page 14. Thank you very much.

In order to show conspicuous expression of target protein, the sections are stained weakly with only hematoxylin (counter staining). This method is quite general in pathology field. Therefore, although the structure is not so clear, it is possible to see difference expression degree between groups. It is also much better than immunofluorescence staining (dark field). In addition, active caspase-3 is always such staining pattern (BMC Cancer, 10:566, 2010). Since phospho-Akt is located in nuclear and cytoplasm, weak nuclear staining with hematoxylin was also performed. Therefore, Figures 7E and F in higher magnification showed clearly location of phospho-Akt in nuclear and cytoplasm. In addition, Figures 7C and D in lower magnification showed apparent differences between control and α-mangostin-treated tumors. For future, the immunohistochemistry should be improved to show background structure as well as target expression as suggested by the reviewer. Possibly, weak cytoplasm staining as well as weak nuclear staining will apply to solve on this point. Thank you very much for your suggestion.
English in overall of the Discussion section has been checked again by a scientist who is a native English speaker.