Author's response to reviews

Title: The involvement of AMPK/GSK3-beta signals in the control of metastasis and proliferation in hepato-carcinoma cells treated with anthocyanins extracted from Korea wild berry Meoru

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Author's response to reviews: see over
Dear James,

I am sorry for the delay in getting back to you with a decision. It is with excitement that I resubmit to you a revised version of manuscript 5718954999889965, Dyadic Perceptions of Goals, Conflict Strategies, and Perceived Resolvability in Serial Arguments for the *BMC complementary and alternative medicine*. Thank you for giving me the opportunity to revise and resubmit this manuscript. In keeping with my last communication with you, I am resubmitting this revision before the agreed upon deadline, October 23, 2013. I appreciate the time and detail provided by each reviewer and by you and have incorporated the suggested changes into the manuscript to the best of my ability. The manuscript has certainly benefited from these insightful revision suggestions. I look forward to working with you and the reviewers to move this manuscript closer to publication in the *BMC complementary and alternative medicine*.

I have responded specifically to each suggestion below, beginning with your own. To make the changes easier to identify where necessary, I have numbered them.

**Point-by-Point response to the reviewers’ comments:**

We wish to thank the reviewers for their useful comments that greatly helped us improve the quality of our study. In this revised manuscript, we provide a significant amount of new references that we believe strengthen the manuscript.

**# Reviewer 1**

**Major compulsory revisions:**

**Background:**

**Line 8: “Several studies----over-activation of β-Catenin”**

This statement as written fosters confusion. Evidence in the literature suggests that the
role of β-Catenin in tumorigenesis is dependent either on the presence of mutation or decreased cytosolic degradation of normal β-Catenin, in part due to aberrant Wnt/APC signaling pathway. The original statement therefore, needs to be revised to include additional published evidence that is specific for the role of over-activation of #-Catenin in tumorigenesis or tumor metastasis.

- We revised the manuscript on background line 8 and added the reference that the number of 29.

: Evaluated levels of β-catenin have been found in many tumors include hepatocarcinomas. Several studies have suggested that evaluated levels of β-catenin in the cytosol activate the Tcf/Lef family of transcription factors that transcript the oncogenes such as c-myc and cyclin D1 is related to cancer metastasis [4, 29].

Methods: The description of the isolation procedure for anthocyanins is adequate. However, it is not clear whether anthocyanin glucosides are the only components present in the extract. Therefore, detailed procedure for the quantitation of specific anthocyanin glucosides and/or other constituents present in the extract need to be included in the Methods Section. Additionally, the results from the quantitation assay should be included in the Result Section of the manuscript.

- We revised the manuscript on method section.

: Isolation of anthocyanins from Meoru

Fruit of Meoru was collected in the middle of September 2007 at Jiri mountain in Korea, freeze-dried and stored in dark glass containers at -20°C until required for analysis. Anthocyanins pigments were extracted by maceration of the fruits (100 g) in methanol containing 0.1% HCl at 5°C for 24 h. The extraction procedure was repeated three times. After concentration under reduced pressure (Rotavapor R-124, Buchi, Switzerland), the extract was diluted with distilled water (100 ml) and partitioned against ethyl acetate (100 ml). The water layer containing the pigments was concentrated to 50 ml. The concentrate was purified according to established procedures by means of ethyl acetate/water partitioning and adsorption chromatography on a bed of Amberlite XAD-7 (Sigma, Younigin, South Korea). The composition of anthocyanidins isolated from meoru (AIMs) was as follows: delphinidin-

Results:

1. Growth Inhibition by Anthocyanins: The statement that in vitro growth inhibition (cytostatic growth arrest and cellular apoptosis) represents chemoprevention is confusing since tumor derived Hep3B cells are used for both in vitro and in vivo assays. These data may be more appropriately described as in vitro and in vivo effects on tumor cell phenotype.

   - We were used Hep3B hepatocarcinoma cells in the *in vivo* xenograft model. Anthocyanins inhibit the growth both *in vitro* and *in vivo*. However, we were not observed different phenotype on *in vivo* tumor cells vs *in vitro* cells.

2. Based on the quantitation of Anthocyanin glucosides present in the extract, it needs to be clarified whether the doses used represent total anthocyanin or a specific glucoside.

   - We are used total anthocyanins. It is that which is included in the method described what kinds of glucoside.

3. Fig. 1 B, 1C: The dose response data for Anthocyanins exhibits a modest inhibition cell survival (80% of control) at 200 µg/mL and about 40% of control at 400 µg/mL (Fig. 1B), while the effect of Anthocyanins on apoptosis is presented at 100 and 200 µg/mL doses (Fig. 1C). What is the status of apoptosis at the higher effective dose of 400µg/mL?

   - We have excluded 400 µg/mL concentration from the simple assumption that 200 µg/mL concentration would reveal the similar trend on apoptotic cells we have selected.

4. Data on AMPK Si RNA and Compound C: The results from the Si RNA experiments are convincing. However, the experiments with the chemical inhibitor of AMPK have
generated distinct data. The interpretation for the observed differences by the two approaches needs to be included.

Discussion:

1. The characteristic effects of AMPK Si RNA and Compound C should be discussed in greater detail in the discussion section.

   - We revised the manuscript on discussion line 22 and 25.

Compound C, a selective inhibitor of AMPK, can inhibit the regulation of biological function for AMPK.

Upon the inactivation of AMPK by AMPK siRNA that knock down of AMPK expression through RNA interference, anthocyanins no longer modulated p-GSK3β implying that AMPK is an upstream signal for GSK3β.

Minor Essential Revisions:

Results:

1. p. 10, Line 5: “but this effect----co-treatment with Anthocyanin” This statement as written is confusing. The data suggests that co-treatment with Anthocyanin inhibits the effect of IGF-1. This aspect needs to be clarified.

   - We revised the manuscript.

   “As shown in Figure 3b, IGF-1 treatment increases cell invasiveness but this effect was reduced by co-treatment with IGF-1 and anthocyanins reduced cell invasion through the Matrigel chamber in Hep3B cell.”

2. p 10, Lines 8-9: “To exclude----- (Fig. C)” This sentence is incomplete and therefore, is confusing. This sentence needs to be revised for clarity.

   - We revised the manuscript

   “We tested the effects of anthocyanins on the gelatinase activity of MMP-2, and found that while IGF-1 increased MMP-2 expression, anthocyanins decreased this IGF-1-mediated increase in MMP-2 activity.”
Discussion:

Line 4: “attenuated transcription oncogenes”. This part of the sentence needs to be expanded to specify oncogenes whose transcription is affected by β-Catenin.

- We changed the discussion line 4.
- “—as well as the attenuated transcription oncogenes such as c-myc, c-jun and cyclin D1.”

Reviewer 2.

1. In the background part, the authors did not provide any introduction on AMPK, and its relation to GSKbeta and beta-catenin. They just jumped to AMPK in the second paragraph of the background part.

- We revised the manuscript on background and added the reference that the number of 30-32.

AMP-activated protein kinase (AMPK) is a cellular energy sensor and activates tumor suppressor signaling pathway such as p53 and pro-apoptotic Bcl-2 family and inhibits tumor survival signaling pathway such as mTORC1 and Wnt pathway [30, 31]. Recent studies have shown that several phytochemicals regulate cell growth by regulating the phosphorylation of GSK3β through activation of AMPK [8, 32], and the possible new GSK3β regulating phytochemicals include anthocyanins.

2. In the background part, the authors did not give a satisfactory description on anthocyanin. Is it a single chemical compound, or a mixture of many different compounds? If it is a mixture of compounds, could the authors make some effort on providing any surmise on which compound/compounds may be the active components in anthocyanin against hepato-carcinoma?

- We revised the method section that contained with anthocyanin components.

“The composition of anthocyanidins isolated from meoru (AIMs) was as follows: delphinidin-3,5-diglucoside: cyanidin-3,5-diglucoside: petunidin-3,5-diglucoside:

3. It is generally believed that in Wnt/beta-catenin signaling, the activity of GSK3beta is not regulated by its phosphorylation. Its activity is mainly regulated by its complex formation with Axin and APC, which is not investigated by the authors. Instead, the authors found that the phosphorylation of GSK3beta is inhibited by the anthocyanin treatment. How would the authors resolve this discrepancy?

- We studied based on the theory that GSK3β acts as a last marker to target β-catenin for ubiquitination through the phosphorylation of the Ser33/37 residue, leading to its degradation by the proteasome. Also, GSK3β can regulate the β-catenin in the mutation of APC, Axin, CK1 complex.

4. The authors declare that anthocyanin activates AMPK through inhibition of GSK3beta. Yes, I understand that promotion of phosphorylation is the most clear-cut result from anthocyanin treatment, but why does it have anything to do with GSK3beta and beta-catenin? Could it be that anthocyanin regulates AMPK and GSK3beta/beta-catenin separately, in two different unrelated pathways? Although the legend of Figure 4 states that “anthocyanins inhibit GSK3beta and beta-catenin in an AMPK-dependent manner”, I still have difficulty understanding the reasoning behind this statement.

- We agree that our mistake. As shown in figure 4D, anthocyanins could not inhibit the GSK3β in Compound C (AMPK inhibitor) treated Hep3B cells. Thus, we suggest that the anthocyanins activate AMPK through inhibition of GSK3β.

We revised the figure legend of figure 4 states that “anthocyanins inhibit GSK3beta in an AMPK-dependent manner”.

I sincerely hope that the manuscript is suitable for publication in your Journal. Please feel free to make any editorial changes, if necessary.
Best Wishes,

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