Author's response to reviews

Title: The induction of activating transcription factor 3 (ATF3) contributes to anti-cancer activity of Abeliophyllum distichum Nakai in human colorectal cancer cells

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Author's response to reviews: see over
[Referee 1]

Reviewer's report
Title: The induction of activating transcription factor 3 (ATF3) contributes to anti-cancer activity of Abeliophyllum distichum Nakai in human colorectal cancer cells
Version: 4 Date: 13 October 2014
Reviewer: Gayathri Chadalapaka
Reviewer's report:
- NA
Level of interest: An article whose findings are important to those with closely related research interests
Quality of written English: Acceptable
Statistical review: No, the manuscript does not need to be seen by a statistician.
Declaration of competing interests:
I declare that I have no competing interests.

[Referee 2]

Reviewer's report
Title: The induction of activating transcription factor 3 (ATF3) contributes to anti-cancer activity of Abeliophyllum distichum Nakai in human colorectal cancer cells
Version: 4 Date: 29 October 2014
Reviewer: Won Ho Kim
Reviewer's report:
accept
Level of interest: An article of limited interest
Quality of written English: Acceptable
Statistical review: No, the manuscript does not need to be seen by a statistician.
Declaration of competing interests:
no
[Editorial comments]
Please include details in your Methods section on who formally identified the plant material used in your study.

RESPONSE: The name who formally identified the plant material used in our study was added in Methods (Sample preparation)
[Referee 2]

Comments:
As you mentioned in introduction, the roles of ATF3 in proliferation, cell cycle, apoptosis, differentiation, etc were complicated and are controversial depends on the experimental condition. As well, to control the expression of ATF3 is too difficult because its expression was highly increased by serum deprivation. Moreover, ATF3 induction was sensitive to oxidative stress. In your manuscript, your suggestion on the purpose of the experiment is not sufficient and your description are not sufficient to understand your data. For example, I don’t know why you examine the induction of ATF3 and the involvement of MAPKs. And also, what are the molecular mechanisms involved in EAFAD-B-mediated apoptosis? At first, you have to suggest the molecular mechanisms involved in EAFAD-B-mediated apoptosis and you have to find the upstream or downstream regulator for these responses.

1. Page 4, in Cell viability, what is GL?
   It was revised

2. Figure 1, Do you have compare the cytotoxic effects of EAFAD in cancer cells with normal cells (primary culture or PBMC, etc)?
   The cytotoxic effect of EAFAD-B on the normal cells was performed and this result was added in Fig. 1

3. Figure 1, although the branch EAFAD has the highest cytotoxic effects in all cancer cells, the cytotoxic effects were too strong. The branch EAFAD almost reduced the viability by 60-80%. This phenomenon will be a necrotic death, not apoptosis. If the phenomenon is apoptosis, please redetermined the concentration and time point of EAFAD showing the reduction (15-30% cytotoxicity). The ratio is the best to see and define your drug’s function.
   We redetermined the concentrations of EAFAD-B and this result was added in Fig. 1B

4. You have already determined the concentration (100 ug/ml) of EAFAD in figure 1, but you have used 200 ug/ml EAFAD in Fig 2 G-K.
   We used the concentration (200 ug/ml) of EAFAD-B because this concentration showed the highest effect on ATF3 expression in Western blot and ATF3 promoter activity.

5. In promoter analysis, you used 200 ug/ml EAFAD in all figures. Do you have a reason to use this dose? If not, you have to confirm by using 100 ug/ml EAFAD.
   See revision (Referee 2 #4)

6. Page 7, line 16 : -1450 change to -1420
   Changed accordingly

7. In Fig. 3, you suggest that EAFAD strongly enhanced ATF3 promoter activity in the region between -1420 and -147 of ATF3 promoter. However, their promoter activities in the cells
using -318 and -147 promoter were significantly reduced compared with those of -1420 and -147. You didn’t explain the reason why they are differing. The region of CREB binding site is located in between -93 and -85 on the ATF3 promoter. Why is different their promoter activities between -1420/-514 and -318/-147? Furthermore, the basal levels of no-treated promoter activity were also significantly different. I think that ATF3 promoter activity can be affected by other transcriptional activity such as AP-1, NF-kB, c-jun, etc. So, you have to explain the reason why the basal levels and its promoter activity by EAFAD are different.

Many transcription factors such as NF-κB, EGR-1, E2F, AP-1, CREB and Ftz have been identified to regulate ATF3 transcriptional activity. We determined that EAFAD-B-responsible sites for ATF3 transcriptional activity might be between the -147 and -85 region in ATF3 promoter because ATF3 promoter activity by EAFAD-B was more decreased in transfection of ATF3 promoter (-85/+34) than other transfection such as pATF3-1420/+34, pATF3-718/+34, pATF3-514/+34, pATF3-318/+34, pATF3-147/+34. Especially, CREB and Ftz are cis-acting elements in ATF3 promoter (-147/-85). To identify the role of each cis-acting element affecting EAFAD-B-mediated ATF3 transcriptional activation, each site-deleted ATF3 promoter constructs were transfected into HCT116 and SW480 cells and we found that EAFAD-B-induced ATF3 promoter activity was significantly decreased when the CREB site was deleted. These data indicated that CREB is an important region in EAFAD-B-induced ATF3 expression. However, we do not exclude the effects of other transcription factors because EAFAD-B-mediated ATF3 promoter activity was gradually decreased in pATF3-1420/+34, pATF3-718/+34, pATF3-514/+34, pATF3-318/+34, pATF3-147/+34 and pATF3-84/+34. Thus, the further experiments for more investigation of the transcription factors associated with EAFAD-B-induced ATF3 activation may be needed. We also observed the basal activity of ATF3 promoter was different in ATF3 promoter constructs. We think that ATF3 promoter activity can be affected by other transcriptional activity such as Sp3, NF-kB and c-Jun. Indeed, Sp3 and c-Jun have been reported to increase basal ATF3 promoter activity.

8. You have suggested the involvement of p38 MAPK and GCK3beta pathway on EAFAD – mediated ATF3 transcriptional activity. However, you didn’t demonstrate the reason why these signaling pathways were examined. Have you been examined the relationship between ATF3 induction and MAPKs or GSK3beta?

It has been reported that ATF3 expression is regulated by MAPK signaling and GSK3β. So, we examined whether EAFAD-B-mediated ATF3 activation is associated with the activation of ERK1/2, p38 or GSK3β. The reason why these signaling pathways were examined was presented in Discussion.

9. Fig.4C and D, the inhibitory effects of p38 inhibitor (SB203580) on EAFAD-B-induced
ATF3 are differing between HCT116 and SW480 cells. Please, explain the discrepancy in both cells. For example, the used doses of EAFAD-B are too high. So, the induced ATF3 may not be overcome by p38 inhibitor. If not, there are some different molecules or signaling pathway in both cells. You should explain this question.

See revision ((Referee 2 #10))

10. In Fig. 4F, you have performed the experiment by using CHX to define induced ATF3 stability. The stability of ATF3 in cells treated with CHX after prestimulation with EAFAD-B was compared with those in cells cotreated with EAFAD-B and CHX. However, I cannot understand the different response of CHX on EAFAD-B-induced ATF3 in pretreatment or cotreatment. You have described their different responses, but you didn’t suggest that why they are different. I think that your experiment is designed too hard to understand. You should be compared between the changes of ATF3 expression in cells pretreated with EAFAD-B (12 h) and those in cells cotreated with CHX and EAFAD-B. Then, you can described follow as: “ATF3 expression induced by EAFAD-B was decreased in time-dependent manner, which was attenuated by CHX treatment, suggesting that de novo protein synthesis may be involved in EAFAD-B –mediated induction of ATF3” From your present data, you cannot describe anything. Two experimented groups are differently regulated, so, what? so, why? : You didn’t explain these questions. So, you should perform the experiment again.

To determine if EAFAD-B affects ATF3 stability against the proteasomal degradation, we firstly pretreated EAFAD-B because of low level of ATF3 in basal. Then, the cells were washed with 1xPBS and subsequently treated with CHX+DMSO or CHX+EAFAD-B. In CHX+DMSO treatment, ATF3 protein was decreased, which indicates that ATF3 proteasomal degradation was induced. But, in CHX+EAFAD-B treatment, decrease in ATF3 protein level was attenuated, which indicates that EAFAD-B may at least in part contributes ATF3 stability.

11. Furthermore, your description and explanations for the key results and discussion are not sufficient to understand. In discussion, you just described as the summary of your results and I can’t find the discussion to solve the key question, main issue, etc. You should describe again.

The discussion was re-described accordingly.

[Referee 3]

Seems results shown in Figure 4C and 4D are not going with the results reported. Authors are reporting that GSK3B inhibition by SB216763 ameliorates the increased level of ATF3, whereas the western blot shows quite opposite and ATF remains same as in DMSO.
It was revised
Authors also report that effect of EAFAD on ATF3 fails to get affected by p38MAPK-inhibitor while western blot doesn’t clearly show that. In figure 4A and B, authors show that ERK1/2 is not involved in ATF transcriptional activation as ERK1/2 inhibitor; PD98059 doesn’t affect ATF activation, whereas in Figure 4E, they show EAFAD increases ERK1/2.

If EAFAD-B affected the phosphorylation of ERK1/2, ERK1/2 inhibition did not affect EAFAD-B-mediated ATF3 activation, which indicates that ATF3 activation by EAFAD-B may be independent on ERK1/2.

The data shown for liver cancer (HepG2) as well as breast cancer (MCF-7 and 231), shows pro-apoptotic effect of EAFAD and it seems ATF-3 is not involved, as ATF-3 has been shown to be pro-proliferating molecule in liver and breast. Authors need to comment on that and discuss. Pro-apoptotic role of ATF3 in colorectal cancer and a therapeutic target, has been shown and is not a novel finding except a target for EAFAD.

ATF3 is both tumor suppressive in early stage tumorigenesis and oncogenic in late stage tumorigenesis in breast cancer cell lines. Although ATF3 has been well known to promote metastasis in the breast cancer, ATF3 expression can induce apoptosis in human breast cancer cells (MCF-7 and MDA-MB-231). In addition, ATF3 has been reported to mediate the suppression of cell viability in human hepatocellular carcinoma cells (HepG-2). In this study, EAFAD-B reduced the cell viability in MCF-7, MDA-MB-231 and HepG-2 cells. Thus, the further elucidation of mechanisms for ATF3 role involved in EAFAD-B-induced apoptosis may be needed in human breast cancer and hepatocellular carcinoma cells.

Minor comments:
The manuscript has some grammatical and syntactical errors.

We revised our manuscript accordingly