Author’s response to reviews

Title: Snake venom toxin from Vipera lebetina turanica induces apoptosis in colon cancer cells via upregulation of ROS- and JNK-mediated death receptor expression

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Version: 3 Date: 30 March 2012

Author’s response to reviews: see over
Dear Editor-in-Chief, Journal (BMC Cancer)

March 30, 2012

Thanks for your letter regarding our manuscript (MS# 1150374584670284) “Snake venom toxin from Vipera lebetina turanica induces apoptosis of colon cancer cells via upregulation of ROS- and JNK-mediated death receptor expression” together with the thoughtful comments from the reviewer. We have carefully considered the reviewer’s comments and have edited our manuscript accordingly.

LIST OF CORRECTIONS ON THE MANUSCRIPT

1st REVIEWER’S COMMENTS

The response to the first reviewer comment:
We authors express our thanks to the first reviewer who have given useful advices on this manuscript.

Comment 1:
In order to make sure that the observed cytotoxicity is related to the toxins contained in the venom, the authors should test the effect after heat-inactivation of the venom.

Author’s response:
Thank you for your comment. We tested the effect of the venom after heat inactivation at 60°C for 30min or at 95°C for 5min in HCT116 cells, but we didn’t show that the cytotoxic effect of the venom as shown in below.
Comment 2:
The authors failed to clearly establish the signaling pathway between ROS production and JNK activation. They only showed the evidence that NAC reduced JNK activation, without testing the effect of JNK inhibitor (SP) on ROS level.

Author’s response:
Thank you for your comment. As your good advice, we tested if ROS level was changed by treatment of JNK inhibitor (SP600125) in HCT116 cells. We found that the ROS level was not affected by JNK inhibitor. This result indicated that the SVT induces apoptosis of colon cancer cells via upregulation of ROS mediated JNK-mediated death receptor expression, not the direction of JNK mediated ROS.

Comment 3:
Another important issue of this study is the lack of temporal pattern of the parameters (ROS, JNK, DR4/5 expression and cell death), as most of the data presented were obtained at one single time-point (24 hrs).

Author’s response:
Thank you for your comment. We checked the ROS after treatment of snake venom toxin for 30 min as described materials and methods. The JNK and DR4/5 expression was checked after 24 hr by refer other study (Sung et al, 2010; Prasad et al, 2011). And we checked cell death in a different time point, but the tendency was almost
same. So the data presented in this manuscript at single time point may have no problem.

References

Comment 4:
The authors failed to highlight the difference between DR4 and DR5. For instance, in HCT116 cells, SP was able to reduce the DR4 protein level, but had no effect on DR5 (Figure 5B).

Author’s response:
Thank you for your comment. In HCT116 cells, SP600125 was able to reduce the DR4 protein level, but had little effect on DR5. We showed DR5 protein level in HCT116 cells after treatment of 10 µM of SP600125. The result indicated that the SVT-induced DR5 level was reduced by treatment of SP600125. This result was changed in revised Fig.5C.

Comment 5:
Throughout the manuscript, the authors misused the word “cell growth”, as they only tested the cell viability/apoptosis, not cell growth.

Author’s response:
Thank you for your comment. We changed the word “cell growth” to “cell viability or apoptosis”.

[Image: HCT116 Western Blot showing DR5 and β-actin levels under different conditions]
Comment 6:
Figure 3: it is more appropriate to state that what was measured is the cleavage of the various caspases, not “expression level”.

Author’s response:
Thank you for your comment. We checked and changed the word “expression level” to “cleavage of caspases” in result section.

Comment 7:
Figure 3: Why there was marked increase of cytochrome C in the whole cell lysate in cells treated with venom?

Author’s response:
Thank you for your comment. We checked the cytochrome c in cytosol extract and mitochondria extract. We showed that the cytochrome c was increased by treatment of SVT in a dose dependent manner in the cytosol extract, but that was decreased in mitochondria extract. This result indicated that the cytochrome c is released from mitochondria to cytosol by treatment of SVT. We could not check the internal control of mitochondria because the antibody delivery was late (porin antibody). In other reports, cytochrome c was detected in cytosol extracts. So, we inserted only cytosol extract data in revised figure 3C.

Comment 8:
Figure 4B: The caspase 3 cleavage data under DR4 and DR5 knockdown were not so convincing. Suggest to check PARP-1 cleavage.

Author’s response:
Thank you for your comment. The reason of this result may be the condition of the cells. In figure 3, the cleavage of caspase-3 of nontreated group is higher than that in
In figure 4, the cleavage of caspase-3 is very weak in control group. As your comment, we checked the PARP cleavage in HCT116 cells as shown in below. We retried western blot and deleted the result of caspase3 cleavage data under DR4 and DR5 knockdown in Fig.4B.

**Fig.4B**

**Comment 9:**

Figure 5A: there was significant increase of p-p38 in HT29 cells. Any explanation?

**Author’s response:**

Thank you for your comment. HCT116 cell has a mutation in codon 13 of the ras protooncogene. But the HT29 cell is ras+ cells. p38 is able to suppress tumor initiation induced by ras oncogene through induction of apoptosis (Dolado et al., 2007). Upon expression of oncogenic Ras, ROS are generated leading to p38 activation and apoptotic cell death. Moreover, p38 MAPK plays an important role in the apoptosis induced by some chemotherapeutical drugs. (Bragado et al., 2007). In
this reasons, the p38 is phosphorylated only in HT29 cells. But the p38 phosphorylation is not changed in both cell lines unlike pJNK.

References

Comment 10:
The manuscript needs careful proof reading for grammatical and spelling errors.
Author’s response:
Thank you for your comment. We revised our manuscript under native speaker’s guidance.

2nd REVIEWER’S COMMENTS

The response to the second reviewer comment:
We authors express our thanks to the second reviewer who have given useful advices on this manuscript.

Comment 1:
In background section, the reference 23 is absent and, consequently, all bibliography is false in the text.
Author’s response:
Thank you for your comment. In the third paragraph of background section, “The death receptors (DRs) are induced through reactive oxygen species (ROS), mitogenic activated protein kinases (MAPKs) and p53 dependent pathway [21-25].” The reference 23 is included.

Comment 2:
In results section "Effect of SVT on the expression of DRs in human colon cancer cells": the authors write that the expression of other DRs such as TNF-R1, TNF-R2, DR3, DR6 and Fas, and DRs ligands such as FasL and TRAIL was not changed after SVT treatment. This is untrue concerning TRAIL for both cell lines, FasL for HCT116
cells and DR6 for HT29 cells (SVT 1 µg/ml).

**Author’s response:**
Thank you for your comment. The expression of TRAIL is changed after SVT treatment, but the tendency is not correlated with cell viability and apoptosis. The expression of FasL is induced after 0.1 and 0.5 µg/ml of SVT treatment, but reduced after 1 µg/ml of SVT treatment. This tendency is also not correlated with cell viability and apoptosis. DR6 is also only changed in HT29 cells. So, we choose and tested the DR4 and DR5 that dose dependently changed after treatment of SVT in both cell lines. We changed this fig.2B to supplementary Fig.1.

**Comment 3:**
The authors analyzed only bax expression but, analysis of bcl-2 expression is necessary to establish a ratio apoptotic because the increase of bax expression is very significative for HCT116 cells but not for HT29 cells.

**Author’s response:**
Thank you for your comment. We showed that the bcl-2 levels in both cell lines (as shown in below), then we established a bax/bcl2 ratio. We changed this data from Fig.3 to Fig.3B.

**Comment 4:**
The authors should be analyzed the expression of cytochrome c in cytosol extracts vs mitochondrial extracts.

**Author’s response:**
Thank you for your comment. As your comment, we checked the cytochrome c in cytosol extract and mitochondria extract. We showed that the cytochrome c was
increased by treatment of SVT in a dose dependent manner in the cytosol extract, but that was decreased in mitochondria extract. This result indicated that the cytochrome c is released from mitochondria to cytosol by treatment of SVT. We could not check the internal control of mitochondria because the antibody delivery was late (porin antibody). In other reports, cytochrome c was detected in cytosol extracts. So, we inserted only cytosol extract data in revised figure 3C.

Comment 5:
For both cell lines, the cleavage of caspase-3 is very important after 1 µg/ml SVT treatment on figure 3. However, on figure 4, for the same condition, this cleavage is detected but it is very weak. The reproductiveness of this result is not sufficient.

Author’s response:
Thank you for your comment. The reason of this result may be the condition of the cells. In figure3, the cleavage of caspase-3 of non treated group is higher than that in figure4. In figure4, the cleavage of caspase-3 is very weak in control group. So, we retried the western blot and deleted the caspase-3 data in figure 4B.

Fig.4B
Comment 6:
The expressions of p53 and PPAR were presented on figure 5A but none result is analyzed in the result section. The research of expression of these two proteins is not necessary in this work.

Author’s response:
Thank you for your comment. We deleted the p53 and PPAR in the figure 5A. In discussion part, we changed “However, we found that the p53 is not induced by snake venom toxin, it may the induction of DR4 and DR5 by snake venom toxin occurs independently of p53 in colon cancer cells” to “However, we found that the p53 is not induced by snake venom toxin (data not shown). Thus, the induction of DR4 and DR5 by snake venom toxin occurs independent of p53 in colon cancer cells. Instead, our data indicate that snake venom toxin-induced upregulation of DR4 and DR5 could be dependent on the ROS and JNK pathway.”

Comment 7:
The authors write in the text that NAC was used at 5 and 10 mM but, on figure 6A, NAC was used at 1 and 10 mM?

Author’s response:
Thank you for your comment. The concentration of NAC 1 and 10 mM is correct. We changed the concentration in the result section.

Comment 8:
Legend of figure 1: the statistical test (*) does not appear on the figure 1A. Legend of figure 2: expression of cleaved DR4? I do not understand! Legend of figure 4: I do not understand the combined treatment SVT + TRAIL? This is not true on the appropriate figure! Legend of figure 5, panel C: only 10 μM SP600125 was used and not 5 μM as indicated in the legend.

Author’s response:
Thank you for your comment. I insert the statistical test (*) on the figure 1A. I changed cleaved DR4 to DR4. We changed the “implemented snake venom toxin was treated (1 μg/ml) combined with TRAIL for another 24 h” to “implemented snake venom toxin was treated (1 μg/ml) for another 24 h” in Figure4 legend. We changed the legend of figure 5 to 10 μM SP600125.

Comment 9:
References section: the reference of Basu et al. is not numbered.
Author’s response:
Thank you for your comment. We deleted the reference in the list.
This revised manuscript consists of 35 pages of text with 6 Figures and 1 Supplementary Figure.

We hope that this revised manuscript would be accepted for publication in the “BMC Cancer”.

Sincerely,
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