Author's response to reviews

Title: Siah1 Proteins Enhance Radiosensitivity of Human Breast Cancer Cells

Authors:

Hai-Tao He (he@med.uni-marburg.de)
Emmanouil Fokas (fokas@med.uni-marburg.de)
An You (youa@staff.uni-marburg.de)
Rita Engenhart-Cabillic (engenhar@med.uni-marburg.de)
Han-Xiang An (an@med.uni-marburg.de)

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

Please find enclosed our revised manuscript entitled:

“Siah1 Proteins Mediate Radiosensitivity of Human Breast Cancer Cells”

by He et al., which we request for revision, upon second submission, in *BMC Cancer*.

We would like to thank the editor and the reviewers for the useful comments and suggestions. We have carefully reconsidered and modified our manuscript based on the latter. We have made several different corrections, marked with red, which are shown below.

Please note that below I report the exact place (page and paragraph) of the modifications performed on the text, as they appear in the version of the manuscript that still contains all the corrections marked with red.
Reviewer #1:

1st comment: In the manuscript ‘Siah1 Proteins Enhance Radiosensitivity of Human Breast Cancer Cells’ by He and colleagues, the authors report that SIAH1 is expressed in MCF7 and overexpression of Siah1L and Siah1 can enhance radiosensitivity of SKBR3 breast cancer cells. This is an interesting finding and has confirmed previous studies that Siah proteins play an important role in apoptosis and cancer progression. However, all the experiments were performed with over-expression of SIAH1. To explore the biological function of Siah proteins in breast cancer, it is more relevant to knock-down the expression of different Siah proteins by RNAi in breast cancer cells such as MCF7 to see if it can affect the cell proliferation, invasion ability, radiosensitivity and Tcf/Lef factor activity. Please keep in mind that there are different isoforms of Siah proteins in mammalian cells.

Answer to the 1st comment: We performed siRNA for Siah1 in MCF-7 cells, as recommended by the reviewer. We addressed the concerns raised by performing the following assays:

1) Western blot: Before proceeding with any further experiments, we tested the efficacy of siRNA for Siah1 by analyzing the expression of Siah1 protein in MCF-7 cells, as described in (marked with red) revised “Materials and Methods” (last paragraph, page 5 and 1st paragraph, page 6). A control siRNA was included as well. We confirmed silencing of Siah1 protein expression in MCF-7 cells after siRNA. Additionally, we analyzed the expression of Siah2 in SKBR3 and MCF-7 cells (requested by the 2nd reviewer). Two new figures were added (Fig. 1C and 1D) together with the corresponding results description (1st paragraph, page 11) and figure legends (1st paragraph, page 26).

2) Apoptosis assay: We analyzed the effect of Siah1 siRNA in MCF-7 cells as described in revised “Materials and Methods” (2nd paragraph, page 7). Siah1 siRNA resulted in decreased apoptosis in MCF-7 cells after irradiation. A new figure was added (Fig. 2B) together with the corresponding results description (1st paragraph, page 12) and figure legend (2nd paragraph, page 26).

3) Clonogenic survival assay: We investigated the impact of Siah1 siRNA in the clonogenic survival of MCF-7 cells, as described in revised “Materials and Methods” (last paragraph, page 7). Siah1 siRNA resulted in increased survival of MCF-7 cells post-irradiation. A new figure was added (Fig. 3B) together with the corresponding results description (2nd paragraph, page 12) and figure legend (1st paragraph, page 27). Please note that Fig. 3B from the initial form of the manuscript (1st submission) was removed and substituted from the new clonogenic assay result derived from MCF-7 cells.
4) **Cell viability assay:** Siah1 siRNA was performed in MCF-7 cells and their cell viability was tested, as described in revised “Materials and Methods” (2nd paragraph, page 8). Siah1 siRNA revealed an increase in the viability of MCF-7 cells. A new figure was added (Fig. 4B) together with the corresponding results description (1st paragraph, page 13) and figure legend (2nd paragraph, page 27).

5) **Cell invasion assay:** The invasive ability of MCF-7 cells was analyzed upon Siah1 siRNA, as described in revised “Materials and Methods” (2nd paragraph, page 9). Siah1 siRNA caused a significant increase in the invasion of MCF-7 cells. Two new figures were added (Fig. 6C and 6D) together with the corresponding results description (1st paragraph, page 14) and figure legend (1st paragraph, page 28).

6) **Tcf/Lef reporter assay:** We explored the potential of Siah1 siRNA to modify the Tcf/LEf luciferase reporter. Siah1 siRNA displayed a significant increase in the latter. A new figure was added (Fig. 7B) together with the corresponding results description (2nd paragraph, page 14) and figure legend (2nd paragraph, page 28).

The abstract and the discussion were modified accordingly in order to describe the new findings. In addition, three more manuscripts were cited and described (References 32-34, 1st paragraph, page 16) to highlight the importance of the proteasome-ubiquitin pathway in response to radiation. A recent study that describes findings similar to our present work in MCF-7 cells has been cited and described as well (Reference 36, 2nd paragraph, page 16).

**Reviewer #2:**

The paper by He et al entitled "Siah 1 proteins enhance radiosensitivity of human breast cancer cells" examines the effect of Siah1, its splice variant Siah1L and the Siah1 mutant with the RING finger deleted (Siah1#R) on radiosensitization of human breast cancer cells. The manuscript in its present form is unacceptable for publication. Data presented by the authors is not very convincing. The authors need to address the following concerns:

1st comment: From the data presented it appears that Siah1#R produces some effect on the breast cancer cells but the authors prefer to indicate that there was no effect.

Answer to the 1st comment: We would like to thank the reviewer for the constructive comment. In the apoptotic assay performed for SKBR3, Siah1ΔR showed a difference in comparison to the control, which however, was not significant due to the relatively high standard deviations. Similar findings for Siah1ΔR were observed regarding clonogenic survival and viability of SKBR3 cells. Moreover, the number of H2AX foci was lower at 3 hours post-irradiation in SKBR3 cells transfected with Siah1ΔR, as compared to the control group. In the invasion assay, however, and as correctly pointed from the reviewer, Siah1ΔR
displayed a decrease in the invasion of SKBR3 cells. The results (last paragraph, page 13) and discussion (2nd paragraph, page 16) were modified accordingly.

2nd comment: What is the status of Siah2 protein in the cell lines listed and what is its contribution.

Answer to the 2nd comment: We analyzed protein expression of Siah2 by Western blot in both SKBR3 and MCF-7 cells, as requested from the reviewer (please see above, on the response to the 1st reviewer). Siah2 protein expression was suppressed in both cell lines. We mainly focused on the role of Siah1 in determining the malignant behaviour of breast cancer cells as well as their response to radiation. A whole series of new assays, similar to the ones performed for the current study, is a prerequisite to study the role of Siah2 in breast cancer and consists a future plan for our lab.

3rd and 4th comment: 3rd - Did the authors try to knockout expression of Siah1 to show its contribution to radiation & 4th - Role of beta-catenin and other proteins activated in invasion needs to be demonstrated in a more convincing manner.

Answer to the 3rd comment: The impact of Siah1 silencing-of-function was investigated in detail, including its effect on the β-catenin-related Tcf/Lef, as suggested (please see above, on the response to the 1st reviewer).

Thank you for considering our revised manuscript for publication. I am looking forward to hearing from you.

Yours Sincerely

Dr Hanxian An
Department of Radiotherapy and Radiation Oncology
Cancer and Radiation Biology Laboratory
Philipps University Marburg
Germany