Author's response to reviews

Title: Epigenetic alterations of the keratin 13 gene in oral squamous cell carcinoma

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Author's response to reviews: see over
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Prof. Santiago Gomez
Journal Editorial Office
BioMed Central

Dear Prof. Gomez,

Thank you for your email of September 25, 2014, regarding our manuscript (MS: 1517799865138998) entitled “Epigenetic alterations of the keratin 13 gene in oral squamous cell carcinoma”, and the valuable comments of the two reviewers. We have revised the manuscript according to the reviewers’ suggestions. In the following pages are our point-by-point responses to each of the comments of the reviewers.

We feel that the revised manuscript is a suitable response to the comments, and is significantly improved over the initial submission. We trust that it is now suitable for publication in BMC Cancer.

Thank you for your consideration.

Sincerely yours,

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Response to reviewers’ comments

Response to Reviewer#1 (Stefan M Willems)

We wish to express our appreciation to the Reviewer for the insightful comments on our paper. The comments have helped us significantly improve the paper.

- Major compulsory revisions

1. Results, paragraph “Alterations of histone H3…”: Why is only the SAS cell line compared with the HaCaT cell line, instead of comparing also the other two OSCC cell lines. If HSC4 cells show different histone H3 methylation patterns than HSC3 and SAS cells, this would make your point that aberrant targeting of PRC2 to the KRT13 promoter may cause the observed KRT13 gene repression in HSC3 and SAS cells, but not in HSC4 cells stronger.

We agree that comparison of histone H3 methylation patterns among the four cell lines as the reviewer suggested would be valuable. We have performed additional experiments and found the difference of methylation patterns between the KRT13 mRNA expressing cells (HaCaT and HSC4 cells) and the KRT13 silencing cells (HSC3 and SAS cells) and no significant difference in PRC2 protein levels among all four cell-lines. These data have been shown in figure 4A and B. Accordingly, we have changed the text of figure legends [page 20, line 463-477] and results sections [form page 10, line 218 to page 11, line 239].

2. Results, paragraph “KRT13 gene reactivation…”: although DZNep restored KRT13 mRNA levels, no information of restoring of protein levels of KRT13 have been shown (yet). Therefore, the statement of “reactivates KRT13 expression” in both this paragraph and the conclusion should be rephrased and correct conclusions should be drawn (or restore of KRT13 protein level by western blot or immunofluorescence should be shown).

In accordance with the reviewer’s comment, we have changed the statement from “KRT13 expression” to “KRT13 mRNA expression”, “KRT13 transcription”, or “the transcription of KRT13 gene” and also changed the subtitle form: “KRT13 gene reactivation by the PRC2 inhibitor DZNep in OSCC cells” to “Reactivation of KRT13 transcription by the PRC2 inhibitor DZNep in OSCC cells”. [from page 11, line 240 to page 12, line 256]

3. Discussion, paragraph two: As mentioned in (1), current data are insufficient to support the statement that additional event such as histone modification is responsible for KRT13 gene silencing in OSCC.

As mentioned above (our response to the reviewer’s comment 1.), we have found the difference in histone H3 methylation patterns between HSC4 cells and the KRT13 gene silencing cells (HSC3 and SAS cells). These data should support the statement: “additional event such as histone modification is responsible for KRT13 gene silencing in OSCC.”
- Minor Essential Revisions

Minor issues not for publication:
1. Methods, Cells and drug treatment; type error “HCS3” should be “HSC3”.

_This error has been corrected in accordance with the reviewer’s comment. [page 5, line 94]_

2. Results, KRT13 promoter methylated in OSCC cells; “Figure 2A” and “Figure 2B” should be “Figure 3A” and “Figure 3B”.

_This error has been corrected in accordance with the reviewer’s comment. [page 10, line 213]_

- Discretionary Revisions
none

**Response to Reviewer#2 (Dawidson Gomes)**

_We wish to express our appreciation to the Reviewer for the insightful comments, which have helped us significantly improve the paper._

- **The only major point** is the necessity to perform densitometric analyses in all western blots.

_In accordance with the reviewer’s comment, we have performed densitometric analysis of all western blots (n = 3–5) using ImageJ 1.47v (National Institute of Health). Relative protein levels have been calculated and shown in bar graphs of each figures (Figure 1, 4, 5 and 6). Accordingly, we have changed the text of figure legends (Figure 1, 4, 5 and 6) and methods sections [page 7, line 139-141]._

- **Minor points:**
  1) Page 6, 123th line: Please include the method and reference used for qPCR data normalization.

_In accordance with the reviewer’s comment, we have added the method and reference used for qPCR data normalization to the methods section [from page 5, line 114 to page 6, line 127]._

  2) Page 6, 127th line: How much protein was used for the western blots?

_The protein amount “5–10 µg” has been described in accordance with the reviewer’s comment. [page 6, line 132]_
3) Page 6, 135th line: Please include the dilution of the antibodies

_In accordance with the reviewer’s comment, we have added the dilution of the antibodies used for the western blot to the methods section [page 7, line 142-147]_

4) Page 6, 142th line: Which negative control was used for the Immunofluorescence?

_We used a normal rabbit IgG (#2729; Cell Signaling Technology) as a negative control, and observed no fluorescence signal in negative control experiments. The image is presented in additional file-1 (Figure S1)._

5) The authors state in page 9, lines 192-194 that “KRT13 protein levels were significantly decreased in the HSC4 and HSC3 cells compared with the HaCaT cells, and almost absent in the SAS cells (Figure 1A)”. It is recommended to perform a densitometry followed by statistical analyses of the western blots results to state that KRT13 protein levels were ”significantly” decreased in the HSC4 and HSC3 cells compared with the HaCaT cells.

_We have quantified band intensity of the western blots (n = 4–5) against KRT13 protein using ImageJ 1.47v (National Institute of Health). The KRT13 protein levels of four cell-lines (HaCaT, HSC4, HSC3 and SAS cells) have been shown in bar graphs of figure 1A._

6) Page 9, lines 195-195. The authors state that “Immunofluorescence microscopy revealed high cytoplasmic expression of KRT13 protein in the HaCaT cells, but not in the OSCC cell lines (Figure 1B)”. The authors used standard immunofluorescence to look at the subcellular localization of the KRT13. It is recommended to use confocal microscopy to state the correct subcellular localization of this protein.

_To confirm the cytoplasmic localization of KRT13 protein in HaCaT cells, we have observed fluorescence using a confocal microscope (LSM710; Carl Zeiss MicroImaging GmbH). A representative image is shown in additional file-1 (Figure S1)._  

7) Page 20, 439th line: How many western blots were performed?

_This experiment has been performed four times, and a representative image is shown. The KRT13 protein levels of four cell-lines have been quantified and shown in a bar graph of figure 1A._

8) About Figure 2: Is the groups compared with HaCat? Please include this information in the legend. The units in the y axis is fold change? Please correct this information in all qPCR graphs.

_The reviewer's comment is correct. To clarify, we have added “Fold change in KRT13 mRNA was normalized to GAPDH and calculated relative to that of the HaCaT cells.” to the legend of figure 2 (page 20, line 449-450), and “Fold change in KRT13 mRNA was normalized to GAPDH and calculated relative to that of the cells under the control._
In accordance with the reviewer’s comment, we have corrected the y axis units of all qPCR graphs (figure 2, 5B and 6A).