Author's response to reviews

Title: S100A16 Promotes Differentiation and Contributes to a Less Aggressive Tumor Phenotype in Oral Squamous Cell Carcinoma

Authors:

Dipak Sapkota (Dipak.Sapkota@gades.uib.no)
Ove Bruland (ove.bruland@helse-bergen.no)
Himalaya Parajuli (Himalaya.Parajuli@k1.uib.no)
Tarig A Osman (Tarig.Osman@k1.uib.no)
Muy-Teck Teh (m.t.teh@qmul.ac.uk)
Anne C Johannessen (Anne.Johannessen@k1.uib.no)
Daniela E Costea (Daniela.Costea@k1.uib.no)

Version: 4  Date: 22 May 2015

Author's response to reviews: see over
Subject: Re-submission of the research manuscript entitled ‘S100A16 Promotes Differentiation and Contributes to a Less Aggressive Tumor Phenotype in Oral Squamous Cell Carcinoma’ to The BMC Cancer.

The authors would like to thank the editor of BMC Cancer for reviewing and providing positive comments to this manuscript. We have modified the original submission according to the suggestions from the reviewers and hope that it is now acceptable for publication. Point-by-point response to the reviewers’ comments has been described below.

All the persons listed as authors have participated to the study to a sufficient extent to be co-authors of the manuscript and all have agreed to the changes made for its re-submission to BMC Cancer.

Sincerely
On behalf of the authors,

Dr. Dipak Sapkota
Department of Clinical Medicine, The Gade Laboratory for Pathology, University of Bergen, Haukeland University Hospital, N-5021 Bergen, Norway
E-mail: Dipak.Sapkota@k1.uib.no
Tel: +47 55973231
Fax: +47 55973157
Response to reviewers’ comments:

Reviewer (Simon A Whawell):

1. **The authors need to comment on the lack of correlation of differentiation, T-stage and clinical stage to survival as this is somewhat surprising.**

Response: Authors would like to thank the reviewer for pointing out this issue. A tendency for well differentiated and early stage tumors to have a better survival probability was observed (Fig. 1), but the correlation was not statistically significant. One possible explanation for this observation could be the limited number of OSCC ($n=65$) specimens used for S100A16 immunostaining in the current study. These results have now been included in the results section of the revised manuscript (page 14, lines 310-312).

Fig. 1: Kaplan-Meier curves for tumor differentiation, clinical stage and T-stage for 10-years survival, $P$ value was determined with Log-rank test.

2. **There is a tendency to over interpret the data in places especially the mechanistic experiments. For example figure 4B shows a modest difference in CK13 expression with no reference to mean fluorescence or number of repeated experiments. Figure 4A has 4 panels which are completely blank and figure 5I shows no difference in MMP-1 for the CalH3 cell line.**
Response: Acknowledging reviewers’ suggestions, we have revised the text describing the results for S100A16-mediated modulation of differentiation markers (page 15, lines 348-353) and MMP1 (page 16, lines 366-372).
Additionally, new analysis for mean fluorescent intensities of CK13 FACS experiment has been included in Fig. 4 (Fig. 4C-new figure). These results are in parallel with the results presented in the original submission.

3. The term 'technical replicates' needs to make it clear whether this relates to number of repeated experiments or repeats within the same experiment. The latter being n=1 and the use of statistics is therefore not appropriate.
Response: We are sorry for this confusion and we would like to thank the reviewer for allowing us to correct/clarify this issue. By ‘biological’ replicates we meant ‘repeated’ experiments and by ‘technical’ replicates we meant ‘repeats within the same experiment (for example three replicates used in qRT-PCR)’ from the same biological sample. We have now revised these formulations throughout the manuscript and used the term ‘repeated experiments’ instead of ‘biological replicates’, to avoid confusion. In addition, we apologize for an error we made in the figure legend (for Fig. 3F and G) of the original submission, ‘3 repeated experiments’ was erroneously stated as 3 technical replicates’. ‘Biological’ replicates have now been replaced with ‘repeated’ experiments in all figure legends as well (page 30, lines 804 and 809; page 31, lines 818-819; page 32, lines 839). Replicates used for qRT-PCR have been specified in the Supplementary material section of the revised manuscript (Page 3, lines 53 and 61).
Minor essential revisions

1. There is a very recent publication on S100A16 in breast cancer which would be good to include (Tanaka M et al BMC Cancer 2015). The authors report that S100A16 promoted tumourigenesis in these tumours which is in contrast to the data presented here. I realize this may have been published after submission of this manuscript.

   Response: Authors would like to thank the reviewer for this suggestion. We have now cited the paper in the introduction section of the revised version (page 4, lines 94-95). Given the differential expression pattern and contextual functional roles of other members of the S100 proteins, S100A16 might also have differential functional roles in breast and oral cancers and it warrants further investigation.

2. Line 284 'performed' spelled incorrectly

   Response: Authors would like to thank the reviewer for this correction. Spelling has been corrected.

3. Line 348 and Figure 4C. P as far as I understand cannot = 0, p<0.001 would be better

   Response: We would like to thank the reviewer for this suggestion. We have now replaced $p=0.000$ with $p<0.001$ both in the manuscript text and in the figure 4C.

4. Table 1 legend, line 665 'patients' spelled incorrectly

   Response: We would like to thank the reviewer for this correction. Spelling has been corrected.

5. Line 838 remove the word 'only', the difference between 5 and 6 mice is not a significant reduction

   Response: We agree with the reviewer and we have now removed ‘only’ from the figure legend.
Reviewer (Tamotsu Kiyoshima):

1. Although authors used a composite score that took into account not only the number of positive cells, but also the grading of the immunostaining intensity to more accurate the interpretation of immunohistochemical analysis, it should be specified followings;

a. how many microscopic fields were investing, the objective used and how many cells were counted (minimum 5000 cells)

Response: Authors would like to thank the reviewer for allowing us to clarify this issue. Three consecutive fields were used for the evaluation of S100A16 immunohistochemistry in NHOM, ODL and lymph nodes. However, 6 fields were used in OSCCs: 3 fields in the tumor center and 3 in the invading front/island. More than >500 cells were evaluated per field using 40X objective (pages 7 and 8, lines 162-167). Based on the published literature and our experience, we consider that the evaluation of approximately 1500 cells (in 3 fields) would be adequate to describe a representative staining pattern and intensity of an immunostained specimen.

b. how the authors decided the criteria for the grading of the immunostaining intensity

Response: We would like to thank the reviewer for comment. As described in the ‘S100A16 IHC evaluation’ in Supplementary Methods section of the original submission, the following criteria was used for the grading of the immunostaining intensity of S100A16: The I score (intensity of S100A16 staining) was calculated as follows: 2, if the proportion of cells with strong to weak S100A16 intensity was >1; 1, if the proportion was equal to 1; and 0.5, if the proportion was <1 (page 1, lines 16-21).
c. how many pathologists performed immunohistochemical evaluation, Authors collected 82 OSCCs (FFPE) in this study. Immunohistochemical data was based on only 65 OSCCs (FFPE). Please let me know the reason.

Response: Authors would like to thank the reviewer for this comment and for allowing us to clarify the number of OSCC cases used for S100A16 IHC. Inter-observer variation was controlled by calibrating the evaluation done by three investigators (DS, TAO and HP). Afterwards, all specimens were evaluated by one investigator (DS) (page 7, lines 163-165).

We are sorry for this confusion regarding the number of OSCC cases. 82 FFPE specimens were collected, but 17 cases were used for laser microdissection, so only the remaining 65 cases were used for the S100A16 immunostaining.

2. S100A16 showed a strong membranous expression in the committed/differentiating epithelial cell layers. Meanwhile negative or weak cytoplasmic staining of S100A16 was found in the basal cell layer (stem cell compartment). Please discuss the mechanism underlying different regulation of membranous and cytoplasmic S100A16 expression between supra-basal and basal layers.

Response: Authors would like thank this reviewer for raising the important issue. At the moment, we do not know the precise mechanism that underlies the regulation of membranous and cytoplasmic S100A16 expression between the supra-basal and basal cell layers. Considering the role of S100A16 in keratinocyte differentiation, it can be speculated that in the supra-basal layers (committed/differentiating cells), the change in localization and expression of S100A16 might be related to its differential interaction with the membranous proteins potentially involved in differentiation pathway signaling. Indeed, we are
currently investigating this possibility. It is worth noticing that one of the main mechanisms of members of S100 proteins for their biological functions is via interaction with other effector proteins.

3. The discussion related to the function of S100A16 in OSCC is too superficial. The putative mechanism underlying down-regulation of keratinocyte differentiation markers by reduced S100A16 expression and/or the relationship between keratinocyte differentiation markers and S100A16 should be discussed in the “Discussion” section. Additionally, if authors have ideas, add the description of the putative mechanism underlying down-regulation of S100A16 expression during OSCC carcinogenesis.

Response: Authors would like thank the reviewer for this suggestion. As described in result and discussion sections of the original submission, we could not find any change in the p38 phosphorylation status with S100A16 over-expression, suggesting that modulation of differentiation markers could be independent of the p38 signaling pathway. This points towards a possible involvement of p38 independent mechanisms. The last statement has now been included in the discussion of the revised version of the manuscript (page 19, lines 433-434).

Authors would like to thank the reviewer for providing us an opportunity to clarify the possible mechanism of S100A16 down regulation in oral carcinogenesis. A recent work from our group reported a loss in the S100A16 locus in OSCC specimens from India and Sri Lanka, indicating that one of the mechanisms for the down-regulation of S100A16 in OSCC could be due to the deletion of S100A16 locus (Lunde ML, Cancer Genomics Proteomics, 2014). The tumor samples in the current study have not been examined for the chromosomal
change for S100A16. However, given the deletion of S100A16 locus in OSCC specimens from India and Sri Lanka, it can be speculated that gene deletion could contribute the observed down-regulation of S100A16 in the current study. This probability has now been mentioned in the revised manuscript (page 18, lines 406-410).

4. **L38:** This part is “Methods” part in the “Abstract”. Could authors please confirm which “was correlated” or “was examined the correlation” is suitable?
   
   **Response:** Authors would like to thank the reviewer for this suggestion. We have rephrased the sentence as suggested by the reviewer (page 1, line 38).

5. **L122:** Authors commented “NHOM, 31 formalin fixed-paraffin embedded (FFPE) and 44 frozen” below. Authors should confirm which “65” or “75” is correct?
   
   **Response:** Authors would like to thank the reviewer for pointing out this error. Yes, the total number of NHOM is 75 (31 FFPE and 44 frozen). We have changed it in the revised version (page 6, line 122).

6. **L299:** Please confirm which “score was down-regulated” or “score was decreased” is suitable?
   
   **Response:** Authors would like to thank the reviewer for this suggestion. We have now replaced ‘down-regulated’ with ‘decreased’ in the revised version.

7. **Add the explanation of the dotted/slashed bars in the Figure legends of the Figure 5.**
   
   **Response:** We would like to thank the reviewer for this suggestion. We have now changed the labels of the Fig. 5 I and J to make it more understandable.

8. **In Tables and supplemental Tables, the space should be inserted between a number and “(“.**
Response: Authors would like to thank the reviewer for this suggestion. Spaces have been inserted between a number and ‘(‘ in the tables both in the main text and in the supplementary tables.

9. In addition, several typos and grammatical errors are still remained in the manuscript. “functions” (L61), “perforemed” (L284), “S100A16- xenografts” (L446), “S00A16” (L809) and so on should be correct in the revised version. Please carefully and carefully check and correct point-by-point.

Response: Authors would like to thank the reviewer for pointing out the errors. The above mentioned errors are now corrected. Moreover, the manuscript has been carefully checked for any errors and corrected accordingly.