Author's response to reviews

Title: PRAF3 induces apoptosis and inhibits migration and invasion in human esophageal squamous cell carcinoma

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Version: 3 Date: 16 February 2012

Author's response to reviews: see over
February 16, 2012

Dear Prof Bjoern Bruecher,

Please consider this revised submission of our manuscript # (1409688418642442), entitled “PRAF3 induces apoptosis and inhibits migration and invasion in human esophageal squamous cell carcinoma”, for publication in the Journal of BMC cancer. We greatly appreciate the thoughtful reviews received from you and from the reviewers. In response, we have prepared and included (below) a detailed description of all changes made to the manuscript, referenced to each of the reviewers’ comments. With reference to the manuscript file, we submit a revised, marked version of the paper with additions indicated in bold underlined text and deletions using strikethrough, and a revised, unmarked version with all changes incorporated.

Again we would like to thank the reviewers for their constructive and professional comments, which help us to improve our manuscript. Your re-consideration of this revised manuscript is much appreciated.

Yours sincerely,

Shengdong Huang, MD, PhD
(corresponding author)
Responses to Reviewers’ Comments:
Reviewer: Alejandro Corvalan
Reviewer's report:
The manuscript entitled "PRAF3 induces apoptosis and inhibits migration and invasion in human esophageal squamous cell carcinoma" by Yuan et al attempts to define the role of PRAF3 which is the only member of the Prenylated Rab acceptor 1 domain family considered as a tumor suppressor gene in SCC.
To this purpose, Yuan et al evaluated 57 clinical cases and 2 ESCC cell lines. Using multiple techniques, Yuan et al demonstrated that a loss of expression in PRAF3 is linked to a worse prognosis in relation to clinical features such as pathological grade, tumor stage and lymph node metastasis.
Furthermore, the overexpression of PRAF3 induces cell apoptosis through both caspase-8 and caspase-9 dependent pathways, along with inhibited cell migration and invasion by suppressing the activity of both MMP-2 and MMP-9.
Taken together, Yuan et al suggest that PRAF3 may act as a negative regulator of tumor progression and metastasis in human ESCC. Although there are many interesting points made throughout the manuscript, the quality of English needs minor improvement (see examples below) and due to an excluded reference, I believe that one final review is needed before its publication.

In the abstract Background the authors should specify in which pathway the role of PRAF3 is relevant to SCC. In the abstract Conclusions, the author should use the word "potential" to refer to PRAF3.
We thank the reviewer for this comment. We have revised the text accordingly (see page 2, paragraph 1, line 2; and page 2, paragraph 4, line 4;).

In the main text, the Introduction is adequately explained. However, in the Methods section, it’s not clear how the samples were obtained. In apoptosis assay, the methodology applied is unsatisfactory since propidium iodide is intercalated in citoplasmic RNA. In order to avoid this problem, I recommend using RNasa treatments.
Thank you for these suggestions. We have added this information in the revised version (page 4, paragraph 1, line 1; and page 7, paragraph 2, line 2).

In the Results section "PRAF3 suppresses the activity of MMP-2 by integratin avb3 signaling in ESCC cells", I would like to know how the authors knew they were dealing with the correct enzyme.
We thank the reviewer for this constructive suggestion. In the present study, we found that the overexpression of PRAF3 could significantly inhibit the activity of MMP-2
and the expression of \( \alpha_\text{v} \) and \( \beta_\text{3} \) in ESCC cells. Since it was reported that integrin \( \alpha_\text{v}\beta_\text{3} \) could activate MMP-2 in some other tumors, we suggest that PRAF3 might suppress the activity of MMP-2 by down-regulating integrin \( \alpha_\text{v}\beta_\text{3} \) signaling in ESCC cells. We have added this information in the revised version (page 11, paragraph 3, line 7).

According to this reviewer, the manuscript of Yuan et al does not have Major Compulsory or Discretionary Revisions. However, several Minor Essential Revisions can be raised:

Q1. In the second paragraph of the abstract, the term "field" is awkward in the context of ".......In recent years, PRAF3 has gained increasing attention in the tumor field...."
   A: Thank you for the suggestion. We have revised the text accordingly (page 3, paragraph 2, line 7)

Q2. In the first paragraph of the Results section, “.....we first scan....” should be “.....we evaluate....”
   A: Thank you for the suggestion. We have revised the text accordingly (page 9, paragraph 1, line 1)

Q3. In the second paragraph of the Discussion section, ......(but not ESCC)....please modify.
   A: Thank you for the suggestion. We have revised the text accordingly (page 13, paragraph 2, line 2 and line 4)

Q4. Missing reference after the text ".....Considering that PRAF3 is a transmembrane protein located at the endoplasmic reticulum....."
   A: Thank you for reviewing our manuscript so carefully. We have added the missing reference in the revised version (page 15, paragraph 3, line 8).
The authors investigated the expression of PRAF3 in oesophageal squamous cell carcinoma and the role of overexpression in oesophageal squamous cell carcinoma cell lines specifically concerning apoptosis and migration. An interesting area for which there is minimal data for squamous oesophageal cancer. The authors use immunohistochemistry, qRT-PCR, western blotting to observe PRAF3 expression in histological specimens from treatment naïve surgical specimens. Over expression of PRAF3 was achieved using infection of replication deficient adenovirus and demonstrated at the mRNA and protein level. Functional effects of PRAF3 overexpression are assessed with MMP-2 and MMP-9 activity, annexin apoptosis assay, caspase-8 and 9 activity, wound healing and cell migration observations. The methods are appropriate and well described.

We thank you for these positive comments.

Minor Essential Revisions

Q1 - Observational data that expression of PRAF3 (immunohistochemically) correlated with pathological grade, tumour stage and lymph node metastasis is very interesting. Did both pathologists agree on the staging / pathological grading of all tumours? Which part of the tumour was used to asses PRAF3 expression in these experiments?

A: We thank the reviewer for these suggestions. In our study, the pathological diagnosis was performed by a pathologist (Zhi-Jun Ge) and subsequently reviewed by a senior pathologist (Jian Zhou). We used tissues near the margin of the tumor to asses PRAF3 expression. We have added this information in the revised version (page 4, paragraph 1, line 9; and page 4, paragraph 3, line 1).

Q2 - PRAF3 overexpression in cell lines induces apoptosis through caspase-8 and caspase-9 dependent mechanisms. What percentage of cells were infected to provoke these results?

A: We thank the reviewer for this suggestion. We have detected the infected efficiency of Ad.GFP in Eca109 and TE-1 at a MOI of 100. Flow cytometric analysis showed that the percentage of GFP+ cells were 84.7% and 79.3% in Eca109 and TE-1, respectively. We have added the information in the revised version (page 5, paragraph 2, line 8).

Q3 - The scale bars are missing from the legend in figure 4.

A: Thank you for reviewing our manuscript so carefully. We have added the scale bars
in the revised version (page 22, paragraph 8, line 1).

**Q4 - There is a reference missing in the final paragraph {ref}**
Thank you for reviewing our manuscript so carefully. We have added the missing reference in the revised version (page 15, paragraph 3, line 8).

**Major Compulsory Revisions**

**Q1 -** The immunohistochemistry in figure 1 suggests that the expression of PRAF3 in normal squamous tissue is expressed as a decreasing gradient from the differentiating squames compared to the transit amplifying and stem cell compartment. PRAF3 has been shown to induce differentiation in certain cell lines and would provide an alternative explanation to the authors conclusion for the observation in figure 1 (1).

A: We thank the reviewer for the insightful comment. PRAF3 was reported to be involved in the regulation of many cellular processes including inducing differentiation in certain cell lines. However, the role of PRAF3 in the regulation of normal squamous cell differentiation has yet to be explored. Considering that the expression of PRAF3 appeared as a decreasing gradient from the differentiating squames compared to the transit amplifying and stem cell compartment, it is tempting to suggest that the down-regulation of PRAF3 in tumor tissue might be associated with the poor differentiation. But further investigation is needed to substantiate this hypothesis. We have added the information in the revised version (page 13, paragraph 2, line 10).

**Q2 -** In subsequent cell line experiments neither cell line is characterised to their initial cell phenotype and differentiation status. Given that the majority of data relates to cell line experiments I think the cell lines need to be characterised at least prior to experimentation.

A: Thank you for the suggestion. It was reported that Eca109* and TE-1** were both derived from well-differentiated ESCCs. We have added this information into the revised version (page 9, paragraph 3, line 4).


Q3 - How was the activity of ad-cmv-null virus tested? Given that a proportion of the results relate to cell death and migration it is important that the ad-cmv-null virus is validated.

A: We thank the reviewer for this suggestion. The activities of adenovirus were determined by plaque assay using BD Adeno-X™ rapid titer kit according to the manufacturer's instruction. We have added the information in the method section (page 5, paragraph 2, line 5).

Q4 - The limitation of applying functional data from cell lines to in vivo is not discussed. Are the results unique to cancer cell lines and what is the effect of PRAF3 overexpression on normal oesophageal cell? If PRAF3 is a lead molecule for the development of ESCC, as suggested by the authors, its role in normal oesophageal cells should be understood.

A: This is an excellent question and we agree with the reviewer that cautions should be taken when applying the data from cancer cell lines to the in vivo situations. Unfortunately, we did not look at the effects of PRAF3 on normal oesophageal cells in this study and we are not aware of any other reports regarding the role of PRAF3 in normal oesophageal cells. We noticed that the expression level of PRAF3 in normal squamous tissue is not homogeneous. It would be interesting to observe the effects of altered expression of PRAF3 on the differentiation of normal squamous cells. We have added this information in the discussion (page 13, paragraph 2, line 10).