Author's response to reviews

Title: Twist and Snai1 expression in pharyngeal squamous cell carcinoma stroma is related to cancer progression

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Author's response to reviews: see over
Dear Editor

BMC Cancer

Dear Editor

We appreciate the opportunity to revise our manuscript entitled "TWIST AND SNAI1 EXPRESSION IN PHARYNGEAL SQUAMOUS CELL CARCINOMA STROMA IS RELATED TO CANCER PROGRESSION" by Jouppila-Mättö et al (BMC Cancer, MS 5885175965250902). We appreciate the constructive remarks and corrections proposed by the Reviewers. All comments and anticipated revisions have been carefully considered and responded. A point-by-point clarification list of the revisions is presented below. The corrections in the manuscript are highlight by using red text colour.

We hope that after these corrections our manuscript would fulfil the strict requirements of The Journal and could be accepted for publication in BMC Cancer.

Sincerely yours,

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A list of changes and a response to the comments of the Referees

Comments to Reviewer Patricia Reis:

Minor essential revisions

1. The authors need to correct the citation of genes and proteins throughout the manuscript: they should use capital letters for human proteins (e.g., TWIST, SNAI1) and capital italicized letters for human genes (e.g., TWIST, SNAII).

   All the citations of genes and proteins have been corrected as requested.

2. The authors should replace “strong protein expression” by strong staining intensity”, as this is more appropriate for studies using immunohistochemistry.

   We have replaced ”strong protein expression” by “strong staining intensity” in several sentences. However, we have not replaced all terms as also the first expression is widely used in literature (Schwock J et al: BMC Clin pathol 2010, 10:1).

3. The study hypothesis needs to be clearly defined in the manuscript.

   The study hypothesis has been defined as proposed (see Introduction, last paragraph, first sentence).

4. Please add details about the patient sample selection - it appears that the patients were randomly selected, or were the patients chosen based on tumor characteristics, disease stage or outcome?

   The patient selection has been specified (see M&M, chapter Patients, first sentence)

5. In Figure 1, Panel D, the authors show positive SNAI1 staining in stromal cells; however there are few cells that are staining for SNAI1 protein. Which score was given for this particular case? Also, please indicate the tumor grade and stage for the samples shown in the figure.

   The figures have been enhanced by adding low power fields (x100) in addition to high power fields (x400). The immunoscores, tumor grades and stages are indicated in the legend.

6. The graphs showing disease-specific survival and overall survival analysis require better resolution and larger size, for improved visualization of the survival analysis results.

   The size and resolution of survival graphs has been improved.

7. In Figure 2, the authors state that survival was poorer according to the number of transcription factors expressed in the stroma. This statement, which is also found in other parts of the manuscript, gives the reader the impression that several transcription factors were analyzed, while there were only two proteins analyzed in the study. The authors should simply state that patient survival was poorer when both transcription factors were positive in the tumor stroma.
The correction has made to Figure 2 legend and to the text according to Reviewers statement (Results, chapter: TWIST and SNAI1 co-expression, second paragraph, first sentence).

8. On Page 8 (Discussion), there are grammatical errors and a typo that needs to be fixed. For example, "this data" should be replaced by "these data". The typo is in the word "conducted", which should be "conducted".

The grammatical errors have been revised.

**Major compulsory revisions**

1. In the Discussion section, the authors state that smoking can modulate the expression of EMT markers. This is an interesting finding or hypothesis that should be expanded and explained in more detail.

   The role of smoking in EMT marker expression is interesting, but not the main aim of our study. However, we have expanded it a bit (Discussion, first paragraph, last sentence).

2. In the Discussion, the authors state that “there is variation between classifying strategies between different publications”, but they do not explain it further. This needs to be expanded and clarified for the reader.

   The variation between classifying strategies has been clarified shortly (Discussion, third paragraph, last sentence).

3. The major point in the Discussion is the lack of a detailed discussion about how TWIST and SNAI1 may drive disease progression and metastasis in cancer and in pharyngeal cancer. In particular, the authors could expand their discussion about the findings of TWIST expression in epithelial cell nuclei. I suggest to include findings from a recently published manuscript entitled “Bmi1 is essential in TWIST1-induced EMT”, Nat Cell Biol. 2010.

   We have discussed more about how TWIST and SNAI1 drive disease progression and cited the proposed study by Yang et al (Discussion, third paragraph).

4. The authors should add data from the work by Schwock et al. BMC Clin Pathol. 2010, which examined SNAI1 protein expression, by IHC, in oral squamous cell carcinomas.

   The work by Schwock has been cited as proposed (Background, fourth paragraph, fourth sentence and Discussion, second paragraph, second sentence).

5. Considering that loss of E-Cadherin protein expression has been correlated with positive SNAI1 and TWIST expression, do the authors have any supporting data regarding E-Cadherin expression in the pharyngeal tumors that show strong staining intensity of TWIST and SNAI1 in the tumor stroma or tumor epithelia?

   To our knowledge there are no published data of E-Cadherin expression and its’ affects on TWIST and SNAI1 staining intensity in oro- and hypopharyngeal SCC. In nasopharyngeal
carcinoma E-Cadherin expression is reported to correlate inversely with SNAI1 expression but not with TWIST expression (Horikawa et al. *Br J Cancer* 2011, **104**:1160-1167). We have cited this in Discussion (third paragraph).

In addition, we have revised the conclusion according to reviewers statement.
Comments to Reviewer Joseph S Pagano:

1. Authors should provide information on which tumors were HPV-positive.

   The association between oro- and hypopharyngeal cancer and HPV is under lively investigation. HPV infection has been shown to exist in up to 80% of the pharyngeal cancer cases. The prevalence of pharyngeal HPV infection seems to be increasing and concomitantly its significance as a pharyngeal cancer risk factor is emphasized (Gillison et al. *J Natl Cancer Inst* 2008, **100**:407-420; Lewis et al. *Am J Surg Pathol* 2010, **34**:1088-1096; Mendelshon et al. *Laryngoscope* 2010, **120**: 1788-1794). The implication of HPV in pharyngeal cancer was pointed out by the Reviewer. At the moment, however, we do not have any data of the HPV status of the present cohort.

2. Fig 1 would be enhanced by showing larger fields at lower power as well as 400X.

   The figures have been enhanced by adding low power fields (x100) in addition to high power fields (x400). The immunoscores, tumor grades and stages are indicated in the legend.

3. Did authors examine any epithelial or mesenchymal markers in tumor tissue by IHC?

   Comparison of epithelial and mesenchymal marker staining to EMT markers in our tumor tissue material is a very interesting conception and is worth to investigate in the future.


   We have cited the study by Horikawa et al as proposed (Discussion, third paragraph).