Author's response to reviews

Title: SNAI1 Expression and the Mesenchymal Phenotype: An Immunohistochemical Study performed on 46 Cases of Oral Squamous Cell Carcinoma

Authors:

Joerg Schwock (jschwock@uhnres.utoronto.ca)
Grace Bradley (bradley@uhnres.utoronto.ca)
James C Ho (jamesho@uhnres.utoronto.ca)
Bayardo Perez-Ordonez (bayardo.perez-ordonez@uhn.on.ca)
David W Hedley (david.hedley@uhn.on.ca)
Jonathan C Irish (jonathan.irish@uhn.on.ca)
William R Geddie (william.geddie@uhn.on.ca)

Version: 2 Date: 27 November 2009

Author's response to reviews: see over
Dear Dr. Burgess,

Thank you for considering our manuscript for publication in BMC Clinical Pathology! We also would like to thank the reviewers for their helpful suggestions!

Please see below for our point-by-point response to the reviewer’s comments. Please note that all changes to the manuscript (with the exception of minor changes in wording) necessary to address the comments raised by the reviewers are highlighted in red font color. Also, sections of the manuscript have been re-structured and non-pertinent information has been removed for increased clarity and focus. Table 2 has been replaced with a new table containing information linked to the Discussion section.

Dear Dr Geddie,

Your manuscript has now been peer reviewed and the comments are accessible in PDF format from the links below. Do let us know if you have any problems opening the files.

Referee 3: http://www.biomedcentral.com/imedia/1645449682316799_comment.pdf
Referee 5: http://www.biomedcentral.com/imedia/1789280838321180_comment.pdf
Referee 1: http://www.biomedcentral.com/imedia/1960018823147721_comment.pdf
Referee 2: http://www.biomedcentral.com/imedia/2075765208316547_comment.pdf
Referee 4: http://www.biomedcentral.com/imedia/1290409449317292_comment.pdf

In addition to the concerns raised by the reviewers please document that the patients who provided tissue samples gave Informed Consent for the study.

Author’s Response:

This retrospective study was approved by the institutional research ethics board of the University Health Network, Toronto, ON (REB protocol #06-0805-T) prior to initiation of any research activities. We confirm that all requirements with regard to retrospective data collection as well as retrieval and use of archived tissue specimens for this type of study have been followed.

We would be grateful if you could address the comments in a revised manuscript and provide a cover letter giving a point-by-point response to the concerns.

Please also ensure that your revised manuscript conforms to the journal style (http://www.biomedcentral.com/info/ifora/medicine_journals). It is important that your files are correctly formatted.

We look forward to receiving your revised manuscript by 27 November 2009. If you imagine that it will take longer to prepare please give us some estimate of when we can expect it.

You should upload your cover letter and revised manuscript through

http://www.biomedcentral.com/manuscript/login/man.asp?txt_nav=man&txt_man_id=3494756673074915. You will find more detailed instructions at the base of this email.
Please don’t hesitate to contact me if you have any problems or questions regarding your manuscript.

With best wishes,

Danielle

Danielle Burgess, PhD
Assistant Scientific Editor
BMC Series
Tel: +44 (0) 20 3192 2013
e-mail: editorial@biomedcentral.com
Web: http://www.biomedcentral.com/

To submit your revised manuscript

When you have revised your manuscript in light of the reviewers' comments and made any required changes to the format of your paper, please upload the revised version by following these instructions:

1. Go to http://www.biomedcentral.com/manuscript/login/man.asp?txt_nav=man&txt_man_id=3494756673074915 and log on with your email address and password.

2. With the 'Manuscript details' tab, please update the title, abstract and author details if they have changed since the previous version. It is very important that all changes are updated on this page, as well as in the manuscript file as the information on this page will be used in PubMed and on BioMed Central if your manuscript is accepted for publication.

3. With the 'Cover letter' tab, please provide a covering letter with a point-by-point description of the changes made.

4. With the 'Upload files' tab, please upload the revised version of the manuscript and press 'Submit new version'. Please wait for the confirmation page to appear - this may take a few moments.
Title: SNAI1 Expression: A Rare Event In Oral Squamous Cell Carcinoma
Associated With Features Of Poor Prognosis
Version: 1 Date: 13 October 2009
Reviewer: Amparo Cano

Reviewer's report:
In the present ms. the authors analyse SNAI1 expression in a series of oral squamous cell carcinomas (OSCC) in primary tumours (n= 46) and nodal metastasis (n=26), and compare to the expression of E-cadherin, FAK and p63. They conclude that SNAI1 expression is a rare event in OSCC associated to poor prognosis, as stated in the title.
The authors previously tested two commercial anti-Snail1 antibodies for specificity and select AF3639 antibody for analysis in the OSCC series. The study is well performed in general terms, and the quality of the IHQ is very high for most studied parameters. The conclusions driven from the study can, nevertheless, be confusing or misleading since the term “rare event” is used to refer to the number of tumour cells positive for SNAI1 expression, and not for positive tumours. Present evidence from different studies support that Snail1 expression should be focal, and likely, transient inside tumours and in this sense it will be a “rare” event in the context of the whole tumour but not when considering a tumor series. This also depends on the “cutoff” for positive staining defined by the authors. Therefore, important clarification in this aspect is required for the present study before publication. The presentation of the statistical analyses is also rather confusing and it is sometimes difficult to follow. Additional controls regarding specificity of AF3639 antibody are also required.

Author’s Response:
We acknowledge that the term “rare event” has been used in a somewhat imprecise manner. To avoid confusion for the reader we have chosen a more descriptive title and clarified our findings throughout the manuscript emphasizing that single SNAI1-positive tumor cells can be found in the majority of carcinomas, although such cells are infrequent. The potential reasons for this, such as the transient nature of EMT, have been discussed.

Specific points:
1. Specificity of AF3639 antibody is only shown by Western blot (Supp Fig. 1C). It needs to be tested by IHQ on the placental samples, xenografts and spindle cell tumors as performed for SC10432 antibody. In this sense, the data on SC10432 antibody do not add substantial information to the ms. (it is not used for analysis of the OSCC series) and can be removed.

Author’s Response:
The specificity of AF3639 has been tested with the same rigor as SC10432. However, no commercial blocking peptide has been available for the former antibody to the best of our knowledge. Thus, SNAI1 staining was performed in parallel with both antibodies and any questionable staining was cross-checked. Antibody work-up and decisions based on it have been clarified with amendments in the “Immunohistochemistry” sub-section of Material & Methods and addition of two images to Supplementary Figure 1.

2. The SNAI1 IHQ staining images with AF3639 antibody should be shown for tumours representative of the different H-score categories used by the authors. Only one case with SNAI1 staining in the stroma is presented in Fig. 1, apart from the two samples with sarcomatoid component (Figs. 4 & 5).

Author’s Response:
Representative images of two different tumors have been added to Figure 1 (A and B). A less important section of this figure illustrating stromal expression of SNAI1 was removed.

3. The most questioning part of the ms., however, is the definition of the term “rare event”. Indeed the data indicate that 22% (10 out of 46) of primary tumors and 19% of nodal metastasis (5 out of 26) are SNAI1 positive (category 2, >5% tumor cells). In addition, “rare” individual SNAI1 staining (<5%) was detected in 30 patients, that is in 65% cases. This figure could indicate that the majority of tumors indeed express SNAI1 even if it is detected in few tumor cells. This could be particularly important if expression is mainly detected at invasive or
inflammatory areas as the authors indicate. Therefore, expression of SNAI1 can not be considered as a “rare event” in the context of the whole tumor series. This analysis thus requires clarification of the term “rare event” and reconsideration of the ms. title.

Author’s Response:

We previously addressed this point and the appropriate changes including the title have been implemented in the manuscript. Also, we chose not to quantify the stromal expression of SNAI1 in terms of percentage of positive cells out of all tumor-associated stromal cells due to inherent problems associated with this approach on complete tumor sections. (No border defines what is tumor-associated and what is not.) Instead we considered stromal staining readily visible at low magnification as “abundant” and readily found at high magnification as “frequent”. Stromal staining was considered “rare/occasional” if multiple HPF had to be screened to find SNAI1-positive stroma cells.

4. Some recent studies by other authors have used a threshold of >1% positive tumor cells (Blechschmidt et al. Br J Cancer, 98: 489-95, 2008; Franci et al. PLoS ONE, 4(5): e5595, 2009). The author should discuss their data also in the context of those previous works.

Author’s Response:

To address this point and also to illustrate the wide range of different methods used to assess SNAI1 in tumor specimens we included a new Table 2 which contains an overview of different studies using IHC on FFPE clinical specimens. A cut-off similar to our 5% level was previously reported by Blechschmidt et al. (Diagn Mol Pathol, 2007) and Peinado et al. (Cancer Res, 2008). We believe that the use of a 5% cut-off is more reproducible, especially considering the focal nature of SNAI1 expression.

5. Presentation of data in Figs. 4 & 5 is very confusing and not easy to follow. Please, distinguish more clearly the information regarding different markers.

Author’s Response:

Amendments have been made to both figure legends to increase clarity.

6. Statistical analysis in Table 2 is also confusing and hard to follow. One problem is that the series is biased towards grade 2 tumors (37 out of 46) while they compare G1&G2 vs G3 tumors. Since G2 present invasive areas, this group should be compare to G1. On the other hand, if no significant differences are found in any parameter, Table 2 could be deleted.

Author’s Response:

Table 2 containing the clinico-pathological data has been removed from the manuscript. Pertinent information is now contained in the text as part of the Results section. It is not quite clear what problem the reviewer is trying to address with sentence 3 in point 6. The grading does not reflect the presence or absence of invasion which is a pre-requisite for the diagnosis of carcinoma.

7. Recent work by Franci et al (PLoS ONE, 2009) has also shown SNAI1 expression in tumor stroma associated to poor prognosis in colon cancer. This study should also be discussed.

Author’s Response:

We included this very recent and interesting study. Also, we extended the statistical analysis of stromal SNAI1 in our cohort. However, no significant results were obtained.

8. Indication that EMT can be a focal and transient event and the participation of different EMT regulators was indicated in Franci et al (2006) as well as in other previous works (i.e, Thiery, Nat Rev Cancer, 2: 442-54, 2002; Huber et al. Curr. Opin. Cell Biol., 17: 548-58, 2005; Peinado et al., Int. J. Dev. Biol., 48: 365-75, 2004) that should also be considered.
Author’s Response:

This point was addressed in the initial version of the manuscript and is emphasized again in the Discussion section of the revised version.

Overall recommendation: Major Compulsory Revisions

**Level of interest:** An article whose findings are important to those with closely related research interests

**Quality of written English:** Acceptable

**Statistical review:** Yes, and I have assessed the statistics in my report.

**Declaration of competing interests:**
We hold a patent on Snail1 as a diagnostic marker, licensed to a biotech company (Advancell In vitro Cell Technologies). Funding for 18,000 € was obtained in 2004 from that company.

I do not have any other financial or non-financial competing interests in relation to this paper.
R2: Reviewer's report
Title: SNAI1 Expression: A Rare Event In Oral Squamous Cell Carcinoma Associated With Features Of Poor Prognosis
Version: 1 Date: 19 October 2009
Reviewer: Juan Carlos C de Vicente
Reviewer's report:
SNAI1 Expression: A rare event in oral squamous cell carcinoma associated with features of poor prognosis.
Joerg Schwock et al.
Authors present here a very interesting study about a relevant topic in head and neck cancer. They immunohistochemically examined SNAI1 expression in 46 tumors and 26 nodal metastasis. Both the design as well as the methodology used here are correct. Results are interesting and their discussion is brief but well performed.

Thus, I recommend the publication of this manuscript. However, I also suggest:

• To remove in the last paragraph of the Background section the results (description of SNAI1 expression, and the antibodies expression profile in the two cases that had a sarcomatoid component). In this section there should not have results.

Author’s Response:
The section pointed out by the reviewer has been removed and was replaced by a paragraph explaining how p63 and FAK relate to the EMT phenotype.

• On Table 2 there were not statistically significant association among SNAI1 expression and other variables included here. I suggest the authors increase the sample size in future studies in order of raise the power of the statistical tests.

Author’s Response:
Our study was intended to explore the feasibility of detecting different EMT-related markers in clinical material. Previous publications had pointed out the lack of commercially available SNAI1-specific antibodies (Rosivatz et al., Virchows Arch, 2006; Franci et al., PLoS One, 2009). Future studies will involve a larger number of cases and possibly take advantage of tissue microarray technology.

Level of interest: An article of importance in its field
Quality of written English: Acceptable
Statistical review: No, the manuscript does not need to be seen by a statistician.
Declaration of competing interests:
I declare that I have no competing interests
Title: SNAI1 Expression: A Rare Event In Oral Squamous Cell Carcinoma Associated With Features Of Poor Prognosis

Version: Date: 1 20 October 2009
Reviewer: Petra Richter

Reviewer's report:

Purpose
The purpose of this study by Schwock et al. was to investigate the tumour progressive role of the EMT inducer SNAI1, known to repress E-cadherin, in oral squamous cell carcinoma (OSCC) by means of immunohistochemistry using paraffin material of 46 patients suffering from OSCC. Moreover, an immunohistochemical analysis of focal adhesion kinase (FAK), E-Cadherin, vimentin, cytokeratin and p63 was added to identify epithelial versus mesenchymal cell phenotypes differentially occurring during the process of EMT.

General comments
Against the background that EMT induced by SNAI1 has been reported to play a special role for tumour invasion and metastasis formation in several carcinomas, the hypothesis of the study that SNAI1 is of tumour developmental importance also in OSCC is of interest for a better understanding of OSCC tumour biology with possible therapeutic consequences for OSCC patients having a poor prognosis even today. Nevertheless, there are reports available in the literature already demonstrating that SNAI1 expression is a rare phenomenon in OSCC occurring in the stromal compartment in association to the myofibroblastic cell phenotype and not contributing to the predict of metastasis occurrence or patient survival.

Author’s Response:

Previous studies showed a wide range of different results as a consequence of disparate methods. It is not yet clear what, if any, conclusions can be drawn with regard to the frequency of SNAI1 expression in different tumor types and its impact on patient prognosis.

Schwock and co-workers present an immunohistochmical study on paraffin sections of OSCC and corresponding lymph node metastases from 46 patients. Selectivity testing based selection of SNAI1 antibodies has been performed very well including xenografts of OSCC cell lines showing different cell phenotypes. Nevertheless there are several concerns.

Major Compulsory Revisions:
1.) The introduction is short but does not sufficiently reflect the level of knowledge concerning SNAI1 and OSCC as available from the literature. Authors should clearly explain why they additionally investigate FAK and p63 and what the known functional linkage within the EMT process is.

Author’s Response:

The last paragraph in the Background section has been replaced to address the lack of information on p63 and FAK.

2.) In the methods section, the first chapter concerning patients and material should be clarified. Either all characteristics should be given in a table or in the text. The one patient with the reported one-time history of OSCC 25 years prior to inclusion should be excluded in my eyes. The semi quantitative scoring system applied by the authors is a little bit to complex. How has the category of staining intensity been assessed? It is indispensable to improve presentation of the scoring system i.e. in form of a nice table. Statistical analysis is well performed.

Author’s Response:

Patient characteristics are given in Table 1. The scoring system used in our study is a common method and, in our opinion, most accurately addresses the heterogeneity encountered in tumor sections. Examples for our assessment of the staining intensity of E-cadherin and FAK have been published previously (Schwock et al., Cancer Res. 2009 Jun 1;69(11):4750-9.)
3.) Results section is explicitly long and unstructured. Authors should concentrate on the main findings and extensively reduce results section in length. Currently there are more than 5 pages! The antibody testing including animal models should be — although performed very well — shortened. The chapter “SNAI1 is a Potential Mediator of OSCC Aggressive Behaviour” should be shortened because there are actually no significances for SNAI1 as the main marker aimed to investigate in the study. The two cases presented at the end of the results do not contribute to the message of the study since these are exceptions and the study was aimed to get a general impression. May be these two interesting cases can be separately presented as case reports.

Author’s Response:

The Results section has been restructured in its entirety for a clearer and more linear presentation of the study’s main findings. The section related to antibody testing has been incorporated into Material & Methods. Section headers have been changed to more accurately reflect the data presented in each sub-section. We disagree with the view to separate the two cases with sarcomatoid component from the main paper since both cases in conjunction with the entire cohort precisely reflect the spectrum of EMT phenotypes that can be encountered in a series of OSCC specimens.

4.) Discussion / Conclusions: Conclusions made by the authors are not completely supported by the data presented in this study. To conclude that SNAI1 contributes to tumour progression in OSCC from 2 cases exhibiting a sarcomatoid phenotype is a little speculative. The statement that its “presence in a significant portion…is associated with …poor prognosis” bases on a minimal number of cases (4 of 46) not allowing such a conclusion in a study where the majority of cases exhibited rare SNAI1 expression levels. Again, this might be a justified conclusion from a case report.

Author’s Response:

The wording in the section criticised by the reviewer has been changed to account for the uncertainties caused by the low number of cases. We previously acknowledged the drawbacks associated with the low case number in our exploratory study. On the other hand, full section analysis on primary tumors and corresponding metastasis as performed allowed for a detailed examination of the individual cases as reflected by the identification of two tumors with EMT-/sarcomatoid component.

Minor Essential Revisions:
1.) The aims section at the end of the introduction is uncommon. Results and conclusions of the study are partially presented here. Is this what the journal structure requires?

Author’s Response:

The paragraph referred to by the reviewer has been deleted from the manuscript.

2.) In general, there are clearly too many figures and tables in the study even if planned to publish as online supplements. Please reduce them to the main findings making it possible to the reader to visually fathom the results adequately.

Author’s Response:

Table 2 has been deleted and the entire manuscript has been re-structured. Five figures accurately illustrate the data presented in the main manuscript. Supplementary figures can be accessed if additional information is desired by the reader.
(Note: Reviewer R3 earlier asked for an additional table to illustrate the scoring system used.)

Discretionary Revisions:
These revisions have been included in the statements above.
Taken together, the study represents only a limited contribution to the knowledge in the field although is contains some interesting data. Authors should restructure the manuscript strictly focussing on the main new results.

Author’s Response:

The authors agree with this comment and the manuscript has been re-structured for increased focus.
Level of interest: An article whose findings are important to those with closely related research interests
Quality of written English: Acceptable
Statistical review: No, the manuscript does not need to be seen by a statistician.
Declaration of competing interests:
I declare that I have no competing interests.
R4: Reviewer's report

Title: SNAI1 Expression: A Rare Event In Oral Squamous Cell Carcinoma
Associated With Features Of Poor Prognosis
Version: 1 Date: 22 October 2009
Reviewer: Takashi T Takata

Reviewer's report:
In this paper, authors examined the expression of SNAI1 and compared with clinicopathological parameters, E-cadherin, FAK and p63 expression in 46 oral squamous cell carcinoma (OSCC) cases by immunohistochemistry. For the immunohistochemical analysis, authors characterized an antibody (Suppl. Fig. 1). By using this antibody, authors found that SNAI1 was positive in 10 of 46 OSCC cases and SNAI1 positive cases showed poor prognosis. However, NAI1 expression was not correlated with E-cadherin, FAK and p63 expression. In two cases with sarcomatoid component, SNAI1(+)/FAK(+)/E-cadherin(-)/p63(-) phenotype was observed. Authors concluded that SNAI1 expression is rare in OSCC, but SNAI1 expression can indicate the presence of a sarcomatoid component. Although so far there are many studies on Snail related EMT in cancer, this paper contains interesting results. As SNAI1 expression is rare, authors should increase the number of cases and check the detailed analysis of sarcomatoid component in OSCC. I think two cases are not enough to demonstrate your hypothesis. Overall, I feel that this paper contains interesting information which merit publication, but authors should perform additional experiments.

Author’s Response:
The study described in our manuscript was intended to explore the feasibility of detecting different EMT-related markers in clinical material. Until recently several authors have pointed out the lack of specific commercially available antibodies for SNAI1 detection in FFPE specimens (Rosivatz et al., Virchows Arch, 2006; Franci et al., PLoS One, 2009), and previous studies have reported conflicting results based on IHC. Thus, we felt that it was appropriate to characterize the specificity of two commercially available anti-SNAI1 antibodies, and to describe their staining pattern in conjunction with other EMT-related markers as a sound foundation for future studies. Further studies are in development and will involve a larger number of cases possibly taking advantage of tissue microarray technology.

Level of interest: An article whose findings are important to those with closely related research interests
Quality of written English: Acceptable
Statistical review: No, the manuscript does not need to be seen by a statistician.
Declaration of competing interests:
I declare that I have no competing interests
R5: Reviewer's report

Title: SNAI1 Expression: A Rare Event In Oral Squamous Cell Carcinoma Associated With Features Of Poor Prognosis

Version: 1 Date: 3 November 2009
Reviewer: Lorenzo Lo Muzio

Reviewer's report:
SNAI1 Expression: A Rare Event In Oral Squamous Cell Carcinoma Associated With Features Of Poor Prognosis

The topic of the paper is interesting and may potentially contribute to the understanding of the oral cancer scenario. Nonetheless, the paper has some minor/major problematic which need to be carefully addressed before it can be considered suitable for publication. In fact, a considerable focus is made on the expression of different molecules, which in some sections, especially in the results, seems to overcome what should be the focus of the paper, according to the title. In addition, relationships among the different reported molecules and SNAI1 expression is not always evident and clear; e.g. "a trend seemed to appear with high FAKc expression... further indicating a link between the kinase and tumor progression. E-cadherin loss (score 2 in >90% area) was associated with a significantly higher proportion of positive nodal status at surgery... E-cadherin loss was not associated with shorter EFS in our cohort". Thus, authors are requested to better target and focus on the issue addressed in the title and its relationships with the other molecules.

Author’s Response:

The manuscript has been restructured to clearly address this comment and visibly place the focus on SNAI1 expression. This, we would expect, is also reflected by the revised title emphasizing the immunohistochemical examination of SNAI1 – in conjunction with three other markers (E-cadherin, FAK and p63) which collectively indicate EMT-like change in epithelial neoplasms.

Here are some other comments for authors use in revising their paper:
- In the Abstract, the Results section should be revised according to the general comment; in particular, lines 49-50 should be removed.

Author’s Response:

The sentence related to E-cadherin and FAK has been moved to the end of the sub-section to more appropriate reflect its position in the context of the entire manuscript. However, it is still pertinent information since it indicates the potential clinical importance of EMT-related phenotypic alterations.

- Lines 92-100: it is not necessary to cite methodological details and the obtained results in this section. Thus, the corresponding lines should be moved in the appropriate section or deleted.

Author’s Response:

This part of the Background section has been replaced with more appropriate information.

- Lines 178-192: these lines should be moved to the methods section.

Author’s Response:

This part of the manuscript has been moved to Material & Methods as requested.

- Conclusions should be limited to results of the present study.

Author’s Response:

The conclusions have been changed to reflect the results of the study and the context associated with it.
Level of interest: An article of outstanding merit and interest in its field
Quality of written English: Acceptable
Statistical review: Yes, and I have assessed the statistics in my report.
Declaration of competing interests: ‘I declare that I have no competing interests

We would like to thank the reviewers for assessing our manuscript and providing thoughtful comments.

With kind regards,

Joerg Schwock DrMed
William R Geddie MD FRCPC

Toronto, November 27 - 2009