Author's response to reviews

Title: Bax Expression Measured by AQUAnalysis is an Independent Prognostic Marker in Oral Squamous Cell Carcinoma

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Author's response to reviews: see over
July 01, 2012

Dr. Christna Chap
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Dear Dr. Chap,

Re: Bax Expression Measured by AQUAnalysis® is an Independent Prognostic Marker in Oral Squamous Cell Carcinoma.

We thank Drs. Chen and Camisasca for their feedback and constructive comments on our manuscript and appreciate the positive review provided by Dr. Coutinho-Camillo. As a result of the reviewers’ suggestions we have made significant changes in the revised version of the manuscript. We hope that these changes are satisfactory and that the revised manuscript can be accepted for publication in BMC Cancer.

The following are our responses to the comments made by the reviewer:

Dr. Wantao Chen (Reviewer 2)

Comment 1: “Since the radiation therapy is one of major treatment of OSCC, which definitely will influence the patients’ prognosis, the author should disclose some detail information about the patients’ treatment method, such as how to decide if radiation therapy is included or not included in the treatment of patients in this study”.

Response: We thank the reviewer for this suggestion and have modified the Methods section (Cohort Identification) to reflect treatment selection in more detail.

Comment 2: “Authors observed a positive correlation between Bax and Ki-67 and then proposed that patients with Bax overexpression maybe benefit from radiotherapy. From the data provided by this paper, this proposal is not suitable. Also, usually high expression of Ki-67 usually is linked to increasing proliferation and malignant behavior of cancer cells, in other words, poor prognosis of patients. The authors should give some explanations on this point”.

Response: We acknowledge that the data presented in the initial version of the manuscript were not sufficient to determine whether patients with Bax overexpression might benefit from radiotherapy. As a result of the reviewer’s comment, we have added Supplementary Figure 3 to the revised manuscript. The figure demonstrates that the association between Bax expression and 5-year disease-specific survival (DSS) was only observed in patients who received adjuvant radiotherapy after surgery. Although, a prospective study is required to establish the predictive nature of Bax overexpression, we believe that the retrospective data provided in this study supports the concept of increased
sensitivity towards radiation treatment in patients with Bax-overexpressing tumors.

We appreciate the reviewer’s comments on Ki67 expression being associated with worse prognosis. Although this might be the case overall, there is evidence in the literature for Ki67 expression being associated with a better response to adjuvant therapy. This might be explained by the observation that both chemotherapy and radiotherapy require proliferation to manifest cell killing. In a recent study performed in a cohort of 121 OSCC patients, we showed that high Ki67 expression is a marker of good prognosis in OSCC (Klimowicz and Bose et al., European Journal of Cancer; Accepted). We also found that this effect was most pronounced in patients treated with adjuvant radiation after surgery.

Comment 3: “In Tab1, since the sample number is not consistent in each group, the authors should include an explanation why the number is not consistent”.
Response: We acknowledge that the inconsistency in sample numbers in groups analyzed for biomarker expression was confusing. We have clarified this variation in sample number in the revised manuscript. As a result of the reviewer’s comments, we have modified the Methods section (Quantitative Fluorescent Immunohistochemistry) to reflect that cores with insufficient tumor area or folded tissue were excluded from analysis. Cores from 64 patients passed such a quality control step for Bax expression analysis, while cores from only 63 patients were suitable for Bcl-2 and Bcl-XL expression analyses. Since a similar lack of clarity also existed in Figure 1 (as mentioned by Reviewer 3), and the reference to Figure 1 precedes Table 1 in the text, we have modified the legend for Figure 1 to better explain the variation in sample number among groups analyzed for biomarker expression.

Comment 4: “In Fig 1, since the size of tumor nests is obviously different between the middle and the lower row, the authors should explain how they justified the variation of the fluorescent intensity between them is not influence by the different size of the tumor nest”?
Response: An AQUA score is calculated by dividing the exposure time-adjusted pixel intensity with the area of the particular compartment. Therefore, AQUA scores represent the average pixel intensity density in the compartment area of interest (e.g. tumor area) and are not affected by tumor nest size variation between cores. We have adjusted the wording in the Methods Section (Quantitative Fluorescent Immunohistochemistry) to further clarify this message.

Minor revisions:

“In this paper, the authors used the median AQUA score in normal OCSE to define if the protein is overexpressed in tumor, but there are only 5 samples in normal OCSE group, the size is too small which may bring some bias”.
Response: We appreciate the reviewer’s concern regarding the small sample size of the normal OCSE group. However, since a 95% CI around the median normal OCSE AQUA score was used to define overexpression, the small sample size
leads to a larger, more conservative, CI range. We believe that by using this definition, we provide a relevant and conservative assessment of the overexpression for a particular biomarker in the tumor.

Dr. Danielle Camisasca (Reviewer 2)

Major Compulsory Revisions:

Comment 1: “page 7 (TMA): Clarify in the manuscript text why pre-treatment FFPE were used. The sample was represented then by biopsy samples? If so, make it clear in the text. Hematoxylin and eosin revision and tumor grading was based in these pre-treatment FFPE slides? Once all patients were submitted to primary surgery, the pathology report should be based on surgical specimens, which better demonstrate tumor biology and probable behavior”.

Response: We recognize that the designation “pre-treatment FFPE” for our tumor samples was potentially confusing. Consequently, we have changed “pre-treatment FFPE” to “surgically-resected, treatment naïve” which better represents the source of our samples. Also, the haematoxylin and eosin revision and tumor grading was based on tissue obtained from surgically-resected specimens.

Comment 2: “page 7 (TMA): Authors state that “blocks of sufficient quality” were selected. Details about this selection should be given – was the front of invasion used in all cases? How many areas were used to represent each case? What were the criteria used in this selection”?

Response: The Methods section (TMA Construction) has been modified to more accurately reflect our FFPE block selection strategy. Briefly, blocks were selected if they contained sufficient tumor tissue to yield at least three cores for TMA construction. Three cores were randomly obtained from the tumor-containing area of an FFPE block for each patient.

Comment 3: “page 7 (TMA): What was the source of the normal epithelia (OCSE)? Explain it in the manuscript text”.

Response: Normal OCSE was obtained from FFPE blocks separate from those containing OSCC. The Methods section (TMA Construction) has been modified to reflect the source of normal tissue.

Comment 4: “page 8 Statistical Analysis: Detail Cox analysis (what was the method employed – Wald/Enter? Different models were used? Why was the one presented in the manuscript chosen?). If univariate analysis (Kaplan Meier) was performed to investigate survival probabilities according to clinicopathologic variables, this should be shown in a table and significant variables could also be added to the Cox model”.

Response: The Enter method was used to generate the Cox model in Table 2. The two clinical covariates most significantly associated with DSS in univariate analysis (see updated Table 1) were included in the Cox model. As explained in
the Methods section (Statistical Analysis), the stage covariate was not included in
the Cox model because it is collinear with pT and pN. Based on the importance
of pT and pN clinically, we believe that this is the most relevant model to use.
Finally, the use of more than two or three covariates in our Cox analysis would
risk over-fitting, considering that we only have 19 events in our cohort as shown

Table 1 has been revised to include univariate analyses for the clinicopathological variables used.

We hope that these changes adequately reflect the helpful suggestions
offered by this reviewer.

Comment 5: "page 10: Ki67 is used as a reference to be compared to Bax, Bcl-2 and
Bcl-X expression. As such, it should be included in the Methods section".
Response: We thank the reviewer for highlighting our omission of the Ki67
staining protocol in the Methods section. The Methods section (Quantitative
Fluorescent Immunohistochemistry) had been revised to include the staining
protocol for Ki67.

Comment 6: "page 11: one or two tables should be added to show the data that are
described as “data not shown”.
Response: Two supplementary figures have been added to present the data that
was previously referred to as “data not shown”. Supplementary Figure 1 shows a
Kaplan-Meier plot for the associations between Ki67 and DSS; Supplementary
Figure 2 shows Kaplan-Meier plots for the associations of Bcl-2/Bax and Bcl-
XL/Bax ratios with DSS.

Comment 7: "page 12: Change the sentence “lack of reproducibility in these studies has
precluded their clinical use”. In order to be accepted and published, methods in every
research should be able to be reproduced. Even semi-quantitative methods are
supposed to be reproducible. At least one study [22] also employs an automated
quantitative IHC, which is further explained by Bernardo V et al. in Microsc Microanal.
Response: “lack of reproducibility...” has been changed to “lack of consensus in
these studies has precluded the use of apoptotic protein expression as a
biomarker in the clinic”.

Comment 8: “Figure 1: What happened to the 5 or 6 cases that were not included for
IHC analysis? Did they fall off the slides? The same 63 patients were analyzed for all
three proteins”?
Response: We acknowledge that the inconsistency in sample numbers was
confusing. We have clarified this variation in sample number in the manuscript.
The legend for Figure 1 and the Methods section (TMA Construction) has been
modified to explain this inconsistency in sample number (also see response to
Comment 3 from Reviewer 2). The same 63 patients were analyzed for each
biomarker. Bax expression analysis included one extra patient that had available
cores for analysis.
Minor revisions:

Comment 9: “Background, page 5, last paragraph: … are expected TO act”
Response: DONE

Comment 10: “Results, page 9 – standardize Bax writing (BAX/Bax)”
Response: DONE

In conclusion, we again wish to thank the reviewers for their careful and insightful comments that have improved both the quality and clarity of our manuscript. We now hope the editors will accept our manuscript for publication in BMC Cancer.

Yours sincerely,

Joseph C. Dort
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