Author’s response to reviews

Title: Expression of ERBB3 binding protein 1 (EBP1) in salivary adenoid cystic carcinoma and its clinicopathological relevance

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Author’s response to reviews: see over
Dear Ms Lacson:

Thank you for your email of August 22, 2012 indicating that our manuscript can be considered further pending incorporation of changes suggested by the Editor and Reviewers. I appreciate the thoughtful and careful critiques. We have revised the manuscript in accordance with the Editorial requests and Reviewer’s comments as follows:

Editorial request(s)

(1) Requesting ethics committee:
We have included the name of the body which gave approval to the current study, with a reference number in the Methods section of the manuscript. Please see attached document from the committee.

(2) Copy editing:
Following your recommendation, we have sent our manuscript to Edanz for editing service as evidenced in the resubmitted manuscript. Based on that version, we have revised and highlighted (with yellow) all changes suggested by the Reviewers. We also received a similar version edited by Dr. Anne Hamburger at University of Maryland, USA (who originally cloned EBP1 gene, ahamburg@som.umaryland.edu). Both Edanz and Dr. Hamburger are acknowledged in the manuscript.

Reviewer #1

1. Figure 1. Magnification of relevant parts of the images is included as insets. Negative control for antibody specificity is originally described in the Methods “Immunohistochemistry” line 13 and shown in Figure 1 D. D is adjacent non-cancerous tissue of solid pattern of ACC, incubated with concentration-matched non-immune rabbit IgG that showed no staining. Since we received similar concerns from Reviewer #2 (MAJOR POINT), I apologize for the confusion. We have clarified the description in the Figure Legend and included adjacent non-cancerous tissues of tubular (B3) and cribriform (C3) patterns of ACCs as negative controls suggested by Reviewer #2 (MAJOR POINT). D is now A3 in the legend. Protein levels of EBP1 are higher in adjacent non-cancerous tissues; we thus use adjacent non-cancerous tissues (B3 and C3) to test rabbit polyclonal anti-EBP1 antibody specificity by incubating concentration-matched non-immune rabbit IgG

2. Figure 2, panels A&B. The Reviewer brings up an important point that the data would be strengthened if a non-functional (mutated) Ebp1 variant is included as negative control. However, this hypothesis is outside the scope of the current investigation to profile the expression levels of EBP1 in primary tumor and adjacent non-cancerous tissues. The EBP1 antibody cannot tell functional from non-functional (mutated) protein variant. Based on the current findings, we are now furthering our study including sequencing the EBP1 gene in those tissues to understand if the IHC staining is normal or mutated protein. We will then compare the impacts from normal and mutated EBP1 variant on cell proliferation and migration in the cell
lines as we discussed in the Discussion. We have received mutated EBP1 cDNA constructs from Dr Hamburger.

3. Figure 2, panel B. I apologize for the confusion. We used the same cell lines as described in Reference #12. In “Cell Culture”, we have thus included “We used ACC-M, ACC-M0 (ACC-M-pcDNA3.1) and ACC-M1 (ACC-M-EBP1-1µg) as Control, Vector and ebp1 (different from the protein and gene names)”. The EBP1 protein levels in Control, Vector and ebp1 were published in Reference #12

4. Figure 2, panel C. The blots are substituted by better quality gels.

5. We do not know yet how EBP1 levels are regulated in tumor lesions versus non-cancerous tissues. It will take years to work out the answer to this very important question. We are currently examining EBP1 mRNA levels change among different tissues as we discussed on page 12 in the Discussion and also please see our reply to 2.

The text has been revised by Dr. Hamburger and Edanz.

Reviewer #2

MAJOR POINT
Fig. 1D: I apologize for the confusion again and please see our reply to Reviewer #1 Figure 1.

MINOR POINTS:

We thank Reviewer for careful proofreading and I apologize for usage and typographical errors.

1. Introduction, page 2, line 15: “...neoplasmas...”. We would respectfully use “neoplasms” as edited by Edanz.

2. Introduction, page 3, line 5: “...in consistent...” “in” is omitted by Edanz.

3. Materials and methods, page 3: We have clearly described the Patient and Specimens.

4. Material and methods, page 4: The pretreatment was the same for all staining. We have specified “at room temperature” and “90°C” as required by the Reviewer. Basically, the text is “Endogenous peroxides were quenched by treatment with 0.3% H2O2/methanol for 30 min at room temperature. Antigen retrieval was accomplished by microwave heating at 90°C for 15 min in citrate buffer (10 mM, pH 6.0).”

5. Results section: We have rounded the results to one significant digit.

6. Results section, page 7, line 11, “...olid...” is edited as “solid” by Edanz.

7. Discussion section, page 10, line 13: ... my interpret... is replaced with “...may suggest...” as highlighted with yellow.

8. Discussion section, page 11, line 10: “...intergrin...” is edited by Edanz as “...integrin...”.

Reviewer #3
Minor Essential Revisions:
1. English revision is done by Dr. Hamburger and Edanz recommended by BioMed Central as we detailed in our replies to Editorial request(s) and Reviewer #1.

2. In the Title we have put the abbreviation (EBP1) after "ErbB3 binding protein" as suggested by Reviewer and highlighted it with yellow.

3. Introduction: We put in parenthesis "(ACC)" in the first line after the whole name of the tumor "adenoid cystic carcinoma", not in the second one.

4. Methods: Immunohistochemistry: This is the first report we stained adenoid cystic carcinoma and adjacent non-cancerous tissues with EBP1 antibody. Previous work, mainly from Dr Hamburger’s group, demonstrated that EBP1 is expressed and localized in both cytoplasm and nucleus of prostate and breast cell lines (Reference #15). Squatrito et al. found that both the N-terminal and the C-terminal regions of EBP1 are required for correct EBP1 localization, and that nucleolar localization is necessary for its growth suppression activity (Reference #27). EBP1 was reported to be mutated in 22% of patients with colorectal cancers (Reference #28). In our current study, we found that in ACC, EBP1 staining was localized predominantly to the cytoplasm of epithelial cells of glands, whereas in the adjacent non-cancerous tissues, abundant EBP1 immunoreactivity was observed in both the cytoplasm and nuclei. What we can specify is the negative control that incubation with purified normal rabbit polyclonal IgG did not result in any staining in adjacent non-cancerous tissues, indicating the specificity for EBP1 antibody. These intriguing findings were discussed in the Discussion line 24. Since no reference for EBP1 staining in the ACC tissues so far, we only can arbitrarily specify that the percentage of cells with positive EBP1 staining was semi-quantitatively assessed using a four-tiered scoring system as described in the Methods Immunohistochemistry.

5. In Result, Discussion, Tables and Figures, we have replaced the whole name "adenoid cystic carcinoma" with the acronimous of ACC and highlighted ACC with yellow.

We thank you again for the constructive review. I believe the suggested changes have considerably strengthened the manuscript. I greatly appreciate your consideration of our work.

Sincerely,

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