Author's response to reviews

Title: Hornerin, an S100 family protein, is functional in breast cells and aberrantly expressed in breast cancer

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Author's response to reviews: see over
Response to Reviewers’ Comments for submission MS# 8599613426869894 entitled “Hornerin, an S100 family protein, is functional in breast cells and aberrantly expressed in breast cancer”

We thank the reviewers for their helpful and insightful comments. We have incorporated the reviewers’ suggestions and comments into the revised and now much stronger manuscript. Our point-by-point response to the reviewers’ comments can be found below.

Response to Reviewers Comments: In all cases, author responses are highlighted.

Referee #1, Sandra Z Haslam

Figure 2.

1. It is stated that based on quantitation of hornerin Ab staining hornerin expression was significantly increased during involution. However, it is noted that nuclear hemotoxyin staining is also increased during involution (Fig 2A). In order to accurately quantify hornerin staining without introducing the nuclear stain artifact it is necessary to do quantitation on sections that are not counterstained. Otherwise it is necessary to convince this reviewer that nuclear stain is not contributing to the interpretation of increased hornerin expression.

To address this comment, we have added a new supplemental figure (new Supplemental Fig. 1), which shows the images of the threshold value that were used for quantitation. As shown in the figure, the conversion of the image to an RGB stack and the analysis in ImageJ 64 removes the possible confounding hematoxylin counterstain. The images also readily show that it is the brown stain, and not the nuclei, that contribute to the threshold value used for the quantitation.

2. In figure 2C the dark staining cells are referred to as macrophages. How were these cells identified to be macrophages – staining with a macrophage specific marker is needed.

As suggested by the Referee, we have performed dual immunofluorescent staining with a hornerin antibody and macrophage specific antibodies for each species. The new data validates that the co-immunostaining is localized within the macrophages and has been added to the manuscript (new Supplemental Fig. 2).

Minor comment: Introducing LPS stimulated blood derived monocytes does not appear to be relevant.

To highlight the observation that hornerin was found in differentiated macrophages, both undifferentiated monocytes and monocytes differentiated into macrophages via LPS-stimulation in vitro were analyzed. To clarify this point, we have edited the manuscript. It now reads on Pg. 7: “Low levels of hornerin were present in the undifferentiated cells, while treatment with LPS/INFγ stimulated a significant increase in hornerin expression in the macrophages. The observed increase in hornerin expression in the differentiated macrophages compared to undifferentiated monocytes suggests the possibility of a functional role for hornerin in phagocytic macrophages.”

Figure 3.

1. For 3C Western blot quantitation: the bands for 245KDa intact and 100KDa fragment of hornerin need to be normalized to tubulin expression and ratio of hornerin:tubulin should be plotted and not “relative units”. The only correct conclusion based on statistical analysis is that the 245KDa was differentially expressed and not the 100KDa fragment. The wording in results needs to be changed.
We thank the Referee for suggesting this alternative analysis. We have calculated the ratio of hornerin to tubulin and replaced the data in the figure with the data generated from the new analysis. We have also updated the wording in the results. The manuscript now reads: “Western analysis also demonstrated posttranslational proteolytic processing of hornerin, similar to previous studies in skin [12-15]. Fragments at 50, 80, and 100 kDa were observed. However, only the 100 kDa fragment showed differential regulation when comparing the premalignant to the malignant cell lines (Fig 3B, C; P<0.05).”

Figure 5

Minor - What expression (RNA, protein) was measured to establish no difference for receptor status among the various cell lines? Please clarify in the text.

The level of ER and PR status was measured via PCR and also referenced via Neve et al., 2006. To clarify, we have updated the manuscript to read: “No correlation was observed with estrogen receptor (ER) and progesterone receptor (PR) status and hornerin expression (MCF7, T47D, ZR75.1, MCF10AI, MCF10Ca1h, MDA MB231, SUM159, MDA MB468 cell lines; analyzed via PCR, data not shown and referenced in [32]).”

1. Fig 5C. It is not clear what the 2 panels each for N-term and C-term are and what they are intended to show. Please explain. For the MDA MB231 and SUM149 images it appears there is significant cytoplasmic staining - how are membrane and cytoplasmic staining distinguished? Need to describe.

For each cell line, the top panel shows hornerin staining (green) and the second image shows hornerin staining as well as nuclear staining with DAPI (blue). This way the reader can vision both the nuclei as well as images without the DNA stain. The authors feel it is important to show the nuclear staining as well as provide an image without the DAPI stain for best visualization of the presence and absence of hornerin in the nucleus. To clarify the image for the reader, we have edited the figure legend to read “For each cell line, the top panel shows hornerin staining (green), while the bottom panel shows both the nuclear (DNA stained with DAPI, blue) and hornerin staining (green).”

Additionally, for the nuclear vs. cytoplasmic/membrane staining, the striking difference is the presence or absence of hornerin in the nucleus. We did not distinguish between membrane and cytoplasmic staining in the immunocytofluorescence. To address the Referee’s comment, we have modified the manuscript to more accurately read: “Confocal microscopy demonstrated a pattern of predominantly cytoplasmic/membrane localization of hornerin using the N-terminus antibody with comparatively low levels of nuclear localization, while the C-terminus antibody demonstrated a comparably stronger nuclear accumulation for all cell lines tested (Fig. 5C).”

Discussion

Minor comment: Too much of the discussion refers to other findings that are speculatively used to support the putative association of hornerin expression with apoptosis/necrosis and less aggressive breast cancers.

Because of our difficulty in producing a recombinant hornerin protein to directly demonstrate whether the increase in mRNA and protein fragmentation promoted cell survival or cell death, we searched the literature for other relevant examples with other S100 family members (of which there are many) to support our data. We appreciate the Reviewer’s viewpoint that the discussion may contain too much speculation; we have condensed this section a bit.
Referee #2, Partha Roy Roy
Overall, the expression data, particularly those involving differential regulation of post-translational fragment are interesting. However, a major weakness of this paper is its descriptive nature. Altered expression of hornerin correlated with malignancy is interesting but does not provide the evidence of a causal relationship between the two. The same criticism goes for apoptosis experiments. The paper will be much stronger if the authors report additional findings on the effects of knockdown and overexpression of hornerin on invasiveness and apoptosis of some of the cell lines.

We thank the Referee for their astute comment that our data is “particularly those involving differential regulation of post-translational fragment are interesting.” Unfortunately, as stated in the paper, we were unable to perform knockdown or overexpression of hornerin due to its complex, repetitive, and tremendously large sequence. Therefore, we are unable to perform the suggested knockdown or overexpression experiments as suggested by the Referee. Our team of proteomic experts will concur with this statement and would be happy to discuss the complexities of this protein in depth with anyone interested. Dominic Esposito Phone: 301-846-7376 Email: espositod@mail.nih.gov

Specific Comments

1) Fig 1C: From the bar graph on the right, it is not clear whether there is any statistically significant difference in 100 kD fragment between the different MCF10A sublines.

Assuming the Referee is referring to Fig. 3C and not Fig. 1C, the amount of fragment decreased within the more aggressive phenotypes. After performing the new analysis based on the recommendations of Referee #1 and using the ratio of hornerin:tubulin, the decreased amount of 100 KDa fragment in the malignant cell lines was determined as significant ($P<0.05$). We have updated the manuscript for clarification. It now states: “Western analysis also demonstrated posttranslational proteolytic processing of hornerin, similar to previous studies in skin [12-15]. Fragments at 50, 80, and 100 kDa were observed. However, only the 100 kDa fragment showed differential regulation when comparing the premalignant to the malignant cell lines (Fig 3B, C; $P<0.05$).”

2) Fig 4D: If lower hornerin expression is correlated with less aggressive phenotype, why do lymph-node positive and higher grade tumors have lower hornerin expression than lymph-node negative and low grade tumors, respectively?

The Referee is correct. The less aggressive phenotypes express higher levels of hornerin. Therefore, the metastatic lymph-node positive and higher-grade tumors have lower hornerin expression.