Author’s response to reviews

Title: Immunohistochemical analysis of ezrin-radixin-moesin-binding phosphoprotein 50 in prostatic adenocarcinoma

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Author's response to reviews: see over
Dear Editors and Reviewers,

We thank you for your consideration of our article, “Immunohistochemical analysis of ezrin-radixin-moesin-binding phosphoprotein 50 in prostatic adenocarcinoma”, and for your helpful suggestions. We address them individually below.

Reviewer #1:

While it is an interesting discovery that expression is highest in HGPIN, goes down somewhat in primary cancer, and goes way down in metastatic cancer, it remains uncertain whether the immunoreactivity in primary prostate cancer is predictive of co-existing or future metastatic potential. This lessens the value of the findings, making the findings just correlative rather than prognostic.

The addition of immunostaining results on primary prostate cancer with known biochemical failure, or with known metastatic outcome, versus known non-failure or known non-progression outcome, would greatly strengthen the paper. An example of this is a paper on EZH2 protein (Varambally S et al. The polycomb group protein EZH2 is involved in progression of prostate cancer. Nature 2002;419:624-9.) The authors examined the increase in this protein by staining 23 primary prostate tissues among 1023 tissue microarray elements from men who died of metastatic prostate cancer. A resource for studying this for EBP50 would be the CPCTR (www.cpctr.info/tma.html) which has, for 21 patients, both the primary and the metastatic lymph node lesion. Since stage and grade do not correlate with staining, is weak staining in the primary cancer tissue predictive of future/concurrent metastasis? Or is staining in the primary tissue relatively strong, and weak staining is solely a function of being in a metastatic site? The authors could demonstrate this without the CPCTR by just using up to 10 primary prostate cancers in which a history positive for metastasis is known, and comparing staining intensity with primary cancer tissue in those men known to have only localized non-progressing cancer.

Unfortunately, this follow-up data was not available for the tumor specimens in our tissue microarrays. Additionally, we do not have enough specimens of matched tumor/mets to make this comparison. We contacted the CPCTR about the TMA in question, and unfortunately, it has been exhausted and is no longer available for order. We now address this possibility in our paper and discuss it as a limitation of our work.

Page 10, 3rd paragraph:

“Despite this, however, it is important to note that these findings may also contain a correlative component that is not prognostic in nature. While all of the tumors in this study were primary tumors at the time of specimen retrieval, definitive follow-up information on these patients was not available. In this sense, from this study it is not possible to rule out that the decreased EBP50 expression, at least in part, may be due to the metastatic location itself. The current results, however, indicate and warrant later phase biomarker studies[20] that will longitudinally correlate EBP50 expression directly
with patient outcomes to further evaluate its potential to predict metastatic risk in prostate cancer.

Figure 2 should be omitted. This is because we have a mean staining of 139, 131, and 137 for stages 2, 3, and 4 respectively, and these groups consist of 39 men, 37 men, and 27 men respectively. The difference between stages 2 and 3, although statistically significant, is not meaningful or logical. The fact that this may be a statistical fluke is handled satisfactorily in the Discussion, but it doesn't deserve its own Figure.

We have now deleted this figure and Figure 3 (for the same reason) and all references to them from the manuscript.

Lastly, I wouldn't make too much of the membranous versus cytoplasmic distinction, as I cannot appreciate such a difference between Fig. 4C (benign) and Fig. 4E (cancer); and the staining in metastases is too faint to determine whether it is cytoplasmic or membranous--giving the impression of 0/36 cases with cytoplasmic staining which may not be valid.

We have now modified our Discussion to reflect this.

Page 14, final paragraph:
“\textit{It is also important to note, however, as this membranous/cytoplasmic distinction was not noted in all comparisons between benign and cancerous specimens, that an alternate explanation may account for these findings. As a general decrease in overall staining intensity was observed between the benign specimens and the cancerous/metastatic specimens, this lack of membranous staining may simply reflect an overall staining decrease, making it difficult to appreciate the true ratio of membranous to cytoplasmic staining from a visual examination. This is especially true in the cases of metastatic cancer, where the overall staining is very faint across all specimens in the first place.}\"

Minor issues:
Abstract line 2 The data was---> were
Introduction line 4 over diagnoses---> one word
paragraph 4 line 3 et al, --> et al.
paragraph 5 line 2 and line 3 over expressed---> one word; also in Discussion page 11
last paragraph line 2 series 11 cases --> series of 11 cases
Methods line 7 35 cases of HGPIN---> just say cases of ISOLATED HGPIN (no accompanying cancer diagnosed)
Page 9, third-last line. co-accompanying---> coinciding, or coexistent
Discussion: Fig. 1 belongs to the Results section, not the Discussion

We have now made these corrections.
How many cases on the microarray were duplicate only rather than triplicates?

We now modify the manuscript to include this information.

Page 6, first paragraph

"This occurred for three cases of the HGPIN, three cases of the Mets, three cases of the NAC, two cases of the BPH, and eight cases of the PCa."

Reviewer # 2

I believe this paper will be of interest to those in prostate cancer biology as few studies have examined this question. I do have some suggestions that I believe will improve the conclusions of the study. First, I would attempt to clarify how the automated scoring was performed. My concern would be that the overall intensity of staining would be related to the relative density of glandular elements in the core since the stroma does not appear to have staining. In other words, could the higher scores be a reflection of more density of glands? If possible, the intensity scores should be proportionate to the glandular area on the slide.

We have now modified the manuscript to address this.

Page 6, last paragraph

"By analyzing the average pixel intensity with a predetermined hue value and width, the stromal tissue and cell nuclei that appear blue and do not feature the immunostain are negated by the software and excluded from the final analysis that determines the average staining intensity. This, in effect, controls for the glandular to stromal tissue ratio present in the TMA cores."

My other recommendation would be that the figures reporting the mean intensity score be shown as dot displays with error bars so readers can see the distribution of values more easily.

For Figure 1, we now show both the means with error bars and as dot displays of the individual data points, as Figure 1a and Figure 1b. Per request, Figure 2 and 3 have now been removed from the results.

We have modified the Figure Legend as follows.
Figure 1 - EBP50 staining intensity by prostatic tissue type

A) Mean EBP50 staining score by prostatic tissue type. Significant differences were seen between Mets and NDP (p=0.027), NAC (p<0.001), BPH (p<0.012), PIN (p<0.001), and PCa (p=0.006), with Mets having the lowest staining of any group. PIN also had the highest staining of any group, and was significantly higher than PCa (p<0.001) and NAC (p<0.009) groups. B) Box plots of EBP50 Staining Intensities by Tissue Type Featuring Individual Cases. It is especially notable that many specimens in the PCa and Mets classifications feature staining below that of the other tissue classifications. Moreover, only one case of Mets featured a score above 155, the lowest limit for high intensity staining.

We have also modified the Scoring of Slides section of our Methods (Last paragraph, page 7)...  


Kevin McDade with assistance in graphical design

Thank you very much for your helpful suggestions and consideration of our manuscript.

Tanner Bartholow