Author's response to reviews

Title: WWOX protein expression varies among ovarian carcinoma histotypes and correlates with less favorable outcome.

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Author's response to reviews: see over
Corrections:

**Referee 1:**

1.) I am unclear as to how the WWOX cutoff levels were decided on. It would be useful if the authors would address this issue. Why was a negative cutoff of 63 decided on?

*The cutoff levels were decided upon evaluation of the 95% confidence interval of staining intensity from the OSE mean values. The cutoff to establish positive and negative staining was determined to be at a mean intensity of 63 (arbitrary units) over a total of 255 (color saturation scale. Cores with values ≤ 63 were negative for WWOX immunostaining, no brown detected.*

2) Why was ER/PR status defined by <5%, 5-40% and >40%. Was this on the authors previous breast ca work? Previous publications have shown histoscores of >120 for ER and >70 for PR to be functionally important for endocrine therapy response in ovarian cancer. It is unclear what the basis for the cutoffs the authors used have been, and this should be explained.


3) Final sentence in the results is wrong. It should read either: “In the PR negative cases 29% (70/240) were WWOX negative, while only 14% (12/83) of the PR positive cases were WWOX negative” or “In the WWOX negative cases 85% (70/82) were PR negative, while only 15% (12/83) were PR positive”

*We agree with the reviewer there was a mistake in the construction of the sentence. We modified the text accordingly and it now reads, “In the WWOX negative cases 85% (70/82) were PR negative, while only 15% (12/83) were PR positive”, as the referee is suggesting.*

4) In the discussion (p15) they state that the association of WWOX with PR but not ER “raise the question of whether the observed positive association between PR loss and WWOX loss is more a consequence of the predominance of WWOX loss in the two aforementioned [clear cell and mucinous] histotypes, rather than a direct mechanistic association between WWOX and PR”.

Since there are only 10 mucinous and 19 clear cell cases (compared to 40 endo and 375 serous cases) this seems unlikely. But it can be easily checked by running the statistics of WWOX and PR association in the serous only (or serous plus endometrioid only).
Two statistical test were running with only serous adenocarcinomas with PR status data: i) ANOVA test comparing WWOX intensity vs. PR status (0:negative, 1:positive)

<table>
<thead>
<tr>
<th>PRCODE</th>
<th>N</th>
<th>Mean</th>
<th>Std. Deviation</th>
<th>Std. Error Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>WWOX_INT</td>
<td>0</td>
<td>41</td>
<td>80.378</td>
<td>14.977</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>16</td>
<td>89.313</td>
<td>12.303</td>
</tr>
</tbody>
</table>

Independent Samples Test

<table>
<thead>
<tr>
<th>F</th>
<th>Sig.</th>
<th>t</th>
<th>df</th>
<th>Sig. (2-tailed)</th>
<th>Mean Difference</th>
<th>Std. Error Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>WWOX_INT</td>
<td>598</td>
<td>.443</td>
<td>-2.120</td>
<td>.039</td>
<td>-8.934</td>
<td>4.215</td>
</tr>
</tbody>
</table>

Such as you can see, WWOX and PR status are correlated in serous adenocarcinomas.

ii) Chi-square test base on WWOX status (0: negative/weak, 1:moderate/strong) and PR status.
Crosstab
Count

<table>
<thead>
<tr>
<th></th>
<th>WWOXCOD2</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>PRCODE</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>7</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>16</td>
</tr>
<tr>
<td>Total</td>
<td>7</td>
<td>50</td>
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</table>

Chi-Square Tests

<table>
<thead>
<tr>
<th></th>
<th>Value</th>
<th>df</th>
<th>Asymp. Sig. (2-sided)</th>
<th>Exact Sig. (2-sided)</th>
<th>Exact Sig. (1-sided)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pearson Chi-Square</td>
<td>3.114</td>
<td>1</td>
<td>0.078</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Continuity Correction</td>
<td>1.731</td>
<td>1</td>
<td>0.188</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Likelihood Ratio</td>
<td>4.985</td>
<td>1</td>
<td>0.026</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fisher's Exact Test</td>
<td></td>
<td></td>
<td></td>
<td>0.173</td>
<td>0.085</td>
</tr>
<tr>
<td>Linear-by-Linear Association</td>
<td>3.060</td>
<td>1</td>
<td>0.080</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N of Valid Cases</td>
<td>57</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Only likelihood ration suggest some association with WWOX status and PR status.

Conclusion: Serous adenocarcinomas cooperate in whole association between WWOX and PR status and it is not exclusively for mucinous or clear cells histotypes.

5) Western blotting: It is not stated how the tissue used for WB was selected and provisionally processed, was it fresh, or snap frozen and used later? The types of tumors studied by WB are not stated, so one cannot assess whether the findings (regarding high and low expression) are concordant with the immuno- results.

The tumors were snap frozen and used later. All the ovarian carcinomas used for Western analyses were of the Serous type or Mixed type with an extensive serous component.

6) Immunostaining: the expression of WWOX was assessed over the whole surface area of each core. It is not clear whether all cores had a comparable amount of the epithelial component of the tumor, which would make a difference. On the same note, the authors mention that for hormone receptor expression, they selected only cores that had at least 10% tumor (page 8) and assessed only 323 cases out of the 444. This gives one the impression that the tumor content across cores was not fairly uniform. Could the authors please clarify these discrepancies?

We understand the reviewer’s concern.
In order to have a fairly good representation of the tumor sample each TMA core was scored individually and the results are presented as the mean of at least two replicate
core samples. Every core was visually inspected for tumor presence and the intensity staining measurements are not influenced by the amount of tumor present in the core. In the case of steroid receptors a minimum number of tumor cells is needed in order to count cells and setting a negative or positive % nuclei.

7) Page 4, third line from the bottom; histotypes and not hystotypes.

*We have modified the text accordingly*

page 10, line 9, corpora and not corpi

*We have modified the text accordingly*

page 15, line 2, the authors call WWOX an enzyme. is this proven?

*Eventhough the activity of WWOX as an enzyme has not biochemically proven. There is little doubt that WWOX is an oxidoreductase enzyme. The characteristics of the protein are extremely telling, it has an extremely conserved central domain almost identical to any Short chain dehydrogenase (SDR domain). This WWOX SDR domain is highly conserved from Drosophila to Humans. All the features for a catalytic activity NAD dependent enzyme are preserved. For more detail we refer the reviewer to Ref.1. It can be predicted with the same degree of certainty as it was predicted that the WW domains are protein-protein interaction domains, a fact already proven in various publications. In fact we are not the only authors who claim that WWOX is an enzyme (even more specifically likely a sex steroid oxidoreductase enzyme), experts on the topic of SDR enzymes have included WWOX among the superfamily of SDR enzymes and published on the topic. For more detail see Ref 16. Duax, W. L. and Ghosh, D. Structure and function of steroid dehydrogenases involved in hypertension, fertility, and cancer. Steroids, 62: 95-100, 1997.*

8) Are the authors sure that the mucinous carcinomas are not of colorectal origin

*We have not doubts that only primary mucinous carcinomas of ovarian origin where included in this cohort of patients. The selection criterion was based on histopathological diagnosis made by specialized ovarian pathologists on whole sections, histochemical staining, immuohistochemical staining, and clinical assessment of the patient. This paragraph has been included in materials and methods section.*

9) It is curious that the proportion of serous and stage 3 to other histotypes and stages is so overwhelming. Could the authors comment on selection bias of the TMA and the inherent problems associated with this when trying to correlate with general clinicopathological variables in ovarian cancer?
Selection and sampling bias is most likely to occur in small lesions and non-solid masses. Examples of the former are very frequently in early stage cancer and for the later in mucinous carcinomas where cells tend to have an infiltrative pattern rather than a solid one. To minimize sampling problems we sampled each case 3 times in specific areas previously selected by a pathologist using H&E stained sections.

However, the proportion of cases described in this study is similar to that of the true incidence of each tumor found in the ovarian cancer literature. (Serous carcinomas 60-80%, mucinous carcinomas 10-20%, endometrioid carcinomas 10-20%, clear cell carcinomas 3-10%, others 1%.)

10) There are no positive and negative controls on the shown western blot. It would be helpful to show that the antibody is specific.

This was simply an oversight on our part. We systematically included in every Immunoblot analysis parallel positive and negative controls as we actually described in the Methods Section. As a negative control for WWOX protein expression we used protein extracts from the ovarian cell line PEO1 that does not express WWOX due to a homozygous deletion affecting this gene. As a positive control we used the same cell line stably transfected with a WWOX expressing vector. In order to demonstrate the antibody specificity we have now modified Figure 1 accordingly to include the aforementioned controls.

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Referee 2:

SCIENTIFIC

1) western blots--were the lysates all from one type of ovarian cancer; ie the common type? Does the fraction of tumors observed to be over and under expressing by western match the results observed by IHC? More details about the tumors analyzed by western would be useful.

See question 5 in referee 1.

2) IHC--what do authors think the overexpression in some cancers is all about?

This is a very interesting and intriguing phenomenon. One could speculate based on our experience with other tumor types, e.g. breast and prostate, that overexpression could be related to the hormonal metabolism or responsiveness within the tumor mass. We also know for instance that the highly hormonally dependent MCF7 breast cancer line overexpresses the WWOX protein. Nevertheless, it is unclear at this point the cause of such WWOX overexpression and we prefer to refrain from speculating further in the Discussion section until more evidence becomes available.
is there variation in level of expression from cell to cell in normal epithelial cells? such that the level of expression might be related to the specific cell type from which the cancer derived?

No, epithelial cells in normal OSE have homogeneous cytoplasmic moderate positive staining. In other words “all cells” from the OSE stained positively for WWOX with the same intensity. Therefore the intra-tumor and inter-tumor heterogeneity in WWOX expression described in this report is a phenomenon intrinsic to the ovarian cancerous condition.

Questions also arise concerning the apparent apical and perimembrane expression. Could subcellular location be due to binding of Wwox WW domains to specifically localized interacting proteins?

*It is an interesting question but this remains to be determined.*

In a specific cancer, was the subcellular localization homogeneous?

*Yes, the subcellular location was homogeneously distributed in every cell in a specific cancer (Figure 3a).*

Also, considering the heterogeneity of the pattern of Wwox expression observed in some of the photos shown, do the authors think that the size of the core sample examined on tissue arrays is enough to conclude that it is representative of the entire cancer?

*The question is valid that is up to certain extent a concern with “every” TMA based study. However, as previously mentioned selection and sampling bias is most likely to occur mostly in small lesions and non-solid masses. (See response to Referee 1 question 9). To minimize sampling problems we sampled each case 3 times in specific areas previously selected by a pathologist using H&E stained sections. We are only including in this study the cases with at least two informative cores as reported in ref 17. Rosen DG, Huang X, Deavers MT, Malpica A, Silva EG, Liu J. Validation of tissue microarray technology in ovarian carcinoma. Mod Pathol. 2004 Jul; 17(7): 790-7*

3) Why is the method of scoring of PR so different from the method of scoring of Wwox? If some of the cancers were scored for Wwox by individual pathologists, would the scoring be similar to that reported? I am asking about this because of the different scoring methods for the hormone receptors and Wwox.

*The most important difference is that WWOX is a scoring of cytoplasmic staining and the steroid receptors is a percentage of positive nuclei. We did perform both, manual and automated measurements, for this study and we found the overall same tendency on the results with both systems. We reported only the
automated due to, in our opinion, higher accuracy.

The automated system (Chromavision’s Automatic Imaging System- ACIS®) that we used for the intensity staining measurements provided two different protocols for cytoplasmic and nuclear antigens. We used the cytoplasmic protocol for WWOX were the average intensity staining over the range for the entire specimen is being assessed regardless the area covered by the positive cells divided by number of cores per case. We used the nuclear antigen protocol for both steroid receptors by determining the intensity staining (brown intensity) and percentage of positivity (brown stained cells) and quantifying that to the total amount of positive and negative divided by number of cores per case.

EDITORIAL (with specific examples)
The MS needs light editing throughout for English usage, particularly for use of articles and some punctuation.

p4, line 3 from bottom, histotypes

*We have modified the text accordingly*
p13, line 8, delete 'this"

*We have modified the text accordingly* p16, line 4, delete "of"; line 3, shows association with?

*We have modified the text accordingly* references 8 and 11 have misspelled Aqeilan and Iliopoulos; authors should check spelling throughout references.

*We have modified the text accordingly* Figure legends--rather than saying 'high power" it would be better to give actual magnification for the IHC photos.

*We have modified the text accordingly*