Author's response to reviews

Title: Differential expression of anterior gradient gene AGR2 in prostate cancer

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Author's response to reviews: see over
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Dr. Ingunn Holen
BMC Cancer

Dear Dr. Holen:

Submitted to your journal for publication is our revised manuscript entitled: Differential Expression of Anterior Gradient Gene AGR2 in Prostate Cancer.

We thank the three reviewers for their helpful comments. We have addressed all of their points and have modified the manuscript where need be. We feel the paper is now even stronger than before. It should also be noted that we added an additional author, Lora Bagryanova, who conducted the gene expression analyses.

As review, in this study we examine the expression profile of anterior gradient-2 protein (AGR2) in prostate cancer. AGR2 is a homologue of the *Xenopus laevis* gene XAG-2 and is thought to be a member of the protein disulfide isomerase family, a group of enzymes that act as molecular chaperones for protein folding. Our entry into considering the expression profile in prostate cancer stemmed from other studies examining the transcriptomes of prostate epithelial and stromal cell components. The striking up-regulation of AGR2 in prostate cancer epithelium compared to appropriate controls was compelling impetus to pursue this study.

One of the technologies that we successfully employed here to effectively consider the expression of AGR2 on a population basis was tissue microarray (TMA). Here too on the protein level, we found a relatively dramatic increased AGR2 protein expression in early and late prostate malignant lesions compared to benign tissue. This of course was validated by gene expression data. Significantly and surprisingly, when we considered outcomes data, a relatively lower expression level of AGR2 identified who had an increased probability of tumor recurrence. In this paper, we discuss the potential significance of this finding, both with regard to potential mechanism of action, but also the opportunity to utilize these findings in a clinical setting.

Thank you very much in advance for considering our revised manuscript for publication.

Sincerely,

Lee Goodglick, Ph.D.
Associate Professor
Department of Pathology and Laboratory Medicine
David Geffen School of Medicine at UCLA
We would like to thank Reviewer #1 for helpful comments. Below we describe our changes based on these comments.

1. The Reviewer comments that we should include and consider additional more recent publications in our treatment of AGR2 expression in prostate cancer. In particular, the Reviewer mentions two works: one by Zhang et al. (2010) and another by Zhang et al (2007). We completely agree with this assessment and have included references that have come out in publication since we submitted this manuscript. This includes the recent article in Cancer Research (2010)¹ and an article that we discussed extensively in our original manuscript published by Zhang et al. in 2007². In addition, a recent article by Bu et al., (2010)³ which came out a week or two ago is highly relevant as well and is discussed in the revised manuscript.


2. The Reviewer asks what the inter-observer variation was for the scoring of the TMA by our two pathologist. The correlation coefficient between the scores of the 2 pathologist was very high (r = 0.95). This has now been included in the Methods section. The scoring method of assessing frequency and intensity of staining is relatively common for such studies.

3 - 4. We have replaced the old Figure 1 with a more extensive evaluation of the gene expression of AGR2 in normal and malignant human prostate tissue (see revised Figure 1, associated methods, figure legend, and Results). The reason for this is that we thought the message would more clear and powerful with only results showing gene expression and protein expression on a population basis.

5. Represented on our TMA is 187 surgical cases. For each surgical case, we have on average three spots per relevant histopathology (e.g., benign versus malignant). Figure 3 and 4 represent spot-level data not case-level. This is now re-emphasized in the Results section and the Figure legends.

6. Figure 4 refers to spot level data while Table 1 refers to the population at the case level. The Gleason grade in Figure 4 is per spot. As stated above, spot-level data has now been emphasized and clarified in the text.

7. As the Reviewer suggested, we have not modified Table 2 and 4 to include T stage.

8. The Reviewer asked about the stages of our cohort. We do in fact have Stage III patients represented on our TMA. (note that 'T stage' does not necessarily define the overall clinical stage). Nevertheless, in Table I we have now included the break-down of Stage I/II and III/IV so that it can be compared with Figure 5. It should also be noted that AGR2 in high stage is not a surrogate marker for pT stage because it shows no association (Table 3).
9. The Reviewer is absolutely correct that a risk in these types of analyses is data-overfitting. Because of this, we have strict statistical parameters for minimizing such effects. Basically, when we conduct our analyses, we scan the complete data set and calculate all possible dichotomization points. To minimize the possibility of overfitting, we only use dichotomized data with a) have adequate representation of the patient population, and b) have a wide dynamic range for dichotomization (i.e., there is not a sharp cut point but rather a range of division points around the 75th percentile that are significant). We do have a figure showing this if the Reviewer thinks this would help. It is probably more appropriate to put into a supplement. We will leave it to the Reviewer and/or editor to give us guidance on whether such a figure would be helpful.

10. As the Reviewer suggested, we have modified the Discussion to make it more balanced and to emphasize the potential utility of AGR2 as a prostate cancer biomarker. We have also included relevant recent publications as well.

11. As the Reviewer suggests, we have tried to emphasize the importance of our results regarding AGR2 expression in prostate cancer.

**Minor Essential Revisions**

1 - 3. We have modified / fixed all sections of the Manuscript recommended in "minor essential revisions". These include a) a clarification of the number of deaths and cases of prostate cancer; b) changing "false negative" to "false positive"; c) writing a more precise and when necessary, more detailed Methods section; d) Figure 2 now shows a representative example of negative control staining.
We would like to thank Reviewer #1 for helpful comments. Below we describe our changes based on these comments.

**Major Compulsory Revision**

1 - 4. We have revised the manuscript by removing the Western Blot and limited transcriptional analyses data (i.e., Figure 1). We have replaced this with a more extensive evaluation of the gene expression of AGR2 in normal and malignant human prostate tissue (see revised Figure 1, associated methods, figure legend, and Results). The reason for this is that we thought the message would more clear and powerful with only results showing gene expression and protein expression on a population basis.

5. Represented on our TMA is 187 surgical cases. For each surgical case, we have on average three spots per relevant histopathology (e.g., benign versus malignant). Figure 3 and 4 represent spot-level data not case-level. This is now re-emphasized in the Results section and the Figure legends. Regarding Gleason grade and stage, as the Reviewer knows, clinical stage is composed of a number of criteria. Therefore, Gleason grade does not, a priori, tell the clinical stage. Therefore, the results from Table 1 and Figure 4 are not in conflict. It should also be noted that we have also added Stage to the revised Table 1.

6. The observation of an apparent inverse relationship between PSA and AGR2 expression is somewhat novel. Zhang et al. (Genes Chromosomes Cancer, 43(3):249, 2005) had previously suggested such an inverse correlation.
We would like to thank this Reviewer for helpful comments. Below we describe our changes based on these comments.

**Major Compulsory Revisions**
1. As the Reviewer recommended, we added more information to the Methods section about tissue and antibody controls for the immunohistochemistry. In addition, in Figure 2, we have added a representative image of a negative control.

2. The Reviewer appropriately pointed out some weaknesses in the original study with regard to the Western blot analyses. Based on these comments, we have decided to replace the Western blots results with greatly expanded gene expression data. We feel that this strengthens this study significantly. Because of this, issues raised by this Reviewer in "Major Compulsory Revisions" point 2 and in "Minor Essential Revisions" point 1, have now been rectified (as the data has been replaced).

3. As suggested by the Reviewer, we have modified the Introduction section by removing any significant mention of array results.

4. Regarding paragraph 2 of the Results section, we have now removed this section.

**Minor Essential Revisions**
1. We have replaced the previous Figure 1 with more extensive gene expression data.

2. The Reviewer is correct that the CD10 / AGR2 subdivision of the population is very interesting. It certainly is relevant to defining subsets of patients and exploring the meaning of these markers. We continue to examine this connection in great detail. We are currently working on a more detailed study of this connection; therefore, we decided to not include the primary data here and have taken out this section of the Discussion so as not to offer this 'teaser'.

3. We respect the Reviewer's comments regarding the last paragraph. We do feel, however, that it is interesting to inform the reader where are heading towards next with the project.

4. Figure 4 shows AGR2 expression (at the spot level) corresponding to Gleason grade 2, 3, 4 and 5.

5. pT are included in Tables 1 and 3 as requested.

6. As requested, we have modified Figure 2 and its associated figure legend to indicate Gleason Score and to show a representative example of control (non-immune) staining.

7. As recommended, we have made the following wording changes:
   a. Outcome was replaced by 'recurrence'
   b. We now use the term "Affymetrix expression array"
   c. The term "bladder cancer" has been removed.
   d. We have modified the Methods section to note temperature more accurately.
   e. In the Discussion, "message" has been replaced by "transcript"
   f. We removed the word "trend"

**Discretionary Revisions**
1 - 2. In the revised manuscript, we now present a much more extensive meta-analysis of gene expression data.

3. The exact function of AGR2 remains mostly a mystery. We and others are trying to more fully characterize the function of this protein. While we understand and respect the Reviewer's point about paragraph 3 of the Discussion, we feel that this small level of background information is relevant to the overall description of AGR2.
4. We have removed the Western blot data from the revised manuscript.