Author’s response to reviews

Title: Correlation of CD44v6 expression with ovarian cancer progression and recurrence

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Author’s response to reviews:

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Dr. Christina Annunziata
Associate Editor
BMC Cancer

Re: MS: 1789195319833820

Dear Dr. Annunziata:

Thank you for the handling of the review of our manuscript. Attached is the revised manuscript entitled “Correlation of CD44v6 expression with ovarian cancer progression and recurrence” (MS: 1789195319833820), which has been revised based on the concerns and comments from the reviewers. Also, we would like to thank the reviewers for their thorough and constructive review of our manuscript.

Our responses to the reviewer’s criticisms and comments are itemized below. Please note that the changes made in the revised manuscript are highlighted in blue.

Reviewer 1

Major Compulsory Revisions:

1. The authors cite several studies that examine differences in the expression of CD44s and CD44v6 in ovarian cancer and further highlight that these studies resulted in conflicting data. To address these discrepancies the authors examine differences in expression of the two variants in patient samples in their
laboratory. Please emphasize what makes the current study unique in comparison to the previous studies, or how it helps clarify prior findings in the literature.

Most previous studies assessed CD44v6 expression using one or two methods. To better address the discrepancies arising from these previous studies, we evaluated CD44v6 expression using three different methods (quantitative real time RT-PCR, western blot and IHC) in this study. Moreover, the number of tumor samples in our study is also bigger than that in most of the previous studies. Thus, we believe that our data are more accurate and reliable.

The reviewer raised an important point. To strengthen the paper, we have revised the relevant paragraph in the Discussion to highlight the point in the revised manuscript (see page 15).

2. The current study focuses on advanced versus recurrent serous adenocarcinoma; please comment on potential differences in mucinous ovarian cancer. Mucinous is distinct from serous and is more similar to GI tract carcinomas and since the CD44v6 was increased specifically in the abdominal cavity, it would be worthwhile to explore any association of this variant with mucinous type.

Thus is an important issue, but the expression of CD44v6 in mucinous ovarian cancer has not been well investigated, probably because of its low incidence. This was the case for us: we only obtained 5 tumor specimens of mucinous ovarian cancer during the course of our study. Nonetheless, we will carry out the study to address this issue in the future once we obtain sufficient numbers of mucinous ovarian cancer samples.

3. Figures 1 and 3 could be combined to underscore the association of CD44v6 with recurrent disease. For the ELISA graph, please indicate number of patients in each group and how many times the experiment was performed.

Figures 1 and 3 have been combined in the revised manuscript. Also, the numbers of patients and the number of the experiments performed have been included in the figure and figure legend, respectively, in the revised manuscript.

4. For IHC please include representative staining image for CD44v6 in normal ovary tissue and lymph node metastases, that was said to be included on the Ovarian Cancer Tissue Chip.

The representative staining images for CD44v6 in normal ovary and lymph node metastases have been added in Figure 3 in the revised manuscript.

5. The conclusions of the study would be greatly enhanced with a functional
assay measuring metastatic potential. For example knocking down CD44v6 with an siRNA in an ovarian cancer cell line and assessing changes in migration or invasion.

This is a very good suggestion. We have performed the suggested assays and we found that knockdown of CD44v6 decreased the adhesion and migration but not invasion of SKOV3 cells. The data from these new assays are shown in Figures 4 and 5 in the revised manuscript.

Discretionary Revisions:

1. page 7: qRT-PCR was repeated once in triplicate. Please comment on why this was not further confirmed (patient tumor tissue limited?)

The assay was repeated only once because the amounts of a small number of tumor samples were not sufficient for additional experiments.

2. page 8: Please comment on which statistical test was performed using SPSS software.

Statistical analysis was performed using SPSS version 13.0. Quantitative real time RT-PCR, Western blot, adhesion assay, migration assay, and invasion assay data were analyzed using Student's t-test and expressed as mean ± SD. The correlation between CD44v6 positive expression and the clinicopathologic parameters was assessed by Chi-square test. Differences were considered statistically significant when P values are smaller than 0.05.

The statistical analysis paragraph in the Methods has been updated with more details in the revised manuscript (page 10).

3. Figure 2: Please comment on why the mRNA is trending down (in A), while the protein appears to be increasing (in B, C) for CD44s

In A, we used primers which recognize cDNAs encoding different variants of CD44 including CD44s and CD44v6 (as such, we label it as CD44). The trending down in A is likely to be caused by the change (decrease) in CD44v6 expression.

In contrast, in B, we used an antibody which specifically recognizes CD44s to assess CD44s protein levels, and thus the data in B show the change in CD44s protein levels.

4. Figure 4: Consider also staining the Ovarian Cancer Chip for CD44s.

Previous reports indicated that CD44s expression is not associated with ovarian cancer recurrence and metastasis. Consistently, our study also showed that
there was no significant difference in CD44s expression among the different sites of ovarian cancer metastasis. Nonetheless, this is an interesting suggestion. We will consider doing this in the future to further validate the negative data.

5. In the last paragraph of the results, please add a comment about the results in table 1 regarding quantity of staining frequency across different grades of tumor differentiation.

We have revised Table1 and added a detailed description about the results in the revised manuscript (pages 8, 13).

Reviewer 2

Minor Essential Revisions

Line 191 "The sections were all quantified by two pathologists in a blinded manner"

Line 248-250 "When the rate of the CD44v6 positive cell more than 5%, we regarded the tissue was expressed CD44v6 positively."

Comment 1) Evaluation of IHC should be in Material & Methods (in connection to line 191) rather than in results. More importantly, this is not a common way to judge standard IHC. Evaluation of standard IHC immunostainings should preferably be performed independently by at least two of the authors. The extent and intensity are most commonly semiquantitatively judged as proportion of tumor cells stained (e.g.: 0, negative; 1, less than one third; 2, between one and two thirds; and 3, more than two thirds) and intensity of immunostaining (e.g.: 0, negative; 1, weak; 2, moderate; 3, intense). If evaluations for tissue chip by IHC differ from standard IHC evaluations, you should add references to other publications using <5% as cutoff.

The sentence in Line 191 has been moved to Materials and Methods in the revised manuscript.

We have also re-analyzed the IHC data using the conventional method as suggested by this reviewer (pages 8, 13). The new results are shown in the revised Table 1. We would like to thank the reviewer for the constructive critique.

Discretionary Revisions

Lines 270-272: "So far, CD44v6 has been shown to be an unfavorable prognostic factor for a variety of cancers including those of the stomach [38], head and neck [39], prostate [40], and lung [22]."

Comment 2) This is not really correct. The same controversies about v6 exist in various cancers, but many studies have found the opposite, see for example

Thanks for the information. To be accurate, we have changed the wording from “an unfavorable prognostic factor” to “a useful prognostic factor” in the revised manuscript (page 15).

Quality of Written English: Needs some language corrections before being published

We have carefully proofread the manuscript to correct spelling and grammatical errors during the revision.

Once again, we would like to thank you and the reviewers for the thorough and constructive review, which has greatly helped improve the quality and accuracy of the manuscript.

Sincerely,

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