Author's response to reviews

Title: Tumor-suppressor activity of RRIG1 in breast cancer

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Professor Stefan Imreh
Associate Editor
BMC Cancer

RE: Resubmission of our manuscript (#MS: 5318618514680292)

Dear Prof. Imreh:

Many thanks for your email and encouraging news regarding our manuscript. We have revised it according to the reviewers’ constructive suggestions and comments. We would like to re-submit it for your re-consideration for publication in MBC Cancer. The revised manuscript has been edited and proofread by the Department of Scientific Publications at The University of Texas MD Anderson Cancer Center.

We hope that this manuscript is now acceptable after this revision, and I look forward to hearing from you soon.

Sincerely,

Xiaochun Xu, M.D., Ph.D.

Our responses to the Reviewer’s comments

First of all, we would like to express our sincere thanks to the reviewers for their time and constructive comments and suggestions.

Reviewer #1: Balazs Györfy

Major revisions:
1. As RRIG1 is not in Pubmed Gene, the authors should add more description. Has the sequence for the primers been blasted?

We fully agree and have added such a description in the introduction and discussion sections. We designed the primers very carefully to avoid amplifying SH3GLB2 by using online Primer 3 software, which was also checked (blasted) to determine whether there are any unwanted amplifications of other genes.
2. There are error bars on the graphs, but the text does not describe whether any repetitions were made.

Most experiments were performed in triplicate and repeated at least once with similar results. The error bars were based on triplicate data from one of the experiments.

3. Is there a possible way to get correlation to survival? (see for example, www.kmplot.com).

We really thank the reviewer for this constructive suggestion. After we searched kmplot.com for SH3GLB2 (since RRIG1 is a novel gene and not in the Genbank database -- although SH3GLB2 and RRIG1 share several exons, their CDs are total different), we found that lost expression of SH3GLB2 mRNA reduced survival rate of breast cancer patients ($p = 0.0054$). However, this may have nothing to do with RRIG1. To date, we do not have any data on RRIG1.

4. "It is unclear whether RRIG1 expression is controlled solely by RAR". Since RAR heterodimerizes with RXR to function, the answer for this question is quite obvious: the authors should add and discuss RXR-related literature.

We fully agree that the function of RAR-beta is to dimerize with RXR-alpha (mostly). However, in our previous study, we found no loss of RXR-alpha expression during breast carcinogenesis and hence did not discuss the relevance of RXR-alpha. In addition, this study was conducted to determine the role of RRIG1 in breast cancer but not to determine cause of the lost RRIG1 expression. Thus, we have removed the statement of "It is unclear whether RRIG1 expression is controlled solely by RAR-beta".

5. Add more description about the patients used (hormone receptor status, survival, HER2 status). ER and HER2 should also be evaluated for any possible correlation to RRIG1, as they also strongly influence the survival.

We fully agree and have added such data in Table 1; however, comparisons between RRIG1 and hormone receptor status did not reach statistical significance.

Minor revisions:
6. Check grammar! ("Next, we therefore used it immunohistochemically" "These cell lines were and grown in" etc).

We have asked the Department of Scientific Publications to review the manuscript before resubmission.

7. The investigation of RhoA is insufficiently addressed. Why not another gene?
We agree and have added more discussion as to why we included RhoA in the study.

Discretionary revisions:
8. Previous results can be discussed in the discussion but do not belong in abstract. We have modified the abstract accordingly.

9. Define aim in abstract. We have added the aim to the abstract.

10. Logical errors in sentences. (like: "It is unclear the underlying mechanism by which the restoration of RRIG1 expression was unable to suppress Stat3 phosphorylation, but knockdown of RRIG1 did so."). We have made corrections as needed.

Reviewer #2: David A Sweetser

In this article the authors provide further evidence for a tumor suppressor-like function of the RRIG-1 gene, which they had earlier identified. Their in vitro studies provide convincing evidence this gene can regulate the proliferation, colony formation, and invasion of breast cancer cell lines. The data they provide in Figure 1 does not convince me of specificity of their RRIG-1 antibody used for immunohistochemistry of breast cancer. There results should be strengthened by the inclusion of additional photomicrographs as supplementary figures.

We have renamed Fig. 1 to be a Supplementary Figure 1. We tested and verified the specificity of our anti-RRIG1 antibody by using three different experiments 5 years ago. Although these three experiments may not be the only means to confirm specificity of antibody, Figure 1 includes sufficient data to verify the usefulness of this antibody for IHC.

Discretionary revisions:

1. Quantitative RT-PCR would be preferable to quantify RRIG1 and MMP9 message levels.

   We fully agree. Because Dr. Guihong Zhang, the visiting scientist in the lab, went back to China some time ago, we do not have the manpower needed to repeat this experiment. The data we presented in the manuscript were semiquantitative RT-PCR. She tried 25, 28, 30, 32, and 34 cycles of PCR and decided to use 32 cycles to semiquantify the changed expression of RRIG1 and MMP9.

2. A standard method of demonstrating antibody specificity is the use of the immunizing peptide for blocking the immunohistochemistry; this would significantly strengthen their verification of specificity.

   We agree. Use of the immunizing peptide as a negative control in immunohistochemical analysis is one of the best methods to verify specificity of the antibody against its peptide. Another method to confirm the specificity of an antibody is to compare
expression of mRNA and protein by using IHC vs. ISH or Northern vs. Western blot. Hopefully, the reviewer agrees. In addition, 5 years ago we paid a commercial company to generate this antibody; however, the company did not provide us with any immunizing peptide to perform the experiment.

Minor Essential Revisions:

3. Include the sequence of the peptide used for RRIG-1 antibody production. We have added it accordingly in the Methods section.

4. Describe the technique for organotypic culture, this is not mentioned in the cited reference [6]. It would help to identify the positive and negative layers of staining cells. We added it accordingly in the Supplemental Methods section.

5. For RT-PCR the number of cycles used for genes and especially GAPDH need to be mentioned to help determine if the results are likely still in the line arrange of amplification to make semi-quantitative comparisons. The intense staining for GAPDH in Figures 3B and 5B suggests saturation and a plateau of the reaction precluding even a semi-quantitative assessment of RRIG1 and MMP9 levels. This is important if the authors want to state they restored RRIG-1 levels as opposed to having over-expressed RRIG-1.

We fully agree. We tried 25, 28, 30, 32, and 34 cycles of PCR analysis of RRIG1 expression in positive and negative cancer cells from our previous study (ref. 5) and used 32 cycles to semiquantify the changed expression of RRIG1 and MMP9 in the current study. The 32 cycles could be a little bit over for GAPDH mRNA.

6. In Table 1 it is not clear if positive RRIG1 expression means positive based on quantitation of intensity of staining or % positive cells, as the text states that both measurements were performed as independent assessments.

We fully understand the reviewer’s concern. The reviewer is correct that the positive cases were based on both intensity of staining and the percentage of positive cells as stated in the Methods section.

7. Discussion, first paragraph states “these effects were through the regulation of additional genes”, since there is no demonstration of the dependence of these phenotypes on changes in expression of these genes it would be more accurate to state “these effects were ASSOCIATED WITH INCREASED EXPRESSION OF...” We have modified the text accordingly.

8. SH3GLB2 related experiments in Figure 6 should be mentioned in results section. We fully agree and have added it to the text.

9. The right two panels of Figure 1D are an enlargement of two panels they previously published in Cancer Res 2007; 67: (4). February 15, 2007, it should be clarified if
permission is needed to republish these.
To avoid any possible copyright issues, we have replaced the photos in the revised manuscript.

10. Poor grammar and lack of evident proofreading throughout the article is very distracting and several passages are confusing. This article needs to be more carefully proofread.

We thank the reviewer for his carefulness. The Department of Scientific Editing at MD Anderson Cancer Center has helped us to edit and proofread the manuscript.

11. Of the 3 methods used to demonstrate specificity of the antibody the organotypic culture is the only relatively convincing study shown. I would not count the Western blot of in vitro transcribed cDNA as demonstrating specificity, given the low complexity of proteins being analyzed. This blot is useful to demonstrate the size of the product on Western, but, as the authors subsequently show, this antibody does not prove suitable for Western blot. As best I can tell from reading the methodology in reference 5 for the RRIG-1 cDNA used for in situ hybridization would cross react with SH3GLB2, and could stain cells expressing SH3GLB2 and not RRIG1.

We thank the reviewer for his careful thought. Although SH3GLB2 and RRIG1 share some exons, their open-reading frames are totally different; thus, the polyclonal rabbit anti-RRIG1 antibody cannot recognize SH3GLB2 protein. In addition, the probe for ISH was specifically designed and prepared; ISH data were quite specific for RRIG1, but not for SH3GLB2.

In the results section paragraph "Specificity of the rabbit polyclonal antibody for immunohistochemistry," and the corresponding Figure 1, the authors mention 10 tumor samples were compared for ISH and ICH and the positive staining matched well but they only selected one negative tumor to show. They should show the other 9 samples including positive staining sections as a supplemental figure.

We fully agree. We re-evaluated the IHC and ISH sections of breast and esophageal cancer tissues and found that the IHC stain on most sections had faded, because the experiments were performed more than 4 years ago. Therefore, we revised the wording in the corresponding text in the revised manuscript. Moreover, it is why we used some old photos from ref. 6 in the current manuscript, after a reviewer from Breast Cancer Res asked us to provide such data.