Reviewer's report

Title: Tumor-suppressor activity of RRIG1 in breast cancer

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Reviewer: DAVID A SWEETSER

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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. Their results should be strengthened by the inclusion of additional photomicrographs as supplementary figures.

Discretionary revisions:

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

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.

Minor Essential Revisions:

3. Include the sequence of the peptide used for RRIG-1 antibody production.

4. Describe the technique for organotypic culture, this is not mentioned in the sited reference [6]. It would help to identify the positive and negative layers of staining cells.

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 linear range 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.

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.

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….”
8. SH3GLB2 related experiments in Figure 6 should be mentioned in results section.

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.

Major Compulsory Revisions:

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.

As a small selection of examples:

• Abstract, methods paragraph “RhoA activation assay was to assess RRIG1-induced…” , insert - was used TO assess, or reword
• Abstract, results paragraph “…tissues, which confirmed previous report”, which confirmed OUR previous report.
• Materials and methods, RRIG1 expression vector and transient gene transfection “started for selection”, change to “were selected”
• Results, Specificity of the rabbit polyclonal antibody for immunohistochemistry, “staining was very clean and showed positive for”, change to was postive
• Results, Specificity of the rabbit polyclonal antibody for immunohistochemistry, “was matched well” change to was similar
• Results, RRIG1 regulation of breast cancer cell growth, migration and invasion, “decreased a number of colonies”, change to “decreased the number of colonies”
• Results, RRIG1 regulation of breast cancer cell growth, migration and invasion, “..caused “induction of colony numbers”, change to increased the number of colonies
• Results, RRIG1 regulation of breast cancer cell growth, migration and invasion, “induced capacity of MDA-MB-435 cells in migration and invasion” change to “increase the migratory and invasion capacity of MDA-MB-435 cells”
• Results “Immunohistochemical analysis of RRIG1 expression in breast tissue specimens” the authors state they “found a statistically significant correlation between RRIG1 expression and lymph node metastasis in breast cancer” this should state a significant INVERSE correlation between RRIG1 expression and lymph node metastasis in breast cancer.
• Discussion paragraph 2 run on sentence starting “In our previous studies [12-14]…” is confusing.

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. 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.

**Level of interest:** An article of importance in its field

**Quality of written English:** Needs some language corrections before being published

**Statistical review:** No, the manuscript does not need to be seen by a statistician.

**Declaration of competing interests:**

I declare that I have no competing interests