Author's response to reviews

Title: TRM2A expression is prognostic in her2+ breast cancer

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Author's response to reviews: see over
Response to reviewers:

General
We appreciate the thoughtful and thorough review of the manuscript by the reviewers. We have now modified the manuscript in response to the suggestions and believe that this is a much improved manuscript. Specifically,

1. We have added a western blot figure supporting specificity of the HTF9C/TRM2A antibodies.
2. Added a table of multivariate analysis.

There was some concern that this report does not address the clinically important question of the relationship of HTF9C/TRM2A expression to response to chemotherapy treatment as well as a lack of rigor regarding her2 measurements. We acknowledge the limitations of this study which uses institutional cohorts in a tissue array format, however, we feel that it is important to publish these relatively early steps in biomarker discovery and validation as access to tissue with clear treatment data requires peer reviewed publication of preliminary studies. We think this manuscript, reporting consistent results from three independent institutional cohorts is an important step towards more definitive studies that can establish the clinical utility of this novel biomarker. The inclusion of three independent cohorts raises this report to an unusual level of rigor albeit short of that allowed by study of clinical trial populations. We appreciate your willingness to reconsider publication.

Lastly please note the official name of this gene has been changed to TRM2A since the initial writing of this manuscript and we have updated the text to reflect this.

Sincerely, Doug Ross MD PhD
Response to Dr. Hartmann

1. It is not entirely clear how the authors defined Her2 positivity. In the RPCI cohort they report that all cases have been detected by immunohistochemistry using the Hercep-Test with the result of 3+. In contrast there are no data indicating the methods to detect Her2 in the CCIH and CCF datasets. Because the authors have a tissue microarray in their hand they should repeat the immunohistochemical staining for all three cohorts and in addition investigate the amplification of Her2 using FISH. These data are absolutely essential because Her2 determination within the described cohorts are not reported according to the guidelines used for daily diagnosis.

The cases from the RPCI cohort where selected because they showed an intense “chicken-wire” pattern of membrane staining that was diffuse and uniform throughout the tumor. The HER2 IHC sections were reviewed to select cases for inclusion in the cohort and construction of the TMA. HER2 staining was repeated on the TMA to confirm that the samples of tumor where positive. The testing was performed with the FDA approved Hercep Test kit following all manufacturers instructions in a laboratory setting where there is much expertise and experience with HER2 analysis. The assay was validated according to the ASCO/CAP HER2 testing guidelines and showed a high degree of concordance with HER2 FISH analysis (>98%). Performing FISH analysis on a TMA with numerous cores is technically very challenging because of the need for fluorescence (dark-field) microscopy at very high magnification. It would be very difficult to correctly identify each core and easy to get out of alignment during the interpretation. Performing the FISH assays on whole sections would likely be more accurate and representative of the HER2 gene status but would be prohibitively expensive.

2. The authors report univariate and bivariate analysis. A multivariate analysis should be done.

Multivariate analysis has now been added demonstrating independence from in models including tumor size, node status and grade in two of three cohorts. In the CCF cohort, tumor size was highly prognostic and TRM2A was not significant in models including tumor size.

3. Two different antibodies were used in the study. For both antibodies no data are reported for the specificity of the staining. The authors should report Western blots which clearly show the specific staining with the used antibodies and in addition should perform blocking experiments using the specific peptides. Because the authors propose this marker as a clinical diagnostic test I think this is also essential for determining the usefulness of this antibody for clinical purposes.

As now detailed in the revised text, the specificity of the antiseras was confirmed by its ability to recognize recombinant TRM2A protein in western blot while not binding equimolar concentrations of non-cognate recombinant proteins. In addition, antibodies generated against two independent peptides from the protein recognize an identical MW band in cell line extracts from cell lines.
Response to Dr. Kilic:

1. what is the specific immunohistochemical staining of Htf9c? The authors have regarded cytoplasmic staining as specific, indeed Htf9c is an cell cycle regulated protein.

As now detailed in the revised text, the specificity of the antisera was confirmed by its ability to recognize recombinant TRM2A protein in western blot while not binding equimolar concentrations of non-cognate recombinant proteins. In addition, antibodies generated against two independent peptides from the protein recognize an identical MW band in cell line extracts from cell lines. We have observed both nuclear and cytoplasmic staining of TRM2A but the former is rare and if present exclusively, not scored as positive. Proteins which cycle between the cytoplasm and nucleus are common amongst cell cycle regulated proteins.

2. Determination of the Her2 status is done by immunohistochemistry (HerceptestTM). More accurate -because of better prediction of response to therapy with TrastuzumabTM- is its determination by FISH analysis.

3. TMA from 2 of the 3 cohorts used in this study were also used in the study of Ring et al. (JCO 2006). Ring et al. have considered tumors as Her2 positive, when they have been scored 2+ or 3+ (table 1). This needs further explanation, then score 2+ in immunohistochemistry means equivocal status and should subsequently tested by FISH. Was Her2 status determined according to the ASCO 2007 guideline?

The cases from the RPCI cohort, the definitive validation cohort, where selected because they showed an intense “chicken-wire” pattern of membrane staining that was diffuse and uniform throughout the tumor. The HER2 IHC sections were reviewed to select cases for inclusion in the cohort and construction of the TMA. HER2 staining was repeated on the TMA to confirm that the samples of tumor where positive. The testing was performed with the FDA approved Hercep Test kit following all manufacturers instructions in a laboratory setting where there is much expertise and experience with HER2 analysis. The assay was validated according to the ASCO/CAP HER2 testing guidelines and showed a high degree of concordance with HER2 FISH analysis (>98%). Performing FISH analysis on a TMA with numerous cores is technically very challenging because of the need for fluorescence (dark-field) microscopy at very high magnification. It would be very difficult to correctly identify each core and easy to get out of alignment during the interpretation. Performing the FISH assays on whole sections would likely be more accurate and representative of the HER2 gene status but would be prohibitively expensive.

The authors respectfully disagree with the reviewers claim that HER2 FISH analysis is more accurate than HER2 IHC. The two tests are complimentary and evaluate different aspects of the biology of HER2 driven breast cancer. If the tissue is properly handled and fixed and the HER2 IHC assays is properly perform and interpreted in a laboratory with experience and expertise in HER2 testing, the results will show a high degree of concordance between the HER2 gene status and the level of protein expression on the tumor cell membrane. In addition, the response to trastuzumab in not relevant for the current study given that these patients were not treated.

4. In the publication Ring et al. the total number of Her2 positive tumors was declared with 68 and 34 for the CCIH and CCF cohort, respectively. Using the
same TMA for this study, the total number of Her2 positive tumors for the CCH cohort is declared with 81. The difference of 47 has to be explained.

In the Ring et al. publication, her2 status from the clinical records was reported whereas in this publication, we repeated staining and re-interpreted on the tissue arrays.

5. In the publication of Ring et al. the univariate association with outcome shows a weak negative correlation with Htf9c in the CCIH and CCF cohort but a positive correlation with the BBCA cohort. Interestingly, in this study the BBCA cohort was replaced by the RPCI cohort.

Her2 status of the BCCA cohort was not available to us and tissue arrays were not available for re-staining. Please note that the Ring et al. manuscript reports associations in ER+ patients regardless of her2 status whereas this report focuses on associations in her2+ patients regardless of ER status and uses the RPCI cohort as an independent validation cohort.
Response to Dr. Sinn.

It is suggested to reanalyze the data with a better definition of tumor recurrence and to analyze the effect of the cytotoxic therapy that was actually given to the patients or not in the knowledge of the antigen expression of this marker.

Dr. Sinn correctly points out the various limitations of performing biomarker discovery studies using institutional retrospective cohorts. However, we respectfully point out that most useful biomarkers begin with such studies and we have taken the unusual step of including data from three independent institutions in a single report to balance these concerns. As pointed out we are excited to study this marker in randomized cohorts treated either with cytotoxic chemotherapy and or her2 targeted therapy. However, access to such cohorts requires peer-reviewed preliminary data supporting biologic subclassification of a clinically relevant patient population which we believe this manuscript demonstrates. It is very difficult to draw conclusions about a predictive relationship between a marker and drug treatment using institutional cohorts therefore we prefer to limit or analysis and discussion to the prognostic question regardless of chemotherapy treatment which is not likely to be confounded by the non-randomized nature of these sample sets.