Author's response to reviews

Title: PAM50 Breast Cancer Subtyping by RT-qPCR and Concordance with Standard Clinical Molecular Markers

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Version: 3 Date: 22 August 2012

Author's response to reviews: see over
Dear Editor,

We again appreciate the reviewers helpful comments for the manuscript titled “PAM50 Breast Cancer Subtyping by RT-qPCR and Concordance with Standard Clinical Molecular Markers” and have addressed each of the reviewer’s concerns.

**Reviewer #1: Thomas Hughes**
The authors have made reasonable efforts to address my points. I have two remaining concerns, which both relate to my previous points.

1) With reference to my original major point 1. The authors have attempted to address my concern of the confounding factor of endocrine therapy. However, the result now shown in Fig 6C is extremely surprising. ER-/esr1+ patients, who were denied tamoxifen although according to their gene expression they should have received it, apparently did not suffer a worse outcome as compared to the ER+/esr1+ patients, who received the appropriate tamoxifen therapy. Could the authors please comment on this? Surely, something is wrong here?

**Response:** The results shown in Fig 6C are a reflection of the better prognosis that “true” luminal/ESR1+ tumors have over the standard ER+ IHC definition. This prognostic value has survival benefit, regardless of therapy. In addition, the patients included in the test set were locally advanced, and received chemotherapy which may have caused chemotherapy induced amenorrhea and a reduction in ovarian function. A reduction in estrogen production may benefit the “true” luminal subtype most. These points have been included in the discussion.

2) With reference to my original major point 6. I would still like the authors to highlight on Figure 1 the 16 non-neoplastic samples as originally requested. A key aspect of this work is that the gene expression panel allows the samples to be clustered, but currently we are unable to assess whether this process can correctly do the most basic differentiation between cancer and normal.

**Response:** The 16 non-neoplastic samples (reduction mammoplasty and grossly uninvolved breast tissue) all Clustered together, away from the invasive cancers. We then applied a statistical method called SigClust to identify the most cohesive samples within the dendrogram and it identified 4 reduction mammoplasty samples that were used to train the subtype of “Normal-like”. We have explained this better in the figure legend with colored bars to designate prototype and non-prototype “normal” samples.
Reviewer #2: Christopher Wong

1. Figure 1 – What are the black samples that cluster together with the 4 normal-like (green) samples? Are they also normal? If they are misclassifications of the other subtypes, they should still be indicated with the respective colours.

Response: Figure 1 has been re-done with colored bars to designate prototype and non-prototype “normal” samples. In addition, the figure legend now emphasizes the “normal” samples and the “Normal” subtype.

2. Missing word “to” – 2nd line under “Prognostic Significance.....” section: .....Her2/ERBB2, we wanted to assess....

Response: This has been corrected.

3. For last line of results, based on fig 6C, authors might be more bold instead and directly claim that the RT-PCR for ESR is more prognostic and accurate than IHC for ER.

Response: Thank you. We have made the bolder statement.