Author's response to reviews

Title: Amplification of HER2 is a Marker for Global Genomic Instability

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Author's response to reviews: see over
Dear Dr. Dunkley,

Thank you for your kind consideration of our manuscript entitled “Amplification of HER2 is a Marker for Global Genomic Instability” (MS: 9569389971919841) for publication in BMC Cancer. We have addressed the reviewer’s comments below (points to address in red, authors’ comments in italics):

Reviewer 1:
This paper describes an evaluation of allelic imbalance in breast tumours in relation to Her2/neu gene amplification.

Major Compulsory Revisions
1. *I am not a statistician, but the analysis of the confounding factors (ER and grade) in relation to AI and Her2 amplification seems not completely correct. Of course the fact that the authors address the possibility of confounding effects of other parameters is very good, but the AI itself is also a confounding factor, especially when occurring on the same chromosome. Furthermore, the analysis itself has not been described in detail. Presumably the samples in both groups (Her2amplified or Her2negative) were matched according to grade and ER status. Of course the groups will then become quite small, since the Her2amplified group contains only 41 cases. Concluding, I may be wrong and the statistics may be well performed, but from my view as a biologist it is not surprising that Her2 amplification associates with genomic instability, because Her2 is mostly amplified in grade III breast tumors, which have a higher degree of AI than low grade tumors.*

Negative ER status and poor tumor differentiation have been associated with HER2+ status. Thus, to determine whether these factors are influencing the relationship between HER2 and AI, statistical analysis was performed holding both ER and grade constant. By performing partial correlations, AI, cannot, be a confounding factor. We have clarified the analysis in the results section with the following sentence:

“Because ER status and tumor grade have been associated with HER2 status and may confounding the relationship between HER2 amplification and AI, partial correlations were calculated at each chromosomal region between HER2+ and HER2- samples, holding ER status and grade constant.”

2. *The authors aim at identifying chromosomal changes that are common in Her2-amplified tumours. They indeed observe a higher incidence of allelic imbalance (AI) for microsatellite markers at 11q, 16q, 18q. However, these are quite commonly deleted regions in breast cancer, so how can these be specific for the 20% tumours with Her2 amplification? It might have been better to look at...*
other regions that are less commonly deleted, to identify real specific loci for Her2. It may be a bit overstated to say that genes in these regions contribute to pathogenesis of Her2 positive tumors (page 9).

We agree with Reviewer 1 that there may be additional modifier regions associated with HER2 status, other than those identified here. However, AI at these regions, although not unique to HER2 amplification, is strongly associated with HER2 status. We have addressed the reviewers concerns by amending the sentence in the discussion section as to:

Thus, chromosomal alterations at these regions may contribute to the aggressive pathology and poor prognosis associated with HER2+ tumors.

Minor Essential Revisions
1. It is not clear from the text or the tables how many tumors were successfully analyzed for AI on all loci, all 194 cases? Please clarify.

Table 3 has been amended to include the number of informative genotypes in each tumor type by marker.

2. The color of the FISH signals in figure 1 should be clarified
The following sentence has been added to the end of Figure legend 1 clarifying the FISH signals:

Figure 1. Images of tumors before and after laser-assisted microdissection with corresponding FISH data. The tumor specimen on the top was taken from a pre-menopausal woman with stage IIb IDCA, without amplification of the HER2 gene. The tumor specimen on the bottom was taken from a pre-menopausal woman stage IIIB IDCA and HER2 amplification of 3.3. Green signals = CEP17 probe, orange = HER2.

3. In table 1 Menopausal should be followed by > 50 years
Correction made to Table 1 such that the row is labeled: “Menopausal (>50 years)”

Reviewer 2:
I have read with interest the article entitled "Amplification of HER2 is a marker for global genomic instability" By Ellsworth et. al. In this study the authors demonstrate that the frequency of AI is significantly higher in HER2 amplified tumors compared to HER2 negative ones. Specific alterations seen in chromosomes 11,16 and 18 appear to show an association to the HER2 status. This paper is well written and the goals and the data are clearly presented. I have the following comments to make on what has been presented.

1. On page 4 in the first paragraph, the authors state that the incidence of HER2 overexpression is 25-30% of human breast cancers. This number really depends on the population of patients being evaluated and the 25-30% range is more relevant to the patient population with metastatic disease that were treated in the
early pivotal clinical trials. In contemporary consecutive series of breast cancer, the incidence is more in the 10-15% range.

Although the frequency of HER2 amplification in unselected tumors is still unclear, we have changed the frequency listed from 25-30%, to 15-25%, in accordance with the ASCO/CAP 2007 publication.

2. In the second paragraph on page 4 the authors make reference to genes that modify the clinical response to trastuzumab, including p27, PTEN IGF1R and TOP2a. I am unaware of any studies that have looked at the response to trastuzumab in terms of TOP2a and there is no reference to this listed in the cited references. the authors should site a reference if one exists or modify this sentence.

References by Fritz et al, 2006 and Cardoso et al 2004 have been added.

3. On page 5, the authors state that amplification of HER2 was defined as a ratio of greater than 2. I am sure that the authors are aware of the new ASCO/CAP guidelines published last year in the JCO and Arch of Pathol that have re-defined amplification as a ratio of greater than 2.2. this new definition creates a new HER2 fish category of equivocal patients who have ratios between 1.8-2.2. Although they are likely to have very few patients in the equivocal category, it would be important for the authors to analyze their results into three categories, neg, equivocal and amplified. the purpose of creating the equivocal category was due to the fact that little was known about this group and more research is needed to better understand the clinical significance and potential benefit of treatment for these patients.

In accordance with the new ASCO/CAP guidelines for scoring HER2 status by FISH analysis, we have re-analyzed the data as suggested by Reviewer #2. Using scores of 1.8 – 2.2 to define equivocal status, did, as Reviewer #2 suggest, result in only 2 cases being changed from HER2+ to equivocal. While examination of genetic changes in this borderline group of patients may, at some point, help better classify and determine treatment options, specimens were chosen for this study based on positive or negative HER2 status, thus we do not have the statistical power to examine genetic changes in equivocal HER2 tumors.

Note, we have updated the methods section (paragraph 2), to reflect the updated ASCO/CAP recommendations and have added the JCO reference as suggested by Reviewer #2. In addition, we included the following statement:

“Patients with either equivocal HER2 status (1.8 – 2.2) or aneusomy were not evaluated in this study.”

4. On page 7 in the second paragraph of the results section, the authors state that 5 samples recieved neo-adjuvant chemotherapy before sample collection
and that none of these patients where HER2+. Was the HER2 status assess on the pretreatment sample or on the post treatment sample? Does the analysis remain the same if these cases are eliminated from the HER2 - group? Are these cases really necessary given that they already have 153 HER2 negative patients?

We agree with the reviewer that given the large sample size in the HER2- group, the inclusion of a small number of samples that had received neoadjuvant therapy is not necessary to the study. These samples have been removed from the analysis. In addition, a sentence has been added to the first paragraph of the methods section stating:

“Samples from patients with a previous history of breast cancer or who had received neoadjuvant therapy were excluded from this study.”

5. On page 9 near the top of the page the authors are refering to trastuzumab resistance and the need to improve our understanding of the biology of aggressive disease and patient response to treatment. I think the authors need to make it clear here that the data presented can not at this point be extrapolated to say anything about predicting which patients will response and which ones will not. the data is however hypothesis generating and this hypothesis is supported by the results obtained in the NSABP B31 study which showed that co-amplification of C-MYC and HER2 predicted for a better response to herceptin (presented by Paik et al at SABCS). did the authors look for and/or see any change in the C-MYC locus?

Reviewer #2 suggests that these data cannot at this time be used to stratify HER2+ patients into those that will respond and those that will not respond to trastuzumab, however, the results are hypothesis generating. We certainly do not wish to imply that this study, with limited clinical data, can be used to predict response to treatment. Rather, we believe the statement on page 9, that research into the molecular changes associated with HER2+ tumors that lead to treatment response and tumor behavior is not specific to this project, but a critical need in the breast cancer community. To make the statement more general, we have changes “alterations of HER2” to “amplification and/or overexpression of HER2”.

Reviewer #2 also asked about co-amplification of c-MYC with HER2. While we do discuss TOP2A as a modifier to trastuzumab response in the introduction, HER2+ and HER2- tumors did not, in this study, differ significantly from one another at chromosome 8q24, the location of the c-MYC gene. Thus, we have not investigated whether c-MYC is altered in these samples.