Author's response to reviews

Title: BRCA1-mutated and basal-like breast cancers have similar aCGH profiles and a high incidence of protein truncating TP53 mutations

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Author's response to reviews: see over
Amsterdam, October 25, 2010

RE: 5263877638291266 - BRCA1-mutated and basal-like breast cancers have similar aCGH profiles and a high incidence of protein truncating TP53 mutations

Dear Dr. Lee,

Thank you very much for your email from August 20th, in which you offer to consider a revised manuscript for publication. We very much appreciate your interest in our work. We would like to thank the reviewers for their helpful and insightful criticism, which has helped us to significantly improve our manuscript. Please find below a point-by-point response to the recommendations and issues raised by the reviewers. We hope that you will find our revised manuscript suitable for publication in BMC Cancer.

Thank you again for your interest in our work. We look forward to hearing from you.

Yours sincerely,

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To the editor:

We would like to communicate following information and changes needed to meet the standards set by BMC Cancer:

1) We have provided an email address for each author on the title page

2) We have included a Methods section in the abstract

3) We have addressed all of the reviewers’ comments in a revised manuscript and with this cover letter we provide a point-by-point response to the concerns (below).

4) In addition to the revised manuscript we also provide a manuscript with ‘tracked changes’, so that the editors can easily see all changes.

5) We have revised the manuscript to conform to the journal style.
General Changes:

In the previous manuscript we compared the TP53 mutations in the BRCA1-mutated tumor group with the luminal-J breast tumors; in parallel we compared the BLBC tumor group with the luminal-H tumors. In the revised manuscript we have constructed a single group of luminal tumors by merging the luminal-J and luminal-H tumor groups. Six tumors overlapped between the luminal-J and luminal-H tumor groups; for these 6 cases we have included the tumors from the luminal-J tumor group. We have redone all analyses (comparisons of TP53 mutations and comparative aCGH analyses with the normalized aCGH data using comparative-KC-SMART) with the combined luminal tumor group. We have made the relevant changes in the manuscript text.

The comparative-KC-SMART analysis of TP53 mutated vs TP53 wild type tumor groups was previously in the Supplementary data. In the revised manuscript we have added this analysis to the manuscript as Figure 3. Therefore, former Figure 3 is now Figure 4 and former Figure 4 is now Figure 5.
Response to reviewers comments:

Reviewer’s report 1
Reviewer: Nicholas C Turner

1. More detail is required on the TP53 sequencing methodology.
We agree with the reviewer and have changed the text on page 8-9 in the Methods section discussing the TP53 sequencing as follows:

“TP53 mutation analyses
For 21 BRCA1-mutated tumors, and 13 luminal-J tumors exons 2-9 of TP53 were previously sequenced [23]. The abundance of the aberrant base was estimated from the sequence chromatogram from both the forward and reverse sequencing runs. When comparing mutation types found in the tumor groups, we minimized the influence of tumor heterogeneity by only including TP53 mutations that had an estimated abundance of >25% in the tumor DNA [23]. For all BLBC and luminal-H tumors TP53 exons 2-11 (including +/- 30 bp outside each exon) were sequenced using AB 3730 DNA Analyzer (reference sequence NM_000546).
Frameshift, splice and nonsense mutations and in-frame insertions/deletions are defined as “complex TP53 mutations”. All missense mutations found in the BRCA1-mutated and luminal-J tumor groups were classified according to their predicted effect on p53 function as determined by the Sorting Intolerant from Tolerant algorithm SIFT; [28, 29], as used in the IARC TP53 database [30, 31]. Because no matched normal/germ-line DNA was available, some benign germ-line variants may have been identified as deleterious somatic mutations by SIFT. All TP53 missense mutations found in the BLBC and luminal-H tumor groups were classified to be deleterious or non-deleterious according to their predicted effect on TP53 function using “EffectGroup3” [32] as used in the IARC TP53 database. The 29 most common hotspot mutations (P<0.001) identified by Walker and colleagues [33] are referred to as “hotspot mutations”: K132, C135, P151, V157, R158, Y163, V173, R175, C176, H179, H193, Y205, Y220, Y234, M237, C238, S241, C242, G245, M246, R248, R249, G266, R273, P278, R280, D281, R282, and E285.”

2. I have some concerns with the comparison of BRCA1 mutated profiles (paraffin extracted DNA) with BLBC profiles (frozen extracted DNA). The profiles with paraffin DNA will have increased noise, and the authors attempt to adjust for this by scaling the data. However this may also compress the profiles of highly genomically unstable cancers. In addition there may be chromosomal regions that are differentially affected by noise between paraffin and frozen, and this cannot be accounted for. There is some evidence presented that this has not grossly effected the results, comparison of luminal J and H (Figure 2D), but is unclear if differences between BRCA1 and BLBC could have been missed.
We agree with the reviewer that identical tumor tissue treatment prior to DNA isolation for both tumor groups is preferable; however, there was no fresh frozen tissue of the BRCA1-mutated breast tumors available and due to low tissue amounts we were not able to paraffin embed the fresh frozen tissue of the BLBC group, isolate DNA from FFPE material and rehybridize the tumors on array. Therefore, we chose to use the aCGH data already present and scale the data. To scale the data, we determined the mean log2 ratio of all data points from all tumors within the FFPE tumor group (i.e. the log2 ratios from all BRCA1-mutated tumors and all luminal-J tumors taken together) and the fresh frozen group (i.e.: all log2 ratios from all BLBC-tumors and all luminal-H tumors taken together), and deducted these mean values from all log2 ratios from each respective group, so that the mean log2 ratio was 0 for both groups. Then we determined the standard deviation of the log2 ratios of from all tumors
within the FFPE and fresh frozen group, and divided the log2 ratios from the FFPE and fresh frozen group by their respective standard deviation. This way, the shift within each tumorgroup does not compress highly genomically unstable regions any differently than genomically stable regions.

To be clearer in the manuscript, we have changed the following text in the Methods section on page 10:

“Therefore, we transformed the log2 ratios from the FFPE aCGH dataset (i.e. all log2 ratios from BRCA1-mutated and luminal-J tumors taken together) and the fresh frozen dataset (i.e. all log2 ratios from BLBC and luminal-H tumors taken together) to have a mean of zero and a standard deviation of one before applying KC-SMART and comparative-KC-SMART.”

3. The BAC array used in this work is of low resolution compared to currently used platforms, and this limits the analysis especially with the increased noise of paraffin extracted DNA. This study will therefore be limited to detecting regional chromosomal alterations, and will have limited sensitivity for focal changes. The authors should discuss these limitations.

We agree with the reviewer that the BAC array used in this work has low resolution compared to current platforms. However, we argue that although this low resolution limits sensitivity for focal changes, the data is nevertheless a sound representation of the tumor groups. The noise level of the paraffin extracted DNA is not affected by the platform used. We have previously developed a robust method for BAC aCGH analysis of DNA from archival FFPE tumor material (Joosse et al 2007, BMC Cancer 7, 43). Using this method, we have successfully generated FFPE-compatible aCGH classifiers for identification of BRCA1-mutated and BRCA2-mutated breast cancers on BAC array platforms (Joosse et al 2009, Breast Cancer Res Treat 116, 479-89; Joosse et al 2010, Breast Cancer Res Treat, Epub ahead of print)

We have added the following text to page 10 in the Methods section:

“Although the 1Mb resolution of the BAC platform limits sensitivity for focal changes, the aCGH data is a sound representation of our tumor groups and can be used to find recurrent differences between tumor groups.”

4. TP53 mutations analysis
The Authors reanalyze TP53 mutation data from Holstege et al Cancer Res 2009, but include mutation data of only 13 of the 37 sporadic breast cancers sequences in this paper. It is unclear why the other 24 cancers have been excluded from the mutation analysis in this manuscript.

The 37 sporadic tumors from the Holstege et al Cancer Res 2009 paper included basal-like, Luminal A/B, HER2- and normal breast like tumors, whereas in this study we wished to compare the BRCA1-mutated tumors with the 13 luminal A/B tumors only, because this comparison parallels the BLBC comparison with luminal tumors.

5. The authors state that there is no difference in non-truncating mutations between BRCA1 mutated and sporadic luminal cancers. The splitting of the two luminal groups is reducing the power of this analysis. Combining the two luminal groups may lead to significantly increased missense and hotspot TP53 mutations in BRCA1 cancers compared to luminal cancers. This analysis should be presented.

We agree with the reviewer, and have therefore have considered making the suggested adjustments. However, the BRCA1-mutated tumor group and the luminal-J tumor group were sequenced by lab A (Dutch authors) and the BLBC and luminal-H tumor groups were sequenced by lab B (Norwegian authors). Lab A used the SIFT algorithm whereas lab B used the EffectGroup3 algorithm to predict whether TP53 mutations were deleterious or not.

The BRCA1-mutated tumor group (lab A) and the BLBCs (lab B) have a similar total amount
of TP53 mutations and a similar amount of complex (truncating frameshift/indel/splice/nonsense) mutations. But the total amount of deleterious missense and hotspot mutations is higher in the BRCA1-mutated tumor group compared to the BLBC tumor group.

The luminal tumors parallel this finding: the total amount of TP53 mutations is higher in the luminal-J tumor group (sequenced by lab A) compared to the luminal-H tumor group (sequenced by lab B). The frequency of complex TP53 mutations is the same in the both luminal tumor groups, but the frequency of deleterious missense mutations is higher in the luminal tumors sequenced by lab A.

In some tumors, lab A finds more than one deleterious TP53 mutation, including hotspot mutations (see Table 1). Therefore, the cause of the differences in numbers could be twofold: lab A may have detected more TP53 mutations in general (all mutations that were present in >25% of the chromatogram peak were included, equal peaks being 50%-50%, heterozygous). Second, it may be that using SIFT as a prediction method finds more "deleterious" missense mutations than using "EffectGroup 3". Together this could have resulted in an overall higher amount of missense TP53 mutation detection in the luminal-J and BRCA1-mutated tumor groups compared to the luminal-H and BLBC tumors.

It is interesting that the amount of complex mutations is similar for the BRCA1 and BLBCs and for the luminal-H and luminal-J tumor groups. With the exception of nonsense mutations, the complex mutations give rise to frameshift mutations which are easily detected in a chromatogram. Also, detection of these mutation types is independent of prediction algorithms. Perhaps this is the reason that lab A and lab B find similar numbers for complex TP53 mutations in tumor groups.

However, the hotspot detection is also independent of prediction algorithms (this concerns a fixed set of mutations defined by Walker et al) and have a higher incidence in the tumor groups sequenced by lab B. Presumably lab A picks up mutations that lab B skips or misses.

For this reason we have decided to keep the tumor groups sequenced by lab A and lab B separate, such that we can be sure to have an honest comparison between the TP53 mutation types.

We have added the following text to page 13 of the Results section:

"The TP53 gene from the BRCA1-mutated/luminal-J tumors and the BLBC/luminal-H tumors were sequenced in different labs with slightly different methods. At the cost of reducing the power of this analysis we wanted to make sure we did not introduce a methodical bias in our comparisons, therefore, the TP53 mutation data for luminal-J and luminal-H tumors were not combined."

6. It would be interesting to report the TP53 mutation rate in luminal BRCA1 tumours vs basal, as these cancers are reported to have fewer TP53 mutations Manie et al Cancer Research 2009.

We agree with the reviewer that this is an interesting aspect. However, since all tumors in our BRCA1-mutated group were basal-like, we were not able to assess TP53 mutation rate in luminal BRCA1-mutated luminal tumors.

7. CGH profiles
The authors comment on a significant difference at chromosome 14 between BRCA1 and BLBC, but it is unclear if this is a chance observation.

The comparative-KC-SMART algorithm is specifically designed to detect significant differences over random (see Holstege et al 2010, BMC Cancer10, 455). Indeed Additional File 5 shows that three of the BRCA1-mutated tumors and none of the BLBC tumors show a gain exceeding the standard deviation of ±1 at this region on chromosome 14.
8. The analysis of Figure 3B is underpowered. It would be useful to have a fourth panel in this figure comparing Luminal-H and luminal J, to assess whether the comparison of Figure 3C is complicated by DNA type.
We agree with the reviewer, this figure has been added. Previously we used the mean amount of aberrations per tumor for each cutoff, however, the median is a better representation of this data as one outlier can have much effect on the mean, whereas the median does not change in the presence of one or two outliers. Therefore, for this new figure we have taken the median of the amount of aberrations per tumor for each cutoff.

The text in the manuscript on page 17 has been changed to:

"Analysis of genomic instability in BRCA1-mutated tumors and BLBCs. To determine the amount of CNAs in the different tumor groups, we used KC-SMART to smooth individual aCGH profiles and counted the amount of CNAs exceeding a range of cutoffs for each tumor separately. We found that the median amount of CNAs of BRCA1-mutated tumors was not different from the amount of CNAs found in BLBCs (Figure 4a). In contrast, we found that the median amount of CNAs is significantly greater in the BRCA1-mutated tumors compared with luminal breast tumors between KSE cutoffs 0.02 and 0.1 and between 0.24 and 0.74 (P<0.01, two sided t-test Figure 4b). Similarly, the median amount of CNAs in the BLBCs was higher compared with luminal tumors for KSE cutoffs between 0.02 and 0.14 and between 0.28 and 0.96 (Figure 4c). We did not detect differences in the median amount of aberrations between luminal-J breast tumors and luminal-H except for KSE cutoffs 0.2 and 0.24 (Figure 4d)."

Figure 4 (previously Figure 3):

9. Figure 5 (Former Figure 4). The clustering of the data in this figure is to a certain extent self-fulfilling. The authors determine which regions differentiate BRCA1/BLBC from luminal tumours, and then on the same data set show this leads to clustering of
the tumours. Demonstration that these regions lead to similar clustering of an independent series would substantially improve the manuscript.

We agree with the reviewer. However, we cannot perform such a validation experiment because we do not have aCGH data from independent BRCA1/BLBC and luminal tumor series that have been profiled on the same BAC array platform. An interesting aspect of Figure 5 is that the BRCA1 and BLBC cases do not form separate clusters but mix together, meaning that only few regions distinguish between BLBCs and BRCA1-mutated tumors. The fact that the luminal-H and luminal-J tumors are mixed, shows that no unwanted biases are introduced by differences in quality of DNA from FFPE vs. fresh-frozen tumor material. As an additional internal control we have used the 6 luminal tumors for which DNA from FFPE and fresh-frozen tumor tissue was both available, and each of the 6 pairs cluster together.

We have added the following text to the Results section on page 18 discussing Figure 5:

"Interestingly, the BRCA1 and BLBC cases do not form separate clusters but mix together, meaning that only few regions can distinguish BLBCs/BRCA1-mutated tumors from luminal tumors. The fact that the luminal-H and luminal-J tumors are mixed, shows that no unwanted biases are introduced by differences in quality of DNA from FFPE vs. fresh-frozen tumor material. As an additional internal control we have used the 6 luminal tumors for which DNA from FFPE and fresh-frozen tumor tissue was both available, and each of the 6 pairs cluster together."
1. Holstege et al compared TP53 mutation frequency and type and aCGH profiles among BRCA1-mutated, basal-like, and luminal breast cancers. The study is well designed with the limitation of comparing data from tumors that differed in preservation (fresh-frozen vs formalin-fixed) but the authors used appropriate statistics to solve this problem.

We thank the reviewer for his positive comments and appreciate the fact that he acknowledges the significance of our work.

2. The author should comment why the frequency of TP53 mutations in the two series of luminal tumors is so different. In this sense, information regarding the histological grade of tumors might be important.

We agree with the reviewer, and this subject has been extensively addressed in point 5 of reviewer 1.

3. Since the authors suggested that TP53 mutations might modulate sensitivity to certain types of chemotherapy, the article should also discuss TP53 mutation as a mechanism of resistance, as was previously reported in some papers by one of the co-authors (A-L Borresen-Dale).

The reviewer is presumably referring to the interesting papers by Stephanie Geisler et al., in Cancer Research 2001 and Clinical Cancer Research 2003. These papers concern the association of distinct mutations in the L2 and L3 domain of TP53 with resistance to anthracyclins, 5-fluorouracil and mitomycin in locally advanced breast cancer. We agree with the reviewer that our article should include these data in the discussion.

The following has been added to the discussion on page 21.

"The high frequency of TP53 mutations in non-hereditary BLBCs might suggest that these tumors are also compromised in homology-directed DSB repair. To test this possibility, it would be interesting to perform functional assays to measure DNA damage response and DNA repair in non-hereditary BLBCs with a (complex) TP53 mutation. It has previously been suggested that TP53 mutations, including complex TP53 mutations, affecting the DNA binding domain of the p53 protein may cause resistance to several different cytotoxic compounds such as anthracyclins, 5FU and mitomycin [43, 44]. However, tumors used in these studies were primarily invasive ductal carcinomas, most of which are likely not compromised in homology directed DSB repair. It is therefore interesting that Silver et al. recently reported a significant association between truncating TP53 mutations and cisplatin response in TNBCs [45]."