Author's response to reviews

**Title:** Quantitative methylation profiling in tumour and matched morphologically normal tissues from breast cancer patients

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

We would like to resubmit our manuscript entitled ‘Quantitative methylation profiling in tumor and matched morphologically normal tissues from breast cancer patients’ by Ilse Van der Auwera et al. We carefully took the comments of the reviewers into account and accommodated their remarks in a revised paper (as indicated below).

In general, concerns have been raised over the sample size of normal breast tissues from unaffected women in this study. Although we acknowledge that the sample size of normal breast tissues from unaffected women is rather small (but similar to prior reports from literature), we do not feel that this raises concerns regarding the conclusions made in our manuscript. There are some arguments to support this. First, none of the normal breast tissues acquired by reduction mammoplasty showed pathological changes. In our experience, no or low levels of methylation are observed in this kind of samples. In a previous study analyzing methylation of the APC gene in 27 normal breast tissues from unaffected women (obtained from reduction mammoplasty specimens), we observed methylation in only 3 samples (Van der Auwera et al., Br J Cancer 2008). Also in this study, methylation in normal breast tissue samples was undetectable or low. Second, methylation profiling of normal breast tissues from unaffected women on a genome scale using the Infinium HumanMethylation27 BeadChips shows that these samples have very little variation in their methylation profile (results will be presented at the upcoming AACR meeting). Third, the reported fraction of methylation-positive breast tumors, determined by using the maximal methylation value in normal breast tissues as a cutoff, highly agrees with other reports from the literature (as indicated in the table below).

Although we acknowledge that there are some previous reports with similar conclusions to ours, we believe that this manuscript could still make an important contribution to the field of epigenetics given its important message regarding the source of normal breast tissue as a comparator for determining the methylation status of tumors. Furthermore, a better understanding of how field cancerization occurs has practical implications for predicting the future risk of local recurrence in breast cancer patients undergoing lumpectomy.
Answers to the comments of reviewer 1

- We have further expanded our discussion of other reports on the subject on page 12.
- We have added references of reports on hypermethylation in atypical hyperplasia and ductal carcinoma in situ in the discussion on page 11.
- We have added the P values indicating the difference between methylation in the normal breast tissues from healthy women and the normal breast tissues from cancer patients in the abstract. We have added the P value indicating the difference between methylation in the cancerous tissue and the normal tissues from breast cancer patients in the abstract.
- We have omitted Table 2 and the reference to the table in the materials and methods section as requested by the reviewer.
- We have deleted Table 5 as requested by the reviewer.
- We have omitted Table 6 as requested by the reviewer.
- We have provided a more detailed legend for Figure 2.

Answers to the comments of reviewer 2

- We have omitted the data regarding the comparison of adjacent normal tissue from mastectomy and lumpectomy samples as requested by the reviewer.
- All normal breast tissues were from unaffected women who underwent breast reductive surgery as was mentioned in the materials and methods section on page 6 and in the results section on page 8. Samples contained no pathological changes.
- Of a total of 6 genes, 3 genes showed P-values of <0.05. This corresponds to a false discovery rate of smaller than 0.1, which is regarded as a valid threshold for significance.
- We have deleted Figure 1 as requested by the reviewer.
- A search in the PubMeth database (http://www.pubmeth.org), which is an annotated and reviewed database of methylation in cancer, revealed following results for relative methylation frequencies in breast cancer:

<table>
<thead>
<tr>
<th>Gene</th>
<th>Average % of methylated breast cancer samples</th>
<th>Range of % of methylated breast cancer samples</th>
<th># ref in PubMeth</th>
<th>% of methylated breast cancer samples in this study</th>
</tr>
</thead>
<tbody>
<tr>
<td>RARB</td>
<td>34%</td>
<td>20-46%</td>
<td>5</td>
<td>29%</td>
</tr>
<tr>
<td>HIN-1</td>
<td>48%</td>
<td>40-57%</td>
<td>2</td>
<td>59%</td>
</tr>
<tr>
<td>TWIST1</td>
<td>56%</td>
<td>32-87%</td>
<td>3</td>
<td>46%</td>
</tr>
<tr>
<td>APC</td>
<td>49%</td>
<td>28-86%</td>
<td>10</td>
<td>55%</td>
</tr>
<tr>
<td>RASSF1A</td>
<td>62%</td>
<td>9-72%</td>
<td>10</td>
<td>77%</td>
</tr>
<tr>
<td>DAPK</td>
<td>52%</td>
<td>13-94%</td>
<td>3</td>
<td>27%</td>
</tr>
</tbody>
</table>
The frequency of gene hypermethylation observed in our study is well within the range of hypermethylation frequencies observed in other breast cancer studies.

The Kappa statistics describes the concordance in methylation status between the cancerous and normal breast tissue samples. We have added this to the head of Table 4 and in the results section on page 9.

- We did not investigate the association between methylation changes and age for normal breast tissues from unaffected women, since the age distribution in this population did not allow for such an analysis. All normal breast tissue samples were derived from patients who underwent breast reductive surgery, which are usually younger women. The mean age of these women in this study was 36 years (+/- 7 years).

Yours sincerely,

Ilse Van der Auwera