Author's response to reviews

Title: A novel germline PALB2 deletion in Polish breast and ovarian cancer patients

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Author's response to reviews: see over
To
Melissa Norton, MD
Editor-in-Chief of BMC Medical Genetics


Dear Dr Norton,

We greatly appreciate the reviewers’ suggestions and we corrected the manuscript accordingly. Enclosed we are sending the revised version of the manuscript, as well as the response to the reviewers.

According to the suggestions we performed additional analyses (immunohistochemical stainings of breast cancers and genotyping of mutation carriers). For the purpose of immunohistochemical stainings we obtained paraffin blocks and we cut sections for HE stainings, too. I, as a pathologist have changed one original diagnosis (tumor 893, atypical medullary carcinoma with a ductal component) into ductal carcinoma. The new diagnosis was also in agreement with negative basal markers. We also corrected English. Therefore some paragraphs may not appear exactly the same.

Sincerely yours
Jolanta Kupryjańczyk MD, PhD
Minor Essential revisions:
Ad1) Page 5. The classification of the familial breast cancer cases should be more detailed. How many families had at least one 1st or 2nd degree relatives affected with breast/ovarian cancer? Did the families with at least one 3rd degree affected relative have any additional selection criteria (such as young age at onset/multiple tumors) for being classified as familial ones, and if not are they really familial cases?

We have added the following text (Materials, Breast cancer patients, page 5):
In 40 families with third-degree relative affected, additional criteria were employed: carcinomas developing in a proband and/or a relative before the age of 50, metachronous or synchronous cancers diagnosed in a proband or a relative, or non-breast and non-ovarian cancers diagnosed in first- or second-degree relatives.

Ad2) Table 1. The identification of different groups (patients/controls) is missing in frequency label. In addition, the different asterisks should be explained.

We have corrected it (this is Table 2 now).

Major revisions:
Please check “basal” markers in the medullary PALB2+ breast cancers – CK5, CK14, EGFR and KIT would be good markers to choose from. HER2 should be completed if at all possible.

Immunohistochemical stainings for all 'basal' markers mentioned, as well for HER2 receptor were completed. For the purpose of immunohistochemical stainings we obtained paraffin blocks and we cut sections for HE stainings, too. JK as a pathologist has changed one original diagnosis (tumor 893, atypical medullary carcinoma with a ductal component) into ductal carcinoma.

We have added the following text:
(Methods, page 8):

Immunohistochemical stainings
Markers of basal/luminal types were evaluated immunohistochemically with the use of the following antibodies: anti-CK5/6 (DAKO, clone D5/16B4), anti-CK14 (Novocastra, clone NCL-L-LL002), anti-CK17 (DAKO, clone IR620), anti-EGFR (DAKO, clone K1994), anti-CD117 (DAKO, clone A4502). All immunostainings were performed against negative controls. Non-neoplastic mammary gland structures served as intrinsic positive controls.

(Results, c.509_510delGA deletion, Breast cancer patients, page 11, 2nd paragraph):
To characterize breast carcinomas with the PALB2 mutation more specifically, we evaluated the expression of CK5/6, CK14, CK17, EGFR and CD117 (Table 3). Two carcinomas were of the basal type and two of the luminal type.
In agreement with data presented by Heikkinen et al. [23], our study suggests that breast cancers with PALB2 mutations are predominantly triple-negative ones; however, their phenotype does not completely overlap with the basal type.

(Table 3):

Table 3 - Characteristics of breast cancers with c.509_510delGA deletion in the PALB2 gene

<table>
<thead>
<tr>
<th>Proband no.</th>
<th>Age</th>
<th>Type, grade</th>
<th>ER, PR</th>
<th>HER2</th>
<th>CK5/6, CK17, EGFR</th>
<th>CK14</th>
<th>CD117</th>
</tr>
</thead>
<tbody>
<tr>
<td>802</td>
<td>53</td>
<td>ductal, G2</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>2076</td>
<td>62</td>
<td>ductal, G3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>893</td>
<td>44</td>
<td>ductal, G3  bifocal</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>1540</td>
<td>47</td>
<td>medullary</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

ER-estrogen receptor, PR- progesterone receptor, HER2- human epidermal growth factor receptor 2, CK- cytokeratin, EGFR- epidermal growth factor receptor, CD117- C-kit receptor

Minor revisions:
Abstract: Methods – I do not think the word “random” in “random ovarian cancer patients” is quite right. What exactly do you mean? Non-consecutive, incident, prevalent, consecutive, non-selected etc? Please clarify.

We have replaced “random” with “non-consecutive” and we use this term consequently throughout the manuscript.


We have added it – reference no. 15.

Methods: page 6 – it is not clear what is meant by “ovarian cancer samples and respective blood samples, as well as control samples were screened for PALB2 alterations...”. Do you mean the DNA from the tumors was screened at the same
time as was DNA from lymphocytes? Or was lymphocyte DNA analysed first with results checked in the tumors? If not, the somatic mutations identified only in the tumors should be presented.

Were the controls screened for all mutations? This should be clarified in this section, rather than later. This is not really made clear in the paper.

We have corrected it as follows (Methods, page 6):
All 13 PALB2 exons with intron/exon boundaries were initially screened for variants in 70 non-consecutive ovarian carcinomas by the PCR-SSCP and sequencing methods. Sequence variants of interest were further studied in larger groups of ovarian carcinomas and in control blood samples (Table 2). The germline origin of changes detected in ovarian carcinomas was confirmed in blood samples from those patients. The analyses were performed in the Department of Molecular Pathology. Blood from breast cancer patients was screened only for the c.509_510delGA PALB2 deletion in the Department of Endocrinology, with the use of the dHPLC method.

Page 7 - the primers should be in a table.

We have added Table 1 with primer sequences.

Page 8 – the sensitivity of SSCP is well known to be well below 95%. Please comment on this and reference several relevant papers.

We have added the following text (Methods, Single strand conformational polymorphism analysis (SSCP), page 7):
In our experience, this method detects 90% of all alterations, and 100% of deletions and insertions [20].

(Discussion, page 14, the last paragraph):
Our study had some limitations. The entire coding sequence of the PALB2 gene was screened in 70 non-consecutive ovarian cancers only; the sensitivity of the SSCP ranges from 70% to 95%, according to different publications [27, 28], and it is 90% in our laboratory [20]. Thus, some PALB2 alterations (particularly of the missense type) could have been missed.

Results - page 10 – was it possible to test any other individuals, or pathology blocks from the family of patient 293? It would be very helpful if the segregation of the two mutations could be better studied. The presence of both a BRCA2 and PALB2 mutation in the same person obviously makes it very hard to interpret the significance of the overall findings with respect to OC risk in PALB2 carriers. Is it clear that the families of the two PALB2 mutation carriers are not fairly closely related?

The patient number 293 died. We have no information where her family members were treated. According to your suggestion we have passed on patient's data to Genetic Counselling Department and if we could do some more analyses it will be publish as a letter.

Page 10: Please show the presumptive haplotype of the seven women with the putative founder mutation
Genotype analysis of all \textit{PALB2} carriers was done with a use of three markers D16S841, D16S403, and D16S417. No common phenotype for all carriers could be found. Therefore, we have replaced the term “founder mutation” by “recurrent mutation”.

We have added the following text (Methods, \textit{PALB2} Haplotyping, page 8):

\textbf{\textit{PALB2} Haplotyping}

DNA extracted from blood samples of \textit{PALB2} mutation carriers was genotyped with the use of three markers applied by Foulkes et al. \cite{8}: D16S841 [UniSTS:2638], D16S403 [UniSTS:150021], and D16S417 [UniSTS:67206]. Primer sequences were obtained from the NCBI \cite{21} UniSTS database. The loci were amplified by PCR (as described earlier, at the annealing temperature of 58°C) with the use of fluorescently labelled (6-FAM dye) forward primer. PCR products were diluted in sterile water, in a volume depending on reaction efficiency. The dilution at the volume of 0,8µl was mixed with 8µl of formamide (SIGMA) and 0,4µl of the standard (Gene Scan 500 ROX, Applied Biosystems). The mixture was denaturated for 5 min at 95°C and then cooled on ice. Electrophoresis was performed in the ABI PRISM 377 DNA sequencer. Data were analyzed using the Peak Scanner Software v1.0 (Applied Biosystems).

(Results, Haplotype analysis, page 11):

\textbf{Haplotype analysis}

Genotyping of the seven c.509_510delGA deletion carriers was performed with three microsatellite markers: D16S417 which is distal to PALB2, and D16S481 and D16S403 that are proximal to this gene. PALB2 mutation-positive individuals had four variants of a haplotype. In particular, patients 293, 893 and the woman from the control group shared 4-2-3 alleles, while patients 375 and 802 shared 4-2-6 alleles. Each of the other two patients had a unique haplotype.

(Discussion, page 14, 2\textsuperscript{nd} paragraph):

However, the genotype analysis of the mutation carriers did not reveal a common haplotype. This indicates that the detected deletion might have arisen two or more times independently as a recurrent hot-spot mutation, and not by founding effect. More detailed analysis is necessary to determine the origin of this alteration.

\textit{Discussion: page 12 – frequency of PALB2 in breast and ovarian cancers was said to be the same, but the presence of one BRCA2 mutation in one of the two cases must be included at this point in the discussion.}

We have corrected it as follows (Discussion, page 13, 3\textsuperscript{rd} paragraph):

In our study, \textit{PALB2} alterations did not associate with ovarian cancer risk. In addition, the \textit{PALB2} deletion was accompanied by a germline \textit{BRCA2} nonsense mutation in one ovarian cancer patient (Moes et al., unpublished data). A similar observation was published by Heikkinen et al. \cite{23} who found two breast cancer patients with a \textit{PALB2} deletion among 104 \textit{BRCA2} mutation carriers. Interestingly, the presence of these two alterations in carcinomas appears to be more frequent than just one occurring by chance, considering the low frequency of \textit{PALB2} and \textit{BRCA2} mutations in control populations.
Page 13: Mention Tischkowitz study at ref 14.

Done -reference no. 15.

Discretionary revisions:
Page 14: it is quite notable that Heikkinen also found 2 breast cancer patients who were double mutants for PALB2 and BRCA2. Based on the gene frequency of each gene in controls in Finland, can the authors speculate on how often this would occur by chance? 2/104 seems somewhat high to me: it would support their own data if the observed ~2% were significantly different from the expected number.

We have added the following text (Discussion, page13, 3rd paragraph):
Interestingly, the presence of these two alterations in carcinomas appears to be more frequent than just one occurring by chance, considering the low frequency of PALB2 and BRCA2 mutations in control populations.

Typographical errors
Page 14, first line: Garcia, not Gracia. There are other scattered typos and an editorial check of English usage would be worthwhile.

We have corrected them.

Editor’s comments:

Research carried out on humans must be in compliance with the Helsinki Declaration (http://www.wma.net/e/policy/b3.htm). A statement to this effect must appear in the Methods section of the manuscript (alongside confirmation of ethical approval and consent), including the name of the body which gave approval, with a reference number where appropriate.

It is included to our manuscript (Materials, Controls, page 6, 2nd paragraph):
Patients gave their written informed consent to be included in the study. Anonymous blood donors gave informed consent. The study was approved by the bioethics committee of the Institute of Oncology (ref. no. 39/2007).

Please include an Authors’ contributions section before the Acknowledgements and Reference list.

It has been added.