**Reviewer’s report**

**Title:** Association of Cytokeratin 5 and Claudin 3 expression with BRCA1 and BRCA2 germline mutations in women with early breast cancer

**Version:** 0  **Date:** 22 Feb 2018

**Reviewer:** Joseph Geradts

**Reviewer's report:**

**Major Points:**

1. Previous publications have reported a wide range in the prevalence of BRCA1 mutations among triple negative breast cancers (TNBC), with an average rate of about 35% [Peshkin BN et al., Breast Dis. 32: 25-33, 2010]. One study found the following frequencies of BRCA1 mutations in TNBC: 16% in the whole cohort, 27% in women under age 50, and 48% in women with a family of breast cancer (similar to Danzinger's cohort) [Fostira F et al., Breast Cancer Res. Treat. 134: 353-62, 2012]. Another publication confirmed that TNBC status is highly predictive of BRCA1 mutation status [Spurdle AB et al., Breast Cancer Res. 16: 3419, 2014]. The latter study also pointed out that age is an important parameter that should be considered. Thus, I suggest that the authors examine the effect of age on their reported associations, perhaps stratifying their patients into two groups (<50 y, ≥50 y). Perhaps more importantly, the body of published literature raises the question whether TNBC status alone may be sufficient to recommend BRCA1 testing (especially in premenopausal patients), and whether any additional biomarkers are actually necessary. Danzinger et al. provide relevant data on this topic. As shown in Figure 3, the AUC for TNBC alone is 0.79, which is quite good already. The authors show that the AUC can be further increased, but is that clinically useful? Can the authors demonstrate that unnecessary BRCA testing could be avoided for patients with TNBC who are negative for CK5 and CLDN3 (who presumably would not have a BRCA1 mutation)? And what percentage of cases would fall into that category?

2. There are several issues related to the interpretation of the immunohistochemical stains:

(a) Regarding E-cadherin, negative staining clearly is aberrant, but down regulation may also be considered abnormal (due to gene mutations, hemizygous deletions, partial promoter hypermethylation etc.). How many cases showed significantly reduced E-cadherin staining? How would the reported associations change if those cases were included in the "negative" (reduced/ down regulated) category?

(b) It would be very informative if the authors could add information about the range of staining seen in the breast cancers for the different claudins. They should also specify the staining in benign breast epithelium and the relative importance of membranous versus cytoplasmic staining (as shown to be important in Ref. 53).
Although many investigators uncritically accept any degree and type of staining as positive for CK5/6, CK14 and EGFR, it can be debated if a tumor with 1% weak staining is biologically equivalent to one with 100% strong staining. Likewise, it is unclear whether this threshold is optimal for claudin expression. As mentioned above, the reader may be able to form a better opinion on this matter if there was more information about the distribution of staining in the reported cohort of TNBC.

As detailed in Ref. 53, heterogeneous claudin staining is common in whole tissue sections of breast cancers. Danzinger et al. used tissue microarrays (TMAs), and it is important to state how many cores per case were evaluated and what the core diameter was. The authors state that claudin staining was "fairly homogeneous", but more details are required. It's not appropriate to assess heterogeneity in individual tissue cores, but it would be informative to describe the concordance rate of multiple cores per case.

The authors report a very low frequency of CLDN7 expression. This is in marked contrast to the published literature reporting significantly higher rates (Refs. 36, 53, 63) and needs to be explained. It would help if the authors could document the sensitivity of their immunohistochemical assay and confirm that benign epithelium was consistently positive.

3. Table 1:
(a) DCIS (n=2) should be eliminated from the analysis because it is a disease that in many important aspects is different from invasive breast carcinoma.
(b) It would be helpful to emphasize in the manuscript text (Results) several important pieces of data:
   - in the BRCA1 group there are no lobular or mixed lobular/ductal carcinomas;
   - E-cadherin is more often negative in tumors with BRCA2 mutations compared to both BRCA1 and non-mutated tumors;
   - 36 of 41 CLDN3-positive cases have a BRCA1 mutation, and 40 of 41 such cases have a mutation in either BRCA1 or BRCA2 (which is remarkable).

4. Figure 1:
Panel a (CLDN3): There is weak cytoplasmic staining but no obvious membrane staining.
Panels b/c (CLDN4): The staining seems to be mostly cytoplasmic.
Panels d/e (CLDN4): There is stromal staining (should be negative), which raises the possibility of nonspecific reactivity.
Panel f (CLDN7): Please pick a field that shows positive benign epithelium, in addition to negative tumor.
Panel i (E-cadherin): Please pick a field that shows positive benign epithelium, in addition to negative tumor.
In addition, I strongly recommend to add pictures showing staining of the four markers in benign breast epithelium (either as additional panels or in a separate figure).

5. Figure 2:
This shows the same data as Table 1 and thus is redundant.

6. Figure 3:
It would be instructive to add another ROC curve: ER/PR/HER2/CLDN3. This may help answer the question whether CK5 adds any information to CLDN3. Moreover, the authors should discuss the overlap in CLDN3 and CK5 staining, and whether the combination is significantly better than either marker alone.

7. While the authors focus on the relevance of claudin testing to detect BRCA1 mutations, they should also discuss the importance (or lack thereof) of CLDN3/4/7 and E-cadherin testing for BRCA2 mutational analysis.

Minor points:

1. The authors should clarify that they studied 239 (should be 237) breast cancer cases, not TMAs.

2. Luminal B breast cancers include ER/PR-positive, HER2-positive cases, but the two are not equivalent (as implied on p.4 l.12 and p.10 l.56), since Luminal B tumors also include HER2-negative cases with low PR or high proliferation.

3. The authors inserted two sentences on p.4 (lines 40-45), but it is unclear what they mean.

4. The described immunohistochemical staining protocols for ER, PR, HER2, CK5, CK14 and EGFR are incomplete. If the data were taken from the pathology reports (however, most breast cancer reports do not include data on cytokeratins or EGFR), no details need to be given. If the stains were performed by the authors, please specify antigen retrieval methods, primary incubation times, detection kit(s), and immunostainer used. For CK5, CK14 and EGFR the primary antibody concentration/titer is not stated.

5. Regarding the interpretation of the HER2 stains, current ASCO/CAP guidelines suggest that the staining is not only in at least 10% of the tumor cells, but it should also be strong and circumferentially membranous. If any cases were considered HER2-positive or -negative as a result of ISH assays, that should also be stated.

6. In the Results, it may be better to mention HER2 positivity (rather than negativity) rates.

7. Regarding the positive controls for CLDN3/4/7 and E-cadherin: Why did the authors not use benign breast tissue for all four markers?
8. CLDN3 was negative in 39% of BRCA1 tumors and in 82% of BRCA2 tumors. On p.8 (l.53) the authors mention that CLDN3 loss was "20 times more likely" in BRCA2 versus BRCA1 tumors, which in my opinion gives the wrong impression.

Are the methods appropriate and well described?
If not, please specify what is required in your comments to the authors.

Yes

Does the work include the necessary controls?
If not, please specify which controls are required in your comments to the authors.

No

Are the conclusions drawn adequately supported by the data shown?
If not, please explain in your comments to the authors.

Yes

Are you able to assess any statistics in the manuscript or would you recommend an additional statistical review?
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