Author's response to reviews

Title: Claudin 1 expression in basal-like breast cancer is related to patient age

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Version: 3 Date: 5 May 2013

Author's response to reviews: see over
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Version: 2 Date: May 5, 2013

Author’s response to reviews: see over
Thank you for considering of our manuscript for publication in your journal. We have reviewed the above manuscript according to your reviewer’s comments.

#1 Reviewer’s report

Title: Claudin 1 expression in basal-like breast cancer is related to patient age

Version: 2 Date: 5 May 2013

Reviewer: Gabriel DiMattia

Reviewers report:

This manuscript describes a great deal of histopathological work looking at the relative levels of claudin1 and 4 in basal-like and non basal-like breast tumour samples using TMAs. They performed an exhaustive and appropriate analysis of the data to determine whether high levels of claudin1 might be associated with specific disease parameters. however found that high levels were only associated with greater than 55 years of age at presentation. They also went on to provide functional data regarding the potential significance of high CLDN1 by generating stable KD of CLDN1 in BT-20 breast cancer cells and found that some EMT markers were reduced in expression and that migration of these cells seemed to be negatively effected. they also observed an unusual subcellular localization in the TMA with claudin1 protein in the cytoplasm instead of being concentrated at the cell membrane. Overall the manuscript is well-written and the data sound. Ideally one would like to see the functional studies consistent across multiple cell lines and western blot data to backup the qPCR data.

Specific Comments:

Given that BLBC is characterized by a mesenchymal phenotype and claudins are considered essential for the overall maintenance of the differentiated state of epithelial cells it seems odd that BLBC cells would show high levels of claudin 1. I would have expected the authors to comment on this in the Introduction or Discussion, unless I missed it.

Yes, the novel observation that high levels of claudin 1 were associated with the BLBC subtype of human invasive breast cancer reported in our previous paper was indeed unexpected. However, we have also observed high endogenous claudin 1 levels in basal-like human breast cancer cell lines, such as the BT20 and MCF10A (unpublished data). The authors have now further commented on this in both the introduction and discussion sections (pages 6 and 18).

The authors indicate in the Introduction that only recently has claudin 1 been shown play an active role in tumorigenesis and I wonder if this included xenotransplantation assays with specific cell lines containing shRNAs?

Yes. Dhawan et al (2005) convincingly demonstrated that changes in claudin 1 expression had significant effects on growth of xenografted tumors and metastasis in athymic mice.
"Although a large cohort of tumors (350 samples) was examined in our earlier study, they belong to mixed pathological lesions. As such, clinical relevance of claudin 1 expression to the BLBCs could not be properly addressed."

The above statement in the manuscript seems to be a major portion of the rationale for the work so I would ask the authors to provide a comparison of their data to the CLDN1 data in the provisional breast cancer TCGA dataset (i.e., cBio website). Does the TCGA dataset agree with their data or are they not comparable?

Although we had not included this information in the discussion of the previously submitted manuscript, we did compare the data with the Cancer Genome Atlas (TCGA) breast carcinoma provisional dataset, accessed through the cBio portal (http://www.cbioportal.org/public-portal/index.do). The RNA sequence analysis revealed that claudin 1 was up regulated in 17/81 (21%) of basal tumors compared with 2/324 (<1%) of the luminal A/B cases. We thank the reviewer for pointing out the relevance of including this in our discussion. We have now done so (page 18).

I would also ask the authors to be more definitive regarding the above statement and define mixed pathological lesions.

Mixed pathological lesions refer to different histologic types of invasive breast cancers: namely ductal, lobular, medullary, papillary and metasplastic, that constitute the TMA analyzed in our previous study. Clarification of this term has now been incorporated into the introduction section (page 5).

Perhaps the authors could be more specific regarding the production of the TMAs used in their studies? Who made them and where? Are BLBC enriched TMAs common or easily generated? If not, perhaps that should be indicated.

The “basal-like” enriched TMA used in this study as well as the TMA used in our previous study were generated by the Manitoba Breast Tumor Bank (MBTB, page 7). In the previous study, the TMA consisted of 350 estrogen receptor +ve/-ve breast tumors, of which only 18 were classified as basal-like. Since generally <15% of breast cancers are basal-like, it is very difficult to generate a very large basal-like cohort for study. We have now indicated this in the introduction (page 5).

Referring to the shRNA expressing lines as clones may not be correct. Are these clones or a mixture of BT-20 cells with independent transgene integration events? Clones implies derivation from a single cell with a unique integration event similar to a limiting dilution cloning assay.

The shRNA expressing cell lines are separate clones derived from a single cell. We have clarified this in the method section (page 9). A minimum of 2 clonal lines were analyzed in each experiment.
H-scores are essential for quantifying subjective assessment of claudin-1 levels in TMAs but what does this really mean in terms of levels of expression? IHC Examples of these data would be useful.

H-scores were derived from a semi-quantitative assessment of both staining intensity (scale 0–3) and the percentage of positive cells (0–100%), which when multiplied, generated a score ranging from 0–300. Breast cancers were considered claudin 1 positive with an IHC-score of >0. We have previously published examples of claudin 1, 3 and 4 IHC staining on TMAs showing various levels of expression reflecting a range of H-scores (Blanchard et al, 2009). See also, Skliris et al, 2008.

The down-regulation of proteins of genes that encode factors regulating EMT is an interesting result; but does this imply that the BT-20 cells have a more mesenchymal phenotype relative to other cell lines?

The BT-20 cells are considered to be of the mesenchymal phenotype (Neve et al, 2006).

Is there any evidence in the literature that this line exhibits a mesenchymal phenotype with loss of EpCAM and E-Cad and specific cytokeratins? Are these cells more "epithelial" upon loss of claudin 1 which doesn't make sense since epithelial cells or adenocarcinoma cells should show tight junctions. I don't think the authors have provide sufficient context for the observations regarding epithelial or mesenchymal phenotype in the BT-20 cells in order for their data to make sense, at least to me.

As mentioned previously, the BT-20 cells are indeed mesenchymal. The authors could not find experiments specifically pertaining to loss of EpCAM and E-Cad and specific cytokeratins in the BT-20 cell line, however a search of Oncomine, for example showed that in the Barretina cell line database E-cadherin and EPCAM were high in the BT-20 cell line. This observation concurs with the unexpected high claudin 1 we see in this cell line.

Moreover the authors could provide a few specifics regarding the PCR array i.e., how genes included in the array given that they saw significant alterations in just seven genes.

This was indeed an oversight and has now been added to the methods section (page 12). The PCR array used in this study profiles the expression of 84 key genes that change their expression during EMT.

MW markers in Fig. 3 western? Should we expect to see a smear? Post-translational modifications?
The omission of MW markers in figure 3 was an oversight and has now been corrected. We had attributed the smearing to the high intensity of the band. However it could also be due to the acetone precipitation prior to the Western blotting.

The authors measure cell migration using a scratch assay but what about cell viability over time or sensitivity to stress i.e. chemoresistance upon down-regulation of CLDN1? Can the authors comment on this?

These are interesting questions and are important components of our future studies. Such work is currently underway.

Level of interest: An article of importance in its field
Quality of written English: Acceptable
Statistical review: Yes, but I do not feel adequately qualified to assess the statistics.
Declaration of competing interests: No competing interests
Review Report for Manuscript entitled ”Claudin 1 expression in basal-like breast cancer is related to patient age.” The authors showed claudin 1 was related to patient age in basal-like breast cancer, and knocking-down of claudin 1 resulted in a significant decrease in migration rate. Moreover, knocking-down of claudin 1 altered the expression of some genes associated with EMT. The authors have already demonstrated that there is a positive association between claudin 1 expression and ER-ve breast cancers in reference 19. ER-ve, one of the criteria for BLBC, is usually found in postmenopausal women. Therefore, the conclusion “Claudin 1 expression in basal-like breast cancer is related to patient age” is lack of innovation. Though it has merit, it is still not enough and not suitable for publication as its current version.

The innovation which sets this study apart from our previous study is that here we are addressing the clinical relevance of high claudin 1 in BLBCs within a much larger cohort of BLBCs. In our previous study, although we found a significant correlation between BLBC and claudin 1 expression only 18 of the 350 breast cancers analyzed were of the BLBC subtype. Since this subtype constitutes only 15% of all invasive breast cancer lesions it is not easy to accrue a large number of patient samples. As well, our observation that high claudin 1 expression is associated with tumors derived from post-menopausal women, hints at a relationship with estrogen levels. Since ER+ve breast cancers (and not ER-ve) are more prevalent in older women as compared to younger pre-menopausal women (Anderson et al, J Clin Oncol, 19:18-27, 2001; Chen and Colditz, Nature Clin Practice 4:415-423, 2007) this revelation could help identify a subset of BLBC. Our study also contributes further insight into a subgroup of aggressive breast cancers which are poorly characterized.

Major comments
Comment 1: The authors observed the expression of claudin 1 in membrane and cytoplasm and referred that ”claudin 1 mislocalization was shown to increase the invasiveness of the cancer cells” in Discussion. However, the BT-20 cell line which chosen to perform the subsequent research only express claudin 1 in membrane (Figure 3A).

This statement may have been taken out of context. We stated that “there have been recent reports of claudin 1 mislocalization in the cytoplasm and in some cases the nucleus in a numbers of other cancers, including melanomas, colon, and oral squamous cancer [11,16-18,31]. In these cancers, claudin 1 mislocalization was shown to increase the invasiveness of the cancer cells [11,16-18,31].” We were therefore referring to what has been shown for melanomas, colon, and oral squamous cancer. However further analysis of our data showed that patients whose tumors retained membrane claudin 1 expression in more than 10% of the tumor cells showed a trend towards increased survival (Kaplan-Meier analysis, p=0.25). We have now included this in our results.
section (page 15). We have addressed our reason for using the BT-20 cells in our response below.

It is confusing that inhibition of claudin 1 membrane expression alone could weaken the invasiveness of breast cancers. I am concerned whether it is suitable for choosing BT-20 cell line as the subject. It is equally possible that high expression of claudin 1 in BLBC is mainly localized in cytoplasm, while the membrane expression of claudin 1 was decreased indeed.

The reviewer is correct in his observation that the BT-20 cells used for these experiments might not be the ideal model system if we were addressing the impact of claudin 1 mislocalization in the cell as they exhibit high claudin 1 membrane expression. However, our focus was to examine the effect of high claudin levels associated with the basal-like subtype, as we observed in the patient samples. It was chosen solely based on its classification as a BLBC cell line (Neve et al, 2006), and its high endogenous level of claudin 1 compared with other breast cancer cell lines (unpublished observation). At the same time, based on our own studies, we have not identified a BLBC cell line that is high in cytoplasmic claudin 1. For example MDA-MB-231 cells, which is another BLBC cell line, is low in endogenous claudin 1 expression and cytoplasmic claudin 1 was undetectable, and thus rendering it inappropriate for these studies. A search of Oncomine has allowed us to identify other potential BLBC cell lines with high claudin 1, however there is no published data on the localization of the protein and therefore no guarantee that any of these may be more appropriate than the BT-20 cell line.

Comment 2: The authors should explain why was the cytoplasmic expression of claudin 1 increased slightly in Cldn1 KD group (Figure 3B). It is confusing that the localization of claudin 1 was altered after treatment with siRNA.

Yes, the reviewer is correct regarding this observation. We had not adjusted for length of exposure time for the two images. In the claudin 1 KD group the signal is overexposed and the specific membrane staining is not clear. We have adjusted these images appropriately.

Comment 3: The authors should perform Invasion assay to detect the impact of claudin 1 on the invasiveness of cancer cells.

Yes, we definitely agree with this suggestion and this will be addressed in the future. However, the wound healing assay is frequently used to demonstrate direct effect on cell motility (Dhawan et al,2005; Leotlela et al, 2007).

Comment 4: The authors provided some data to show that claudin 1 may be involved in regulating EMT and each discussion presented is intriguing, but overall the work falls short of demonstrating the mechanism by which claudin 1 regulates EMT.
Yes we agree more functional studies are warranted, but at the present time the authors feel that this is outside the topic of this paper. Future studies will be carried out to address the mechanisms and impact of claudin 1 localization in breast cancer.

Minor comments
Comment 1: The authors referred 151 breast cancer samples including 79 cases of BLBC and 72 cases of “non-basal” breast cancers in Methods, but only 144 cases (79 cases of BLBC and 65 cases of “non-basal” breast cancers) were analyzed for claudin 1 and 135 cases (73 cases of BLBC and 62 cases of “non-basal” breast cancers) were analyzed for claudin 4 in Table 1. The authors should explain the inconsistency in these positions.

The TMA consisted of a total of 151 tumor biopsies. However, only those tumors from which we were able to retrieve interpretable data (intact, unfolded tumor sections) were considered for our analysis. Additionally, some tumor cores were not available for analysis due to exhaustion of the TMA, and therefore interpretable data could not be derived from these samples. This has been added to the methods section (page 8).

Comment 2: The authors referred 79 cases of invasive breast cancers were categorized as BLBC, but only 73 cases were analyzed for the correlation between claudin 1 and Node status, 76 cases were analyzed for the correlation between claudin 1 and tumor size in Table 2. For claudin 4, only 67 cases were analyzed with the Node status, 73 cases were analyzed with the patient age and tumor grade, and 71 cases were analyzed with the tumor size in Table 2. The authors should explain the inconsistency in these positions.

While patient age and tumor grade were available for all cases, the nodal status and tumor size was not assessed in a small number of cases resulting in the variations in the sample sizes used in the analyses reported in Table 2.

Comment 3: Scale should be added in Figure 2 and 3C.
Scale bars have been added as requested by the reviewer.

Level of interest: An article of limited interest
Quality of written English: Acceptable
Statistical review: No, the manuscript does not need to be seen by a statistician.
Declaration of competing interests: declare that I have no competing interests