Reviewer's report

Title: CA1 Contributes to Microcalcification and Tumorigeneic Process of Breast Cancer

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Reviewer: Vimla Band

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Mammary microcalcification is frequently associated with poor survival in breast cancer patients, and occurs in about 30% to 50% of breast cancer. Based on the literature, authors tested if enzyme Carbonic Anhydrase (CA1) is up-regulated in breast tumors and thus stimulates calcium precipitation. The authors assessed the levels of CA1 in tissues and blood from breast cancer patients by IHC, WB, q-PCR and ELISA. Authors induced calcification in the mouse breast tumor cell line 4T1 with ascorbic acid and #-glycerophosphate. MCF-7, a ER+ breast cancer cell line was treated with anti-CA1 siRNA, and then proliferation, Annexin V cell apoptosis, and transwell migration assays were performed. Tag SNP rs725605 in the CA1 locus was genotyped using TaqMan® genotyping.

The authors observed an elevated calcification and increased CA1 levels in mouse mammary adenocarcinoma cell line 4T1 upon induction of osteogenic condition. Furthermore, authors observed increased migration and apoptosis in the anti-CA1 siRNA-treated MCF-7 cells. The PCR array detected androgen receptor up-regulation and X-box binding protein 1down-regulation in the treated MCF-7 cells. A significant difference in allele and genotype frequency for rs725605 was detected in the cohort of patients with breast cancer. The authors conclude that CA1 plays an important role in the calcification, apoptosis and migration of tumor cells and thus contributes to tumorigenesis of breast cancer by regulating AR and XBP1 expression.

Major Questions/Comment

1. Authors compared fibroadenomas with breast cancers for CA1 levels. The better controls should be normal tissue from the same patients or from reduction mammoplasty specimens. Fibroadenoma are not right controls for breast tumors of epithelial origin.

2. The authors mentioned the prognostic significance of micro-calcification in breast tumors however did not examine the functional role of micro-calcification in human tumors. They conclude that CA1 plays an important role in regulating breast tumors cells in driving calcification, apoptosis, and migration. However, they have shown this effect in only 2 cell lines -4T1 (calcification) and MCF-7(apoptosis, migration) where one of them is mouse cell line and may not be a physiologically relevant model. Inclusion of a panel of breast cancer cell
lines representing various subtypes of breast cancers to show expression of CA1 will help in generalization of their results and conclusions.

2. The genotyping data for SNP analysis is only written in the text. Data should be included.

3. The images in Fig. 4, are not very clear. Authors please include better quality images. Also, is this effect valid for MCF7 or other human breast cancer cells?

4. Figure 8. Level of CA1 after siRNA knockdown in WB (8B) and its intensity quantititation (8C) does not seem to correlate. SiRNA knockdown does not seem to be significant. Authors should use atleast two siRNAs to account for off target effect.

6. In figure 9-11 authors show that increase in apoptosis (Fig. 10) and migration (Fig. 11) is observed when CA1 is knocked down in MCF7 cells and there is no difference in cell proliferation upon CA1 siRNA knockdown (Fig.9). These results are contradictory. Authors please explain how increased apoptosis is not affecting cell proliferation. How does apoptosis correlate with increased migration ability of the cells?

7. Results of Figure 12A (except for XBP1 and AR) do not correlate with those of Figure 12B. Inclusion of a PCR array with more no. of genes would be better to demonstrate significance of these results. Also, transcription factor SNAI2 which is down regulated in CA1 knock down (Fig.12A) plays an important role in regulating EMT and/or migratory ability of cells and this does not match with the results in Fig.11. Authors please explain?

Minor Questions/Comment

1. The authors show that CA1 negatively regulates AR expression and further discuss that AR plays an important role in bone formation (discussion 447-456) and that CA1 regulates AR expression and thus regulates micro-calcification in tumorigenic process in breast tumors. Can the authors stain the invasive tissue section which show increased CA1 staining (Fig. 2) with AR and also perform Calcium staining (using staining agent used in Fig. 4).

2. Pl renumber all the figures in the text (i.e. 1a, 1b,....)

3. Pl include better quality IHC images should be included.

4. Pl Change Health in Figure 3- “Healthy”

5. Pl Correct mRNA expression scales in Figure 4.

Level of interest: An article of importance in its field

Quality of written English: Needs some language corrections before being published
**Statistical review:** Yes, but I do not feel adequately qualified to assess the statistics.

**Declaration of competing interests:**

I have no competing interests