Author's response to reviews

Title: CA1 Contributes to Microcalcification and Tumourigenesis in Breast Cancer

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Author's response to reviews: see over
Reviewer's report 1

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

Version: 3 Date: 16 April 2015

Reviewer: Vimla Band

Reviewer's report:

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 β-glycerophosphate. MCF-7, an 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 1 down-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.

Answer: I agree the suggestion of this reviewer. The better controls should be normal tissue from the same patients or from reduction mammoplasty specimens. However, it is difficult to obtain normal breast tissue during the operation. It is not easy to determine the health part in the breast with tumor during the operation. Normally, another breast of the patient is normal, but it violates ethics to collect health control from the health breast. Additionally, women like to increase breast size by mammoplasty operation in China. It is difficult to get health breast tissue from reduction mammoplasty specimens. Furthermore, benign breast lesion also has calcification (Breast Cancer. 2011 Jan;18(1):33-6.) as breast cancer does. Thus, it is better for this study to use fibroadenoma breast tissue as a control to study calcification in breast tumors. We addressed concern of this reviewer in the revised manuscript.

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.

Answer: So far, ten cell lines were established from either breast primary tumor, pleural effusions or various metastatic sites in individual patients. Deborah et al. summaries the molecular characterizes of these ten cell lines (Breast Cancer Res. 2011 Aug 12;13(4):215.). MCF-7 is ER+, PR+/– as other 5 cell lines and HER2– as other 8 cell line. Thus, MCF-7 represents most features of breast tumor cell lines and is largely used in the breast tumor study. In the present study, we used MCF-7 as a cell model to explore the pathogenic role of CA1 to breast tumor, not just to determine the pathogenic effect of CA1 for calcification. Because Cox et al. have recently reported a novel in vitro model of mammary mineralization using 4T1 cells, we used 4T1 cells to focus on the involvement of CA1 in calcification. In addition, there is a report that calcium hydroxyapatite promotes mitogenesis and matrix metalloproteinase expression in human breast cancer cell lines (Mol Carcinog. 2001 Nov;32(3):111-7.). We addressed concern of this reviewer in the revised manuscript.

This reviewer provides a good idea to use a panel of breast cancer cell lines
representing various subtypes of breast cancers to show expression of CA1. We will process the next study based on the idea of this reviewer. Because these ten breast tumor cell lines have possible heterogeneity in tumorigensis of breast cancer, we could find different role of CA1 in breast cancer.

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

**Answer:** In the revised manuscript, we provided a new table (Table 2) to represent genotyping data to respond to the suggestion of this reviewer.

4. (1) The images in Figure 4 are not very clear. Authors please include better quality images. (2) Also, is this effect valid for MCF-7 or other human breast cancer cells?

**Answer:** (1) In the revised manuscript, we prepared Figure 4 with high definition. The transition of ppt file to pdf file caused the bad quality. (2) We detected CA1 transcription and translation of CA1 in MCF-7 cells. MCF-7 was reported to express alkaline phosphatas and bone sialoprotein (BSP) that enhance bone mineralization and calcification (Bone. 2011 Oct;49(4):830-8; Bull Cancer. 1997 Jan;84(1):17-2). Thus, MCF-7 may produce calcification. We tried many times to induce mineralization, but the result is not stable as 4T1 cells did. We are finding the best condition to induce mineralization. In the present study, we determined the role of CA1 to mineralization by inducing calcification in 4T1 cells, because Cox *et al.* have established *in vitro*
model of mammary mineralization using 4T1 cells. On the other hand, we thoroughly investigated how CA1 is involved in tumorigenic process including cell proliferation, apoptosis, cell migration and regulatory pathway by treating MCF-7 cells using anti-CA1 siRNA. Although calcium hydroxyapatite promotes mitogenesis and matrix metalloproteinase expression in human breast cancer cell lines (Mol Carcinog. 2001 Nov;32(3):111-7.), our study suggests that CA1 contributes to tumorigenesis of breast cancer by more than a few pathways. The carbonic anhydrases catalyze the rapid interconversion of carbon dioxide and water to bicarbonate and protons, which can affect physiological process of the cells by pH, proton concentration, membrane permeability and other signaling pathway, not just bio-mineralization. We addressed the concern in the revised manuscript.

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

Answer: We tried three anti-CA1 siRNAs to inhibit the CA1 expression. The current one had best target effect. Based on the criticism, we repeated the CA1-knock out experiment within MCF-7 cells and checked the expressions of CA1. The transcription and translation of CA1 were considerably declined (as we showed in the new Figure 8). The results of cell proliferation, apoptosis and cell migration as well as XBP1 and AR expressions were similar to the previous experiment.
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. (1) Authors please explain how increased apoptosis is not affecting cell proliferation. (2) How does apoptosis correlate with increased migration ability of the cells?

**Answer:** This is very interesting question. Our study indicated that the down-regulation of CA1 expression significantly stimulated cell apoptosis and cellular migration, but did not influence cell proliferation in MCF-7 cells. The apoptosis is not always correlated with cell proliferation. For example, Gálfí et al. investigated a set of cell lines as to their sensitivity to proliferation inhibition by the core histone deacetylase inhibitors butyrate and trichostatin A. They found that there is no correlation between the sensitivities to proliferation inhibition and the sensitivities to apoptosis induction by the histone deacetylase inhibitors (Cancer Lett. 2002 Dec 15;188(1-2):141-52.). In addition, we detected decreased expression of XBP1 in the anti-CA1 siRNA treated MCF-7 cells. XBP1 can activate the anti-apoptotic gene BCL2. Thus, it is possible that decreased expression of CA1 in the cultured breast cancer cells caused the reduction of the anti-apoptotic role and resulted in the increased level of apoptosis of the treated MCF-7 cells. We addressed the concern in the revised manuscript.

Increased migration and apoptosis were observed in the anti-CA1 siRNA treated MCF-7 cells. CA1 seemingly has a contradictory role in malignancy as this reviewer
mentioned. Apoptosis is disrupted during the progression of many solid tumors. Studies demonstrated that apoptosis doesn’t always have negative correlation with tumorgenic progress, especially metastasis. For example, Termuhlen et al reported that isogenic metastatic colon adenocarcinoma cells displayed significantly higher levels of staurosporine-induced apoptosis compared to their nonmetastatic counterparts *in vitro* (Cancer Biol Ther. 2002 Jan-Feb;1(1):58-63.). Their data suggest that the molecular events associated with acquisition of the metastatic phenotype sensitize tumor cells to some pro-apoptotic stimuli. In the present study, we detected an increased expression of AR in the anti-CA1 siRNA treated MCF-7 cells. Many studies reported that AR stimulated tumor cell migration, such as prostate cancer cell invasion (Oncotarget. 2015 Mar 30;6(9):6862-76.) and esophageal cancer cell migration (Tumour Biol. 2015 Feb 28.). We discussed the contradictory dual role of CA1 in malignancy in the revised manuscript.

7. (1) Results of Figure 12A (except for XBP1 and AR) do not correlate with those of Figure 12B. (2) Inclusion of a PCR array with more no. of genes would be better to demonstrate significance of these results. (3) 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?

Answer: PCR array is very sensitive skill, and we thus verified the result with real time PCR as manufacturer’s requirement. Our experience and other’s indicate that the
PCR array result is not always accurate and consistent with verification of the real time PCR. The PCR array results must be verified with the real-time PCR. In addition, the PCR array detected decreased transcription of SNAI2 in CA1 knock down MCF-7 cells. Although the real time PCR also detected the similar result, there was no significant mean and we thus did not make a further analysis.

We prepared a new Supplementary material 1 that shows all genes expression results in the PCR array.

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).

Answer: This is very good idea. Alizarin Red-S staining is used to stain calcification in the cultured cells. We used hematoxylin-eosin and Kossa staining to localize calcium hydroxyapatite calcifications in tumor tissue sections and found CA1 and osteopontin (OPN), a bone matrix protein, were closed in the tumor tissue. But we cannot co-localize CA1, AR and calcification, indicating that CA1 doesn’t directly regulate AR in the breast tumor tissues. We are investigating relation among CA1, OPN locations and metastasis based on the idea of this reviewers. We also tried to
immune-stain bone sialoprotein (BSP), another bone matrix protein, with CA1 expression. We have to examine a number of breast tumor samples to determine relation among CA1, AR, OPN, BSP and metastasis. This work will be introduced in the next report about pathogenic role of CA1 in breast cancer. Differential expressions of osteopontin and bone sialoprotein are implicated in bone metastasis of breast carcinoma. (Clin Exp Metastasis. 2003;20(5):437-44.)

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

Answer: We renumbered all the figures in the text (i.e. 1a, 1b,....) in the revised manuscript.

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

Answer: In the revised manuscript, we prepared Figure 2 with high definition. The transition of ppt file to pdf file caused the bad quality.

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

Answer: We change Health in Figure 3- “Healthy” in the revised manuscript.

5. Pl Correct mRNA expression scales in Figure 4.

Answer: Figure 4 shows calcification in 4T1 cells cultured with ascorbic acid and β-glycerophosphate. The cell cultures were stained with cetylpyridinium chloride and quantified by measuring the absorbance at 562 nm. Figure 4B represents the
quantification of induced calcification, which does not indicate mRNA level of CA1.

We really appreciate this reviewer for his/her suggestion and comment to our study.

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

**Reviewer's report 2**

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

Version: 3Date:9 April 2015

Reviewer: Rajini Rao

Reviewer's report:

*This is an interesting and potentially significant study on the role of carbonic anhydrase I in breast cancer and microcalcifications. Several novel approaches are used, including a biomineralization assay. However, the link between CA1 and calcification is correlative in the absence of knockdown and inhibitor studies.*

Major Compulsory Revisions:

1. Although there is a correlative link between induction of CA1 transcript and
biomineralization, causality is not established. Knockdown experiments on 4T1 cells should be performed to evaluate the role of CA1 on calcium deposits. What is the effect of using the CA1 inhibitor acetazolamide on the 4T1 cells (the authors refer to the effect of this drug on a different cell line Saos-2 in a previous publication). Surprisingly, the authors only perform CA1 knockdown in MCF7 cells, which were not evaluated for calcium deposits. This disconnect is a major logical flaw in the study.

**Answer:** We completely understand the opinion of this reviewer. Because Cox et al. have established a good *in vitro* model of mammary mineralization using 4T1 cell, we induced calcification in 4T1 cell line to determine a correlative link between CA1 and bio-mineralization. We treated 4T1 with acetazolamide and detected a significantly decreased calcification in the cultured 4T1 cells after the treatment. But we can’t sure if acetazolamide specially inhibited the expression of CA1, although acetazolamide considerably inhibited the expression of CA1, because CA family has 20 members. In addition, 4T1 is mouse cell line and may not be a physiologically relevant model with human breast cancer. To thoroughly investigate how CA1 is involved in tumorigenic process including cell proliferation, apoptosis, cell migration and regulatory pathway, we treated MCF-7 cells using anti-CA1 siRNA. We detected CA1 transcription and translation of CA1 in MCF-7 cells. MCF-7, originated from human breast tumor epithelium, was reported to express alkaline phosphatas and bone sialoprotein (BSP) that enhance bone mineralization and calcification (Bone. 2011 Oct;49(4):830-8; Bull Cancer. 1997 Jan;84(1):17-2). Thus, MCF-7 may produce calcification. We tried
many times to induce mineralization, but the result is not stable as 4T1 cells did. We are finding the best condition to induce mineralization. The carbonic anhydrases catalyze the rapid interconversion of carbon dioxide and water to bicarbonate and protons, which can affect physiological process of the cells by pH, proton concentration, membrane permeability and other signaling pathway, not just bio-mineralization. Although calcium hydroxyapatite promotes mitogenesis and matrix metalloproteinase expression in human breast cancer cell lines (Mol Carcinog. 2001 Nov;32(3):111-7.), our study using MCF-7 cell model suggests that CA1 not only contributes to calcification of breast cancer, but also plays a role in tumorigenesis by several signaling pathways such as AR and x-box. We addressed the concern in the revised manuscript. We know that the study result is much better than the present result if we can induce and inhibit calcification by mediating CA1 expression in MCF-7 cells. Although the logic of this study is not very smooth, the finding itself is very interesting and meaningful as two reviewers indicated. Please agree to publish our study. We will consummate our study. In our next study, we will focus on relation of CA1 to metastasis in addition to inducing calcification in MCF7 cells and other human cancer cell lines.

2. CA1 knockdown in MCF7 cells is largely ineffective and should be improved. (1) How much does “considerably reduced” mean (line 375, pg 18)? Please specify in Results what positive and negative controls mean in this experiment. (2) Also, x and y axis labels in Figure 8 need clarification and relabeling. Scientific notation may be
better for y-axis, or use a log scale or % of control. In light of the weak knockdown, it is difficult to interpret significance of growth data that show no difference.

**Answer:** In the revised manuscript, we specified control, positive and negative controls in Figure 8, Methods and Result sections. Control means the cells without siRNA treatment and negative control means the cells treated with Allstars siRNA. We repeated the CA1-knock out experiment in MCF-7 and the expressions of CA1. The transcription and translation of CA1 were considerably declined compared with the controls (as we showed in the new Figure 8). We normalized mRNA level and protein level of CA1 with the levels of the control and relabeled mRNA level and protein level of the control as 1 in Y-axis. The results of cell proliferation, apoptosis and cell migration as well as XBP1 and AR expressions were similar to the previous.

**Major Essential Revisions:**

1. In Results, please begin with an introduction of the SNP under evaluation. Where is it located (intron, coding region?), and what are the predicted consequences if known (expression or activity changes). How was this specific SNP chosen for analysis among the many (29) CA1 SNPs previously reported in Table 1 of Chang et al. (*Arthritis Res. Ther.* 14:R176; 2012).

**Answer:** In the previous study, we genotyped 13 tag SNPs in CA1 gene to determine the potential association of CA1 gene to AS using a custom-designed Illumina 96-SNP VeraCode microarray (Illumina). We chose the tag SNPs with significant association to the disease to perform Taqman genotyping in a larger cohort. Both
microarray and Taqman genotyping demonstrated that rs725605 had statistically significant evidence of allele frequency ($P = 0.022307$) and gene frequency ($P = 0.007731$) for association with AS. At that time, we tested five SNPs to perform Taqman genotyping, only rs725605 site selection and the primer design worked very well. Normally, it is not easy to find a suitable SNP site to process Taqman genotyping. Successful Taqman genotyping is dependent on successful Taqman primer design and SNP location. Thus, we continued to genotype this tag SNP to determine the possible association with various tumors. In the introduction section of revised manuscript, we introduced reason why we chosen tag SNP rs725605 for analysis among the many CA1 SNPs. rs725605 is located in the intron 1 region of CA1 encoding gene.

2. **Figure 9: did you forget to label?**

   **Answer:** We labeled the columns of Figure 9 in the revised manuscript according to the requirement of this reviewer.

3. **Figure 10: Axes need labels.**

   **Answer:** We labeled Axes of Figure 10 in the revised manuscript according to the requirement of this reviewer.

4. **Figure 11: Panels have no labels (A, B, C, etc.)**

   **Answer:** We numbered panel of Figure 11 in the revised manuscript according to the
requirement of this reviewer.

5. Overall, figure quality needs to be improved. Labels should be in uniform font and legible, with correct spacing and capitalization. Typographical errors should be corrected throughout (e.g., Fig 12, replace “alternations” with “alterations” and “raltive” with “relative”).

**Answer:** We corrected typographical errors, labeling, font and figure numbers in the revised manuscript according to the requirement of this reviewer. We also prepared new Figure 8 and Figure 4 with high solution.

We really appreciate this reviewer for his/her suggestion and comment to our study.

*Level of interest: An article of importance in its field*

*Quality of written English: Needs some language corrections before being published*

*Statistical review: No, the manuscript does not need to be seen by a statistician.*

*Declaration of competing interests: I declare that I have no competing interests.*