Author's response to reviews

Title: Elevated 14,15-EET associated with upregulated cytochrome P450 2C8, 2C9 and 2J2 and aggressive in human breast cancer

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Dafne Solera
Executive Editor,
BMC Cancer

Dear Dr. Dafne Solera,

Thank you very much for providing us the opportunity to revise our manuscript (MS: 1590356043128170) entitled “Elevated 14,15-EET by increasing of cytochrome P450 2C8, 2C9 and 2J2 and decreasing of soluble epoxide hydrolase associated with aggressiveness of human breast cancer”. We have carefully read reviewers’ comment and revised the manuscript according to their suggestions. The revision was made with the highlighted point-by-point responses to the reviewers for your consideration. I hope the revised version will satisfy with reviewers and be beneficial to the readers of the journal BMC Cancer.

I am looking forward to hearing from you soon.

Sincerely,

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Reviewer(s)' Comments:

To Reviewer 1
1. Title, change ‘by’ to ‘associated with’. By implies cause which is not shown.
   Response: Thank you for your suggestion, we corrected the title and make it clear. The new title is “Elevated 14,15-EET associated with upregulated cytochrome P450 2C8, 2C9 and 2J2 and aggressiveness in human breast cancer”

2. Eliminate use of ‘epoxygenases’, this is commonly used and miss used in the field. There are some bacterial P450s that make fatty acid epoxides exclusively. They can be called epoxygenases. Miss using the term in this context implied the P450 listed specifically make epoxides is wrong. They do omega and omega-1 OH, allylic hydroxylation and many others. It also implies other P450s do not make epoxides of fatty acids (1A1, 2D6, etc) this also is wrong.
   Response: Thank you for your comment on the term. Yes, we deleted ‘epoxygenases’ in new manuscript.

3. Page 3 the statement “EETs have been linked with cardiovascular disease, diabetes and several cancer diseases.” Can be interpreted that the like is not favorable. Actually EETs reduce CV and diabetes, pain and inflammation. On cancer the EETs and sEHI can be pro angiogenic or strongly anti angiogenic. See two recent papers by G. Zhang. The wording should reflect biological reality.
   Response: Yes, we changed the word “linked” to “associated”.

4. Page number 5, line 78 and 79: Mentioned EETs as inflammatory whereas they are anti-inflammatory. Page number 6, line 96: "...CYP2J2 expression, and the molecule.." should be written as "CYP2J2 expression, and the enzyme..."
   Response: Yes, we corrected.

5. Be consistent in how references are referred to consumption[12]. A word [12]. And A word. [12] are all used.
   Response: Yes, we corrected.
6. Do not say ‘five folds’ rather five fold. This is nutty English convention.

**Response:** Yes, we corrected.

7. Page 8 ask if selectivity of antibodies was evaluated at least on SDS page. Santa Cruz Biotechnology is known for antibodies that detect multiple proteins.

Response: Yes, we had checked the specificity of antibodies on SDS PAGE and shown in the following figure 1.

8. Page 12. Limit to 3 significant figures.

Response: Yes, we corrected.

9. Page number 14, line 274: reference number 10 and 3 should be placed/written in a common bracket.

Response: Yes, we corrected.

10. Page 15. Be careful using the term ‘specific’ there is no such thing as receptor specific or specific EET receptors. One could say EET receptor or selective EET receptor. Specific is a good term to avoid.

**Response:** Yes, we corrected.

11. Statistics are fine at this level. Page number 21, line 451: "P value calculated by..." should be written as "P value is/was calculated by .."

**Response:** Yes, we corrected.
To Reviewer 2:

1. Epoxide hydrolase is higher in non-cancerous tissue and could hydrolyze EETs causing artifact during the recovery, leading to lower EET measurements because of hydrolysis after biopsy from the patient or excision from a mastectomy specimen. Was an epoxide hydrolase inhibitor used to protect against sEH influence on results? Was there an internal standard used to control for recovery of EETs from tumors vs. non-tumor tissue? If not, and the results were normalized to protein, is there a difference in protein per wet weight of tumor compared to protein per wet weight of tumor? How was the specimen protein measured?

Response: We appreciate your valuable suggestion. Indeed, EET and DHET might be decrease because of oxidation and hydrolysis after biopsy from the patient or excision from a mastectomy specimen. Thus, in order to avoid the oxidation, we isolated and homogenized 30mg breast tissue and grinded in 0.4 mL of H$_2$O, and 0.001 mg TPP (triphenylphosphine), which acts as an antioxidant, strictly following the protocol of 14,15-DHET ELISA kit (Detroit R&D, USA. http://www.detroitrandd.com/pdf/CATDH2.pdf). All procedures were performed on ice and finished within 10 minutes to keep the level of EET and DHET as stable as possible.

Our previous report shown that sEH inhibitor, such as 1471 and 1709 could be affectively inhibit sEH activity and maintain EET levels in vivo and in vitro (Circ Res. 2012;110(6):808-17; Proc Natl Acad Sci U S A. 2009;106:564-9; Proc Natl Acad Sci U S A. 2007;104:9018-23.). In the present study, we did not use any sEH inhibitors. According the protocol of EET and DHET measurement, EET+DHET can be measured after totally and effectially changing EET to DHET by the mixture of ethanol and acetic acid. If we can not detect the levels of EETs, EET was totally hydrolyzed to DHET, which could be used as an internal standard for the recovery of EET in tissue.

Breast tummor tissue is complated and composed of fibrous connective tissue, lobular breast duct system and several breast tissue, which would effect EET and DHET detection. Thus, total protein is better to normalize the measurement of EET and DHET detection. Thus, total protein is better to normalize the measurement of EET and DHET than the weight of tumor tissue. After the tissue (30mg) was grinded in H$_2$O, total protein concentration was measured with BSA kit according to the protocol (Takara Biotechnology [DALIAN] Co.) and used as the normalization of measurement of EET and DHET.
2. The method of EET hydrolysis to DHET was not described. How was it ascertained that the EET hydrolysis went to completion? The EET is being measured as the difference of DHET before and after hydrolysis of the extract, ascertainment that the hydrolysis went to completion is important. If the hydrolysis was performed before extraction, efficiency of conversion of EET to DHET would be very difficult to determine. On the other hand if hydrolysis was performed on the ethyl acetate breast cancer tissue extract or the ethanol-dissolved eicosanoids, then control experiments can be performed to confirm that full hydrolysis occurred under the conditions used to obtain the data in this manuscript. What method was used for hydrolysis-acid or base?

**Response:** You are absolutely right. According to your suggestion, we added the detail method of EET hydrolysis to DHET in the section of “Methods”.

Episomal and esterified 14,15-DHET (including episomal and transformed DHET from 14,15-EET by sEH) in BC tissues was determined by use of an ELISA kit (Detroit R&D, USA). Briefly, After the tissue (30mg) was grinded in H$_2$O, total protein concentration was measured with BSA kit according to the protocol (Takara Biotechnology [DALIAN] Co.) and used as the normalization of measurement of EET and DHET. Lethanol and acetic acid for 18 h at room temperature to allow EET totally hydrolyzed to DHET. Then, 130 uL of Sample Dilution Buffer was added to make stock solution. The stock sample solution can be diluted in a proper range of concentration for ELISA test. Check the final pH (should be pH 7.4). Use the 14,15-DHET ELISA kit to measure DHET, which includes DHET converted from EET. At the same time, measure the DHET level without hydrolysis of EET in the same sample. Subtract that value from the EET + DHET level and you will obtain the EET level in the sample. If we could not detect the levels of EETs, it means that EET was totally hydrolyzed to DHET. The efficiency of conversion of EET to DHET according to the free of EET formation activity measurement after the EET hydrolyzed to DHET by ethanol and acetic acid.

3. How was it determined that the margins of the resected tumor were free of cancer? How was it determine that the whole cancer specimen submitted for this study contained cancer? Although it is not necessary to examine the entire specimen, standard of care would be to establish a margin by histopathology. If ascertainment of a margin wasn’t performed for lumpectomy or mastectomy it would be difficult for the results in this manuscript to be reproduced because there could be play of chance regarding the amount of tumor in the tumor specimen even in a study of 40 paired
specimens.

**Response:** Thank you for your concerns on the margin of cancer. In fact, all patients in the study have undergone modified radical mastectomy or mastectomy, but not lumpectomy. Fresh breast cancer tissues and paired noncancerous tissues with margins of at least 5cm away from malignant lesions were collected by experienced pathologists. A piece from each tissue sample was immediately frozen in liquid nitrogen and stored at -80°C. Remaining tissue samples were fixed with hematoxylin-eosin staining were reviewed and confirmed by two pathologists (Xiaolong Wei and Guojun Zhang). Statistical analysis involved use of SPSS 16.0 and each experiment was performed at least in triplicate. P < 0.05 was considered statistically significant.

4. Does “non-cancerous” mean no DCIS, no atypical hyperplasia and no benign breast disease?

**Response:** Yes. The non-cancerous tissues confirmed by pathologist were defined as not presented with DCIS, atypical hyperplasia or benign breast disease.

5. How were the tumor and non-cancerous tissues obtained? Did all of the patients undergo lumpectomy, all mastectomy or a mix? If lumpectomies were performed how was the “non-cancerous tissue” obtained? Were patients consented for an additional biopsy and if so what kind of biopsy? If mastectomy specimens were used how was the tumor was removed from the mastectomy along with a biopsy of “non-cancerous tissue?” How were the distances between the tumor and the “normal” tissue ascertained? What was the longest time tissue sat at room temperature before being frozen? How were the breast specimens stored and at what temperature and method?

**Response:** Informed consents were obtained for all patients in this study, and all patients underwent breast needle biopsy but not lumpectomy for initial pathological diagnosis before surgery. The procedure of tissue collection was that all the malignant tumor tissues and noncancerous tissues were collected and stored (-80°C) in less than one hour after operation. Before storing, the specimens were treated with RNAse inhibitor. The other duplicates of the corresponding tissues were fixed immediately in 4% paraformaldehyde for 24 hours, and then embedded in paraffin and sectioned at 4 µm for IHC staining.

Cancer and non-cancerous tissues were obtained from surgical specimens of breast cancer. All patients underwent modified radical mastectomy or mastectomy but not lumpectomy. The
procedure for tumor tissues were sampled vertically along the linking-line between tumor and nipple. Paired noncancerous tissues with margins of at least 5cm away from malignant lesions were also collected.

6. Were the specimens homogenized before ethyl acetate extraction?  
Response: Yes, we homogenized for 30 mg for all the tumor and non-tumor tissues before ethyl acetate extraction.

7. All red scoring or 0 through +++ staining would help to quantify the IHC.  
Response: Yes, the scoring for quantify the IHC was according to previous report (Liu Y. Prostate 2012, 72(6):690-701), which was widely used in this field.

8. In the tumors was there CYP expression in the blood vessels or tumors?  
Response: Yes, the expression of CYP and sEH was found in both of blood vessels or tumors and shown in Figure 2.

Minor concerns:
1. In Table 2, ErbB2 staining is pooled for ++ and ++++. This is not useful because ++ is equivocal and +++ is positive. How many +++ patients were there?  
Response: C-erbB-2 ++, weak or moderate circumferential complete membrane staining>10% of tumor cells or strong complete circumferential membrane staining in ≤30% of tumor cells; and ++++, strong complete circumferential membrane staining in >30% of tumor cells. C-erbB-2 ++ is also useful for determination of Her-2 positivity with FISH/CISH method, though it can be judged as positive by IHC alone. The exact number of patients with C-erbB-2 staining “+++” were 7 and shown in table 2.

2. In Figure 2, the CYP mRNA levels are all set to 1. The absolute levels of CYP mRNA should be presented.
Response: The expression of CYP mRNA was positive in all tissues (Cycle threshold value <32 in real time PCR). The relative expressions of CYPs and sEH were normalized to their corresponding normal control tissue.
3. The IHC measurement of CYP2C8, CYP2C9, CYP2J2 and sEH should have larger fields of view because the microscopic images presented were of poor quality for CYP2J2 and marginal for the others. The counter stain of the nuclei cannot be easily seen in some of the images presented.

Response: Thank you for your comment. We replace corresponding IHC images with high quality and larger views (200X) in the new Figure 2E.

4. There should be positive controls for the IHC staining process.

Response: Yes, since high level of CYPs and and sEH expression in human hepatocellular carcinoma tissue (Enayetallah AE. J Mol Histol 2006, 37(3-4):133-141), we used human hepatocellular carcinoma as the positive control for IHC staining assay in the new figure 2E.

5. Were there were correlations between CYPs? Is there exclusion such that if one CYP is expressed, another isn’t? What proportion of all tumors express at least one CYP?

Response: There was positive correlation only between CYP2C8 and CYP2C9 protein expression (R=0.395, P=0.012). Herein, we analyze the correlation of each CYPs and clinicopathological variables. The percentages of tumors and non-tumors expressed at least each one CYP were 73.73% and 40.91%, respectively. Furthermore, there were no correlation if one CYPs expression and clinicopathological variables.

6. It is notable that only 30% of tumors expressed CYP2C8 mRNA and 30% expressed CYP2J2 mRNA, whereas 60% expressed CYP2C9. This comparison should be stated in the abstract or discussion. We need to know what percentage of tumors expressed no CYP.

Response: In fact, the percentages of CYP in tumors were protein level by immunoreactivity staining, not mRNA levels. The percentages of tumors and non-tumors expressed no CYP were 27.27% and 59.09%, respectively.

7. The CYP siRNA knock down experiments lack Western blot controls to establish that the mRNA level was decreased relative to control.

Response: Yes, we added the data of western blot assay to confirm the effect of siRNA knock down, and shown in the following figure 1.
Figure 1. Western blot analysis of expression of CYP2C8, CYP2C9 and CYP2J2 by si-RNA in MDA-MB-231 cells, were transfected with 40-nM siRNA for CYP2C8, CYP2C9 and CYP2J2 or scramble siRNA control (si-Ctrl) for 12 hr. β-actin was an internal control. *P<0.05 vs si-Ctrl transfection.

8. Statistical analysis of the cell migration experiments should be performed.

Response: Statistical analysis was performed for cell migration experiment, shown in new Figure 3D.