Reviewer’s report

Title: Elevated 14,15-EET by Upregulated Cytochrome P450 2C8, 2C9 and 2J2 and Downregulated Soluble Epoxide Hydrolase Associated With Aggressive Human Breast Cancer

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Reviewer: David Potter

Reviewer’s report:

The question posed by the authors is whether 14,15-epoxyeicosatrienoic acid (14, 15-EET) content is increased in breast cancers compared to adjacent non-cancerous tissue. This is a timely and important question. This manuscript has several strengths. It addresses an important unanswered question regarding EET content in breast cancer by examining EET content indirectly in tumor tissue and adjacent non-cancerous tissue. The relationship between expression of CYP epoxygenases that synthesize EETs and tumor histology is addressed. The data in Tables 1 are important because they relate CYP2C, CYP2J and soluble epoxide hydrolase (sEH) gene expression levels in tumors and non-cancerous tissue and linking the CYP expression to tumor demographics and histologic type. These are confirmatory data. The data in Table 2 relate CYP2C, CYP2J and sEH gene expression to demographics of the patients and their tumors, which is valuable information. While the authors don’t measure EET directly, they can indirectly measure 14,15-EET, an important regioisomer, by measuring its hydrolysis product 14,15-dihydroxyeicosatrienoic acid (14,15-DHET) using a commercially available ELISA assay. By measuring the difference between 14,15-DHET content before and after hydrolysis, the authors estimate 14,15-EET as the difference between the two measurements. A major weaknesses is the methodology for measurement of 14,15-EET, which is indirect and doesn’t protect against hydrolysis of EETs by sEH, which hydrolyzes EET to DHET. The authors show that sEH is present in much higher amounts in the non-cancerous tissue in which they measure lower EET levels. If the process of tissue homogenization results in activation of sEH, then EET recovery in the non-cancerous tissue is likely to be reduced and there was no internal standard to address this potential pitfall. In Figure 1C there is no difference between the 14,15-DHET levels in the non-cancerous tissue and the tumors and if there was a problem with EET loss due to higher sEH levels in the non-cancerous tissue, the higher 14,15-EET levels in the tumors may be an artifact of the measurement. Furthermore, the margins of the tumors are not established as negative by histopathology and the non-cancerous tissue is not established as morphologically normal breast tissue and could contain DCIS, atypical hyperplasia or other pre-malignant tissue unless otherwise specified by a pathology review.

Major concerns:
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?

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?

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.

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

6. Were the specimens homogenized before ethyl acetate extraction?

7. Allred scoring or 0 through +++ staining would help to quantify the IHC.

8. In the tumors was there CYP expression in the blood vessels or tumors?

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?
2. In Figure 2, the CYP mRNA levels are all set to 1. The absolute levels of CYP mRNA should be presented.

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 counterstain of the nuclei cannot be easily seen in some of the images presented.

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

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?

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.

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

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

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 have no competing interests.