Reviewer’s report

Title: Low expression of hTFPI-2 associated with poor survival outcome in patients with breast cancer

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Reviewer: Vagner Bernardo

Reviewer’s report:

• Major Compulsory Revisions

1- Patients Section

The authors randomly selected 156 breast cancer patients and assessed h-TFPI-2 expression by immunohistochemistry. The follow-up data were available for 118 patients. So, 38 losses are expected. However the authors stated that only 33 patients were lost to follow up.

2- Digital Image Analysis Section

A- The main purpose of digital image analysis is to increase reproducibility. All slides are imaged under optimal conditions and a continuous variable is produced (e.g. IOD, area index, labeling index), being most often used in survival curves. The authors used a hybrid approach. Slides were assessed by two independent pathologists and only tumors showing more than 10% of positive cells were assessed by DIA. Why 10% of tumor cells was the cut-off point? Why were neither 11.5% nor 20% of tumor cells chosen? This must be clarified.

How will borderline “negative” tumors (e.g. 9% of positive cells) be distinguished from borderline “low-expression” tumors (e.g. 11% of positive cells)? Remember that manual counting is tedious and time-consuming. Visual estimates are biased and non reproducible.

B- The stoichiometric principle must be followed when Integrated Optical Density is used. Walker states: “…One problem with using diaminobenzidine as a chromogen is that there is only a linear relationship between the amount of antigen and staining intensity at low levels of the latter. Image analysis systems assess the amount of staining by measuring absorption, so the non-linear relationship that occurs at higher levels between amount of antigen and intensity can result in inaccurate readings.” (Walker RA - Histopathology 2006, 49, 406–410)

DAB is not a stoichiometric stain. So IOD is not proportional to the amount of the target protein in the whole range of protein expression. It is further hampered by signal enhancers (e.g. Envision).

C- More details about DIA must be supplied. Was Koehler illumination
implemented before image acquisition? Was shading correction used? The area of the microscopic field (in #m2) must be supplied, allowing other researchers to reproduce these findings. Field area can vary enormously between different DIA systems under the same magnification.

D- I think that the best approach to assess h-TFPI-2 expression by immunohistochemistry is:
1- Negative cases are cases without staining
2- Quantify all remaining cases assessing the stained area (area index = stained area/total area).
3- Ensure that all slides are imaged under optimal conditions (Koehler illumination, shading correction…)

- Minor Essential Revisions
1- Images are dark. Ensure Koehler illumination before image acquisition.
2- IOD stands for integrated optical density and not for integrated option density
3- Several spelling mistakes are present in the manuscript
4- Table 2 – The following variables (LN metastasis, ER, PR and HER-2) do not sum 121 patients

Level of interest: An article whose findings are important to those with closely related research interests

Quality of written English: Not suitable for publication unless extensively edited

Statistical review: Yes, but I do not feel adequately qualified to assess the statistics.

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