Reviewer's report

Title: ESR1 Gene Promoter Region Methylation in Breast Cancer Patients: Correlation with Tumor Hormone Receptor Status and Luminal Phenotypes

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Reviewer: Heidelinde Fiegl

Reviewer's report:

The authors describe in their paper ESR1 DNA methylation in peripheral blood cells from 110 patients with non-metastatic breast cancer and correlate their findings with the estrogen receptor expression in the corresponding tumor tissues. ESR1 DNA methylation was measured by means of quantitative real-time PCR. The authors identified a significant association between ESR1 DNA methylation in peripheral blood cells and ESR1 phenotype in the tumor. Furthermore they observed the non significant trend that a higher proportion of patients with tumor types associated with a poorer prognosis showed ESR1 DNA methylation in peripheral blood cells.

Major Compulsory Revisions

Methods:

- Collection and processing of samples and DNA preparation:
  Commentary 1: In this section the authors should also include the DNA isolation for the peripheral blood cells, which is described now in the following section. The description of the immunohistochemical staining should be shown in a separate section. It is described, that the results were expressed as percentage and intensity of positive cells. Since in the entire manuscript the estrogen expression is described as positive or negative, the cut-off points should be indicated.

- Conventional methylation and specific analyses:
  Commentary 2: In the last paragraph of the bisulfite modification section the authors describe already specific issues of the methylation specific qPCR. This paragraph should be moved to the next section “Quantitative MSP PCR methylation analysis”.

  Commentary 3: The authors describe the usage of a methylation-positive control for standard curve preparation. But since the authors also measured the unmethylated DNA as indicated in the result section (“correlation of ESR1 non-methylated promoter in DNA extracted from peripheral blood ...”) the standard curve preparation for unmethylated DNA should be shown, otherwise it could be assumed that this was not conducted.

- Quantitative MSP PCR methylation analysis:
Commentary 4: In every conventional qPCR a reference gene is included. Also in methylation specific PCRs like MethyLight PCR a gene region without any CpGs of a special gene is used as an input control for the bisulfite modified DNA. Here in this manuscript the usage of such a reverence gene is not shown wherefore it can be assumed that this important step was not conducted.

Results and discussion:

Commentary 5: In the first paragraph of this section the definition of the cut-off points for ESR1 methylation is described. But unfortunately no data are shown. Although ROC curves are described none of them are shown. In addition it is not clear how ROC curve analysis was conducted.

Commentary 6: The authors describe that “differentiating between methylated and non-methylated ER with maximum efficacy ... together with the minimal probability of error, is a frequent clinical problem ...”. To my knowledge the ESR1 methylation status is not used already in the clinic. Therefore I would not agree with the authors that this is a frequent

Commentary 7: In the second paragraph of the results section the authors describe the association between DNA methylation analysis in peripheral blood cell (pbcDNA) and the ER expression in the tumor measured by immunohistochemistry. This paragraph is really confusing. First the authors describe an association between unmethylated ESR1 in peripheral blood cells and the ER expression. Then in the following sentence the authors write “we did not observe correlation between non-methylated ER in pbcDNA and ER+ in tumor tissues”. Then they analysed the association of the methylated ESR1 in peripheral blood cells and the lack of ER expression in the corresponding tumor tissues. Again, first the authors describe an association, and then a few sentences later they negate an association. Finally they summarize, that they identified an association between the presence of methylated ESR1 in DNA from peripheral blood cells and the ESR1 phenotype in the tumor tissues.

This whole paragraph must be rewritten and should be summarized in 3-4 sentences.

Commentary 8: It is not clear in which part of the manuscript the DNA methylation analysis of plasma samples is shown. In the material section it is described that plasma DNA was analyzed and also in the discussion section free circulating DNA from the tumor is described. But in the result section only pbcDNA methylation analyses are shown. Generally ESR1 DNA methylation analysis of tissue samples should also be included and this should be compared with the detection rates of methylated DNA in plasma samples.

Commentary 9: There a several papers dealing with cancer risks and DNA methylation of specific genes in peripheral blood cell DNA. Methylation analysis of peripheral blood cells would implicate a general predisposition for a cancer disease. But in this manuscript plasma DNA analysis and pbcDNA methylation analysis cannot be distinguished. According the statements shown in the
discussion section it can be assumed that the authors describe mainly the association of free circulating tumor ESR1-DNA and the tumor tissue ER-phenotype. But based on the descriptions in the manuscript this is not clear.

Commentary 10: The authors write that there are no studies that correlate the epigenetic profile of methylation and its relationship with ER expression status. The authors quote Li et al with reference number 25. But reference 25 is a work from Yang et al. Nevertheless the manuscripts with reference number 24 (Widschwendter et al) and 25 (Yang et al) describe DNA methylation and mRNA expression analyses in tumor tissues. In the present manuscript the authors should accentuate that they compared ESR1 DNA methylation in peripheral blood cells with ER expression in tumor tissues.

Commentary 11: A clear description of ESR1 methylation analysis in plasma samples, peripheral blood samples and tumor DNA (should be added) as well as a description of ER immunohistochemical ER expression in tumor and blood samples (if whole blood samples are still available and suitable for RNA expression analysis) should be given.

Commentary 12: Based on the mixture of ESR1 DNA methylation results from plasma samples and from peripheral blood cells is not clear if the title and abstract accurately convey what has been found and if the discussion and conclusions are well balanced and adequately supported by the data.

Minor Essential Revisions

Methods:
- Collection and processing of samples and DNA preparation:
  Commentary 13: First paragraph: The conditions for the centrifugation should be stated as the relative centrifugal force (x g) and not as rounds per minute.

Commentary 14: Second paragraph: The abbreviation RT-PCR is generally used for Reverse-transcription PCR. The generally applied abbreviation would be qPCR (quantitative real-time PCR). Since the authors use in one sentence the wording QMS-PCR (quantitative methylation specific PCR), they should use this abbreviation consequently throughout the whole manuscript.

- Quantitative MSP PCR methylation analysis:
  Commentary 15: The ER-alpha gene should be named as ESR1 throughout the whole manuscript.

Commentary 16: primer “dimers” instead of “dimmers”

Results and discussion:

Commentary 17: In the second paragraph “RE” instead of “ER” is used.

Commentary 18: In the last paragraph of the results and discussion section Table 5 is indicated although there are only 4 tables.
Abbreviations:

Commentary 19: In this section several abbreviations are shown which are not used in the manuscript: 14-3-3-sigma; DFBC; MBC; HC; BBR. They should be removed.

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

**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.