Author's response to reviews

Title: ESR1 Gene Promoter Region Methylation in free circulating DNA and its Correlation with Estrogen Receptor Protein Expression in Tumor Tissue in Breast Cancer Patients

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Author's response to reviews: see over
Authors’ response to reviewers’ comments

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Deadline date: 4th October 2013

2nd of Oct. 2013

Dear Editor,

Thank you for your positive response to our manuscript (ID #1223676754943296).

In submitting this revised version of the manuscript we have taken into account all the criticisms of the reviewers. It has taken us a considerable time to accommodate the extensive (and intensive) suggestions/criticisms of the reviewers but, nevertheless, we have been able to return the revised version of the m/s to you before the deadline of 4th Oct.

Our responses are itemized on the accompanying sheets and, where necessary, we have made appropriate changes in the main text.

We hope that the revised m/s meets with the quality requirements for inclusion in the Journal.

Yours sincerely,

Joaquina Martínez-Galán, MD, PhD
jmgalan22@hotmail.com
Reviewer ≠ 1 (Dr. Heidelinde Fiegl)

MAJOR COMPULSORY REVISION:

1. Methods:
   - Collection and processing of samples and DNA preparation.

Commentary 1:

Point: In this section the authors should also include the DNA isolation for the peripheral blood cells, which is described now in the following section.

Response: Sorry about the confusion. The DNA was not isolated from peripheral blood cells as previously stated. The method was that of the QIAGEN kit which used columns to isolate DNA from plasma following separation of EDTA whole blood by centrifugation. For clarification, the m/s has been amended as:

DNA isolation: DNA from plasma samples (2 ml per column) was obtained using QIAmp DNA Blood kit (QIAGEN Inc., CA) according to manufacturer’s recommendations. A final elution volume was 200 µl and the extracted DNA was quantified spectrophotometrically. The amount of DNA recovered, measured as µg/sample, was 0.431 ± 0.019 (mean value ± standard error of the mean). The fcDNA samples were stored at -80°C until needed for analysis. The above statement has been included in the revised section “Collection and processing of samples and DNA preparation” in the revised m/s. (Page 7. Lines 15 to 20).

Ibid: The description of the immunohistochemical staining should be shown in a separate section.

Response: OK. Done.

Ibid: The cut-off points of estrogen receptor expressions as positive/ negative should be indicated.

Response: The analyses of ER and PR expression were performed using the DAKO HORIZON automatic processor (Techmate Horizon) Monoclonal antibody kits were purchased from the manufacturer (DAKO M 7047 Clone 185 for ER and DAKO M 3569 Clone 636 for PR, and used according to the manufacturer’s instructions. The immunohistochemistry results are noted as positive or negative to the nuclear stain and expressed as a weak (+), moderate (++) and strong (+++) and the percentage of positive-staining cells. Subsequently, HER2 amplification in the tumor sample was with the DAKO K5206 kit. In those cases with HER2 (++), further analysis was with FISH using the DAKO K 5331, HER FISH PharmDxTM kit and hybridized in the DAKO HYBRIDIZER.

We have rewritten this paragraph page 9 line 23 to page 10 line 8.
- **Conventional methylation and specific analyses.**

**Point 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”.

**Response:** OK. Done. The statements have been clarified and, as recommended by the reviewer, the “Conventional methylation and specific analyses” has been moved (page 7 line 22 to page 8 line 20.

**Point 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.

**Response:** We accept that the paragraph contained errors and was not clear. In the study we had only measured methylated DNA. For clarification we have extended (and simplified) the statements as:

DNA bisulfite modification. Identical DNA sequences that differ only in methylation status can be amplified by means of QMS-PCR. Reagents required for the bisulfite modification of DNA were supplied in the CpGenomeTM DNA Modification Kit (Intergen, MA). The process was performed according to manufacturer’s recommendations. Sufficient DNA can be recovered to perform QMS-PCR from an amount of starting material as small as 0.001 µg.

In brief, 100 µl of extracted DNA was treated with sodium bisulfite for 16h, thereby converting all unmethylated cytosines to uracils, but leaving methylcytosines unaltered. After purification, the DNA obtained was dissolved in 20 µl of TE buffer and the modified DNA was spectrophotometrically quantified. Efficiency of DNA recovery after bisulfite modification was around 55% (data not shown). Recovered bisulfite-treated DNA (1 µl) was used in each well for SYBR green reaction. Modified DNA of standards and samples are stable for at least 2 months at -80°C. A sample of bisulfite-modified universally-methylated genomic DNA, (CpGenomeTM Universal Methylated DNA, Intergen, New York, NY) treated in the same way as patient samples and the concentration adjusted, after modification, to 2 µg/ml (quantified spectrophotometrically), served as internal standard in preparing serial dilutions (from 1 to 1/128 with MiliQ water) to construct a standard curve for Real-Time QMS-PCR. Each multi-well plate contained patient samples, serial dilutions of completely methylated DNA for constructing calibration curves, positive controls, and two wells with water used as negative controls (“blanks”). In all experiments, correlation coefficients for the calibration curves were >0.98, slopes ranged from 3.2 to 3.4, and PCR efficiencies were around 100%.

As found by other authors,[19, 20, 23, 24] some gene promoters were frequently observed, in the serum of cancer patients, to have methylated DNA; albeit traces of methylated DNA may also be found in serum of patients without cancer when highly sensitive quantitative techniques are used. Hence, cut-off points for the ESR1 methylated promoter was established from the ROC
curves i.e. selecting values that gave the maximal likelihood ratio (in current case the cut-off value was 0.02 relative units).

Assuming levels of methylation < 0.02 relative units, “test of methylation (-)”, were indicative of absence of the disease (physiological) while levels of methylation of ESR1 of > 0.02 relative units measured in the plasma “test of methylation (+)" were indicative of presence of breast cancer (pathological level of methylation). Once the distribution of cases was established in the two groups: “test of methylation (-)" and “test of methylation (+)" the study proceeded to assess whether this characteristic was associated with the phenotype ER(+) and ER(-) in tumor tissue (page 7 line 23 to page 9 line 8).

- **Quantitative MSP PCR methylation analysis:**

**Point 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 reference gene is not shown wherefore it can be assumed that this important step was not conducted.

**Response:** We apologize, again, for this inexplicable error. We have corrected this section (see above) and we hope that the statement is clear and unambiguous.

- **Results and discussion:**

**Point 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.

**Response:** Please see our detailed response (above). Description of ROC curve analysis is tedious on repetition so we have included a reference to a publication by our own group [Martinez-Galan J, et al. Quantitative detection of methylated ESR1 and 14-3-3-sigma gene promoters in serum as candidate biomarkers for diagnosis of breast cancer and evaluation of treatment efficacy. Cancer Biol Ther. 2008 Jun; 7(6):958-65] which contains these data that establish cutoff points.

**Point 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.

**Response:** We agree, ESR1 methylation status is not used in standard clinical practice, as yet. What we had intended to highlight was that the identification of numerical cutoff point that differentiates between a level indicative of absence of disease and a level indicative of disease with a maximum efficacy and low error, is a problem that is frequently encountered in different clinical scenarios e.g. the
level of arterial pressure beyond which the patient is considered hypertensive, or the level of glycemia that indicates diabetes. In our case, the problem was to determine a cutoff that distinguishes between the methylated \( ESR1 \) level indicative of presence of breast cancer from a level that indicates absence of breast cancer, and which can explain, at least in part, the absence of ER expression in tumor tissue. To do this we used the ROC curve analysis and have introduced a reference to our own previous work on this issue [Martinez-Galan J, et al. Quantitative detection of methylated \( ESR1 \) and 14-3-3-sigma gene promoters in serum as candidate biomarkers for diagnosis of breast cancer and evaluation of treatment efficacy. Cancer Biol Ther. 2008 Jun;7(6):958-65.] We have rewritten this paragraph in the revised m/s so as to avoid confusion (page 10 line 19 to page 11 line 4)

**Point 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.

**Response:** OK. Done. In accordance with the reviewer’s wishes we have re-written and reduced the length of this 2nd paragraph. What has caused confusion is that we had neglected to emphasize that we had measured methylated \( ESR1 \) DNA in free circulating DNA (fcDNA) in plasma from peripheral blood collected into tubes with EDTA as anticoagulant. We then correlated the methylation with ER receptor status in breast tumor biopsy tissue using immunohistochemistry. These concepts have been re-written for clarity in the revised m/s. (page 11 line 5 to 12)

**Point 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.

**Response:** In this study we did not analyze methylation in circulating tumor cells (as the reviewer rightly states, these are very few and far between). Methylation was measured in free circulating DNA (fcDNA) in plasma of patients with breast cancer. Neither did we assess methylation in tumor tissue, but we did study estrogen receptor expression in the biopsy tissue using immunohistochemistry. From these results we correlated the fcDNA methylation of \( ESR1 \) and the non-expression of ER in tumor tissue.
Point 9: There are 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 to 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.

Response: Again, we apologize for the confusion. As we have stated, we measured ESR1 methylation in fcDNA using QSM-PCR, and correlated the findings with the ER receptor status measured in tumor tissue using immunohistochemistry.

Point 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.

Response: OK. Done (see response to point #3, above). We apologize for claiming “priority” of our research findings; always a dangerous exercise.

Point 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.

Response: The study quantified methylated DNA in plasma aspirated following centrifugation of EDTA-anticoagulated peripheral blood. We did not study DNA methylation in breast tumor biopsy. The breast tumor tissue was used to determine the expression of ER using immunohistochemistry. The results from the two sets of analyses were found to be significantly correlated. RNA analyses, although attractive, were not part of the original protocol and, unfortunately, no samples from the study are available for these analyses.

Point 12: Based on the mixture of ESR1 DNA methylation results from plasma samples and from peripheral blood cells, it 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.

Response: Sorry. But the confusion results from not being clear in our methodology descriptions. Peripheral blood cells (pbc) were not isolated for the methylation studies. We assessed the level of methylation of the promoter region of the ESR1 gene using DNA isolated from the plasma of whole blood which had been collected into EDTA tubes and, from which, the plasma was isolated by centrifugation. The objective of the study was to determine the level of methylation in fcDNA and to relate this to the expression of ER in breast cancer tissue using immunohistochemistry. The results obtained showed
significant correlations between \textit{ESR1} methylation and ER-, and non-methylation with ER+ in tumor tissue. 

For clarification we have considerably re-written the text and, as well, we have changed the title to reflect the content of the study. The revised title reads: 

\textit{ESR1 Gene Promoter Region Methylation in free circulating DNA and its Correlation with Estrogen Receptor Protein Phenotype Expression in Tumor Tissue in Breast Cancer Patients} 

MINOR ESSENTIAL REVISIONS 

1. Methods: Collection and processing of samples and DNA preparation: 

Point 13: First paragraph: The conditions for the centrifugation should be stated as the relative centrifugal force (x g) and not as rounds per minute. 
Response: Quite right. The statement now reads: 
“Samples for methylation analysis were centrifuged at 2000 g for 10 min at room temperature…” (page 7 line 12) 

Point 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. 
Response: We agree. There is almost universal confusion between reverse transcriptase PCR and real time PCR. We have clarified the concept by using QMS-PCR throughout the text

- Quantitative QMS PCR methylation analysis: 

Point 15: The ER-alpha gene should be named as \textit{ESR1} throughout the whole manuscript. 
Response: We have used the italic \textit{ESR1} to refer to the promoter region of the estrogen receptor gene, and non-italic ER to refer to the protein expression in tumor tissue. 

Point 16: primer “dimers” instead of “dimmers”. 
Response: We would have made a correction for this error but the paragraph in which it had occurred has been deleted, and the point is moot. 

- Results and discussion: 

Point 17: In the second paragraph “RE” instead of “ER” is used. 
Response: Correction done 

Point 18: In the last paragraph of the results and discussion section Table 5 is indicated although there are only 4 tables. 
Response: Sorry for the error. There were, indeed, only 4 tables. 

- Abbreviations:

Point 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.

Response: OK. Done
Reviewer ≠ 2 (Dr. Luca Magnani)

MAJOR COMPULSORY REVISIONS:

Point 1: The manuscript is poorly written. The authors should reformat the manuscript, streamline and simply \textit{sic} the prose and communicate the, \textit{sic} results in an ambiguous \textit{sic} way.

Response: We have sought the help of a native-English-speaking professional provider of editorial assistance to revise the complete manuscript with a view to improving clarity of presentation (scientific and orthographic) while eliminating any errors of grammar, style and syntax.

Point 2: The authors fail to mention until the very end of the discussion the rationale of their design. PBC cells do not express ERalpha hence the promoter should be methylated. I assume their results imply that difference in \textit{ESR1} promoter methylation observed in pbc is accounted for by difference in the nature of tumor circulating cells. However no reference is provided and there are serious concern about the results. Indeed, the authors report that only 28\% and 36\% of Luminal A and Luminal B patients have pbc with \textit{ESR1} methylated. This number seems too low considering the limited amount of tumour \textit{sic} circulating cells in the plasma. Could the authors describe in better details how the \textit{sic} prepare the pbc cells? Do they enrich for tumour \textit{sic} circulating cells?

Response: Perhaps we were nor clear in our methodology statements. The study established the \textit{ESR1} methylation status using free circulating DNA (fcDNA) in plasma of patients with breast cancer. We related the findings to the ER expression in tumor biopsy tissue using immunohistochemical techniques. We did not isolate peripheral blood cells (pbc) nor circulating tumor cells. In the revised m/s we have clarified these concepts wherever they had arisen.

Point 3: It is not clear what is the real advancement in the field. The author states in page 12 that “In our literature search, we did not find any studies that correlated the epigenetic profile of methylation and its relationship with ER expression status”. This statement leads this reviewer to think that the authors perform they \textit{sic} search quite superficially. Here \textit{sic} few examples of previous work that looked specifically at the DNA methylation profile of \textit{ESR1} promoter in breast cancer tissues: Maybe the author stated that there are not \textit{sic} studies that linked pbc \textit{ESR1} methylation and ERalpha status? What is the benefit of their approach compared to normal histo-pathological staining?

Response: We thank the reviewer for highlighting the dangers of claiming priority for one’s findings without having conducted as exhaustive a literature search as would seem desirable. What is new in our study is that it was conducted in Caucasian patients, and that we analyzed the methylation of \textit{ESR1} as a factor involved in the silencing of ER receptor, not only triple negative but Her2, as well. Further, in comparing the subgroups with better prognosis (i.e. luminal A and B) we found that the methylation was significantly lower than the subgroups of poor prognosis. Further, the results point towards the possibility of each phenotype
of patients with breast cancer can have subgroups with a greater or lesser level of methylation of ESR1 that could have an effect on overall survival with treatment. These findings were significant despite a low number of patient samples.

Finally, our results highlight a possible future field of research such as the use of de-methylating agents as therapeutic tools which may reverse the expression of ER- to ER+ and, as such, not only generate a new hormone therapy but also, perhaps, improve the prognosis of these patients by converting them to ER+ expression.

Point 4: The statement “Hence, it appears that the molecular variant of the promoter of the ESR1 gene…” Page 13 is not supported by any results. Table 5 is missing.

Response: Perhaps we had overstated our claims, and these have been omitted in the revised m/s.
The table 5 had been deleted in an earlier version of the manuscript. Sorry for the error.

Point 5: The final conclusion “The results are promising in terms of early diagnosis, monitoring of response to treatment as well as a prognostic factor predictive of response” Page 14 is a gross overstatement not supported by any data.

Response: As with Point 4 (above), we may have been a trifle over-optimistic. We have toned down these statements in the revised text and have confined ourselves merely to a low-key interpretation of our findings.