Author's response to reviews

Title: Caspase 8 and Maspin are downregulated in Breast Cancer Cells due to CpG Island Promoter Methylation

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Author's response to reviews: see over
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Diana Marshall, PhD
Scientific Editor
BMC Cancer

Dear Dr. Marshall,

Thank you for the opportunity to submit our revised manuscript # MS: 2107853832871531, entitled “Caspase 8 and Maspin are downregulated in Breast Cancer Cells due to CpG Island Promoter Methylation. Authors: Yanyuan Wu, Monica Alvarez, Dennis Slamon, H Phillip Koeffler and Jaydutt V Vadgama.

We thank you and the reviewer’s for the constructive suggestions on our manuscript. As per your instructions, we have addressed the reviewer’s comments in our revised manuscript. We have provided in this cover letter point-by-point response to their concerns.

In addition, our revised manuscript conforms to the journal style.

I thank you for your support and consideration of our revised manuscript.

Sincerely yours,

Jay

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Response to reviewer’s comments:

Reviewer 1: Suhu Liu:

We thank Dr. Liu for constructive suggestions.

Major Compulsory Revisions

1. The abstract is too long and lack focus. Need to be reorganized to be more concise.
   
   Response: We have reorganized the abstract with better focus.

2. On page 10, the authors chose 4 breast cancer cell lines? with different phenotype? for methylation screening. Please further specify the meaning of different phenotypes?. Are they cell lines representing different cancer stages or metastasis potential or ER status?
   
   Response: We have clarified this information in the text in the methods section under Cells and Culture. These cell lines represent the different breast cancer subtypes with different receptor status and tumor properties. Each cell type exhibit unique morphology. The breast cancer properties of these cell lines are well described in the literature and in ATCC. In summary, MCF-7 cells were derived from pleural effusion from a 69 years old breast cancer patient with estrogen receptor positive tumor. This line is determined to be negative for HER2 or Erb2 receptor. MDA-MB231 was also derived from pleural effusion from a 51 years old breast cancer patient with tumor that was determined negative for ER. This cell line is also negative for HER2-receptor and is often characterized as “triple negative” line (ER/PR and HER2 negative) with highly metastatic properties. This line is determined to be positive for Epidermal Growth Factor receptor (EGFR) and for Transforming Growth Factor receptor-alpha. SKBR3 was derived from pleural effusion from a 43 years old breast cancer patient whose tumor was determined to be
HER2/c-erbB2 positive. This line is ER/PR negative, and is well established to study the mechanisms associated with HER2 gene and protein overexpression. HCC1937 was derived from 23 years old breast cancer patient with tumor stage: TNM stage IIB, and grade 3. The cell line was established from breast tissue that was determined to be ER/PR negative, HER2 negative, but positive for BRCA1 mutation at 5382C. This line is also positive for mutations in the TP53 gene.

3. Methylation of maspin in breast cancer has been reported previously. So the authors should be cautious about the claiming that they are the first to report this.

Response: Thank you for the clarification. We have modified our statement in the text.

4. There are quite a few statements in the paper that require re-iteration. For example, on page 17, the author said they are the first to shown maspin methylation in breast cancer while in fact research about maspin methylation in breast cancer had be published as early as 2000 (Epigenetic silencing of maspin gene expression in human breast cancers. Domann FE, Rice JC, Hendrix MJ, Futscher BW. Int J Cancer. 2000, 85(6):805-10.)

Response: As stated in comment 3, we have modified our statements both in the abstract and the discussion section. We also included the publication from Domann et al (2000) in reference and compared our results with theirs in discussion on page 21

5. There are also some ambiguous and descriptive statements that lack scientific conciseness and clearance and need to be re-phrased. For example, Methylation of the promoter regions of CpG-rich sites in genes is the major mechanism for the silencing of many genes in tumors? On page
17. Methylation might be an important mechanism of gene silencing but there is no confirmative scientific data to show that methylation is the major mechanism. Also how many genes can be described as? Many? genes?

Response: The statement has been deleted.

6. In the second paragraph of discussion, the author listed and described several genes that were reported to be methylated in breast cancer. This part did not related to the content of this paper and not necessary at all and should be shrink back and only keep the first one or two sentences. Also the author gave a small review as to what caspase 8? is on page 18 for discussion, which was not related to the findings in this research and can be cut back significantly.

Response: We have modified the discussion according to the suggestion from reviewers. In addition, the parts not related to the findings of our study, are deleted.

7. While there is quite some unrelated discussion, there are also some interesting findings in this paper which the authors seem to fail to notice or give further confirmation and discussion. One of the example is the finding that 5-Fu seems to lead to de-methylation of Caspase 8 promoter. This interesting finding surely require further validation and in depth discussion.

Response: We have discussed these important findings in our paper.

8. Another novel and interesting finding of this paper is that one oncogene, Survivin, is found hyper-methylated in all these breast cell lines. The authors should give further investigation and discussion about this discovery. It is actually recently reported that surviving is also hypermethylated in endometrial cancer (Oncogene. 2009 May 14;28(19):2046-50.)
Response: Thank you. We agree. Survivin was methylated in all breast cancer and non-tumorigenic breast cell lines. These data were obtained from Panomic methylation array and confirmed with promoter methylation specific PCR using Methylation promoter PCR kit (Panomic). To better understand why Survivin was methylated in both breast cancer and non-breast cancer cells more functional studies need to be performed. These studies are ongoing in our laboratory.

9. The discussion about Caspase 8 and maspin is mixed up in the ?DISCUSSION? part.

Response: The discussion section has been reorganized according to reviewer’s comments. The discussion about CASP8 and maspin has been separated into two parts in discussion section. Other concerns regarding written English have been addressed.

Reviewer 2: Vladimir Strelnikov

We thank Dr. Vladimir Strelnikov for the constructive suggestions on our manuscript. Here are our responses:

2.1 The selection of biological material to be studied in the contest of this research is rather poor and insufficient for some of conclusion made. Thus, the conclusion that the two most prominently methylated genes were the proapoptotic gene Caspase 8 (CASP8) and tumor suppressor gene maspin is ridiculous when based on the study of four cell lines.

Response: This statement in abstract and discussion has been deleted.
2.2. Another pitfall in this section is the selection of normal breast cells MCF12A and MCF10 for control ....

Response: Initially we selected MCF12A and MCF10 cells as control cell lines. In the revised manuscript we have modified our text and changed the “normal breast cell lines” as “non-tumorigenic breast cell lines” according to reviewer’s comments. Those non-tumorigenic breast cell lines as well as breast cancer cell lines were used for screening promoter methylation of those 82 genes.

2.3. Indeed, their own results demonstrate discrepancies in methylation status of several (importantly, cancer-related) genes between MCF12A and MCF10. This should have been given a more detailed discussion in the manuscript.

Response: Our results are demonstrated that IRF7, NME2, TFF1, Tastin and Survivin were methylated in both MCF10 and MCF12A as well as in breast cancer cell lines. Except for Survivin, we did not further confirm or validate the methylation status of those other genes. Therefore, we did not provide additional discussion in the original manuscript. However, in the revised manuscript we have discussed the results in discussion section. In this study we have observed differences in methylation status of POU3F1, HOXA2, and VHL between MCF10 and MCF12A. POU3F1, HOXA2 and VHL were methylated in MCF10 but not in MCF12A. We have also confirmed HOXA2 promoter methylation in MCF10 using Promoter methylation PCR kit (Panomic); however, POU3F1 may need further conformation (figure 1). mRNA levels of HOXOA2 and POU3F1 were examined in 30 non-cultured breast cancer tissues and 10 non-cancer tissues by RT-Q-PCR. Our data showed that mRNA levels of both HOXOA2 and POU3F1 were higher in non-cancer tissues than in cancer tissues (data not shown). Nonetheless
there were two to three non-cancer tissues that had low mRNA expression of HOXOA2 and POU3F1. We have discussed these results in the discussion section of the revised manuscript (on pages 17-18).

2.4. A part dedicated to the screening of CpG islands methylation is controversial as well. The authors use Promoter Methylation Array (Panomics, Fremont, CA). The probe sequences are blinded and neither the authors themselves nor the readers a fortiori can locate them in the genome. Panomics states that the promoter methylation array spots range from -800 to +200? Which, first, does not necessarily mean that the probes cover veritable functionally significant sites, and, second and most important, does not allow comparisons with other studies performed on the same genes.

Response: The sequences of CASP8, maspin and other genes listed in Table 1 have been provided in supplement 1. These sequences were provided by Panomics technical support. The information on the promoter methylation array spots range from -800 to +200 is also obtained from Panomics technical support.

2.5. It is not clear to which extent the primers used for promoter methylation PCR, MSP, and bisulfate sequencing match each other and the Panomics probes.

Response: Yes. They do match each other. The sequences of primers for promoter methylation PCR used in the manuscript are highlighted in blue in supplement 1. The primers used for MSP and/or bisulfite sequencing for CASP8 and maspin were designed based on the sequences used for Panomics probes. The primers that covered regions on the promoter are demonstrated in supplement 2.
2.6. It would be useful to supplement figure 2D with the indication of the positions of all primers and probes used in the study.

Response: We have provided supplement data indicating the positions of the primers used in this study and in the revised manuscript (supplement 1 and 2). However, we are not able to provide the position of probe sequences used in this study since the array was purchased from Panomics. This is proprietary information only known by the manufacturers.

3. Most data give no rise to doubt. It is surprising, though, to see a PCR product in the last lane (water control) for Survivin on figure 1. Mind, also, to correct the Survivin gene name at the same picture.

Response: The data has been corrected on figure 1.

4. Does the manuscript adhere to the relevant standards for reporting and data deposition?
Yes.

Response: Thank you. No response needed.

5. The conclusions begin to surprise from the very first pages. See Abstract: we show for the first time that CASP8 and maspin were the most common genes methylated in breast cancer cells. Such a conclusion is unacceptable when the study is performed on limited sample (four cell lines). Note that this conclusion is also present in the Discussion section. The limitations of the sampling also apply to the statements involving ER/HER2 positive/negative status of the cell lines. Unlike the ER negative and HER2 positive SKBR3 cells, most CpG islands of CASP8 were
methylated in ER positive and HER2 negative MCF-7 cells (page 18) pretends to provoke some conclusions, and those may be confusing. In the same cited sentence CpG islands should be substituted with CpG sites? Other conclusions, based on the results of analyses of individual cell lines, seem to be well supported and reasonable.

Response: The statement in Abstract and Discussion has been deleted. The statements involving ER/HER2 positive/negative status of the cell lines were also modified. “CpG islands” in the text has been replaced with “CpG sites”.

6. The limitations posed by the material studied (number of samples, extent of normality of the control cells) are not stated at all. Limitations produced by blind nature of the methylation array might also have to be mentioned.

Response: We have described the study limitations in the discussion section on page 25 of the revised manuscript. For instance, our study is limited to cell lines. Another limitation of the study could be the choice of genes immobilized on the Panomic Array membrane. There may be other genes not included on the array, whose promoter methylation and subsequent gene silencing may be associated with breast cancer.

Studies using large sample size of clinical breast tumors will provide better understanding of the clinical relevance of CASP8 and/or maspin promoter methylation in breast cancer. Furthermore, methylation array screening could be extended to larger selection of known and unknown genes.

8. The title conveys the findings. The statements “the two most prominently methylated genes were the proapoptotic gene caspase 8 and the tumor suppressor gene maspin” and “we show for
the first time that CASP8 and maspin were the most common genes methylated in breast cancer cells” should be either excluded from abstract or modified as being confusing.

Response: These statements have been deleted in the manuscript.

9. This is the article, which is easy and interesting to read. There are about a dozen places in need of grammar correction, and the authors will easily identify them by use of an automatic grammar corrector in their text editor. Gene names will need a closer, personal attention. Some misprints I noticed are in the table and figures (e.g., table 1 tasting instead of tastin; figure 1, survivin instead of surviving).

Response: We have corrected these errors in the revised manuscript.