Author's response to reviews

Title: p-Glycoprotein ABCB5 and YB-1 expression plays a role in increased heterogeneity of breast cancer cells: correlations with cell fusion and doxorubicin resistance

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Version: 6 Date: 17 July 2010

Author's response to reviews: see over
Dear The BioMed Central Editorial Team:

I thank the editors of the BMC Cancer for taking their time to review our article. We have made corrections after going over the editorial adviser’s comments. According to the comments to the authors by the advisers, we carefully revised this manuscript. The responses to adviser’s comments are detailed below and the changes were made in the manuscript.

I hope the revised manuscript will better meet of the BMC Cancer for publication. I thank you again for the constructive review.

Looking forward to receiving your decision on this manuscript.

Yours Sincerely,

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**Reviewer's report**

**Title:** p-Glycoprotein induced by YB-1 expression plays a role in increased heterogeneity of breast cancer cells: correlations with cell fusion and doxorubicin resistance

**Version:** 3  **Date:** 10 February 2010  
**Reviewer:** Yuji U Basaki

**Reviewer's report:**

Major Compulsory Revisions

Yang and colleagues describe the role of YB-1 on drug-induced cell fusion event in breast cancer cell lines. Furthermore, the authors investigated the involvement of YB-1 and p-glycoprotein, ABCB5 on drug sensitivity for doxorubicin and unique morphological appearances.

**Comments:**

1. Results section: The IC50 values of resistant clone for anticancer drugs including doxorubicin should be presented.
   
   **Answer** As the reviewer recommended, we presented the IC50 values.

2. The results section is difficult to follow and needs to be rewritten.
   
   **Answer** As the reviewer recommended, the results section was rewritten.

3. It would be helpful to the data of microarray for reader.
   
   **Answer** Microarray data was presented in supplementary Table 1.

4. Condition of 35 cycles in RT-PCR was too much for quantitative analysis. Real time PCR analysis was recommended.
   
   **Answer** RT-PCR results are deleted and dot-blot array results are added instead.

5. In page 11, line 10, “As demonstrated in Figures 4B, C, D, and E, cell fusion was induced in doxorubicin-treated MCF-7/YB-1 transfectants (doxorubicin
sensitive),” could be deleted.
Answer) As the reviewer recommended, we deleted it.

6. Does expression of ERK1/2, ERK3, MDR1, and ABCB5 decreased or increased in microarray analysis?
Answer) Increased, but below our cut threshold (x<1.5 fold).

7. If overexpression of ABCB5 was introduced, does drug sensitivity (or morphological changes) change in MCF7 cells?
Answer) Drug resistant cells are known to frequently resist over the threshold concentration of accumulation-reduction (mediated by MDR1) suggesting there’s another mechanism(s).

8. Discussion: In some reports, gene expression profile regulated by YB-1 in ovarian cancer and in breast cancer was demonstrated (Oncogene, 2007; 26: 2736-46, Cancer Res, 2008; 68: 1504-12). Please comments the differences in the discussion.
Answer) Unlike in ovarian cancer, breast cancer is composed of more diversified subpopulation of cells suggesting stem cell like cells may exist.

9. In Figure 4, difference of D and E is difficult to follow.
Answer) We added guide lines of gate1

10. In Figure 5C, what was relative level of protein expression standardize for?
Answer) Clarified as “Numbers indicate a relative level of protein expression based on the level of intensity of β-actin after normalization.”

Level of interest: An article of importance in its field
Quality of written English: Acceptable
Statistical review: No, the manuscript does not need to be seen by a statistician.
**Reviewer's report**

**Title:** p-Glycoprotein induced by YB-1 expression plays a role in increased heterogeneity of breast cancer cells: correlations with cell fusion and doxorubicin resistance

**Version:** 3  **Date:** 22 February 2010  
**Reviewer:** Karsten Jürchott

**Reviewer's report:**

The authors generated MCF7 cells resistant to doxorubicin by a one step selection procedure. They observed the formation of bi- and multinucleated MCF7 cells as a result of this process. Utilizing stable transfected MCF7 cells, they confirmed a dominant nuclear localization of YB-1, a protein linked to drug resistance, in the drug treated cells. Moreover, using different tags for YB-1 they were able to demonstrate and quantify cell fusion events induced by the drug treatment. They analyzed the differentially expression of a number of genes, which were suspected to be involved in cell fusion events. Using dot blots and quantitative RT-PCR they demonstrated a up-regulation of YB-1, c-Kit, MAPT and GST as a response to drug treatment. Moreover, they found an increased expression of ERK3 in the resistant cells and an expression of ABCB5 in the YB-1 over expressing, drug treated cells.

This study is interesting and raise important questions regarding the influence of cell fusion on the development of drug resistance and genomic heterogeneity. However, the presentation of some of the data needs to be improved and some of the conclusions seems not to be supported by the data. Therefore, this manuscript is not suitable for publication in BMC Cancer in this version, but I think that a proper revised version might have a good change.

**Major comments:**

Page 10: The calculation of the IC50 is not clear. Based on the graph in Figure 1A, I would estimate an IC50 of 1 nM. Some additional information, how the IC50 was calculated would be helpful.

Answer) Figure 1A and C shows percentage vitality of MCF-7 cells. The dose-effect of doxorubicin (0.01, 0.1, 1, 10 and 1000 nM) on cellular growth rate of MCF-7 was observed over the period of two weeks. Our proximal 50% growth inhibition (IC50) of doxorubicin for parental MCF-7 cells was 68 nM and for one of the pre-selected
doxorubicin resistant clones was 120nM. 10-20 nM doxorubicin was employed based on the previously known reports (23,24).

Page 10: “The ratio of MCF-7 cells with the multiple nuclei is significantly increased among doxorubicin-resistant MCF-7 clones compared to the drug sensitive control MCF-7 cells.”
What means significantly increased? The authors should indicate the fraction of cells with multiple nuclei prior and after the selection process. Otherwise there are no data which support this statement.
Answer) The ratio of MCF-7 cells with the multiple nuclei is significantly increased (inserted table in Figure 3), before doxorubicin-resistant MCF-7 clones emerged, compared to the drug sensitive control MCF-7 cells.

Page 11: The authors classified cells with two or more nuclei stained with the same color as homotypic cell fusion. Even this is maybe true, I did not found data which exclude mitotic defects as a cause for multinucleated (“homotypic”) cells. The authors should either present additional data, or be more moderate in this conclusion.
Answer) There is inserted small table in Figure 3.

Page 11: “Since the appearance of the polyploidy cells with increased genomic instability is the result of cell-to-cell fusion”
As long as mitotic defects cannot be excluded I would suggest to be more moderate in this statement.
Answer) However, we cannot exclude that some of these homotypic multinucleated cells may also associate with mitotic defects or with cytokinesis failure.

Page 11: “A significant fraction of MCF-7 cells contained the multiple nuclei after doxorubicin treatment compared to non-treated cells.”
See Page 10 – comment two.
Answer) There is inserted small table in Figure 3.

Page 12: “These results demonstrate a novel function of cell fusion events, followed by genome rearrangement might have triggered the de-differentiation of cancer cells.”
What are the criteria for de-differentiation in this case? I did not see any clear evidence for a de-differentiation.

Answer) The results demonstrate cell fusion events, followed by genome rearrangement. The origin of cancer-initiating cell (cancer stem cell) remains elusive and the fusion of genetic and cytoplasmic material between cells could be important in the development of the cancer stem cell [26].

Page 12: The authors point to a microarray experiment, but did not show any data. I suggest to indicate either that the original data are planned to be published elsewhere or to include at least the data of the selected genes in a supplementary.

Answer) Based on the results of Sentrix Human-6-V3 Expression Bead Chips on cell fusion (Supplimentary Table 1) and literature review….Supplementary Table 1 is added.

Page 12: The authors list Actin and GAPDH as differentially regulated genes during drug treatment. How far I understood, these genes were included as controls and to allow normalization of the different hybridizations. I did not found any information regarding the normalization process and the data processing. It is clearly necessary to provide a proper description of this.

Answer) In the time course study, GAPDH could not be acceptable for normalization because it was regulated in doxorubicin resistant MCF-7 cells. In the pre-chemotherapeutic (doxorubicin sensitive) MCF-7 cells, total 4 genes (YB-1, c-Kit, MAPT, GST) out of 14 were differentially expressed….normalization process and the data processing described in Methods as “The house keeping genes (Actin, GAPDH) were employed for normalization. For background subtraction and normalization, the Excel (Microsoft) program was used. Spots below a threshold intensity were eliminated and the expression ratio data were median-centered”

Page 12: “This result was verified by RT–PCR analysis with drug-resistant MCF-7 cells (Figure 5B).” Since the data in Figure 5A were obtained using the sensitive cells, it is not possible to verify this data with different (drug resistant) cells. To avoid any confusion I would suggest to include all data from the dot blots and the RT-PCR for the sensitive as well as the resistant cells.

Answer) Figure 5B was changed.

Page 13: “Unlike the pattern of c-Kit and MDR-1 expressions, YB-1 was highly
expressed following the drug treatment.”
I did not understand the term “unlike”, because c-Kit as well as MDR-1 were highly expressed following the drug treatment.
Answer) Changed as “YB-1, c-Kit and MDR-1 was noticeably expressed”.

Page 13: “Figure 5C demonstrates that MCF-7 parental population does not exist among the pre-selected doxorubicin resistant MCF-7 clones,”
I did not understand this conclusion.

Answer) Figure 5C demonstrates ……drug-induced stimulations was changed as “and ABCB5 expression was not induced in the drug untreated sensitive MCF-7 cells and in the pre-selected doxorubicin resistant MCF-7 cells (Figure 5C). Additional efforts are needed to adequately address this disparity. A specific role of ABCB5 in cell fusion, through membrane potential regulation, was reported [22]. If it is the case that the transcription factor YB-1 regulate ABCB5, doxorubicin treatment may correlate with cell fusion, however, further study is needed.”

Page 13: “We generated multi-nucleated cells, through cell fusion possibly mediated by YB-1, in doxorubicin treated MCF-7/YB-1 cells (doxorubicin sensitive).” Changed as “Multi-nucleated cells were generated in doxorubicin treated MCF-7/YB-1 cells (doxorubicin sensitive)”

I think it is an interesting idea that YB-1 is involved in mediating the fusion process, but I did not found any data supporting this.
Answer) Added “A specific role of ABCB5 in cell fusion, through membrane potential regulation, was reported [22]. If it is the case that the transcription factor YB-1 regulate ABCB5, doxorubicin treatment may correlate with cell fusion, however, further study is needed”

Page 17: “MCF-7 cells exhibited differentiated-progenitor cell like behavior, suggesting that acquired doxorubicin resistant cells may be originated from the self-renewed hybrid cells with progenitor characteristics.”
Answer) Added , but additional studies should address this possibility.
The authors demonstrated, that a small portion of the cells exhibited alterations in cellular morphology, but no evidence for other molecular changes corresponding to the new proposed types of cells. At least the new expression
of typical markers would support this statement, otherwise the conclusion should be more moderate.
Answer) Deleted Figure 6

Page 18: The present study involving drug induced cell fusion, triggered and mediated by YB-1 and ABCB5 respectively.
There are no functional data supporting this conclusion. All data are correlative, but not functional.
Answer) Changed as “Drug resistance could not be explained only by reductions of drug accumulation. The present study shows a correlation between doxorubicin induced transcription factor YB-1 and the transiently regulated fusogenic factor ABCB5 [22], suggesting cell fusion and clonal selection may involve as an additional mechanism in the progress of acquired drug resistance in MCF-7 cells.”

Minor comments:

Figure 2: Figure 2D is a little bit puzzling. Why is there one nuclei stained with both colors, and one only stained in green? I would expect that after cell fusion, both plasmids are expressed in the same cell, and therefore, both nuclei should be stained with both colors. Maybe I missed this point, but are there any explanation?
Answer) Added “Confocal images of the two fluorescent tagged YB-1 proteins localized to each of the nuclei of non-fused MCF-7 cells and localized in one nucleus in fused MCF-7 cells.”

Figure 4 and 5: The authors started the first experiments with 10 nM doxorubicin, but in both of the Figures 20 nM are indicated. There is no explanation and no information in the text, why the concentration was changed in these experiments.
Answer) Added “10-20 nM doxorubicin was employed based on the previously known reports (23,24)” and “In order to increase cell to cell fusion ratio, 20 nM doxorubicin applied (Figure 4E and Figure 5C).” in the Results section.

Figure 6L: The pictures demonstrate changes in the cellular morphology, but did not indicate, if there are really “Neurons”, “Astrocytes” and “Oligodendrocytes”. For that, the analysis of the expression of typical marker genes would be helpful.
Since the data did not directly support the schema, I suggest to change it or skip it.

Answer) Figure 6 was deleted since we do not detect the markers yet.

I would like to encourage the authors to recheck the language and spelling.

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:

Changed as 'I declare that I have no competing interests'
Reviewer's report

Title: p-Glycoprotein induced by YB-1 expression plays a role in increased heterogeneity of breast cancer cells: correlations with cell fusion and doxorubicin resistance

Version: 3 Date: 11 March 2010
Reviewer: Roberto Mazzanti

Reviewer's report:
Authors are trying to show the role of ABCB5 (p-glycoprotein) induced by the YB-1 expression in mediating cell to cell fusion in doxorubicin resistant MCF-7 cell line.

The ms deals with a new and interesting phenomenon that concerns cell to cell fusion and drug resistance. Experiments are nicely done; however according to this referee:

a. the title it is now misleading: referring to p-glycoprotein and drug resistance usually scientific community thinks to the MDR1 (ABCB1) phenomenon; the fact that the sequence of ABCB5 is 73% homologous to ABCB1, the real P-glycoprotein involved in conferring drug resistance, does not allow to talk of MDR1 or ABCB5 indifferently.

Answer) Title changed as “p-Glycoprotein ABCB5 and YB-1 expression plays a role in increased heterogeneity of breast cancer cells: correlations with cell fusion and doxorubicin resistance”

b. MCF-7 express in normal condition P-gp (the MDR1 gene product) (see also figure 5) thus it is not surprising that they show some degree of drug resistance to doxorubicin (Figure 1) as said by Authors.

Answer) It is true, but we attempted to find out another mechanism of drug resistance, because resistant cells are known to frequently survive over the threshold concentration of accumulation-reduction (mediated by MDR1) of anti cancer drugs.

c. Finally, to be sure that the effect on ABCB5 is mediated by the expression of YB-1 protein, it should be done some form of experiments where the YB-1 expression is abolished by siRNA or by inhibitors.

Answer) In our time frame, it was difficult. Instead, we sited a correlated paper [22]

d. English requires review by a mother language expert.