Author's response to reviews

Title: MMP-9 expression varies according to molecular subtypes of breast cancer

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Author's response to reviews: see over
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Ms. Cherry Battad
Journal Editorial Office
BioMed Central

Subject: MS: 6632970051263567- MMP-9 expression varies according to molecular subtypes of breast cancer- revised manuscript and reply to the reviewer’s comments

Dear Ms. Battad,

Please find enclosed a copy of the revised manuscript as well as a point-by-point reply to the insightful and very constructive comments of the reviewers. We are grateful to the reviewers and agree that their comments improved the quality of our manuscript. We are confident that you will find the proposed changes to be satisfactory.

Please do not hesitate to contact me if you have any other questions regarding the new version of the manuscript.

Kind Regards

[Signature]

Dr. Louis A. GABOURY
Principal Investigator
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Reply to reviewer comments:

A. 1st reviewer

1. It would be very helpful if the statistical tests used to determine significance were identified in the figure legends where appropriate (in addition to the Methods).

   Reply:
   
   1- Figure No.1 legend: Lines No. 623, 624 were added.
   2- Figure No.5 legend: Lines No. 652, 653 were added
   3- Figure No.6 legend: Lines No. 664-666 were added.

2. In Figure 1, it is not clear to what the numbers 388, 103, 443, 116, 210 correspond (in the row between molecular sub-type and MMP-9 expression). Are these the total number of patients in each molecular sub-type category? This does not seem consistent with the text indicating that over 3400 microarrays were analyzed.

   Reply:
   
   1- These numbers 388, 103, 443, 116, 210 are corresponding to the total number of patients in each molecular subtypes.
   2- We did a mistake in the number of patients as we used 3400 patients instead of 3,063 patients (Jézéquel et al. Breast Cancer Res Treat (2012) 131:765–775).
   3- Explanation of the difference between the 3,063 and the total number of patients used in the table:

   This website (http://bcgenex.centregauducheau.fr) presents results of molecular subtype determination based on three single sample predictors (SSPs) and three subtype clustering models (SCMs) for a large cohort of patients, and proposes robust lists of molecular subtyped patients to increase performance of molecular assignment. Only patients with concordant molecular subtype assignment for the three SSPs (robust SSP classification [RSSPC]), for the three SCMs (robust SCM classification [RSCMC]), or for all molecular subtype predictors (robust MSP classification [RMSPC]), are kept.
So, the number of patients in each subtype changes between classification methods as there are always patients left out because they could not be assigned a subtype.

3- To resolve this issue:
We decided to change the total number of patients from 3,063 to 1210 (380+103+443+116+210).
3,063 corresponds to the initial number of patients in the study.  
1210 is the total number of patients that could be assigned to each molecular subtype.
In the material and methods: the following sentence was added at line no. 109, 110 “However, only 1210 patients could be assigned precisely to each molecular subtype”. Also on lines no. 217 and 341, the number 3,063 was changed to 1210.

3. Figure 6 is derived from the review of 200 patient charts, but it is not indicated how many of these patients had some versus no metastasis. It is also not reported what proportion of the 200 patients had high versus low MMP-9. Can the authors show on the plot the distribution of high and low MMP-9 expression in the patients without metastasis as well as those with metastasis? Also, can the legend more clearly describe what comparison is being made that leads to the determination of statistical significance for LN, L.V., and Lung?

Reply:
1- Out of 200 patients, 121 (60.5%) patients have high MMP-9 expression and 79 (39.5%) patients have low MMP-9 expression. (Lines No. 295, 296)
2- Since the plot that includes all the data would be illegible, we preferred to depict all the numbers in a table. (Additional file 1).
3- The results were considered significant when the percentage of patients who developed metastases significantly differed in terms of low and high levels
of MMP-9 expression. Only lymph node, lymphovascular and lung metastases show significant difference. Lines no. 297-299, lines 302-303.

4. The description of the multivariate analysis model displayed in Table 2, and its interpretation, should be more clearly described. If I understand correctly, it appears that adjusting for the presence of metastasis eliminates the significant association of the HER-2 positive subtype with MMP-9 expression, but strengthens the significant association of the triple-negative subtype with MMP-9 expression. What are the possible reasons for this? What are the implications?

Reply: Lines No. 308-312. The writing of the interpretation in the results was modified accordingly. We are not sure about the reason and feel that future studies might be needed to address this question.

(Discretionary Revisions)

5. In Figure 7, on the Kaplan-Meier plots it would be more usual to report time in months or years on the x-axis.

Reply: The time was changed from days to years.

6. At the bottom of page 14 the authors state, “Multivariate analysis could not identify any other significant factors involved in case of relapse.” The meaning of this statement is not clear.

Reply: this line is modified, line no. 319-321

7. In the Discussion the authors state that there has been no previous report in the literature that specifically correlated MMP-9 expression with individual breast cancer molecular sub-types. It would be appropriate for the authors to update their literature survey, as our group has very recently published a new study in Oncotarget that is highly complementary to the present work, and corroborates their observations on the significance of MMP-9 up regulation in triple-negative breast cancers.
Reply: we included this study as a reference in the discussion (ref. No. 39) and modified this part in the first paragraph (lines no. 336-338). However, at the time we submitted the manuscript (April 14) this paper was not published as yet. Of note, an abstract of our work was published in the Archives of Pathology and Laboratory Medicine, volume 137, Abstracts page 1511 (poster no. 103) in October 2013

B. 2nd reviewer

**Major Compulsory Revisions:**

A correlation between MMP-9 (gene and protein) expression and breast cancer progression has been found in many studies (including recent ones). The authors should cite these papers and compare their study. For example:

- Van ‘t Veer et al., 2002, Nature (PMID:11823860). (MMP-9 is significantly unregulated in the poor prognosis signature in breast cancers) Ref. 44

- Mehner et al., 2014, Oncotarget (PMID: 24811362). (Production of MMP-9 by tumor cells and progression and metastasis of basal-like triple negative breast cancer) Ref. 39

- Wu et al., 2014, Plos One (PMID:24845596). (MMP-9 is a potential marker for breast cancer progression) Ref. 50

Reply: These references were included in the discussion. References no. 39, 44 & 50.

Background: MMP-9 cleaves denatured collagens (gelatins). This implies that MMP-9 only digests collagens which have first been cleaved by collagenases (e.g. MMP-1, MMP-8,...). See: Van den Steen et al., 2004 (PMID:15311942) and Rosenblum et al., 2010 (PMID: PMID:20585385). Please adjust this in the manuscript.

Reply: This part was modified accordingly in the background and the two references
The MMP-9 protein expression data solely rely on the detection of immunoreactivity with antibody ab38898, a polyclonal antibody against MMP-9. The authors should test whether or not this antibody cross-reacts with closely related MMPs (e.g. MMP-2). Otherwise, it cannot be excluded that the effect is due to the upregulated expression of a combination of MMPs. In addition, the authors should consider confirming the MMP-9 protein expression data by gelatin zymography (see PMID 23443633).

Reply:

1. This antibody has been used in more than 50 published articles on MMP-9 expression in mouse and murine tissues.
2. This Ab does not cross-react with MMP-2, the only other member of the MMP family that is capable of degrading collagen type IV; it also does not react to MMP-1 and MMP-3.

Figure 1 needs more explanation. For example: define low, intermediate and high MMP-9 expression. What can be concluded from the top representation (colored bars)?

Reply: the gene expression map is showing the MMP-9 gene expression according to molecular subtypes in 3,063 patients (as determined by the different predictors [robust or not]).
The gene expression data is given for those patients that could be assigned to certain molecular subtype (robust classifications for 1210 patients). In figure 1, the table indicates for each subtype the proportion of patient with low, intermediate, and high gene expression. Gene expression values were being beforehand split in order to form three equal groups. This means that "high expression" is the 1/3 of the patients with highest expression of MMP-9 and "low expression" is the lower 1/3 of the patients.

This part was included in the results, lines no. 218-223.

**Figure 2:** The authors state that the MCF7 cell line is the only luminal cell line with elevated MMP-9 levels. However, from figure 2 it can be seen that KPL1 also exceeds the mean expression value. Please address this concern.

**Reply:** KPL1 cell line was added in the text as a luminal cell line associated with a modest increase in the level of MMP-9 (Line No. 237, 630)

**Figure 5A:** Please add ‘n = ‘ for each molecular subtype and define the p-value for ***.

**Reply:** The numbers were added in the figure and p-value was added (p-value <0.0001).

**Figure 6:** Please define the p-value for *** **Figure 7:** Please label the y-axis

**Reply:** p-value was defined in figure 7. The label for the y-axis (Cumulative probability) was added in the diagram.

**Minor Essential Revisions:** •

**Abstract, Results:** remove the red semicolon

**Reply:** the red semicolon was removed (Line No. 40)

**Materials and methods:** Tissue Microarray, last sentence: (IHC) should be
defined immediately after immunohistochemistry.

**Reply:** Line 142: the place of the abbreviation (IHC) was changed from the end of the sentence to be immediately after the word immunohistochemistry.

**Results, Overexpression of MMP-9 is associated with a higher incidence of metastases, last paragraph: p-value = 0.001**

**Reply:** Line 306, Line 310: the p-value was changed from =0.00 to =0.0001

**Results, first line page 16: please correct:** ‘As for as the expression...’

**Reply:** Line 345: the word (As for as) was removed and used instead the word (when)

C. 3rd reviewer (all the comments are done)

1) The reviewer comment is to keep MMP-9 either regular or italic in the manuscript.

   **Reply:** we used the italic font only when we are talking about MMP-9 mRNA.

2) Line No. 132: the word donor was removed.

3) Line No. 148: The location of Abcam Company was added.

4) Line 265: the sentence (to that end) was removed.

5) Line 428: The capital letters JML were removed.