Author's response to reviews

Title: Human breast cancer and lymph node metastases express Gb3 and can be targeted by STxB-vectorized chemotherapeutic compounds

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Version: 3  Date: 1 September 2014

Author's response to reviews: see over
Dr Maggie Cheang  
Editor  
BMC Cancer  

**MS: 1383022758140769 / MS: 6853997901021436**  
Paris, September 1, 2014  

Dear Dr Cheang,  

We are pleased to resubmit our manuscript entitled “Human breast cancer and lymph node metastases express Gb3 and can be targeted by STxB-vectorized chemotherapeutic compounds” by Stimmer et al. With the help of the reviewers' comments, we significantly modified our work.  

As you requested, we included a continuous line and page numbering in the revised manuscript.  
Additionally, we improved the title page. It contains now the names, institutions, countries and email addresses of all authors, and the full postal address of the corresponding author.  

Furthermore, in response to the reviewers' comments, we have performed many new experiments, and have introduced substantial modifications throughout the manuscript. The most important change concerns a point that was raised by both reviewers who indicated that we were apparently presenting two contrary hypotheses: that increased Gb3 expression was linked to a less differentiated state in primary tumors, and to re-differentiation in metastasis. We agree with both reviewers that this part of the discussion needed to be clarified. The mechanisms that control Gb3 expression and the functions of Gb3 in primary tumor tissue and metastasis are not known yet. In the revised version of the manuscript, we speculate that Gb3 has different functions in both tissue contexts, possibly depending interacting partners. The same molecule could thereby be linked to apparently opposing phenotypes. While this is pure speculation at this stage, it creates an interesting paradigm in a field — the molecular mechanisms of glycosphingolipid functions — in which even the most basic aspects are still unexplored.  

This change and many other are detailed in the rebuttal letter (see below).
Because our findings are applicable for the development of novel chemotherapeutic conjugates, we believe that they are general interest to researchers and clinicians who read your journal.

This manuscript describes original work and is not under consideration by any other journal. All authors approved the manuscript and this submission.

In conclusion, we hope that you will find our work represents a valuable contribution to these topics. Many thanks in advance for your consideration.

Yours sincerely,

Ludger Johannes
Director, U1143 INSERM — UMR3666 CNRS at Institut Curie
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We thank the reviewers for their constructive and helpful comments. Our responses are written below in the text of the initial comments.

Reviewer’s report

Title: Human breast cancer and lymph node metastases express Gb3 and can be targeted by STxB-vectorized chemotherapeutic compounds

Version: 2 Date: 3 November 2013

Reviewer: Toshiyuki Yamaji

Reviewer’s report:

The manuscript “Human breast cancer and lymph node metastasis express Gb3 and can be targeted by STxB-vectorized chemotherapeutic compounds” makes several points.

1. Gb3 expression in primary breast tumors and lymph node metastases was analyzed using a novel method and showed several correlations as below.
   a. The expression of Gb3 and estrogen receptors.
   b. The absence of Gb3 in primary tumors and the presence of metastases
   c. Up-regulation of Gb3 expression in lymph node metastases.

2. Gb3-positive breast cancer xenografts in mice were detected by intravenous injection of STxB subunit.

The expression of Gb3 in breast cancer cells was already reported (LaCasse et al, Blood (1999), Johansson D et al, BMC Cancer (2009)) as authors described, therefore the novelty of this work is dependent on the relationship between Gb3 expression and metastasis, and the novel technique (ex vivo Bioaccumulation (EVB)). The manuscript is suitable for publication if the following points are clearly addressed.

Major revisions:

1) Description of the relationship between Gb3 and metastasis is confusing in Discussion. Authors showed that the absence of Gb3 was linked to frequency of lymph node metastasis. On the other hand, the expression of Gb3 was up-regulated in lymph node metastases compared with the primary lesions. As an interpretation of these data, they supported two contrary hypotheses that Gb3 is linked to less differentiation and well differentiation, and combined the hypotheses tandemly. The discussion should be described more clearly and satisfactorily.
Response to reviewer's remark:
We agree with the reviewer that this part of the discussion needed to be clarified. The mechanisms that control Gb3 expression and the functions of Gb3 in primary tumor tissue and metastasis are not known yet. We speculate that Gb3 has different functions in both tissue contexts, possibly depending interacting partners. The same molecule could thereby be linked to apparently opposing phenotypes (i.e. a decrease of Gb3 expression with increased metastatic spread in primary tumors, and an increase of Gb3 expression with epithelial tumor cell differentiation in metastatic tissue). While we agree that this is pure speculation at this stage, it creates an interesting paradigm in a field — the molecular mechanisms of glycosphingolipid functions — in which even the most basic aspects are still unexplored. This hypothesis is now better explained in the revised version of the manuscript discussion (page 21-22 lines 511-533).

2) Discrimination of tumor cells from normal tissue is clinically important. Is normal breast tissue around tumors negative for Gb3? And how much is the intensity of STxB-positive signal in breast cancers (or their xenografts) compared with kidney or other Gb3-positive normal tissue?

Response to reviewer's remark:
1. Is normal breast tissue around tumors negative for Gb3?

   The discrimination of tumor cells from normal tissue is indeed very important, especially for cytological diagnostics in the clinics. A clear cut differentiation between normal, hyperplastic, or tumor epithelial cells is not possible on a cytological specimen separately from clinical examination, because of the lack of overall histological structure. This question could therefore not be answered for the examined group of patients. Furthermore, for ethical reasons there was no possibility to perform a second cytological or histological biopsy in the healthy parts of the patients' breasts. Our preliminary studies of total Gb3 expression (using an extraction method) showed that overall Gb3 levels were increased in breast carcinoma versus healthy breast tissue, confirming previously published results [4] even if in our study we did not reach statistical significance. We included these data in reviewed version of the manuscript (page 13, lines 306-319, and Fig. 1).

2. And how much is the intensity of STxB-positive signal in breast cancers (or their xenografts) compared with kidney or other Gb3-positive normal tissue?

   Human specimens: We could not directly compare breast cytology with kidney or liver cytological specimens in examined patients. However, our preliminary results showed that overall Gb3 level in human breast carcinoma is 2.3 times lower compared to normal kidney tissue (page 13, lines 306-319, and Fig. 1).

   Mouse specimen: Cytological specimens from xenografted mice showed similar STxB-staining patterns as those observed on patient tissue. In histological specimen of STxB-
Cy3 injected mice we could observe the following patterns in overall stain intensity: Tubular epithelial cells of kidney medulla showed constantly diffuse and intense staining, whereas liver parenchyma showed constantly low staining (mainly in cells with endothelial appearance). Tumor tissue (xenograft) showed heterogeneous staining with uneven intratumoral distribution, including tumor lobules with intense signal and with no or low staining. These observations are now reported in the revised version of the manuscript (page 19, lines 453-464).

3) In xenograft model, is the expression level of Gb3 maintained in comparison with the original tumors?

Response to reviewer’s remark:
For this study we used established xenografts, which were maintained for several years in our institute. The tissues from original tumors are not available any more. Recent studies with these and similar breast cancer xenografts completed in our institution show that human breast cancer xenografts maintain the overall histologic, genomic and gene expression profile of the corresponding patient tumors and remains stable throughout sequential in vivo generations [5, 6]. This point is now explicitly mentioned in the revised version of the manuscript (Result part page 18, lines 420-425).

4) The term of “bioaccumulation” is usually used as the accumulation of chemicals in the tissue. The new method “ex vivo Bioaccumulation (EVB)” that authors described is confusing because the method indicates the isolation of cancer cells through a density gradient. Another understandable term might be used. And authors need to explain the merit of the method more closely.

Response to reviewer’s remark:
We have replaced the term “ex vivo Bioaccumulation” by “ex vivo STxB labeling” (ESL).

Breast cancer is a heterogeneous disease with large differences in the biological behavior of each individual cancer and its drug response. Therefore it is clear that novel clinical tools are needed to select patients for most appropriate therapy, to monitor the development of the disease, and to change the treatment, if necessary. The ESL tool should be useful early in routine medical consultation, to facilitate treatment decision. Our ESL tool, based on routine cytological diagnostic methods, should allow selection of Gb3 positive patients, suitable for STxB-based targeted therapy. This aspect is now better explained in the revised version of the manuscript (page 22-23 lines 535-544).

Minor revisions:
1) In Methods (p7), the source of the antibodies and STxB used in this study should be described.

Response to reviewer’s remark:
This information was added, as requested by the reviewer (page 8, lines 182-187 for the
antibodies, and page 9, lines 213-222 for STxB).

2) In Methods (p7), correct crbB2 to cerbB2. And also unify the term "Her2/neu" (Results p11 middle, Discussion p17 1st sentence) and "CerbB2".

Response to reviewer’s remark:
We unified these terms. Only the abbreviation HER2 was used for human epidermal growth factor receptor 2 in the revised version of the manuscript.

3) In Methods (p8) etc, correct FC analysis to FACS analysis or define FC analysis (flow cytometry).

Response to reviewer’s remark:
We replaced “FC analysis” by “flow cytometry analysis” in the method and results sections as well as in table 2 and the legend of figure 3.

3) In Methods (p9), correct GB3 to Gb3.

Response to reviewer’s remark:
We corrected this typo. Thanks for having pointed it out to us.

4) In Results (p11), the explanation of RO and RP is required (estrogen receptor and progesterone receptor).

Response to reviewer’s remark:
RO and RP were replaced by more appropriate abbreviations ER for estrogen receptor and PR for progesterone receptor. These explanations were added to the result section (page 14, lines 333-336).

5) In Results (p11 middle), correct asses to assess.

Response to reviewer’s remark:
We corrected this typo. Thanks for having pointed it out to us.

6) In Fig.3 legend, C-E should be described correctly.

Response to reviewer’s remark:
As requested, we improved the legend of Fig. 3 (Fig. 4 in improved manuscript) for the points C to E.

7) In Table 2, BEV? EVB?

Response to reviewer’s remark:
EVB is the right abbreviation. As mentioned above, we replaced the term “ex vivo Bioaccumulation” by the term “ex vivo STxB labeling” (ESL). This was corrected in the table 2.
Level of interest: An article whose findings are important to those with closely related research interests

Quality of written English: Acceptable

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
Reviewer's report

Title: Human breast cancer and lymph node metastases express Gb3 and can be targeted by STxB-vectorized chemotherapeutic compounds

Version: 2 Date: 29 July 2013

Reviewer: J. Chuck Harrell

Reviewer's report:

In this manuscript, the authors’ goals were to determine if Shiga toxin conjugated therapeutics could be utilized for cancer therapy. Developing novel therapeutic approaches that specifically target cancer cells, as opposed to all cells in an organism, is an important objective to eradicate malignant cells. They developed novel approaches using clinical samples and xenografts models to determine the type of breast cancers that would be most susceptible for this targeting. While the concept and goals are important and should be addressed in future studies, there are a significant number of areas that need to be addressed in this manuscript and with the research performed.

First, page numbers are very helpful when reviewing papers as it allows the reviewer to directly comment on specific items.

Response to reviewer’s remark:
Page numbering was added to our manuscript. We apologize for this oversight.

When describing the features observed in the text, the authors need to better guide the reader to the points they are trying to make. Instead of saying in Figure 2, say in figure 2A we saw this, and figure 2B this.

Response to reviewer’s remark:
This point was improved throughout the results section.

Table 1. Progesterone is misspelled. Table 1. CerbB2 is usually written C-ErbB-2

Response to reviewer’s remark:
We corrected this point in the table 1. Furthermore, throughout the manuscript, CerbB2 was replaced by its synonym HER2.

What are other gene names for Gb3. A4GALT or CD77?

Response to reviewer’s remark:
Globotriaosylceramide is a ganglioside also known as CD77, Gb3, and ceramide trihexoside [8]. It is formed by the alpha linkage of galactose to lactosylceramide catalyzed by A4GALT [9]. We
have now added this information to the revised version of the manuscript (page 5, lines 116-119).

Lymph node metastases are usually not fatal, are there any insights if Gb3 is expressed in distant metastases?

Response to reviewer’s remark:
In the current manuscript, we provide the first description of Gb3 expression in lymph node metastasis of breast cancer patients. Gb3 expression was studied in human colon cancer and in their liver metastases. Previously, we had found that compared with normal tissue, the expression of Gb3 was strongly increased in colorectal adenocarcinomas and their metastases, but not in benign adenomas [10]. To our knowledge, there are no descriptions of Gb3 expression in distant metastases of human breast cancer. Metastases, including hepatic, pulmonary and bone metastases are not tested yet in our laboratory, and should be tested in future studies. We develop STxB-based therapies to prevent multimetastatic spread of the primary tumor in the early phase of the disease. Therefore, we are particularly interested in the characterization of lymph node metastases and in micrometastases in the lung and liver. However, these metastases are not available for routine cytological diagnostic because of their size and anatomic position.

The first sentence of the final paragraph in the introduction needs to be re-written.

Response to reviewer’s remark:
We have re-written this sentence, as requested (page 7, lines 148-151).

In the second paragraph of the methods c-ErbB-2 is misspelled.

Response to reviewer’s remark:
In the revised manuscript, we only use the abbreviation HER2 for human epidermal growth factor receptor 2.

In the second paragraph of the methods you mean to state the estrogen and progesterone RECEPTORS were evaluated. Which antibodies were used? “appropriate monoclonal antibodies” is not specific enough.

Response to reviewer’s remark:
We replaced the statement “Estrogen and progesterone and crbB2 expression were evaluated…” by the following:

Estrogen and progesterone receptors as well as HER2 expression were evaluated using a set of monoclonal antibodies: ER (clone 6F11; 1/200; Novocastra, Rungis, France), PR (clone 1A6; 1/200; Novocastra) and HER2 (clone CB11; 1/1,000; Novocastra). Proliferation index was assessed using monoclonal anti-Ki67 antibody (Ki67, clone MIB-1; 1/75; DAKO, France) (page 8, lines 182-187).

Why were the breast cancer xenografts grown in a non-orthotopic site?
Response to reviewer’s remark:
The direct implantation of patient tumor fragments into sub cutaneous interscapular fat pad of mice was chosen as we have observed that this site was more favorable than the orthotopic site for tumor take and growth. Furthermore, we observed less traumatic lesions due to scratching and biting in interscapular grafts compared to the orthotopic site. However, these observations are not published yet.

Why did the mice receive supplemental estrogen? Usually mice are injected with estrogen in oil, or injected with an estrogen-releasing silastic pellet. Why was it diluted in the drinking water? Is there any evidence that the estrogen produced a biological response (expanded uterus)?

Response to reviewer’s remark:
We decided to supplement grafted mice orally mainly for reasons of animal facility management. Estrogen serum levels were verified in previous studies (data not published). Indeed, estrogen serum levels rise after the beginning of treatment and then remain stable throughout. Furthermore, we observed a minimal to mild uterus hypertrophy during the whole period of supplementation. Mice without estrogen supplementation showed normal uterus appearance without hypertrophy.

For the EVB method, were there any supplements in the DMEM?

Response to reviewer’s remark:
There were no supplements added to the DMEM medium.

In the EVB method section, the sentence that starts “Cancer cell-fraction…” should be “The cancer cell fraction…”

Response to reviewer’s remark:
This was corrected (page 10, line 235).

Where/how was the STxB-Cy3 produced? How was 1µM of STxB-Cy3 chosen as the dose used?

Response to reviewer’s remark:
Recombinant STxB-Cys was produced as previously described [7, 11]. We have now briefly outlined the expression and purification protocol (pages 9-10, lines 213-222). Covalent coupling of STxB to Cy3 (cyanine 3; Amersham Biosciences) was carried out according the instructions of the supplier. This is also mentioned now (page 9, lines 219-221).

The concentration of 1 µM of STxB-Cy3 was chosen empirically during the setup of the protocol. Different concentrations of STxB-Cy3 were tested on cytological specimens of Gb3 positive xenografts. 1 µM of STxB-Cy3 represent the optimal concentration to saturate the maximum of Gb3. Concentrations above 1 µM did not increase the labeling efficiency on epithelial cells on xenografts samples.
Roughly how many cells were subjected to the EVB method?

Response to reviewer’s remark:
Roughly 100 to 500 cells with epithelial morphology were subjected to this analysis. Patients with lower cell counts were excluded from the study. Cell density and morphology were judged on MGG stained slides, and on EVB (which is now termed ESL) slides using DAPI nuclear stain.

Results
Table 1:
In the text, it says 106 patients were analyzed by the FNA and EVB techniques, however, Table 1 has 107 patients listed. Which is correct?

Response to reviewer’s remark:
Thanks for pointing this out to us. 107 patients participated in this study. The corresponding number was corrected in the abstract and in the results section (page 14, lines 327-329).

How were the molecular classifications for the patients determined?

Response to reviewer’s remark:
All data were reviewed and primary tumors were classified according to the St. Gallen International Expert Consensus of 2011 [12]. This classification distinguishes the following categories: Luminal A (ER and/or PR positive, HER2 negative and Ki67 low), Luminal B/HER2- (ER and/or PR positive, HER2 negative and Ki67 high), Luminal B/HER2+ (ER and/or PR positive, HER2 positive), HER2+ (ER and PR negative, HER2 positive), Triple Negative (ER, PR, HER2 negative).

Define RO and RP prior to abbreviating.

Response to reviewer’s remark:
RO and RP were replaced by more appropriate abbreviations ER for estrogen receptor, and PR for progesterone receptor. The explanation was added to the result section (page 14, lines 333-335).

The claim that “Gb3 expression is enhanced in lymph node metastases compared to the primary tumor”, is not accurate. According to the data, only 40% showed relative increases, where as 50% had no change, and 10% lost Gb3 expression.

Response to reviewer’s remark:
We agree with the reviewer that this formulation was misleading. We have replaced it by: “Gb3 expression is enhanced in lymph node metastases of 40% of patients compared to the primary tumor” (page 15, lines 356-357).

The use of decimal points or commas should to be consistent when used for p-values.

Response to reviewer’s remark:
We reviewed all decimal numbers and used decimal points for all p-values. The decimal comas
were replaced by decimal points in the result section.

Figure 2.
The immunocytochemistry looks good. The stains appear to be strong and specific. What data is used to draw the conclusion that the structures that are not positive for cytokeratins are capillary structures?

Response to reviewer’s remark:
We used morphological criteria to describe endothelial cells, including fine tubular structures lined with small, elongated cells with small oval to cigar-shaped nuclei. We agree with the reviewer that these criteria did not prove that observed cells were indeed endothelial cells. For this reason, we changed “capillary structures” and “endothelial cells” to “capillary-like structures” and “cells with endothelial morphology”, respectively (page 17, lines 411-412 and Fig. 3F in reviewed manuscript).

Table 2.
Given that most ER+ tumors express Gb3, do the xenografts also express Gb3 in a similar ER-dependent manner? Adding ER status to this table or to the text would help.

Response to reviewer’s remark:
We tested all xenografts for ER expression. However, only one xenograft was ER positive. Thus there is no correlation between ER expression in xenografts and Gb3 expression.

Furthermore, we added ER, PR, and HER2 status to the xenografts description in the table 2 and in the result section.

Figure 3.
Without the presence of a co-stain for fibroblasts or endothelial cells, how is it possible to conclude which cells are binding the STxV-Cy3?

Response to reviewer’s remark:
We used morphological criteria to describe endothelial cells, including fine tubular structures lined with small, elongated cells with small oval dense nucleus, often containing erythrocytes (structures with capillary morphology). Fibroblasts present similar elongated appearance. These cells constitute compact tissue with streams and waves, however.

Discussion
In the fourth paragraph is unclear. Originally the authors had stated that Gb3 expression is associated with ER+ tumors, which are not as aggressive as the ER- tumors which spread more rapidly to the lymph nodes. In this paragraph, they suggest that initially Gb3 is upregulated with the tumor cell dedifferentiates which may coincide with increased invasive properties...then with further dedifferentiation Gb3 is down regulated which increases metastasis. Then once the cells they have metastasized, they re-differentiate, and have increased Gb3 expression. There is no data to support this hypothesis. If figure 1, could be analyzed in the context of ER- and ER+ tumors, maybe, this hypothesis would have a little more support.
Response to reviewer's remark:
We agree with the reviewer that this part of the discussion needed to be clarified. The mechanisms that control Gb3 expression and the functions of Gb3 in primary tumor tissue and metastasis are not known yet. We speculate that Gb3 has different functions in both tissue contexts, possibly depending interacting partners. The same molecule could thereby be linked to apparently opposing phenotypes (i.e. a loss of Gb3 expression with increased metastatic spread in primary tumors, and an increase of Gb3 expression with epithelial tumor cell differentiation in metastatic tissue). While we agree that this is pure speculation at this stage, it creates an interesting paradigm in a field — the molecular mechanisms of glycosphingolipid functions — in which even the most basic aspects are still unexplored. This hypothesis is now better explained in the revised version of the manuscript (page 21-22 lines 511-533).

Level of interest: An article of limited interest

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.
References


