Author's response to reviews

Title: Epstein-Barr virus infection and clinical outcome in breast cancer patients correlate with immune cell TNF-alpha/IFN-gamma response

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Author's response to reviews: see over
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To the Editor, BioMedCentral Cancer

You will find enclosed the 2nd revised version of our article entitled “Epstein-Barr virus infection and clinical outcome in breast cancer patients correlate with immune cell TNF-\(\alpha\)/IFN-\(\gamma\) response”. We have taken into account all the reviewers’ comments, and wish to sincerely thank them for helping us improve the manuscript (our reply in red).

**Reviewer #1**

**Discretionary revisions:**

1. **Reword the introductory section in two areas,** a. “definitely demonstrating that EBV is restricted to tumor epithelial cells” and b. “clear that EBV expression is restricted to tumor epithelial cells and that the cellular source of the PCR EBV DNA was the epithelial tumor cell” since other groups found EBV in lymphocytes but not in epithelial cells.

   **We have modified the sentences:** “…demonstrating that the EBV could be restricted to tumor epithelial cells (Bonnet \textit{et al.}, 1999, Hippocrate \textit{et al.})…” (lines 88-89) and “…although the detection of EBV (protein expression and DNA detection), in terms of it being restricted to tumor epithelial cells, is still a debated issue.” (lines 99-100).

2. **Line 51 of abstract,** it is not clear you are measuring “latent” virus so leave out this qualifier.

   **We have modified the sentence:** “Those who recovered from their disease were found to have a measurable EBV DNA load…” (now line 45)

3. **Line 50,** replicative EBV correlates with worse outcome but does not necessarily have a role in driving that worse outcome.

   **We have modified the sentence:** “The replicative form of EBV, as investigated using anti-ZEBRA titers, correlated with poorer outcomes, whereas the latent form of this virus…” (now lines 50-51)

4. **It is confusing to use the term “expression” unless you mean RNA or protein expression,** e.g. in line 98 of page 5. **So clarify throughout the introduction if you mean DNA detection or RNA/protein expression.**

   **Please see above (Item 1).**

5. **Misspelled word on line 128**

   **We have replaced this with** “histopronostic Scarff-Bloom-Richardson classification (SBR)” (line 128)

6. **Line 139,** was the frozen section checked for tumor proportion by H&E stain prior to extraction of DNA? What was the minimal tumor content input to the assay?

   **Initially, the block weighed 25mg. The DNA was extracted from 10\(\mu\)m paraffin section (dewaxed in xylene for 30 min)(see Herrmann & Niedobitek 2003). We did not verify tumor proportion prior to DNA extraction.**

7. **Which ribosomal DNA served as the normalizer gene,** and was is present at only 2 copies per cell? Concern is that (perhaps abundant) rRNA contaminating the specimen might also amplify by Q-PCR. Another control gene might be a better choice, and results of this human gene quantification might also serve as a normalizer that is more accurate than is spectrophotometry for calculating the denominator (cell number) for viral load
We used the Eukaryotic 18S rRNA Endogenous Control of Applied Biosystems (see line 157) and verified using another control (β-actin). The reviewer’s query is valid, yet we would like to draw the editor’s attention to the fact that we have demonstrated that the amount of EBV viral load (tumor and peripheral blood) is not relevant for predicting the disease outcome. All in all, we believe that our internal controls remain valid.

8. Line 234, add results of EBV load in non cancer control PBMC here.
   We have added the sentence: “In comparison, three PBMC samples from 16 healthy control individuals contained EBV DNA (median: 0 copies/µg DNA).” (lines 243-244).

9. Line 277, spell our Lymp and RE/PR.
   We have altered this to read: “lymph node invasion” “and estrogen/progesterone receptors” (lines 288-289).

10. Line 369, the tumor type is properly named Hodgkin lymphoma, not Hodgkin’s disease.
    The modification has been made (line 380).

11. Line 379, remove the word “clear” given that you (properly) use the word suggest.
    The modification has been made (line 389-390).

12. Line 390, change “obvious” to “possible”
    The modification has been made (line 402).

13. Line 411, did you measure single cells? If not, change this wording.
    The modification has been made (lines 420-421).

14. Line 432, reword since there there is an association with high ZEBRA titer and poor outcome but the high ZEBRA could be the effect of late stage cancer and not the cause of poor outcome.
    We have modified the sentence: “There is an association between high anti-ZEBRA titers and poor outcome, though the high anti-ZEBRA response could be the result of late-stage cancer rather than the cause of poor outcome.” (lines 440-442).

15. Table 2, clarify which grade.
    The modifications have been done. We added this sentence in tables 1 & 2: “95% CI” stands for the confidence interval at 95%. The variable “Grade” includes all grades.

16. Table 2, clarify the meaning of explicative “Explicative variables” and “variables” have the same meaning

17. Table 2, clarify if the entries on this table and what they mean. For example, the first entry is EBV-t+ and the relative risk is 2.36 (poor risk), but in table 1 the univariate risk was 0.795 (better risk). What is table 2 showing???
    We added the “relative risk” (confidence interval) in table 1, as well as the table 2. The relative risk (RR) >1 corresponds to a negative effect, i.e. a decrease in patient survival, while an RR <1 corresponds to a positive effect, i.e. an increase in patient survival (cf.lines 746-747)

18. Line 147-148 imply your extracted DNA from serum but no data is presented on this topic. EBV DNA levels in serum (copies per mL) would be useful with respect to this survival analysis
    The PCR was consistently negative in the serum of all patients.

Reviewer#2
1. Latent form of EBV infection had abundant EBER. EBER in situ hybridization
is recommended to demonstrate EBV infection on the tumor cells rather than in non-tumoral cells.

We did not perform EBER detection, as it had been previously stated that this marker is inconsistent in the breast cancer setting (see references: Glaser et al. 1998, Deshpande et al. 2002, and the review published by Arbach & Joab, 2005, especially the table 5).

2. Authors found a high frequency of IFN-\# and TNF-\# producing PBMCs in BC patients with EBV infection and recovered from the disease, and jumped into a conclusion “The survival advantage in BC patients might be through activation of non-specific anti-tumoral immune responses” A gap was still present between the finding and the conclusion. It was necessary to realize tumor specific and EBV specific immune response in the EBV-infected BC patients. Therefore, PBMCs should be stimulated at least by dominant tumor antigens and EBV antigens in addition to non-specific stimulation with iono/PMA

In this paper, we focused only on the non-specific responses. We have since conducted further research on specific immunity, and will provide you with an overview of the key experiments we performed in order to understand the mechanism of action of EBV in this context:

(i) We investigated the production of granzyme B through co-culture of the PBMCs with (i) the breast cancer cell line MDA-MB-231 or (ii) the EBV-producing B-cell line (B95-8) serving as antigens to stimulate the specific immunity for breast cancer or EBV, respectively. In this context, we showed that the granzyme B production was primarily provided by NK T cells: A higher granzyme B production of CD56^+CD57^+ cells was found when compared to CD56^CD57^+, but this difference between the two cell subsets was not statistically significant.

(ii) To summarize, we retrospectively compared granzyme B expression in PBMC cells taken from 35 breast cancer patients, focusing on EBV status. Patients who relapsed exhibited a lower granzyme B production than those who did not, though this difference was not statistically significant. When comparing other patient groups according to EBV status, we found that the granzyme B production profile by NK T cells followed a very similar trend to the one we obtained when exploring non-specific immunity.

In a more general sense, our laboratory is now interested in the effects of EBV (B95-8 lytic cycle proteins) on antigen-presenting cells (APC). Interestingly, we have demonstrated that EBV from the B95-8 cells (high copy number) and generally lytic cycle proteins (ZEBRA, BGLF5, BALF2) drastically reduce the expression of HLA-DR, whereas B95-8 cells (low copy number) increase this expression.

Two articles are currently being written addressing this topic.

Yours sincerely,