Author's response to reviews

Title: Characterization of Beta2-Microglobulin Expression in Different Types of Breast Cancer

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Author's response to reviews: see over
Dear Dr. Remoto:

On behalf of my co-authors, we thank you very much for giving us an opportunity to revise our manuscript, and appreciate reviewers very much for their positive and constructive comments and suggestions on our manuscript entitled “Characterization of β2-Microglobulin Expression in Different Types of Breast Cancer” (ID: 2073262619133320). Those comments are all valuable and very helpful for revising and improving our paper, as well as the important guiding significance to our researches. We have studied reviewer’s comments carefully and have made correction which we hope meet with approval. Revised portion are marked in red in the paper. The main corrections in the paper and the responds to the reviewer’s comments are as follows:

Reviewer 1:

Minor points:

1. It needs to confirm and correct the sample numbers of breast cancer patients in Tables.

   We are very sorry for our negligence of “Tables”. Columns of “Age (years)” in Table 1 (page 23 line 474) and “Basal-like” in Table 4 (page 24 line 480) were corrected.

2. It needs to add the PCR primer sequences and sequences for β2-M siRNA.
The PCR primer sequences were listed in Table 2 (page 23 line 476). As Reviewer suggested that β2-M siRNA sequences were listed in Table 3 (page 23 line 478).

3. In order to avoid confusion, “breast benign tumors” should be replaced with “benign breast tumors”.

   Considering the Reviewer’s suggestion, “breast benign tumors” was corrected as “benign breast tumors” (page 6 line 108 and 110, page 12 line 240 and 244, page 14 line 293, page 23 line 474 (Table 1), page 25 line 485 (Table 6)).

4. “liquid tumors” also called blood cancer, which include leukemia and lymphoma, in this MS, it is best to replace “liquid tumors” with “leukemia”.

   As Reviewer suggested that “liquid tumors” was replaced with “leukemia” (page 3 line 43, page 5 line 86 and 93).

5. There are some spelling mistakes need to be corrected, for examples:

   “B cell lymphoma/leukemia-2 (Bcl-2)” should be B-Cell Lymphoma/Leukemia 2 (Bcl-2)” in Abstract, “straining” should be “staining” on Page 8 (two places) and in Table 1 (one place), and so on.

   We are very sorry for our incorrect writing “B cell
lymphoma/leukemia-2 (Bcl-2)” and “straining”. “B cell lymphoma/leukemia-2 (Bcl-2)” was corrected as “B-Cell Lymphoma/Leukemia 2” (page 3 line 47, page 18 line 367). “straining” was corrected as “staining” (page 8 line 163, page 9 line 169, page 23 line 474 (Table 1)).

Special thanks to you for your good comments.

Reviewer 2:

Major Compulsory Revisions

The authors Kesheng Li et.al. identify expression of a well-known housekeeping gene, β2-microglobulin in breast cancer molecular subtypes. They show differential protein expression but not gene expression of β2-M in the different subtypes. However, the expression has no correlation with patients’ clinico-pathological parameters. The study has several limitations.

We thank the second reviewer’s critical comments. We have done our utmost in improving the manuscript and trying to make it as near perfect as possible. We hope that the revised manuscript is suitable for publication in the journal.

1. It is stated in the abstract that the purpose of the study was to investigate the mechanism of β2-M action in breast cancer. However, the authors have not done any functional analysis to show the
mechanism of β2-M action which is warranted.

We appreciate the reviewer’s correction, the purpose of the study is to characterize β2-M expression in breast cancer molecular subtypes, thereby investigating the mechanism of β2-M action in breast cancer. These have been discussed in the Discussion (page 16 line 340-343, page 17 line 344-353, and page 17 line 355-359).

2. Conclusion in the abstract is usually drawn from the results of the study which is ambiguously stated.

We have made correction according to the Reviewer’s comments. Our study indicated that: (1) the expression of β2-M had a significant difference in four breast cancer molecular subtypes, and had a negative correlation with ER protein expression and positive correlation with p53; (2) β2-M siRNAs have different silencing effects in the different breast cancer molecular subtypes; it significantly inhibited Bcl-2 mRNA expression and did not inhibit the ER, PR and HER-2 mRNA expression in MCF-7 cells (ER+, PR+ and HER-2-); however, there was significant up-regulation in the Bcl-2 and HER-2 mRNA expression levels in MDA-MB-231 cells (ER-, PR- and HER-2-), which is also consistent with the silencing effect at the protein level. These results indicate that the mechanism of β2-M action was different in breast cancer molecular subtypes, and the
β2-M may be concerned with apoptosis signaling pathways of breast cancer. Therefore, the “Conclusion” in Abstract was replaced by following, “β2-M expression demonstrated a significant difference in the four breast cancer molecular subtypes, and may be related to apoptosis regulation in breast cancer.” (page 4 line 73-74).

“Conclusion” in the manuscript was revised as following: “The expression of β2-M is significantly different in four breast cancer molecular subtypes, and the β2-M siRNAs have different silencing effects in the different breast cancer molecular subtypes. β2-M may be involved in apoptosis regulation of breast cancer, and understanding the regulation of the β2-M signaling pathways will help to identify new targets for the treatment of patients with breast cancer.” (page 17 line 361-365).

3. Primer sequences in the methods section for real-time PCR have not been verified. Usually the sequences are verified using BLAST or BLAT after they are designed. Surprisingly, the β2-M primers sequences that the authors have designed do not belong to β2-M.

Primer sequences of real-time PCR had been verified. We are very sorry for our incorrect writing the β2-M primers sequences. Table 2 (Page 23 Line 476), the “Forward primer” of β2-M was corrected as “5’-CGGGCATTCCTGAAGCTGA-3’”, the “Reverse primer” of
β2-M was corrected as “5’-GGATGGATGAAACCCAGACACATAG-3”.

4. The authors have not mentioned the purpose of using p53 and ki-67 antibodies for their experiments.

We thank the second reviewer’s critical comments. The p53 is an important anti-oncogene, that may induce tumor cells apoptosis via breaking combining of Bak/Bcl-2. The p53 mutant gene may has anti-apoptosis function and promote proliferation of tumor. The Ki-67 protein is a cellular marker for proliferation. The purpose of detecting p53 and Ki-67 protein in the study is to confirm the interrelation of β2-M and p53 protein, and that of β2-M and Ki-67 protein, thereby investigating whether the β2-M is concerned with apoptosis of breast cancer or it is directly concerned with proliferation of breast cancer.

5. In the Result section (silencing effect), it was mentioned that 3 siRNAs were used. However, the authors did not elaborate on the three siRNAs and the rationale behind using three. Why only siR-3 showed significant downstream effect?

We are very sorry for our negligence of the three siRNAs, their sequences was added in Table 3 (Page 23 Line 478). In addition, “All siRNAs are detailed in Table 3.” was added (Page 9 Line 178-179).
Three siRNAs targeting different regions of β2-M mRNA were designed and purchased from Commercial Company, and Scrambled siRNA that does not target any gene was used as the negative control siRNA. The results showed that all three siRNAs showed a significant silencing effect (P<0.01) and knocked down 80 to 98% of β2-M mRNA in comparison with the scrambled siRNA. Among the β2-M siRNAs tested, only the siR-3 siRNA showed a significant effect on downstream genes and therefore this siRNA was selected for silencing the β2-M gene. We can not explain the silencing mechanism of three siRNAs and why only the siR-3 siRNA showed a significant effect on downstream genes. That question need further study.

6. What is the clinical significance of β2-M expression in this study?
   Was there any correlation with overall survival and treatment status of the patients?

β2-M has been demonstrated as a growth factor and signaling molecule in breast cancer and liquid cancers. The levels of serum β2-M have become one of the most important prognostic factors and predictors of survival in patients with some tumors including breast cancer. The aim of this study is to characterize β2-M expression in the different breast cancer molecular subtypes, thereby investigating whether β2-M is involved with apoptosis regulation in breast cancer. The results of this study will be useful in confirming β2-M-mediated
signaling as a new target for breast cancer therapy.

7. The results have not been adequately discussed. The only reason suggested was that β2-M might be regulated by different signaling pathways. Which pathways and how are they regulated? There is repetition of sentences in the discussion. The study did not provide enough information about these. If the title states “characterization of β2-M-microglobulin…”, I think clinical significance and functional analysis would make it a complete study.

We have made correction according to the Reviewer’s comments.

The discussion has been revised. In our study the results showed:

(1) the expression of β2-M protein have shown significant differences in the four breast cancer molecular subtypes, and have significant differences between ER⁺ and ER⁻ breast cancer groups, but no significant difference between HER-2⁺ and HER-2⁻ groups;

(2) the β2-M protein expression has a negative correlation with ER protein expression, a positive correlation with p53 protein and no correlation with Ki67 protein. According to the aforementioned results, we deduced that the expression of the β2-M protein may be regulated by other signaling pathways beside the ER signaling pathway; the mechanism of this regulation needs to be further defined.

In discussion the repeated sentences “β2-M siRNAs have different
silencing effects in the different molecular subtypes of breast cancer cells; it significantly inhibited the Bcl-2 mRNA expression, but did not inhibit the ER, PR and HER-2 mRNA expression in breast cancer cells with ER$^+$, PR$^+$ and HER-2$^-$ status. In contrast, it significantly up-regulated the Bcl-2 and HER-2 mRNA expression in breast cancer cells with ER$^-$, PR$^-$ and HER-2$^-$ status.” were replaced by “β2-M siRNAs significantly inhibited Bcl-2 mRNA expression, but did not inhibit ER, PR and HER-2 mRNA expression in breast cancer cells with ER$^+$, PR$^+$ and HER-2$^-$ status. In contrast, there was significant up-regulation in Bcl-2 and HER-2 mRNA expression levels in breast cancer cells with ER$^-$, PR$^-$ and HER-2$^-$ status” (Page 16 Line 323-327).

The discussion about β2-M being concerned with apoptosis signaling pathways of breast cancer was renewed. “β2-M may accelerate human renal cell carcinoma cell growth via activation of PI3K/Akt and ERK, and induce phosphorylation of the Bcl-xL/Bcl-2-associated death promoter (Bad). The β2-M antibody may induce the human renal cell carcinoma cells apoptosis by inhibiting the phosphorylation of Akt and ERK, and activating JNK, resulting in the phosphorylation of Bcl-2 and decreased phosphorylation of Bad, leading to apoptosis [22]. Thereby, we deduced that β2-M may resist apoptosis by activating
PI3K/Akt and ERK. Moreover, the β2-M siRNAs inhibited Bcl-2 mRNA expression by inhibiting the phosphorylation of Akt and ERK in breast cancer cells with ER overexpression. HER-2 may increase the antiapoptotic proteins survivin and Bcl-2 via activating the ERK and PI3K signaling pathways [23]. Accordingly, β2-M may promote apoptosis by inhibiting HER-2 expression, resulting in inhibition of PI3K/Akt and ERK signaling pathways. In addition, β2-M siRNAs may up-regulate the Bcl-2 mRNA expression via increasing HER-2 expression in breast cancer cells with ER−, PR− and HER-2− status.” was added (Page 16 Line 340-343, Page 17 Line 344-353). “Briefly, the results of this study indicate that expression of β2-M is significant differences in four breast cancer molecular subtypes, which may lead to different functions of apoptosis regulation in breast cancer. These results will also be useful to understanding β2-M signaling pathways regulation, and help to identify new targets for the treatment of breast cancer patients.” was added (Page 17 Line 355-359).

Minor Essential Revisions

1. The quality of English could be better. The manuscript was not spell checked. The authors confused between “straining” and “staining” a number of times. In the abstract methods section “lewkmia” should
have been spell checked.

As Reviewer suggested that we confused between “straining” and “staining” a number of times. “strained” was corrected as “stained” (Page 8 Line 163), “straining” was corrected as “staining” (Page 9 Line 169, Page 23 Line 474 (Table 1)). In the abstract methods section “lewkmia” was corrected as “leukemia” (Page 3 Line 43 and 47).

2. Figure 5 could be avoided as it does not add any value to the manuscript.

The Figure 5 (It should be Table 5) showed the association of β2-M transcripts expression with Bcl-2 transcript expression. The statistical result showed that 2-M transcript expression has a positive correlation with Bcl-2 transcript expression. Consequently, the overexpression of β2-M transcripts may up regulate the Bcl-2 transcripts in breast cancer, and restrain apoptosis of breast cancer cells. Therefore, the figure 5 was a necessary figure in the manuscript.

3. Statistical analyses were performed with an older version of SPSS 11.5 while IBM-SPSS version 22 is mostly used nowadays.

The data has been statistically analysed by IBM-SPSS version 22 in the manuscript.
Special thanks to you for your good comments.

Other changes:

1. Page 6 Line 111, “(FFPE)” was added.

2. Page 18 Line 370, “FFPE, formalin-fixed, paraffin-embedded;” was added.

3. Page 18 Line 374, “Bad, Bcl-xL/Bcl-2-associated death promoter” was added.

4. Page 22 Line 456 and 459, references [22] and [23] were added.

5. The statements of “molecular subtypes of breast cancer” were corrected as “breast cancer molecular subtypes” in the manuscript.

6. The statements of “groups of breast cancer” were corrected as “breast cancer groups” in the manuscript.

We tried our best to improve the manuscript and made some changes in the manuscript. These changes will not influence the content and framework of the paper.

We would like to express our great appreciation to you and reviewers for comments on our paper, and hope that the correction will meet with approval.
We look forward to hearing from you at your earliest convenience.

Yours sincerely,

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