Author's response to reviews

Title: Ki67 expression and the effect of primary systemic chemotherapy on luminal breast cancer -a new concept for determining an institution-specific Ki67 cut-off value-

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Author's response to reviews: see over
Dear Editors,

We are submitting a revised version of our manuscript, originally entitled *Ki67 expression and the effect of primary systemic chemotherapy on luminal breast cancer - a new concept for determining an institution-specific Ki67 cut-off value*. We changed the title of the manuscript in accordance with the changes in the contents and added a co-author who helped with the extensive revision.

We deeply appreciate the reviewers for their comments based on careful reading, which we found to be most constructive and helpful. As suggested, to minimize any confusion for readers, we decided to radically change the composition of the manuscript using exactly the same data but with less speculative discussion. As revised in the Introduction and Discussion, we completely deleted the "hypothesis" regarding our effort to distinguish between luminal A and B, focusing instead on our investigation of a Ki67 cut-off value for identifying a subpopulation within luminal HER2-negative breast cancer cases that might benefit from chemotherapy.

Our specific responses are detailed below. Reviewers’ comments are in **bold type**. We sincerely apologize for any inconvenience due to the extensive changes made in presenting the contents of our study. Also, please forgive minor corrections in the text following these major revisions. All revised portions are in blue text.

**Reviewer1:**

#1) INTRODUCTION: While I agree, that different cut-off levels for Ki-67 should be explored, the rationale presented in the introduction does not seem to meet the clinical need. There might be different cut-off levels for several therapies, while the cut-off of 14% is based purely on biological behavior. The introduction could be adjusted concerning that.

We totally agreed with the reviewer’s suggestion. We have now revised the Introduction and Discussion and completely removed the “hypothesis” part from this manuscript including the former Figure 1, which caused confusion and made the manuscript difficult to understand. A Ki67 cut-off value distinguishing between luminal A-like and B-like is based on biological features, as the reviewer pointed out. Thus, we have now focused on seeking a Ki67 cut-off value to predict pCR cases since the real clinical need is to find a subpopulation that would benefit from chemotherapy.

#2) INTRODUCTION/METHODS: As only immunohistochemistry was used to determine the molecular subtype it might be necessary to point out, that you mean the approximation of molecular subtype when you mention luminal (e.g. luminal like...) This is of importance as a high Ki-67 could be indicative of a basal breast cancer with positive hormone receptor status thus flawing the hypothesis of this paper.

We tried to avoid the terms ‘luminal A’ and ‘luminal B’ as much as possible in the revision. If necessary, we used the term, ‘luminal-like’. We have taken the reviewer’s advice and revised the Background and purposes. It now reads: “According to biological differences, hormone receptor positive breast cancer is categorized into two groups: luminal A-like and B-like tumours with good and poor prognoses, respectively. These two groups can be approximately distinguished based on low and high expressions of Ki67.”

#3) METHODS: While I support the idea, that Ki-67 should have different cut-off for different phenotypes, it is difficult to understand, why the cut-off for the differentiation between luminal A like and luminal B like tumors should be determined by the responsiveness to chemotherapy. There can be different cut-offs for all kind of phenotypes. Additionally the definition of luminal brings some more problems concerning your hypothesis (see comment #2)

We totally agree with the reviewer’s comments. Please see the response to #1.
#4) METHODS: The hypothesis looks like being positioned at the 50% probability in the logistic regression model to predict a pCR. This must not necessarily implicate the best cut-off for actually predicting a pCR.
   We removed the hypothesis itself.
   (See also the response to #1)

#5) METHODS: How was ER positivity and how was HER2 positivity defined?
   ER and PR were judged to be positive when more than 10% of the nuclei of cancer cells were stained. HER2 was judged to be positive if strong staining of the complete cell membrane in more than 10% of tumour cells was observed, or HER2/neu gene amplification was confirmed by fluorescence in situ hybridisation. These definitions were originally included in the Matherials and Methods and we have now added them as explanations in the footnotes of Tables 1 and 2.

#6) METHODS: While the hypothesis is described in detail, the statistical section is missing the information, how this aim is reached.
   We have removed the “hypothesis” from the manuscript and the statistical analysis part in the Materials and Methods was revised with the provision of more detailed information.
   (See response to #8)

#7) RESULTS: Please numerate the tables in the text.
   We have now numbered both tables in the text.

#8) RESULTS: Now it seems as if ROC was used to determine the predictive value of Ki-67. The methods section should clearly describe, what was aimed at and how it was achieved.
   We have now revised, with more detailed information, the ‘statistical analysis’ part in the Materials and Methods and the ‘Ki67 cut-off value’ section in the Results. We removed the Youden index which can confuse readers and analysed the data employing the distance to the perfect classification. This method has been widely used and is highly versatile. We concluded “35%” to be optimal Ki67 cut-off value for distinguishing pCR from non-pCR cases among our 114 patients.

#9) RESULTS: Please consider exact wording. The DFS can be higher or lower but a median progression free survival can be longer or shorter.
   We thank the reviewer for this advice and have now revised this expression in the Results and the legend of Figure 2. It now reads: “the pCR group had higher rates of DFS and OS than the non-pCR group”.
   Also, we have added newly written parts to the revised Results in the abstract and the Discussion, i.e. ‘lower’ was used for DFS, in accordance with the reviewer’s advice.

#10) DISCUSSION: There seem to be original data, which are presented in the discussion. Why not present them right away in the results section.
   We have now moved these data into the Results section with Table 2.

#11) DISCUSSION: It still does not become clear, why a cut off for Ki-67 can differentiate between luminal A and luminal B tumors.
   Please see the response to #1

Summarized there are several concerns with this work. The hypothesis, that a cut-off for a response to chemotherapy might differentiate between luminal A and B tumors is somewhat difficult to understand. There are completely different classification strategies for both phenotypes. However I think the study data deserves presentation, as this data is needed for current treatment strategy developments. There is a study of ours with a larger sample size in
luminal tumors, that is not referred to in this study, and which might be worth mentioning (Fasching et al. BMC Cancer 2011)

We appreciate the reviewer’s suggestion and have now referred to this issue in the Discussion as reference no.20.

We believe that this study using Japanese patient samples can contribute to enhancing the value of Ki67 expression for clinical use, although the contents of our manuscript do indeed follow from the previous pioneering work of Fasching et al., and our sample size is smaller than theirs.

Reviewer2:
Major: The definition of cut point values for Ki67 is very important. However, the authors should validate their cut point in an independent neoadjuvant cohort, otherwise it is not clear if the selected cut point is the result of overfitting in this moderately sized cohort.

To validate our identified Ki67 cut-off value, we recruited an additional 196 patients in this study. These included all luminal HER2-negative tumour patients during the prior 18-month period, regardless of whether or not they had received neo-adjuvant or adjuvant chemotherapy. We acknowledged that the Ki67 cut-off value should be assessed in a larger cohort with a longer observation period. Unfortunately, the median 29-month follow-up period was probably not long enough but this is all that data that we could collect and show at present.

Also, we changed the definition of the Ki67 cut-off value in this revision as mentioned above. We thus believe the current study population to be acceptable for the aim of this study.

Furthermore it would be important to include data on long term outcome of these patients in larger cohorts. In the analysis that is shown in the manuscript, there is no evidence of a significant relation between Ki67 and patient survival. This might be critical for clinical utility.

During the median 58-month follow-up period, there was no significant relationship between any Ki67 cut-off value and outcomes in the 114 patients who all received neo-adjuvant chemotherapy, as the reviewer pointed out. However, we observed that DFS was significantly lower for tumours with Ki67 higher than 35% in the 196 patients of the other dataset, recruited for validation analysis. Thus, we speculate that a certain fraction of the 114 patients with high Ki67 expression were salvaged by neo-adjuvant chemotherapy and thus remained recurrence-free and this is why no relationship between Ki67 expression and patient survival was seen in our 114 patients.

Minor: It would be interesting to provide some more details on the method used for Ki67 evaluation.

We accepted the reviewer’s suggestion and have revised the Materials and Methods section accordingly. It now reads: “Cells positive for nuclear Ki67 were counted in at least 500 cancer cells in one hot spot on each of the biopsy specimens.”

We thank you very much in advance for your consideration.

Yours sincerely,

Yoshiya Horimoto