Author's response to reviews

Title:Surgery Time Interval and Molecular Subtype May Influence Ki67 Change after Core Needle Biopsy in Breast Cancer Patients

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Version:5 Date:29 March 2015

Author's response to reviews: see over
Dear Editor and Reviewers,

Thank you for your review and helpful suggestions. We have revised our manuscript according to reviewers’ comments and give a point-by-point response to the concerns as follows:

Response to Editor’s Comments:

Q1: Given the narrow range of the surgery time interval, the question is how well the conclusions are resting on solid grounds. To address this I suggest further analysis based on alternative categorization, as already suggested by one of the reviewers. If possible, 5 groups of approximately the same size as the first category (1-2 days, n=55) could be used. If the conclusions are not affected, the new results could be presented as supplementary.

A1: Ok, we have re-categorized surgery time interval into 5 groups according to your suggestion and listed as a supplementary figure 1: 1-2 days (n=55), 3 days (n = 50), 4 days (n = 63), 5 days (n = 52), and ≥ 6 days (n = 56). ANOVA analysis showed that Ki67 change after CNB was significantly associated with surgical time interval (P = 0.01). Compared with patients of STI 1-2 days, 5 days and ≥ 6 days STI were both associated with higher Ki67 increase after CNB, with P value 0.022 and 0.003, respectively. (Lines 228-231)

Q2: The fixation intervals ranged from 6 to 24 hours for CNB and from 6 to 48 hours for SRS samples. Is the percentage of Ki67-positive cells affected by fixation time within these intervals?

A2: A good question. We have no data to show whether there was an association between Ki67 change after CNB and fixation time difference. We have added this point in the discussion session. (Lines 277-279)

Q3: Line 192: The numbers should be revised according to the table.

A3: Thank you, we have corrected it!
Q4: Table 5, Luminal A: The Ki67 values for CNB are exactly the same as before the refined definition of the subtype. Are the values correct?

A4: Thank you. We have calculated and the values are correct.

Q5: Copyediting:

A5: We have received help from David, Garfield, who is a native English-speaking professor of medical oncologist, to improve our written English for this paper.

Response to Dorthe Grabau’s Comments:

Q1. The present work is largely a copy of a previous article (Chen et al. BMC Cancer 2013, 13:390) from the same group. A reference to the previous article is made to details of selection of patients and immunohistochemical procedures but some differences are not explained.

The hospital where the patients are treated and the enrolment period is exactly equal but the number of patients differ with 298 patients in Chen et al. BMC Cancer 2013, 13:390 and only 276 in the present work. The reason to exclude some patients is not commented.

Answer 1: Yes, thank you for your suggestion. We excluded 22 patients in this study due to their surgery time intervals more than 60 days. We have added this criterion in text: “Twenty-two patients with STI more than 60 days were further exclude in current study.” (Lines 132-133)

Q2. Breast cancer molecular subtype classification second paragraph.

2-a: ……However the 2013 StGallen guideline use cut-off of 20% of PgR to distinguish between Luminal A and B in low-proliferative patients and the present study use 1% to define PR positive patients which means, even though claimed so, the study do not present StGallen 2013 subtypes……. The data presented in Table 1 also appears in tables in the article by Chen et al. BMC Cancer 2013, 13:390 except for the above mentioned change in the number of patients and inclusion of ER+/PR-/Ki67<20% in the Luminal B group instead of the Luminal A group.

Answer 2-a: Yes, our previous criteria for molecular subtype classification were not
considered the 20% PR cutoff value for Luminal B disease. We have re-classified molecular subtype according to your suggestion: Luminal A (ER+/HER2-; Ki67<20% and PR ≥20%), Luminal B-HER2- (ER+/HER2-; Ki67≥20% or ER+/HER2-, PR<20%, or ER-/PR+/HER2-). In addition, the number of Luminal A and Luminal B-HER2-patients were changed with this new definition, please find new results with this new definition in the “Results” part. Importantly, molecular subtype and STI were still associated with Ki67 change after CNB.

*(Lines 166-167, and Tables 1, 3 and 5)*

**2-b:** The present study is expanded with time between core needle biopsy and surgery compared with the publication by Chen et al. BMC Cancer 2013, 13:390. However the mean time between core needle biopsy and surgery was 4.5 days with 55 patients receiving surgery 1-2 days after core needle biopsy, 115 patients receiving surgery within 3-4 days and 108 patients waiting more than 4 days. (The sum of the number of patients in the text is 278 and not 276, the table is correct).

**Answer 2-b:** Thank you for your correction. We have revised 115 to 113 in the text.

*(Lines 197)*

**2-c:** In the discussion is mentioned that the time between core needle biopsy and surgery is very short compared with routine procedure in most institutions but the authors do not explain why the interval is so short in their own institution. In everyday practice it will be impossible to have the results including immunohistochemistry and eventually FISH for HER2 of the core needle biopsies, decide the recommended treatment, inform the patient and get her acceptance of the treatment and plan and do the surgery within one day.

Results of time between core needle biopsy and surgery where only 39% have an interval of more than 4 days is not interesting or useful for routine practice in our part of the world. If readers miss the short time interval and extrapolate the results to their own patients, the conclusions might even be misleading.

**Answer 2-c:** Yes, thanks a lot for your great advice. We agree with you that IHC and FISH take much longer time than our cohort's average STI period. However, in our
center, we firstly did hematoxylin and eosin stain diagnosis for tumor within 24 hours after biopsy in the in-patient ward, and further surgery was arranged for majority patients when invasive breast cancer was presented. ER, PR and HER2 status analysis was not mandatory before surgery. We should be caution to interpret our result on the basis of this short time interval. For other centers with longer period after biopsy, which IHC or FISH analysis was done before surgery, we need to know this STI difference and to be careful to explore our results to their patients. We have added this explanation in the discussion section.

*(Lines 324-328)*

**Q3.** However your results might be interesting for institutions using the same routine procedures as you do. So in order to get your results published a careful and meticulous description of your routine practice will be necessary. Also you must find the time between core needle biopsy and surgery for the missing 22 patients or at least explain why the patients are excluded. Consigning the StGallen subgroups you can either choose to use the 2011 data and refer to your article Chen et al. BMC Cancer 2013, 13:390 or you can re-score the PR receptor and use the 2013 subgroups.

**Answer 3:** Ok, we have described our routine practice (see “Answer 2-c”) and missing 22 patients (see “Answer 1”) according to your advice. The 2013 St.Gallen subgroup classification criteria were used with PR 20% positivity cutoff value for further calculation (see “Answer 2-a”).

**Response to Linda Sofie Lindström’s Comments:**

**Q1.** Patient cohort definition

The patient inclusion is unclear. “Consecutive breast cancer patients with paired CNB samples and SRS.”

**1-a:** When were the patients diagnosed and which patients had paired samples (unclear even after reading your previous article in BMC Cancer 2013)?
**Answer 1-a:** Ok, we have mentioned this in the revised manuscript as “Consecutive breast cancer patients who received CNB and following surgery in Ruijin Hospital, Shanghai Jiaotong University School of Medicine between Oct. 2009 and Feb. 2012 were retrospectively analyzed. All enrolled patients need to have paired CNB and SRS samples.”

*(Lines 122-124)*

**1-b:** Were the samples taken because of study enrolment including selected patients (and tumor characteristics) or was this done for most patients at that time? The tumor characteristics (Table 1) points at more aggressive breast cancer disease than in a general adjuvant breast cancer cohort. This needs to be described in detail in the Patient and Methods section.

**Answer 1-b:** I agree with your point that most patients received CNB are with relatively large tumor than general adjuvant population, and whether patients would receive CNB was decided by surgeon’s choice. We have added this point as “Patients with large tumor were likely to receive CNB by surgeon’s choice.”

*(Lines 125-126)*

**1-c:** It’s unclear to the reader what analysis/analyses were done retrospectively. If the immunohistochemical analyses for ER/PR/HER2 and Ki67 were redone by you, please clarify this along with the year(s) this was done and other relevant details (including antibodies used etc). Helpful for the reader to have all the information about the marker assessment in the same paragraph.

**Answer 1-c:** IHC analysis for ER, PR, HER2, and Ki67 was done prospectively, which was scored by the pathologist. We retrospectively collected data and two pathologists were re-scored ER, PR, HER2 and Ki67 for this analysis. All methods for IHC analysis information were described in our previous report and we have added our previous paper citation in the new manuscript.

*(Lines 131)*

**1-d:** Did time to surgery depend on initial core biopsy tumor characteristic or patient
characteristics? It would be interesting to see a table with time to surgery categorized versus initial (core biopsy) tumor characteristics as well as patient characteristics (as found in Table 1).

**Answer 1-d:** OK, we have added a new table (table 2) regarding surgery time interval and patient as well as tumor characteristics.  
*(Lines 198-200, and Table 2)*

**1-e:** Age selection? Percentage of women below 45 or 40? Helpful with an age cutoff at 40 or 45 in the table to understand the age distribution in the patient cohort.

**Answer 1-e:** We have re-categorized age with your recommendation and set cutoff age as 40 for young age group, please find this revision in Table 1.  
*(Tables 1)*

**Q2.** Time to surgery.

Most patients were operated within a few days. However, a smaller proportion of the patients waited much longer (up to 37 days). It’s important to understand how the surgery interval is associated with proliferation change especially for the patients with a longer time to surgery. Are patients with long time to surgery impacting the results the most?

**Answer Q2:** Thank you for your advice. We agree that we need more patients with longer surgery time interval to find out whether long time to surgery would impact the Ki67 change. However, there are only 10 patients with STI more than 10 days, and it is hard for us to find out this point in our cohort of patients. Moreover, Gandini (ref. 13) reported that Ki67 change after CNB was more obvious in TN or HER2+ breast cancer with median STI 41 days, but that there was no significant Ki67 increase in the luminal subtypes. We have mentioned this in “discussion” part and listed this as our work’s limitation as follows: We have not categorized patients into another group with longer STI because there were only 10 patients with STI of more than 10 days. This made our study unable to answer whether Ki67 change would be decreased in specific subtypes with a prolonged surgery waiting period.  
*(Lines 302-306; 331-335)*
2-a: How were the categories for the time to surgery defined?
The categories should either be chosen due to a general accepted definition (i.e. most
studies categorize in this way or the categorization is biologically reasonable), or
alternatively a statistical definition (generally from the distribution).
A clear explanation and reasoning behind the current categorisation of time to surgery is
needed, alternatively if this is not clear my recommendation is to re-categorize the time to
surgery from a statistically reasonable point of view (using the distribution of time to
surgery categorizing by quartiles for example).

Answer 2-a: In our study, we classified STI according to its distribution. The first,
second, and third quartile days of STI in our cohort were 3, 4, and 5 days. So, we
categorized STI as follows: less than 3 days, 3-4 days, and more than 4 days. We have
revised our manuscript and added this point in the text as: “Ten patients had STI more
than 10 days. The first, second, and third quartile days of STI in our cohort were 3, 4, and
5 days, respectively. Then, we categorized STI with following groups: less than 3 days
(55 patients), 3-4 days (113 patients), and more than 4 days (108 patients) ”.

(Lines 194-198)

3-b: First, to understand the distribution of time to surgery and proliferation, it would be
interesting to see a graph of the included patients’ tumor proliferation (as a continuous
variable of Ki-67 percentage) at core biopsy and at surgery (as two different graphs)
versus patients’ time to surgery (as a continuous variable), i.e. time to surgery as X and
proliferation as Y.

Answer 2-b: Thank you, we have added a new figure according to your suggestion as
figure 1A (Ki67 at CNB vs. STI) and 1B (Ki67 at SRS vs. STI) in the text.

(Lines 200-202, Figures 1A and 1B)

3-c: Second, it would be interesting to see a graph of the included patients’ tumor
proliferation change between core biopsy and surgery (as a continuous variable of Ki-67
percentage) versus patients’ time to surgery (as a continuous variable) i.e. time to surgery
as X and proliferation as Y.

Answer 2-c: Thank you, we have added this figure as figure 1C (Ki67 change after CNB
Q3: Ki-67.

Given the large variability of Ki-67 it’s vital for the authors to describe the details of their assessment of Ki-67. It should be clear to the reader how the core biopsy/surgery specimen was handled for the complete process including handling (time to fixate and how the specimen was stored contrasting core biopsy and surgery specimen), antibody/protocol used and how the pathologist scoring was done (for instance number of cells scored for the core biopsy versus the surgery specimen including whether hot spots were considered and if so what strategy was chosen). In addition, the core biopsy selection (representative regions or hot spots) is very important, especially when investigating proliferation change as done in this work, and should therefore be described.

Answer Q3: Yes, we do agree with you that sample handle and scoring were very important for Ki67 scoring, and we added these points in the text. For samples dealing process, CNB and SRS samples were fixed in 10% neutral buffered formalin within 30 minutes after tumor removal, and fixation intervals ranged from at least 6 hours to 24 hours for CNB samples and at least 6 hours to 48 hours for SRS samples. For Ki67 expression scoring, we used the same method for calculating CNB and SRS samples. Cell distribution over the whole slice was firstly reviewed and 500-2000 cells were chosen from different microscope views if the Ki67 expression distribution was uniform. Otherwise, 2000 cells were equally counted in both hotspot and negative areas in slice. Ki67 expression was scored as the percentage of positive invasive tumor cells with any nuclear staining and recorded as mean percentage of positive cells.

Q4. Statistical methods need to be revised and clarified.

The statistical methods need to be clarified, tests (why used and in what way helpful to answer the hypothesis) and assumptions described (normality assumption etc checked) and variable inclusion reasoned for.

It’s very important for the reader to be able to follow between the Methods section, the
Results section and the Tables. What finding was the result of which test and what does this mean.

For instance, the use of ANOVA/multivariate ANOVA/subgroup test need to be described together with why and what the results actually say about the data. What variables were included in the specific analyses and why?

**Answer Q4**: Thank you for your advice, we have revised our manuscript and describe the statistical methods used in results section and tables.

*(Lines 172-175, 206, 212, 219, 237, 239; Table 2, 3)*

Thanks again for your great advice and looking forward to your response.

Best Regards,
Xiaosong Chen
Kunwei Shen