Author's response to reviews

Title: Nucleostemin expression in invasive breast cancer

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Author's response to reviews: see over
Dear professor Solera,

We were pleased to learn that our manuscript (4072674271109233) could be reconsidered for publication pending major revisions. We have responded the reviewers’ comments, and have revised extensively the manuscript, table and figure as follows:

**Reviewer: Dr. Semir Vranic:**

**Major compulsory revisions:**
The authors explored nucleostemin, a novel stem cell marker, in a subset of breast cancer cases.
1. Did you run separately positive and negative controls for NS (apart from the positivity within the normal mammary epithelium)? It is not mentioned in Materials and Methods. How would you justify cytoplasmic expression of NS in tumor cells?

   **As a positive control, we separately run the assay by using a case of esophageal carcinoma (Nakajima et al. Cancer Sci. 103: 233-8, 2012). As negative controls, reactions without the primary antibodies were used.** We have added these sentences in the section of Methods on page 9, lines 13-15 in the revised manuscript. In our study, the expression of NS was only limited to the nuclei of the cells, and the cytoplasmic expression of NS was not seen in almost all cases. We have added the sentence that “cytoplasmic staining was not observed” in the section of Results on page 12, line 14 in the revised manuscript. That staining pattern was compatible with that reported in the previous studies (Yoshida et al, Cancer Sci. 102: 1418-23, 2011; Nakajima et al, Cancer Sci. 103: 233-8, 2012).

2. Why did did you use a 10% threshold for NS positivity? It is not supported by any reference. What was the mean (or median) NS positivity in breast tumors?

   What happens with your results when the data are dichotomized according to
the NS mean/median/ value?

Initially, the expression levels of NS were classified as negative (0%), weak (1% to <10%), moderate (10% to <30%), or strong (30% or more). The number of cases categorized into the negative, weak, moderate, and strong groups was 62, 16, 55, and 87, respectively. From these results, we judged that NS expression showed bimodal distribution and used a 10% threshold for NS positivity between negative and positive groups. We have added these sentences in the section of Results on page 12, lines 4-9 in the revised manuscript. Because we did not accurately count the percentage if positive cells, we cannot show mean or median values. Median or mean values should consist in 10% to <30%.

3. How would you explain higher NS expression in ER+ and HER2+ breast tumors and correlation with p53 given that triple-negative breast carcinomas typically exhibit TP53 abberations (followed by p53 expression)?

Currently, we cannot explain the correlation between NS expression and p53 expression. Although several studies have shown that NS modulates the expression of wild-type p53 (Ma et al, Mol Biol Cell. 18: 2630-35, 2007; Dai et al, Mol Biol Cell. 28: 4365-76, 2008), the role of NS in breast cancers with mutant p53 has not yet been evaluated. Further research is needed to elucidate the correlation. We have added these sentences in the section of Discussion on page 17, line 16 to 20 in the revised manuscript.

We already discuss the correlation between NS- and ER- or HER2- expression in the section of Discussion on page 18, line 4 to 11. However, we had not referred the survival impact of NS expression status on each subtypes, so have added the sentences in the section of Discussion on page 18, line 11 to 15, as follows; “We found no survival impact of the NS expression status among patients with triple-negative tumors, who show higher rates of mutated p53 than patients with luminal-type or HER2-type tumors. NS can function in the presence of wide-type p53; therefore, the expression status of NS may have survival impact only for the luminal-type and HER2-type tumors”.

4. Did you have any special histotype among 220 breast cancers you studied? If so, did you notice different NS expression in these cases?

According to the reviewer’s comment, we have added the results of correlation between NS expression and histological subtype in the revised Table 1 and have added the sentence that “NS expression was detected in 50% or more in any histological types except for tubular carcinoma (20%), and the positive rate was 100% (6 of 6) in mucinous carcinoma” in page 13, lines 4-7 in Results in the revised manuscript.
Minor essential revisions:
1. Proof-reading of the manuscript, preferably by a native English-speaking, is mandatory as some typo and grammatical errors are seen throughout the draft.

   According to reviewer’s comment, our revised manuscript has re-checked by a professional native English-speaking editor, qualified to PhD level and specializing in biomedical science.

2. Some statements are not supported by the appropriate references (e.g. the sentence on NS as a marker of „stemness”).

   According to reviewer’s comment, we have cited an article (novel reference #6) in order to support the statement of NS as a marker of “stemness” in page 5, line 3 in the revised manuscript.

3. Results section in the Abstract should begin with the classification of the tumors on the basis of ER, PR, and Her-2/neu.

   According to the reviewer’s comment, we have begun with the classification of the tumors on the basis of ER, PR and HER2 by moving the sentences “Among the 220 patients, 154 were hormone-receptor (HR)-positive, 22 HER2-positive/HR-negative, and 44 HR-negative/HER2-negative.” to the top of Results in Abstract in the revised version.

Reviewer: Dr. Caterina Marchio

The paper by Kobayashi and colleagues investigates Nucleostemin as a potential prognostic factors in breast cancer. The results are potentially interesting, since the prognostic relevance is maintained also in the multivariate analysis, however some points need clarification.

Major compulsory revisions:
- about the cohort: the analysis was conducted in a cohort of 220 carcinomas, but it is not clear whether this was a consecutive series or there had been a selection of cases. In addition, the patients have not been treated homogeneously, so this is a bias and should be mentioned in the discussion as a word of caution. Finally the ER, PR, HER2 were taken from records of a previous study, but it is important to clarify whether these markers were re-assessed or not in order to have a more homogeneous evaluation: what were the cut-offs for positivity for ER ad PR? The cases belong to the ’90, when HER2 was not an established routine as yet, therefore was HER2 performed on new sections?
Lastly, why ki-67 has not been performed?

We used a consecutive series. So, we have added the phrase "consecutive" in the Abstract on page 2, line 13, and in the section of Methods on page 7, line 5 in the revised manuscript.

The limitations of the present study included the retrospective analyses and the heterogeneity of adjuvant treatments. Therefore, one should pay careful attention when interpreting these results. Further studies using a uniformly treated patient cohort are required to clarify the role of NS in breast cancer stem cells. We have added these sentences in page 16, lines 20 to page 17, line 4 in Discussion in the revised manuscript.

ER, PgR, and HER2 were re-assessed on new sections in our previous study using standardized testing kits and methods. Therefore, we have replaced "analyzed" with "re-assessed on new sections" in page 10, line 4, and have added the phrases "according to the methods recommended by the manufacturer " in page 10, line 7 in Methods in the revised manuscript.

As the reviewer mentioned, Ki-67 is a promising prognostic factor. However, we think it is not established as yet. Actually, the threshold of Ki-67 high and low groups changes from year to year in St. Gallen consensus meeting. From here onwards, we did not include Ki-67 results in the present study.

- about the IHC of the marker: it is not 100% clear whether Nucleostemin is positive also in the normal tissue, the authors state in the introduction that nucleostemin not in the differentiated somatic cells of most adult tissues, however at some point in the results they state "Unremarkable mammary glands showed nuclear NS immunoreactivity in almost all luminal epithelial cells": are they referring to unremarkable CANCER mammary glands or to normal mammary gland? Figure 1D is the reference for this statement, however from the area they took it is not trivial to understand whether it is normal breast or cancer, as the illustrated structures are glandular but a bit distorted and the field is not very representative, it is difficult to figure out. Please clarify this point.

Was nucleostemin correlated in particular to any histological type?

According to the reviewer’s suggestion, we have removed the part "but not in the differentiated somatic cells of most adult tissues" from the section of Background in the previous manuscript in order to avoid misunderstanding. The statement in Results that “unremarkable mammary glands showed nuclear NS immunoreactivity in almost all luminal epithelial cells” is correct. The image presented as Figure 1D is non-neoplastic unremarkable mammary glands with uniform nuclei with two-layer structure of epithelial cells although the glands are a bit distorted. In order to avoid misleading, we have replaced Figure 1D into novel one that appears more representative of unremarkable mammary glands.
in the revised version.

According to the reviewer’s comments, we have added the results of relationship between NS expressions and histological types in the revised Table 1, and have added the sentences “NS expression was detected at 50% or more in all histological types studied except tubular carcinoma (20%), and the positive rate was 100% (6 of 6) in mucinous carcinoma” in page 13, lines 4-7 in the revised manuscript.

- about the prognostic relevance of nucleostemin: curiously, two strong prognostic factors have not been taken into account in this analysis, however I believe they shouldl, namely the proliferation index (as also mentioned above) and the histological grade. Correlations should be performed with these two parameters and the multivariate analysis run again.

We have described the reason why Ki-67 labeling index was not included in the present study in the reply to Major Compulsory Revisions as above.

We showed the results of relationship between nuclear grade and NS expression in Table 1. Nuclear grade, composed of nuclear atypia and mitotic counts, is a variant of histological grade and is one of the most powerful prognostic factor (Tsuda et al, Jpn J Clin Oncol. 28:486-91,1998). In Japan, this grading system is widely used in daily practice as well as in several prospective clinical trials (Watanabe et al. J Clin Oncol 27:1368-72,2009; Hozumi et al. Ann Oncol. 22:1777-82,2011), and it is considered that nuclear grade and histological grade are interchangeable. Therefore, in our study, we chose nuclear grade instead of histological grade.

Minor essential revisions:
- the authors inappropriately use some adjectives: for example "breast cancer is one of the most serious and prevalent diseases worldwide": serious disease is not very scientifically sound.

As the reviewer mentioned, "serious" disease does not sound very scientific. Therefore, we have removed this word from page 4, line 2 in Background in the previous version

- is the formatting of the manuscript in accordance with the Journal's guidelines? Please double check.

We would like to apologize for our format error, and have corrected the style of our manuscript in accordance with the guidelines of BMC Cancer.
**E-mail of Jan 31, 2014**

The authors addressed most of the comments of the previous reviewers. However, there are two points to modify before accept the paper for publication:

1. Page 15: Eight patients with HR-positive and HER2-positive tumors were included and analyzed as HER2-positive patients. This is not correct because these tumour in all classifications are considered luminal. So, I suggest that the authors include these cases among the luminal cases and redoing the statistical analysis.

   **According to the reviewer's comments, eight HR-positive and HER2-positive tumors were included and analyzed as luminal-type tumors, and we have changed main-text, figure 4, and figure legends.**

2. Please modify the conclusions for:

   In summary, our results indicate that the expression status of NS, abundant in stem cells, is a prognostic indicator in breast cancer patients, especially for those with HR-positive or HER2-positive tumors, and that the coexpression of NS and p53 correlates with poorer prognostic outcomes. Examination of NS expression may be useful for the stratification and management of breast cancer patients in future daily practice.

   **According to the reviewer's comment, we have modified Conclusions as above.**

In order to facilitate the editorial process and make our changes readily understandable, we are also enclosing another file of the manuscript in which the points of revision have been highlighted, and deletions have been indicated with strikethrough.

We thank the editor and the reviewers for their comments. We believe the contents of the revised manuscript have been much improved, and hope that the corrections we have made are satisfactory to you. If you feel any further amendment is needed, please let us know. We look forward to hearing from you soon.

Sincerely yours,

Hitoshi Tsuda,

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