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:

Replies to the comments of reviewer Dr. Semir Vranic:

Major Compulsory Revisions:

1. 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 10, 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 13, 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. 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 13, 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. 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 18, 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 19, 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 19, 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. 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 14, lines 4-7 in Results in the revised manuscript.
Minor Essential Revisions:
1. 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. 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 6, line 3 in the revised manuscript.
3. 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, 146 were hormone-receptor (HR)-positive/HER2-negative, 30 HER2-positive, and 44 HR-negative/HER2-negative” to the top of Results in Abstract in the revised version.

Replies to the comments of the reviewer, Dr. Caterina Marchio

Major Compulsory Revisions:
1. We used a consecutive series. So, we have added the phrase "consecutive" in the Abstract on page 3, line 11, and in the section of Methods on page 8, 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 17, lines 20 to page 18, 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 11, line 4, and have added the phrases "according to the methods recommended by the manufacturer " in page 11, 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.

2. 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 14, lines 4-7 in the revised manuscript.

3. We have described the reason why Ki-67 labeling index was not included in the present study in the reply to Major Compulsory Revisions 1 by the reviewer.

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:

1. As the reviewer mentioned, "serious" disease does not sound very scientific. Therefore, we have removed this word from page 5, line 2 in Background in the previous version.

2. 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.

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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