Title: Expression of nerve growth factor and heme oxygenase-1 predict poor survival of breast carcinoma patients

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Version: 2 Date: 16 October 2013

Author’s response to reviews: see over
Thank you for your letter of October 2, 2013, informing us that our manuscript ID# MS: 5874205961005971 “Expression of nerve growth factor and heme oxygenase-1 predict poor survival of breast carcinoma patients” by Noh et al. has been invited to submit a revised version of the manuscript.

We agree with all the issues raised by the reviewers and in response, we have introduced all the suggested changes and revisions. We thank the reviewers and the editor and feel that the comments and suggestions have significantly strengthened the paper. Enclosed, please find a point-by-point response to each of the concerns posed by the two reviewers.

Revisions introduced into the text are red-colored.

Thank you for your kind consideration.

Sincerely yours,

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**Response to reviewer 1**

We thank the reviewer for the insightful comments and for finding our study to be “well written.”

**Comments to the Author:**

**Reviewer’s report**

**Title:** Expression of nerve growth factor and heme oxygenase-1 predict poor survival of breast carcinoma patients

**Version:** 1 Date: 15 July 2013

**Reviewer:** Gary Tse

**Reviewer’s report:**

This is an interesting study on NGF and HO1 expression in a series of breast cancer.

We very thank the reviewer for this comment.

**Major Compulsory Revisions**

1. Please confirm the TM tissue core size is 3mm in diameter per core. It is well documented that intratumoral heterogeneity existed in breast cancer. As only a single core was selected from each case, there could be potential selection bias.

We thank the reviewer for this comment and agree with the reviewer. As the reviewer points out, breast cancer is heterogeneous and could differ according to the area sampled. In response to the reviewer’s comment, we established one additional tissue microarray (TMA). Thereafter, we did additional immunohistochemical staining for NGF and HO1. Furthermore, we analyzed two 3.0 mm tissue cores per case. To confirm the number and size of TMA cores, we revised the sentence below in METHODS section:

To establish the TMA, we reviewed all of H&E slides and took two 3.0 mm tissue cores from the paraffin-embedded tissue blocks per case at the area of highest tumor grade.

In addition, when we compared the expression status of NGF and HO1 between two TMA cores, 32 cases with NGF and 28 cases with HO1 immunostaining showed different results with a cut-off points of 5 for NGF and 7 for HO1 staining. The immunohistochemical staining score ranged from zero to eight and the same cut-off values were used in the two sets of TMAs. The precise scoring method is described in answer to the reviewer’s next comment. However, despite some difference of the expression of target markers between first and second TMA cores, there were no significant differences in the prognostic impacts of these markers for the breast cancer patients. Therefore, we thought that the evaluation of the expression of NGF and HO1 is helpful for the prediction of the prognosis of breast cancer patients despite the limited sample size and heterogeneous nature of tumor tissue.

Briefly, the $P$-values for overall survival (OS) and relapse-free survival (RFS) in the first TMA core (TMA1), and second TMA core (TMA2), and final results with the combination of first and second cores (TMAs) were as follows: NGF [$P$-value for OS in TMA1 ($< 0.001$), TMA2 ($< 0.001$), and TMAs ($< 0.001$); $P$-value for RFS in
TMA1 (< 0.001), TMA2 (< 0.001), and TMAs (< 0.001]), HO1 [P-value for OS in TMA1 (< 0.001), TMA2 (< 0.001), and TMAs (< 0.001); P-value for RFS in TMA1 (0.003), TMA2 (< 0.001), and TMAs (< 0.001)].

2. The 30% cutoff criteria were used previously for other markers and in other tumors. It may not necessarily applicable for HO-1 and NGF as well as for markers in breast cancers. What is the basis for this criteria in the current study?

We thank the reviewer for this comment. In response to the comment of reviewer, we modified the immunohistochemical scoring. We evaluated the staining intensity and staining area and evaluated by the sum of staining intensity scores and staining area scores in each TMA core. The staining intensity was scored as 0 (no staining), 1 (weak staining), 2 (intermediate staining), and 3 (strong staining) and the area of positive staining was evaluated in each TMA core using the following scale: 0 (no staining cells), 1 (1% of the cells stained positive), 2 (2-10% of the cells stained positive), 3 (11-33% of the cells stained positive), 4 (34-66% of the cells stained positive), and 5 (66-100% of the cells stained positive). Thereafter, a combined score obtained by adding the sum scores of two different TMA cores was used for further analysis. The maximum combined score was 16 and the minimum sum score was zero. Subsequently, the expression of NGF and HO1 were grouped as positive or negative by receiver operating characteristic curve analysis at the highest positive likelihood ratio point for the death of BRCA patients. The cut-off point for NGF expression was 9 and was 14 for HO1 expression. The expression of NGF was considered positive when a combined score was greater or equal to nine, and HO1 expression was considered positive when a combined score was greater than or equal to fourteen.

According to the new scoring for the immunohistochemical staining and new results, we revised METHODS section and most of the results (RESULTS, DISCUSSION, FIGURES, and TABLES).

METHODS section

Immunohistochemical staining for NGF and HO1 were evaluated by the sum of the staining intensity scores and the staining area scores in each TMA core [26,27]. The staining intensity was scored as 0 (no staining), 1 (weak staining), 2 (intermediate staining), and 3 (strong staining). The staining area was scored as 0 (no staining cells), 1 (1% of the cells stained positive), 2 (2-10% of the cells stained positive), 3 (11-33% of the cells stained positive), 4 (34-66% of the cells stained positive), and 5 (66-100% of the cells stained positive). Thereafter, the combined score (obtained by adding the sum of the scores of two different TMA cores) was used for further analysis. The maximum combined score was 16 and the minimum sum score was zero. Subsequently, the expression of NGF and HO1 were grouped as positive or negative by receiver operating characteristic curve analysis at the highest positive likelihood ratio point for the death of BRCA patients. The cut-off point for NGF expression was 9 and was 14 for HO1 expression. The expression of NGF was considered positive when a combined score was greater or equal to nine, and HO1 expression was considered positive when a combined score was greater than or equal to fourteen.

3. Some details (including mean/ median/ range of follow up time, no of cases of breast cancer specific death, number of cases of relapse, etc) on follow up data are missing.

We thank the reviewer for this comment. As a response to the Reviewer’s comment, we have added sentences below in the METHODS section:

The median follow-up duration was 144.9 months (range, 7.7 - 192.6). Among the 145 BRCA patients, 21 patients experienced local relapse, 33 patients had latent distant metastasis, and 44 patients died from BRCA at the follow-up endpoint. The median survival was 192.0 months and the five- and ten-year survival rates for the entire BRCA patients were 81% and 74%, respectively.

OS duration was measured as the time from diagnosis to date of death from BRCA and the patients who were
alive at last contact or died from other causes were treated as censored. RFS was calculated as the time from diagnosis to the date of relapse, death from BRCA, or last contact. Patients who were alive at last contact or died from other causes and who did not experience the relapse were treated as censored for RFS analysis.

4. The author suggested the co-expression of NGF and HO-1 has the worst survival and could have synergistic effect. However, it is not very convincing. The survival curve of NGF positive is very similar to that of NGF+HO-1+. In their analysis, cases expressed either HO-1 and NGF was grouped together. In that group (either HO-1/NGF pos), majority was HO-1 positive (34/47). The difference in survival may just reflect the difference due to HO-1 and NGF. Though a higher HR for NGF+HO-1+ than NGF positive, the former is compared to the double negative cases while the latter is referenced to NGF negative cases. Therefore, cases with expression of a single marker should be analyzed as two separate groups.

We thank the reviewer for this comment and agree with the reviewer. As a response to the reviewer’s comment, we re-analyzed our data according to the new results and have revised the results for the combined expression pattern of NGF and HO1. Below is the revised paragraph in the RESULTS section, DISCUSSION section, and FIGURES (Figure 3 and 4). As we showed in the response to reviewer 2, we also revised the TABLES.

RESULTS section

Thereafter, to investigate the prognostic effect of the combined expression pattern of NGF and HO1 (NGF/HO1 expression), we analyzed the prognostic effect of the expression of one marker in two separate groups according to the positivity of another marker. In the NGF group, the expression of HO1 significantly associated OS (Log-rank, \( P < 0.001 \)) and RFS (Log-rank, \( P = 0.004 \)) (Figure 3A). However, HO1 expression did not affect for the survival of patients in NGF+ group (Log-rank, OS; \( P = 0.514 \), RFS; \( P = 0.831 \)) (Figure 3B). However, NGF expression significantly associated with shorter OS of BRCA patients in both HO- group (Log-rank, OS; \( P = 0.011 \), RFS; \( P < 0.001 \)) and HO1+ (Log-rank, OS; \( P = 0.045 \), RFS; \( P = 0.071 \)) group (Figure 3C and 3D). Based on these results, we divided the BRCA patients into three groups according to the NGF/HO1 expression pattern as shown in Figure 4. The NGF/HO1+ expression was significantly associated with shorter OS (Log-rank, \( P < 0.001 \)) and RFS (Log-rank, \( P < 0.001 \)) (Figure 4A). The NGF/HO1- group showed favorable prognosis and the NGF+/anyHO1 group showed the poorest prognosis. The ten-year survival rate of the NGF/HO1+ group, the NGF-/HO1+ group, and the NGF+/anyHO1 groups were 94%, 71%, and 47%, respectively (Figure 4B).

DISCUSSION section

Our study has also showed a significant correlation between the expression of HO1 and NGF. 89% (40/45) of NGF-expressing BRCA co-expressed HO1. These results suggest the possibility that NGF and HO1 mediated pathways are involved in the progression of BRCA. However, interestingly, HO1 expression associated with shorter OS and RFS in the NGF-negative group, but not in the NGF-positive group. This finding suggests the possibility that HO1 may have its own role in the progression of BRCA-independent of an NGF-related mechanism.

Another interesting finding of this study is that the combined expression pattern of NGF and HO1 is helpful for the prediction of the prognosis of BRCA patients. The patients with tumors expressing NGF had the shortest OS and RFS; furthermore, the patients with tumor which did not express NGF or HO1 showed the longest survival time. Multivariate analysis revealed NGF/HO1 expression as an independent prognostic indicator of OS and RFS. Therefore, this result suggests that the combined expression pattern of NGF and HO1 might be usable as a prognostic indicator of BRCA patients.

FIGURES

Figure 3 Kaplan-Meier survival analysis between the expression of NGF and HO1. Overall survival (OS)
and relapse-free survival (RFS) according to the expression HO1 in the NGF-negative group (A) and the NGF-positive group (B). OS and RFS according to the expression NGF in the HO1-negative group (C) and the HO1-positive group (D).

Figure 4 Prognostic significance of the combined expression pattern of NGF and HO1. A. Kaplan-Meier survival analysis for overall survival and relapse-free survival between the NGF/HO1-, the NGF/HO1+, and the NGF/anyHO1 subgroups of breast carcinoma patients. B. An algorithm for the sub-grouping of breast carcinoma patients into three sub-groups according to the expression patterns of NGF and HO1. 10 yr; ten-year survival rate.

5. 110/145 cases were having both chemotherapy and hormonal therapy. Less than 20 cases have received only single therapy. It is not very meaningful for the additional analysis based on different therapies. The current data mainly based on patients received combined treatments and did not support the discussion on the relationship of NGF and chemoresistance.
We thank the reviewer for this comment. As a response to the reviewer’s comment, we deleted some discussion sentences and shorten our discussion. Below are the revised sentences in DISCUSSION section.

**The poor prognosis of the patients with NGF-expressing tumor** might be related with the ability of NGF-TrkA signaling to induce chemoresistance [31]. Therefore, there is a possibility that NGF-targeted therapy with a combination of conventional chemotherapy could be helpful for BRCA patients. In agreement with our findings, the expression of the NGFRs TrkA and p75NTR was associated with poor prognosis of pancreatic cancer patients [4].

6. **The role of NGF on breast cancers is dependent on its receptors.** There are reports in the literature of TrkA/NFGR expression in breast cancer (e.g. J Clin Pathol 2013;66:4 291-296). It would be of interest to the readers if the results of the current study and its receptor expression are discussed.

We thank the reviewer for this comment. As a response to the reviewer’s comment, we have added below sentences in the DISCUSSION section

In addition, similar results were found in a recent report which demonstrated that the expression of NGFR significantly associated with the higher histologic grade of BRCA, suggesting the expression of NGFR as a potential indicator of poor prognosis [32]. In this large cohort study in BRCA, the expression of NGFR was negatively correlated with the expression of ER and indicative for the basal-like BRCA or luminal B subtypes [32]. Our result has shown a negative correlation between NGF expression and ER expression.

**Level of interest:** An article whose findings are important to those with closely related research interests

**Quality of written English:** Acceptable

**Statistical review:** Yes, but I do not feel adequately qualified to assess the statistics.

**Declaration of competing interests:**

‘I declare that I have no competing interests’
Response to reviewer 2

We thank the reviewer for the insightful comments and for finding our study to be “well written.”

Comments to the Author:

Reviewer's report

Title: Expression of nerve growth factor and heme oxygenase-1 predict poor survival of breast carcinoma patients

Version: 1 Date: 20 September 2013

Reviewer: Xuefen Le Bourhis

Reviewer's report:

This study is nicely realized in general and appears important in the frame of the growing importance of neurotrophins in the cancer field. Its publication in BMC cancer is appropriate. Nevertheless, some points should be precised.

We very thank the reviewer for this comment.

Major points:

1) For immunohistory analysis, the authors used an antibody against NGF, which recognizes also proNGF. As proNGF has been shown to favor tumor development, this should be taken into account in the text.

We thank the reviewer for this comment. As a response to the reviewer’s comment, we have added sentence below in the DISCUSSION section:

Moreover, the precursor of NGF was overproduced in BRCA compared with benign breast tissue and involved in the stimulation of the invasion of BRCA cells [30].

2) Benefits of multivariate analysis compared to univariate analysis should better argued in manuscript. This should be also reflected in the title.

We thank the reviewer for this comment and agree with the reviewer. During the revision of our manuscript, we established one more tissue microarray set and performed additional immunohistochemical staining. Finally, we analyzed two cores per case. Thereafter, as a response to reviewer 1, we modified the immunohistochemical scoring and re-analyzed our data. As we have shown below in Table 3, the expression of both NGF and HO1 was an independent poor prognostic indicator for overall survival, although it was not significant for relapse-free survival in HO1 expression, by multivariate analysis. Based on these new results, we did not change the title of our manuscript. Below is the revised Table 3 for multivariate analysis.
### Table 3 Multivariate Cox proportional hazards regression analysis for overall survival and relapse-free survival

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>OS</th>
<th>RFS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR</td>
<td>95% CI</td>
</tr>
<tr>
<td>TNM stage, * I</td>
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<td></td>
</tr>
<tr>
<td>II</td>
<td>3.542</td>
<td>1.241-10.109</td>
</tr>
<tr>
<td>III and IV</td>
<td>7.933</td>
<td>2.441-25.787</td>
</tr>
<tr>
<td>HER2, * positive (vs. negative)</td>
<td>1.98</td>
<td>1.132-3.464</td>
</tr>
<tr>
<td>NGF, * positive (vs. negative)</td>
<td>2.174</td>
<td>1.073-4.404</td>
</tr>
<tr>
<td>HO1, * positive (vs. negative)</td>
<td>4.847</td>
<td>1.990-11.807</td>
</tr>
<tr>
<td>NGF/HO1, ** NGF-/HO1-</td>
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<td>&lt; 0.001</td>
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<tr>
<td>NGF-/HO1+</td>
<td>6.542</td>
<td>2.381-17.979</td>
</tr>
<tr>
<td>NGF+/anyHO1</td>
<td>11.206</td>
<td>4.595-27.330</td>
</tr>
</tbody>
</table>

* The variables included in the multivariate analysis were age, TNM stage, and the expression of HER2, NGF, and HO1. ** The variables included in the multivariate analysis were age, TNM stage, HER2 expression, and the combined expression pattern of NGF and HO1 (NGF/HO1).

Minor points

References number 1, 3, 18 are not appropriately cited in the text.

We thank the reviewer for this comment. According to the reviewer’s comment, we carefully checked citation of references and corrected mistake as below.

In addition to its neurotrophic effect, NGF is also known as a stimulator of cancer cell proliferation and tumor angiogenesis, and participates in tumor cell growth and invasion [1-3].

All the cases were reviewed and classified by two pathologists (KY Jang and SJ Noh) according to the World Health Organization Classification [24].

High expression of HO1 is associated with poor prognosis of non-small cell lung cancer [18].

Level of interest: An article of importance in its field

Quality of written English: Acceptable

Statistical review: Yes, but I do not feel adequately qualified to assess the statistics.