Author's response to reviews

Title: Localization of phosphorylated ErbB1-4 and heregulin in colorectal cancer

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Author's response to reviews: see over
Dear Dr. Milo Frattini

Enclosed is a re-submission of MS: 1771760274130307, titled “Localization of phosphorylated ErbB2-4 and heregulin in colorectal cancer” by Mitsui, Yonezawa, Tatsuguchi, Shinji, Gudis, Tanaka, Fujimori, and Sakamoto.

We would like to thank the reviewers for their thoughtful and helpful comments. We have addressed each of the reviewers’ comments in the revised manuscript and trust that the manuscript is improved from the original submission. We highlight all changes with red coloured in the text. At first we changed the title to “Localization of phosphorylated ErbB1-4 and heregulin in colorectal cancer”. A point-by-point explanation of how the comments were addressed follows.

We believe that the BMC Cancer readership will recognize the importance of this work and receive the paper with significant interest.

Sincerely,
Atsushi Tatsuguchi, M.D., Ph.D.
Compulsory revisions: In Figure 1, the authors stimulate cultured colorectal cancer cells with heregulin, fractionate the cells and then assess the distribution of phosphorylated ErbB2, ErbB3 and ErbB4 in the cell fractions. However, the methodology is poorly described. Were the nuclear fractions analyzed by immunoprecipitation/western blotting or just by straight western blot?

We agree with the reviewer that the methodology was poorly described. The nuclear fractions were analyzed by western blotting after immunoprecipitation by anti-phosphotyrosine antibody (PY-20). Therefore anti-ErbB2 of anti-ErbB3 antibody could detect only the phosphorylated form of ErbB2 or ErbB3 by western blotting. We revised the Methods section on page 8 line 15 to page 9 line 15 accordingly.

The authors do not demonstrate the purity of their cell fractions by western blotting for known nuclear/cytoplasmic proteins. This is an essential control.

We can understand the reviewer’s point. However, The protein from nuclear fractions was analyzed by western blotting after immunoprecipitation by PY-20. Therefore the fractions contain only the phosphorylated form of proteins in the nuclei/cytoplasms. We did not know which protein is adequate as a control in such a condition. Therefore we could not show a control.

It is unclear what percentage of p-ErbB2 and p-ErbB3 are in the nuclear fraction? The western blot in Fig. 1A seems to suggest that ~50% of the proteins are nuclear, but it is unclear whether the nuclear and cytoplasmic fractions represent the same number of cell equivalents, or whether the fractions were normalized by total protein amount, which could be misleading. This should be clarified.

Again, the proteins from both nuclear fractions and cytoplasmic fractions were analyzed by western blotting after immunoprecipitation by PY-20. Therefore the pellet contain only the phosphorylated form of proteins in the nuclei/cytoplasms. The nuclear and cytoplasmic fractions were normalized by total protein amount (1 mg) before immunoprecipitation. However, after immunoprecipitation, we do not know whether both pellets contain the same cell number or the same amount of proteins.

Does immunocytochemical staining of these cells with antibodies against p-ErbB proteins yield similar results as the cell fractionation?
We think that it is a very important points. Unfortunately, we did not perform immunocytochemistry against p-ErbB proteins on any cancer cell lines in the study.

Discretionary revisions: The authors should at least discuss how they think their data on the prognostic value of p-ErbB3 and p-ErbB4 fits into the context of EGFR signaling in this indication.

We performed immunohistochemical analyses to assess the expression of EGFR and pEGFR proteins in 155 surgical resections from colorectomy patients and showed each pEGFR and pErbB4 expression had an independent prognostic value by multivariate analysis. We added the above mentioned data about EGFR and pEGFR immunohistochemical analysis.

Then, we tried to examine whether heregulin stimulates EGFR nuclear translocation. However, we failed to get good results due to some technical difficulties. The DLD1 cells express EGFR, ErbB2, and ErbB3 strongly. On the other hand, the Caco2 cells express ErbB2 and ErbB3 weakly, EGFR negative according to our previous data (Yonezawa 2009). In this study, pErbB2 was observed under basal conditions in the nuclear fractions of DLD-1 cells, suggesting that ErbB2 was phosphorylated under a steady state. On the other hand, pErbB2 and pErbB3 were detected in the nuclear fractions of Caco2 cells only after heregulin stimulation. It suggests that heregulin stimulated ErbB2 and ErbB3 phosphorylation in the nuclear fractions without participation of the EGFR signal crosstalks in Caco2 cells since they express low level of EGFR. We added above sentences in the discussion section on page 22 line 1-7.
Reviewer's report
Title: Localization of phosphorylated ErbB2-4 and heregulin in colorectal cancer
Version: 3
Date: 15 July 2014
Reviewer: Myung-Geun Shin

Reviewer's report:
Major comments: First, author should check corresponding adjacent and surrounding normal colon tissues.

We include the findings regarding corresponding adjacent normal colon tissues from page 14 to 15. In the end, heregulin and ErbB1-4 were nearly absent in glandular epithelial cells of colorectal mucosa adjoini ng cancer tissue.

Second, they tried to keep objectiveness for the interpretation of immunohistochemical stainings. However, for confirmation and scientific interesting, the application of confocal microscopy seems to be mandatory in some cases.

We agree with the reviewer’s comment that confocal microscopy method is mandatory. In fact we have tried to do that but got no good results. Instead we performed standard immunohistochemical analysis using different antibody for each protein in some cases to confirm the results. Although data not shown we got the similar results.

Third, it's strongly recommended to check serum value of their targeted proteins because the quantification of ErbB2/Her-2 protein was already commercialized and also commonly used in clinical laboratory using serum.

The study is retrospective. Since we have not collected the patients’ blood, we couldn’t check serum value of each protein.

Minor points: First, overall shortening manuscript, especially introduction, discussion is needed.

Done. We shortened the introduction and discussion about 80%.

Second graphic display of survival analysis is recommended.

Done. We added a new figure: figure 3.

Third, Tables 1 and 3 put into supplementary ones.

Done.
Reviewer's report
Title: Localization of phosphorylated ErbB2-4 and heregulin in colorectal cancer
Version: 3
Date: 9 July 2014
Reviewer: Cornelis Sier

Reviewer's report: The number of patients does not allow thorough multivariate statistical analyses, especially because colon and rectum tumors could/should be considered as different tumors for survival prognosis. The uni/multivariate Cox analysis model seems awkward. Why are tumor grade or location (colon/rectum) not integrated?

There is no significant difference of patient prognosis between colon and rectum in the study. About tumor grade, there includes only 7 cases of poorly differentiated adenocarcinoma in the study. Because of small size of the subgroup, statistical analysis is difficult to be performed without bias.

Considering the limited message of the paper, it could be more compact, especially the introduction and discussion.

We shortened the introduction and discussion about 80%.

Minor points:
Introduce heregulin better in the first paragraph of the abstract.

The first line of the background of the abstract has been changed as the reviewer indicates. Page 3 line 2-3

The choice for HCT116 is described, but for DLD1 and Caco2 not. Discuss and explain the different results between the cell lines.

The DLD1 cells express EGFR, ErbB2, and ErbB3 strongly. On the other hand, The Caco2 cells express ErbB2 and ErbB3 weakly, EGFR negative. The phosphorylation of these receptors are not obseved in serum-free medium. We thought that these two cells were useful for evaluate the effect of HRG stimulation. In this study, pErbB2 was observed under basal conditions in the nuclear fractions of DLD-1 cells, suggesting that ErbB2 was phosphorylated under a steady state, even though heregulin stimulated both receptors phosphorylation furthermore. On the other hand, pErbB2 and pErbB3 were detected in the nuclear fractions of Caco2 cells only after heregulin stimulation. It suggests that HRG stimulated ErbB2 and ErbB3 phosphorylation in the nuclear fractions without participation of the EGFR signal closstalks in Caco2 cells since the cell express low level of EGFR. We added above sentences in discussion section on page 22 line 1-7.

Figure 1A should contain HCT116 as well.
We didn’t get good results about ErbB2 and ErbB3 phosphorylation after stimulation of heregulin in HCT116 cells.

Because of the similar MWs, describe for this figure whether 1 blot is re-probed (plus striping conditions) or whether separate gels are used for each antibody.

One blot is re-probed. Although similar MWs, we think that the band correspond to each protein, since these antibodies don’t cross-react with other proteins.

Indicate why 100 ng/ml HRG was used (response curve?) and incubation time(s).

Prior to this study, we evaluated the optimum concentration of rHRG stimulation. The phosphorylation of EebB3 was observed by dose-dependent manner, and it was saturated with 100 ng/mL rHRG. We also evaluated the optimum incubation times of rHRG stimulation. The phosphorylation of ErbB3 in the nuclear fractions observed most strongly at 1 h.

Indicate the sample period for the patients.

We added the sample period for the patients as the reviewer indicates. Page 9 line 23.

Indicate kappa statistics between observers.

We added kappa statistics between observers as the reviewer indicates. Page 10 line 23-25

Indicate in Table 4 and Table 5 how many patients are analyzed in total (155/95) and how many are in the groups.

We added patients number in Table 2 (old Table 4) and Table 3 (old Table 5) as the reviewer indicates.

Figure 1, Indicate magnifications and put higher magnification inserts and arrows where multiple forms are indicated.

We added magnifications and put higher magnification inserts in Figure 2.

Figure 2, change pErBB3 into pErbB3

Change made by the reviewer indicates in Figure 2.

Table 1, correct polyclonal, rabbit IgG = monoclonal?

Yes, rabbit IgG = monoclonal. Correct and change made by the reviewer indicates in additional file 1/Table S1 (old Table 1).
Table 4, officially Depth is not a continuous variable (numeric)

Change made by the reviewer indicates in Table 2 (Table 4) and Table 3 (Table 5).