Author's response to reviews

Title: An association of a simultaneous nuclear and cytoplasmic localization of Fra-1 with breast malignancy

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Author's response to reviews: see over
Dear Dr. Edmunds,

We are very grateful to your hard work and the comments from all reviewers. Now, according to the reviewers’ criticism, we revised our manuscript. All revised contents are marked in red.

Thank you very much. Looking forward to hearing you soon.

Sincerely yours,

Ning Guo

The major revisions are as follows:

For reviewer M Mitzi Brentani

In this study, we analyzed the expression of Fra-1 in benign and malignant breast tumor tissues by immunohistochemistry. We didn’t find the correlations of cytoplasmic Fra-1 expression with ER, PR and ErbB2. We didn’t find the overexpression of Fra-1 affected the proliferation of MCF-7 cells either (data not shown in this study). Cytoplasmic localization of Fra-1 in malignant tumors was the major finding in this study. The recent study showed that nuclear p21Cip1/WAF1 translocates to the cytoplasm and this translocation event is accompanied by resistance to various apoptotic stimuli. We have cited this work in our manuscript. It is important to know the potential significance of cytoplasmic Fra-1 expression. However, the non-transcriptional mechanism of Fra-1 remains to be further investigated.

For reviewer Karin Milde-Langosch:

1. In some studies, Fra-1 expression was analyzed in breast cancer cell lines. We also investigated Fra-1 expression both at transcription and protein levels in some breast cancer cell lines. Our data are very similar to the ones published (please see Fig. 1). The study by Bamberger et al. showed that high Fra-1 expression was undetectable in receptor-positive, highly differentiated cells, but strong in poorly differentiated tumor cells by Western blot. In our study, we investigated the expression of Fra-1 in breast cancer tissues by immunohistochemical staining with different antibody (ab22837, abcam co.) from the one (Santa Cruz) used by Bamberger et al. We can see significant differences between normal and cancer tissues or between poorer differentiated region and well-differentiated adjacent peritumoural tissues in the same sections. In our experiences, the data from IHC were more consistent and reproducible than the ones from Western blot. It is difficult to evaluate the different findings. We have cited these articles and discussed the differences (please see discussion).
2. In this retrospective study, our samples are paraffin-embedded tissues. So, an immunohistochemical method was chosen to analyze the expression of Fra-1. Our data obtained by Western blot were similar to the ones from previous studies by others (please see Fig. 1). The antibody used in this study can be trusted.

3. The reason why we didn’t chose to count only the percentage of positive tumor cells was that Fra-1 nuclear positive was observed in all breast cancer samples. The evaluation of the staining results was done by two pathologists.

4. ErbB2 overexpression can be found in about 25 – 30% of breast cancer patients in clinics. In this study, the results of ErbB2 staining were evaluated by the Department of Pathology of 307 Hospital. The evaluation standard is similar to the HercepTest kit scoring guidelines, that is, scores of 0 or 1+ were considered negative for HER-2/neu overexpression, 2++ was weak positive, and 3+++ was strong positive. Only membrane staining intensity and patterns were evaluated. Complete membrane staining of more than 10% of tumor cells was considered as 2++ and 3++++. In this study, both 2++ and 3++++ were included in positive (please see the revised “assessment of Fra-1, ER, PR and ErbB2 expression in Methods and Table 4).

5. There is disagreement in the evaluation of Fig. 2 among the reviewers. As matter of fact, it happens very frequently among pathologists. In this study, the sections were analyzed by two pathological specialists and some sections were reevaluated by two pathologists at 307 Hospital.

6. Table 2 was correct (please see the revised Table 3). We are sorry for making the mistake.

7. The positive nuclear immunoreactivity for Fra-1 in all tumors is unexpected indeed. In some benign tumors, Fra-1 was also highly expressed. However, cytoplasmic Fra-1

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Fig. 1 The expression of Fra-1 in human breast cancer cell lines analyzed by Western blot.

MCF-7  SKBR3  T47D  MDA231  MDA435

MCF-7  SKBR3  T47D  MDA231  MDA435
staining in carcinomas was obvious. That is why we didn’t consider studying the correlation of nuclear staining intensity with ER, PR, or ErbB2.

8. We did find that Fra-1 staining intensity was heterogeneous in the same samples, especially in different status of differentiation. Fig. 3 shows an example. In the same section, Fra-1 staining was weak in a well-differentiated region, but stronger in poorer differentiated region. Because such kind of sections was not sufficient to evaluate the correlation between Fra-1 expression and differentiation of carcinomas, we only describe that Fra-1 staining tended to be correlated with differentiation.

9. In Fig. 1-3, the magnification has been added in revised manuscript.

10. The statistical data in page 9 was analyzed by using standard statistical software SPSS version 13.0. Following table showed the process of analysis. The \( P \) value in our study should be considered significant.

### Case Processing Summary

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### Chi-Square Tests

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a. Computed only for a 2x2 table
b. 0 cells (.0%) have expected count less than 5. The minimum expected count is 6.89.

For reviewer SEkhar P.M. Reddy

1. In the abstract, “Fra-1 protein levels in the cytoplasm may be an indicator for the grading of human breast cancer” is changed to “Fra-1 protein accumulation in the cytoplasm may play an important role in human breast cancinogenesis”.

2. The negative control was done in the absence of primary antibody. Rabbit serum was also used as a control. Since the microphotograph of negative control using PBS is more harmonious with the positive one in color, we chose it in Fig. 1.

3. We have made revision and provide more details of immunohistochemistry (Please see the revised version).
4. Dr. Ning Guo and Ming Shi performed the immunohistochemistry analysis of tumor sections. Prof. Guomin Li and Xiaobing Li at the Department of Pathology of 307 Hospital also helped to evaluate some sections.

5. We have cited the studies published in Horm. Res. and Int. J. Cancer in the discussion.

6. There is disagreement in the evaluation of Fig. 2 among the reviewers. As matter of fact, this dissension happens very frequently among pathologists. Cytoplasmic Fra-1 staining in carcinomas is obvious in this study.

7. The wrong word has been corrected.

8. Reference 37 should be 36.

For reviewer Dany Chalbos

1. There is disagreement in the evaluation of Fig. 2 among the reviewers. As matter of fact, this dissension happens very frequently among pathologists. Cytoplasmic Fra-1 staining in carcinomas is obvious in this study.

2. In submitted manuscript, we state that “strong positive nuclear staining for Fra-1 was evident in all types of breast carcinomas”, but not in all breast cancers. Now, this sentence is changed to “strong positive nuclear staining for Fra-1 was easily seen in all types of breast carcinomas”. Table 2 is correct. So, in text, “a cytoplasmic staining observed in 78 % of breast cancers” should be “in 90 % of breast cancers”. We are sorry for making the mistake (please see the revised Table 3 and text).

3. In Fig. 1A, the negative control was done in the absence of primary antibody. Rabbit serum was also used as a control. Since the microphotograph of negative control using PBS is more harmonious with the positive microphotograph in color, we chose it in Fig. 1.

4. We investigated Fra-1 expression in different human breast cancer cell lines by Western blot (Fig. 1). Out results are very similar to the data reported by others. The specificity of antibody used in this study can be trusted.

5. We didn’t do an anatopathological analysis of slides.

6. Other mistakes were corrected (please see the revised manuscript).