Author’s response to reviews

Title: Coexpression of VEGF-C and cyclooxygenase-2 and its Association With Lymphangiogenesis in Human Breast Cancer

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Author’s response to reviews: see over
1. The mere immunohistochemical expression of lymphatic markers did not mean lymphangiogenesis. The use of a proliferation marker, e.g., Ki-67 could help the authors to ascertain this phenomenon. 
Answer1: In this revised paper, we carried out double immunostaining with antibodies to D2-40 and Ki-67 to observe the occurrence of dividing nuclei among lymphatic endothelial cells. The results confirmed Ki-67-positive nuclei in a proportion of lymph vessel endothelial cells(Fig e-f), suggesting that there is indeed lymphangiogenesis in breast cancer, the most compelling evidence being the presence of proliferating lymphatic endothelial cells.

2. The authors have classified their cases as “breast cancer”. This is not acceptable because breast cancer is presently a broad denomination. There are several histological breast cancer phenotypes which express different markers. It is essentially to clarify this point in order to avoid errors of interpretation and biases. 
Answer2: All carcinomas were classified in accordance with the criteria of the World Health Organization( Tavassoelii FA, Devilee P: Pathology and Genetics: Tumors of the Breast and Female Genital Organs Lyon: WHO Press; 2003. IARC WHO Classification of Tumours, No. 4) and were recorded as invasive ductal or invasive lobular as well as invasive medullary-like carcinomas. There are 59 patients (84.3%) had invasive ductal carcinomas and 8 patients had tumors histological typed as invasive lobular carcinomas as well as 3 patients had medullary-like carcinomas.

3. English and typing should be extensively revised. 
Answer3: The spelling and typing errors have been checked and corrected.

4. Figures 1a and 1b are of poor quality and need to be improved. 
Answer4: The quality of Figures 1a and 1b have been improved.
Answer to referre2 comment:

Major Revisions:

1. The authors should describe how the histopathological examination was done.

   Answer1: Routine histological examination was performed with hematoxylin–eosin staining. All carcinomas were classified in accordance with the criteria of the World Health Organization (Tavassolli FA, Devilee P: Pathology and Genetics: Tumors of the Breast and Female Genital Organs Lyon: WHO Press; 2003. IARC WHO Classification of Tumours, No. 4.) and were recorded as invasive ductal or invasive lobular as well as medullary-like carcinomas. The combined histological grade was obtained according to a modified Scarff–Bloom–Richardson histological grading system with guidelines as suggested by Nottingham City Hospital pathologists (Robins P, Pinder S, de Klerk N: Histological grading of breast carcinomas: a study of interobserver agreement. Human Pathol 1995, 6:873-879.). Tumor size and lymph node status were evaluated separately. The clinicopathological characteristics of the series are shown in Table 1.

2. The survival curves should show the sensors

   Answer2: In this revised paper, we have revised this point as requested (Figure 2).

Discretionary Revisions:

1. p4, Evaluation of Staining. Authors classified the intensity of COX-2 and VEGF-C expression only by the percentage of positively stained cells. Author should describe how the cells with moderate expression of COX-2 or VEGF-C (expression intensity was between positive control and negative control) were dealt with. Some authors classified COX-2 expression level by scoring method, in which both expression intensity of cancer cells and positivity rate (percentage of positively stained cells) are taking into consideration.

   Answer1: The criteria for evaluation of Staining depends on antibody used, staining protocol and the intensity of positively stained cells. In our present study, most of the positively stained cells have the same expression intensity. This may be due to the same antibodies used as well as the same staining protocol, and we did all the staining at the same time. Each stained specimen was evaluated according to gross percentages of cells demonstrating cytoplasmic immunoreactivity on the whole tumor section. Criteria for evaluation of Staining was performed according to Su, as reported previously (Su JL, Shih JY, Yen ML, Jeng YM, Chang CC, Hsieh CY, Wei LH, Yang PC, Kuo ML: Cyclooxygenase-2 induces EP1- and HER-2/Neu-dependent vascular endothelial growth factor-C up-regulation: a novel mechanism of lymphangiogenesis in lung adenocarcinoma. Cancer Res 2004, 64(2):554-564.)

2. p9, Discussion, the 3rd paragraph, the 2nd line from the bottom. Although the reference 21, a similar study, showed different result by studying VEGF-C in human breast cancer, authors of the present study commented
only "the difference is due to the use of different antibody". Author should discuss much more deeply about the cause of their different results (number of the cases, patient’s background, evaluation method of immunohistochemistry, statistical analysis, and so on) in detail.

Answer2: We have revised this point as the reviewer suggested.

3. p2, Introduction, first paragraph the first line. Authors commented “spread of tumor cells by lymphatic vessels to regional lymph node is the initial step of further dissemination and....”, which is not always true.

Answer3: In this revised paper, we have changed this paragraph into “The lymphatic vasculature is an important route for the metastatic spread of human cancer. And the presence of tumour foci in lymph nodes is the most important adverse prognostic factor in a variety of human cancers”.

4. p8, Result, the last paragraph, the 4th line from the top of the page. Authors should show a hazard ratio and P value of COX-2 and VEGF-C expression by multivariate analysis, even if the differences are not statistically different.

Answer4: We have revised this point as suggested, please see Table 3.

5. p8, Discussion, the first paragraph, the 9th line. It can not be suggested by showing only the positive correlation between COX-2 and VEGF-C expression that COX-2 regulated lymph angiogenesis. If the relationship between COX-2 and VEGF-C expression has already shown in some basic researches (DNA transfecting study, etc), the document has to be included in the Reference.

Answer5: The relationship between COX-2 and VEGF-C expression has already shown in one basic research(Su JL, Shih JY, Yen ML et al. Cyclooxygenase-2 induces EP1- and HER-2/Neudependent vascular endothelial growth factor-C up-regulation: a novel mechanism of lymphangiogenesis in lung adenocarcinoma. Cancer Res. 2004,64: 554–564.). And the document has been included in the Reference(Ref 8).

6. p11, Discussion, the last paragraph. It is a considerable speculation that a COX-2 inhibitor prevents lymph node metastasis of breast cancer in clinical use.

Answer6: In this revised paper, we have revised this point as suggested(Discussion, the last paragraph).
Answer to referee comment:

Major Revisions:

1. **Evaluation of lymphangiogenesis by "hot spot method" is not unacceptable, as estimation LVD should go on Chalkley gridding method (Br J Cancer 95:1611-25, 2006).**

   **Answer1:** Chalkley gridding method involves the use of an eyepiece graticule containing 25 randomly positioned dots, which is rotated so that the maximum number of points is on or within the vessels of the vascular ‘hot spot’. Thus, instead of counting the individual microvessel, the overlaying dots are counted. And the Chalkley count offers a suitable alternative for LVD assessment according to Weidner’s guidelines. In the other hand, tumour-associated LVD is most frequently assessed by counting the number of immunostained vessels in tumour sections (hot spot method). In some studies of breast cancer, quantitation of immunohistochemically highlighted microvessel ‘hot spots’ has been shown to be a powerful prognostic tool. Our present study confirmed that estimation of lymphangiogenesis by "hot spot method" had independent prognostic value in breast cancer patients. Honestly, there is not an eyepiece graticule containing 25 randomly positioned dots in our department neither our colleagues’ departments which are in different geographical areas. Although we have tried to do exactly the same as Prof Nakamura suggested, it seems to be difficult up to now. If the reviewer consider estimation LVD must go on Chalkley gridding method but not other techniques, such as hot spot method. We would try to get an eyepiece graticule for Chalkley counting as hard as we can.

2. **Selection of cases sounds inappropriate, as median.**

   **Answer2:** In our study, the criteria for selecting cases lies below:

   Paraffin-embedded archival specimens from 70 patients with unilateral, invasive breast cancer, who were diagnosed and treated in the Department of Oncology, The First Affiliated Hospital of Wenzhou Medical College, from January 2000 to October 2001, were included in the study. We excluded patients with in situ carcinoma, distant metastases at the time of the diagnosis, synchronous or metachronous bilateral breast cancer, malignancy other than breast cancer in history, and the patients who had received neoadjuvant chemotherapy or radiation therapy before surgery, which left 70 patients for the analysis.