Author's response to reviews

Title: Expression and clinical significance of extracellular matrix protein 1 and vascular endothelial growth factor-C in lymphatic metastasis of human breast cancer

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Author's response to reviews: see over
Dear Christna:

Many thanks for your comments concerning our manuscript entitled “Expression and clinical significance of extracellular matrix protein 1 in lymphatic metastasis of human breast cancer” (ID: 1899149268567887). We are very grateful for your consideration to accept our revised manuscript.

Your comments were highly insightful and enabled us to greatly improve the quality of our manuscript. We have sent our manuscript to Edanz for language editing service, and got a great deal of helpful suggestions in improvement of the English used. We checked the format of our revised manuscript again to make sure that it conformed to the journal style. In the following pages are our point-by-point descriptions of the main changes. To ensure the revised manuscript read fluently, we only upload the final copy of the revision.

I promise that these changes will not influence the content and framework of the paper. We hope that the revisions in the manuscript and our accompanying responses will be sufficient to make our manuscript suitable for publication in *BMC Cancer*.

We shall look forward to hearing from you at your earliest convenience.

Thank you very much for your consideration.

Yours sincerely,
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The point-by-point descriptions of changes in the revised manuscript:

1. Title page:

According to the suggestions from Edanz, the title of the revised manuscript “The expression and clinical significance of extracellular matrix protein 1 and vascular endothelial growth factor-C in the lymphatic metastasis of human breast cancer” was changed to be:

“Expression and clinical significance of extracellular matrix protein 1 and vascular endothelial growth factor-C in lymphatic metastasis of human breast cancer”

In addition, the email addresses of all co-authors were deleted to make sure to conform to the journal format.
Details were as follows:
The expression and clinical significance of extracellular matrix protein 1 and vascular endothelial growth factor-C in the lymphatic metastasis of human breast cancer

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2. Manuscript text:

We modified some sentences to improve the English used in the revised manuscript according to the suggestions and added four keywords in the final section of Abstract. These changes would not influence the content and the framework of the manuscript. The main modifications were as follows:

Abstract

Background: Extracellular matrix protein 1 (ECM1) and vascular endothelial growth factor-C (VEGF-C) are secretory glycoproteins which are highly associated with lymphangiogenesis; and thus these proteins could, therefore, play important part roles in the lymphatic dissemination of tumors. However, their roles are still very little is known about their potential roles in lymphangiogenesis. The aim of this study was to investigate whether correlations exist between the expression pattern of ECM1 and VEGF-C in human breast cancer, lymphangiogenesis, and their correlations with the clinicopathological characteristics of the disease.

Methods: The mRNA and protein expressions of ECM1 and VEGF-C mRNA and protein expression levels in 41 patients were examined using real-time reverse transcriptase polymerase chain reaction (RT-PCR), or immunohistochemical (IHC) staining in the breast cancer tissues, the matched noncancerous breast epithelial tissues, and one of the suspicious metastatic axillary lymph nodes from 41 patients. Lymph vessels were labelled by D2-40 labelled lymph vessels and lymphatic
microvessel density (LMVD) was counted. The correlations between ECM1 or VEGF-C protein expression levels, LMVD, and relative clinicopathological parameters were also evaluated statistically tested.

**Results:** The positive rate of ECM1 staining in breast cancer tissues was higher (31/41, 75.6%) than that in the corresponding epithelial tissues (4/41, 9.8%, $P < 0.001$) and the lymph nodes (13/41, 31.7%, $P < 0.001$). Similarly, the VEGF-C expression rate in cancer specimens was higher (33/41, 80.5%) than that in the other two types of epithelial tissues (19/41, 46.3%, $P < 0.01$) and lymph nodes (15/41, 36.6%, respectively, $P < 0.01$). Higher mRNA expressions of ECM1 and VEGF-C mRNA expressions levels were also detected in the tumor tissues, compared to the other two non-cancerous tissue types or lymph nodes ($P < 0.05$). The ECM1 protein expression was positively correlated with the estrogen receptor status ($P < 0.05$) and LMVD ($P < 0.05$, respectively). LMVD in the ECM1- and VEGF-C-positive tumor specimens was higher than that in the ones with both negative stainings ($P < 0.05$).

**Conclusions:** Both ECM1 and VEGF-C were overexpressed in breast cancer tissue samples. The protein expression of ECM1 expression was positively correlated with estrogen responsiveness and the metastatic properties of breast cancer. We conclude, therefore, that ECM1 and VEGF-C may have a synergistic effect on lymphangiogenesis to facilitate lymphatic metastasis of breast cancer.
Keywords: lymphangiogenesis, breast cancer, extracellular matrix protein 1, vascular endothelial growth factor-C

**Background**

(Page 4, paragraph 1 in the revised manuscript) Recent studies have demonstrated that there is expansion of lymphatic networks within the lymph nodes prior to the onset of metastasis [3]. Thus, the status of lymph node metastasis cannot be predicted by the presence of early lymphatic invasion early. Lymphatic microvessel density (LMVD) reflects the status of lymphangiogenesis and lymphatic vessel remodeling. It represented the chance of increased numbers of lymphatic microvessels provide more opportunities for tumor cells to disseminate to the lymphatic system; hence, and LMVD has been shown to be correlated with lymphangiogenic factors, the occurrence presence of lymphatic metastasis and a poor prognosis in breast cancer [4].

(Page 5, paragraph 2) The present study was designed to investigate the expression pattern of ECM1 and VEGF-C in the tumor specimens, their peritumoral normal counterparts and axillary lymph nodes from 41 breast cancer patients. The correlation between We also evaluated whether ECM1 and VEGF-C these protein expression and correlated with lymphatic microvessel density or the clinicopathological characteristics and LMVD were also evaluated of the disease.

**Methods**

(Page 7, paragraph 1) The house-keeping gene GAPDH mRNA were was used as reference, since because it is the product of a house-keeping gene, and continuously expressed to at a constant amount-level in cells. Its The GAPDH mRNA primers used were as follows: Melting curves were run analysed to identify ensure only single amplicons the specificity of the expected size were quantified PCR products.
The results of ECM1 IHC staining were expressed in two ways [10]: (1) the percentage of cells staining on a graduated percentage (0-100%): ++: 10-30% of tumor cells in the section were positive (+): +++: 30–60% of tumor cells were positive (++): ++++: 60–100% of tumor cells were positive (+++). For analysis as a dichotomous variable, staining <10% was classified as ECM1-negative and ≥10% was classified as ECM1-positive; this allowed comparisons to be made against previous studies. (2) The percentage of positive staining = (the numbers of positive samples/the numbers of samples tested) × 100%. The semi-quantitative assessment of VEGF-C staining referred to the ways of was conducted as described for the ECM1 staining assessment.

Results

Despite it has been reported that the basal epithelial cell layers of the epidermis and the myoepithelial cells of human breast tissue, prostate and salivary gland can also be stained by D2-40; but the morphology of these cells are different from the characteristic morphology of lymphatic endothelium [25, 26].

Between the two groups, the difference of in the LMVD in the metastasis and non-metastasis groups was not found to be statistically significant in the cancer tissues, neither in nor the normal breast epithelium (P = 0.409 and P = 0.377, respectively) (Table 1).

ECM1 and VEGF-C mRNA and protein expression

We used real-time RT-PCR to determine the mean relative expressions expression levels of ECM1 mRNA (Table 2) and VEGF-C mRNA by real-time RT-PCR (Table 3) in the breast cancer specimens, the normal epitheliums and lymph nodes were respectively shown in Table 2 and Table 3 from the patients. The differences of in the
ECM1 mRNA expression levels among these tissues were statistically significant (one-way ANOVA, P < 0.01) (Table 2). Multiple comparison analysis (Tukey’s test) showed that ECM1 mRNA expression levels in the breast cancer samples were overall significantly higher compared to that in the normal tissue (P < 0.05) or in the lymph nodes (P < 0.05, respectively); while there were no differences found between the normal tissues and the lymph nodes (P > 0.05). In general, the results of VEGF-C mRNA expression among the three tissue types of tissues were in idem showed the same trends as those obtained for ECM1 (Table 3).

(Page 14, paragraph 2 and 3) Differences in ECM1 and VEGF-C expression between metastatic and non-metastatic groups

Differences in ECM1 expression in tumor tissues between the metastasis group and the non-metastasis group were not significant found (Mann-Whitney test, P = 0.314); neither no statistically significant differences were found for that in the normal breast epithelium tissues, nor that in or the lymph nodes, between the metastasis and non-metastasis groups (P = 0.754 and P = 0.178, respectively; Table 4). The results of VEGF-C mRNA expression between the metastasis group and the non-metastasis group were in idem showed similar trends as those observed for ECM1 (Table 5).

…… Likewise, there was no differences apparent in VEGF-C expression in the above three tissues (i.e., cancer tissue, normal tissue and lymph nodes) between the metastasis and non-metastasis groups (Fisher’s exact test, P > 0.05, Table 5).

(Page 15, paragraph 1) …… Of In two of the cases, we found that the primary tumor was ECM1 negative in the primary tumor, whilst ECM1 was expressed in the corresponding lymph node metastases. In the same way Similarly, the difference in the VEGF-C positive staining rate between the primary tumor and the metastatic focus……
was not significant \((P > 0.05, \text{Table } 5)\). In addition, the VEGF-C staining rate in the two tissue types (i.e. cancer tissues and lymph nodes) were both 68.4% (13/19), although the cases that had the staining-positive cases staining did not all coincide with each other (Table 5).

(ECM1/VEGF-C protein expression and Correlations between LMVD and ECM1 or VEGF-C)

We first found that histological these sections which that were ECM1-positive had higher LMVDs (Figure 4). ……

However, LMVD in the lymph nodes of the different assemblies in terms of both ECM1 and VEGF-C staining (i.e. E-V-, E-V+, E+V- and E+V+) was showed a statistically different difference (one way ANOVA, \(P = 0.025\)), but did not show a significant tendency (Figure 5B).

Discussion

Therefore, like VEGF-C, ECM1 appears to be a potent enhancer of tumor lymphangiogenesis and may contribute to an increased rate of metastatic spread of breast cancer cells to the lymph nodes. ……

The differences among observed between our findings and those reported by Wang et al. [10] and Lal et al. [8] may be due-related to the different number of the cases and the differences between the studies; for example, differences in the number of cases (i.e., sample size effects), use of antibodies from different producers/suppliers, as well as the different compositions of the histological types. The objects of Participants in the Lal et al. [8] and study, as well as our study were own, mainly presented with infiltrating breast cancer, while but Wang et al. [10] did not illustrate specify the breast cancer stage. ……
There are some limitations for this study, including the relatively small sample size. It is possible that ECM1 alone is not sufficient to facilitate lymphangiogenesis, which may require multiple lymphangiogenic factors. VEGF-C is the most extensively studied molecule that for tumor lymphangiogenesis and we found that VEGF-C has potential synergy with ECM1 to facilitate lymphatic metastasis. One limitation of this study was the relatively small sample size. Nevertheless, our findings supported a potential role of ECM1 in the lymphatic progression of breast cancer, an area that will require further study to explore the mechanisms involved.

Conclusions

Our data demonstrated that both ECM1 and VEGF-C mRNA and protein of ECM1/VEGF-C were overexpressed in the breast cancer specimens, in comparison to their corresponding normal counterparts and axillary lymph nodes. The protein expression of ECM1 protein expression was positively correlated with estrogen responsiveness and LMVD, but was not correlated with the status of the lymph node metastasis in this study. ECM1 and VEGF-C may have a synergistic effect on lymphangiogenesis to facilitate the lymphatic metastasis of breast cancer.

Figure legends

(Figure 1) A-C Invasive ductal breast cancer: A. Stained lymphatic microvessels within a peripheral tumor (long arrow) were with dilated tubes, while the capillary vessel (short arrow) and tumor cell clusters were not stained; B. The tumor central vascular structures appeared linear, small and flattened, cluttered and densely arrayed; ……