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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Abstract

Background

Extracellular matrix protein 1 (ECM1) and vascular endothelial growth factor-C (VEGF-C) are secretory glycoproteins which are highly associated the lymphangiogenesis and thus may play important part in lymphatic dissemination of tumors. However, their roles are still little known. The aim of the present study was to investigate the expression pattern of ECM1 and VEGF-C in human breast cancer, and their correlations with the clinicopathological characteristics and lymphangiogenesis.

Methods

The mRNA and protein expressions of ECM1 and VEGF-C were examined by 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 and lymphatic microvessel density (LMVD) was counted. The correlation between ECM1/VEGF-C expression and relative parameters was also evaluated.

Results

The positive rate of ECM1 staining in the breast cancer tissues (31/41, 75.6%) was higher 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 (33/41, 80.5%) was higher than that in the other two types of tissues (19/41, 46.3% and 15/41,
36.6%, respectively; \( P < 0.001 \)). Higher mRNA expressions of ECM1 and VEGF-C were also detected in the tumor tissues, compared to the other two tissue types (\( P < 0.05 \)). The ECM1 protein expression was positively correlated with the estrogen receptor status 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 (\( P < 0.05 \)).

**Conclusion**

Both ECM1 and VEGF-C were overexpressed in the breast cancer tissues. The protein expression of ECM1 was positively correlated with estrogen responsiveness and the metastatic properties of breast cancer. ECM1 and VEGF-C may have a synergistic effect on lymphangiogensis to facilitate lymphatic metastasis of breast cancer.
**Background**

Breast cancer is one of the most common cancers and the leading cause of cancer death among females in economically developing countries [1]. Compared with hematologic system, the lymphatic system is the primary pathway to metastasis [2]. Recent studies have demonstrated that the expansion of lymphatic networks within the lymph nodes was prior to the onset of metastasis [3]. Thus, the status of lymph node metastasis can not predict lymphatic invasion early. Lymphatic microvessel density (LMVD) reflects the status of lymphangiogenesis and lymphatic vessel remodeling. It represented the chance for tumor cells to disseminate to the lymphatic system and was correlated with lymphangiogenic factors, the occurrence of lymphatic metastasis and poor prognosis in breast cancer [4].

Extracellular matrix protein 1 (ECM1) was originally derived from the osteogenic mouse stromal cell line MN7 [5]. It is a glycoprotein of 85-kDa and has a close association with vascularity. Recent study which suggested a homozygous frameshift mutation in *ECM1* led to a failure of human mucocutaneous lymphangiogenesis [6], indicating a possible role of *ECM1* in the lymphangiogenesis. ECM1 was overexpressed in various malignant epithelial tumors [7-10] and was identified as a marker of poor prognosis clinically [7-9]. However, possible correlation between ECM1 and malignant lymphangiogenesis/lymphatic metastasis was still little known.
Lymphangiogenesis is also closely correlated with vascular endothelial growth factor-C (VEGF-C) expression, demonstrated by abundant of previous investigations [11-12]. VEGF-C initiates the activation and the phosphorylation of VEGFR-3 (Flt-4), and then leads to PI3K-dependent Akt activation and PKC-dependent activation of the p42/p44 MAPK pathway. This process can protect lymphatic endothelial cells from apoptosis and stimulate their proliferation and migration in vitro [13-14]. Genetically engineered mice conditionally overexpressing VEGF-C showed hyperplasia of lymphatic vessels, whereas VEGF-C-null mouse embryos completely lack lymphatic vasculature [15]. Preclinical studies provided direct evidence that increased level of VEGF-C promoted intratumoral and peritumoral lymphangiogenesis and lymphatic tumor spread to regional nodes [16-17].

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 these protein expressions and the clinicopathological characteristics and LMVD were also evaluated.

**Methods**

*Patients and specimens*

Fresh surgical specimens from 41 randomly female patients who had undergone surgery in the breast surgery department, the first affiliated hospital of Xiamen University, from
February 2009 to February 2010. The average age at time of diagnosis was 53 years (ranged from 29 to 76 years). None of the patients had received preoperative treatment, such as radiotherapy or chemotherapy. Metastatic tumors from other tissue origins were excluded from this study. All cases had three parts of samples: their cancer tissues, the corresponding noncancerous breast epithelial tissues (located more than 5 cm away from the tumor margins) and one of the suspicious metastatic lymph nodes from the same side of armpit by naked eye view. These specimens above were all divided into two parts: one was quickly frozen in liquid nitrogen for RNA extractions, while the other was fixed for immunostaining and routine histological characterizations.

The institutional ethics committee approval for the project was achieved before the study and was in compliance with the Helsinki Declaration. Approval to conduct this study was obtained from the Human Subjects Office of the Institutional Research Board at the Xiamen University. Written informed consents were obtained from the patients or their relatives. Cases were evaluated for histological type, tumor grade, histological grade (according to the Nottingham histological score) [18], and the status of lymph node metastasis, estrogen receptor (ER), progesterone receptor (PR), and HER2/neu status, according to the American Joint Committee on Cancer (AJCC, seventh).

Real-time RT-PCR
Total RNA was extracted following the Trizol reagent (Invitrogen, USA) manufacturer’s instruction. Reverse transcription of total RNA to cDNA was carried out using TaKaRa Reverse Transcription Reagents (TaKaRa Bio, Japan) at 37°C for 15 min, and then 85°C for 5 sec. Primers were designed by the Primer Premier 5.0 software (Premier, Canada) and synthesized (Invitrogen, USA). Sequence specific primers for ECM1 mRNA [GenBank Accession No. NM 004425.3] were designed as follows: Forward (F):
5’-CAAATCTGCCTCCTAACC-3’; Reverse (R): 5’-AAGCAGGAGAACCAGCC-3’.
Primers for VEGF-C mRNA [GenBank Accession No. NM 005429.2] were as follows: F:
5’-GGGAAGGAGTTTGAGGT-3’; R: 5’-GCATCGGCAGGAAGT-3’. GAPDH mRNA were used as reference, since it is the product of a house-keeping gene, and continuously expressed to a constant amount in cells. Its primers were as follows: F:
5’-GAAGGTGGAAGGTCGGGTC-3’; R: 5’-GAAGATGGTGATGGGATTTC-3’.
Real-time quantitative PCR was performed with the TaKaRa SYBRR® Premix Ex Taq™ II PCR kit (TaKaRa Bio, Japan) in a Roche Lightcycler 480 (Roche, Switzerland) PCR machine. The reaction was performed in a 10µl volume and by denaturing at 95°C for 5 s, annealing at 58°C for 15 s, and extending at 72°C for 20s for 40 cycles. The system automatically monitors the binding of a fluorescent dye to double-strand DNA by real-time detection of the fluorescence during each cycle of PCR amplification. Melting curves were run to identify the specificity of PCR products. To determine the fold change in expression and to normalize the ECM1/VEGF-C expression, triplicates for the cycle threshold (Ct) from the target gene were averaged and divided by the average of the triplicate from GAPDH in the same specimen.
**IHC staining and evaluation**

IHC staining was carried out following the manufacturer’s instructions. Briefly, 4-µm sections of formalin-fixed, paraffin-embedded tissues were deparaffinized, stepwise rehydrated and endogenous peroxide was blocked. For both ECM1 and D2-40 staining, the slides were processed with antigen retrieval by being boiled in citrate buffer (pH 6.0) for 1.5 min; for VEGF-C staining, the slides were processed by being boiled in EDTA solution for 20 min, and then cooled. Nonspecific binding was blocked by using 10% non-immune goat serum (Santa Cruz, USA.) for 10 minutes. Then, sections were incubated either with anti-ECM1 antibody (Abcam, UK, clone SC-05) at 1: 50 dilution or with anti-VEGF-C antibody (Abcam, UK) at 1: 200 dilution or with D2-40 antibody (Abcam, UK, clone D2-40) at 1:40 dilution, for 120 minutes at room temperature. After rinsing, the sections were incubated with EnVision™ Detection Systems (Dako, Denmark), then counterstained with haematoxylin, dehydrated, and mounted. The negative controls were processed with the same procedure except that 10% nonimmune mouse serum (Santa Cruz, USA.) was used in place of the primary antibody. No detectable staining was observed in all negative control slides. H&E stained slides of all cases were reviewed to confirm the diagnosis and the histopathologic characteristics.

LMVD was assessed by counting the number of D2-40 immunostained vessels on tissue sections. Morphometric analyses were estimated independently by two observers, without
knowledge of the patients' clinicopathologic data. As previous reported [19], we firstly identified the area containing the most stained vessels (“hot spots”) by scanning the sections at low magnification (40×); then counted the number of positive vessels in two high magnification fields (200×). We defined those vessels as lymphatics if they were lined by a single layer of immunopositive flattened endothelial cells with a vascular lumen, in the presence or absence of lymphocytes and absence of erythrocytes [20]. LMVD in tumor sections was determined by averaging the number of total lymphatic vessels in all the fields of each slide, including within the tumor or at the periphery of the tumor. The mean visual microvessel density was calculated as the average of four counts (two authors and two microscopic fields). Discordant cases were recounted, and consensus resolved any discrepancy of more than 10% of the microvessel count.

The results of ECM1 IHC staining were expressed in two ways [10]: (1) 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 to allow comparison to previous studies. (2) percentage of positive staining = (the numbers of positive samples/the numbers of samples tested) × 100%. The semiquantitative assessment of VEGF-C staining referred to the ways of ECM1 staining assessment.
Statistics

Data were analyzed by using SPSS (ver. 17.0, Chicago, USA). The distributions of quantitative variables were tested for normality using Kolmogorov-Smirnov test. Data which were normal distribution were applied to Parametric statistics and expressed as mean ± standard error of the mean (S.E.M.). Unpaired Student’s t-test was used to compare two sets of data and one-way analysis of variance (ANOVA) with Dunnett’s post-test for comparisons of more than two data sets. Non-parametric statistics were applied to the data which is abnormal distribution. The Chi-square test or Yates’ correction or Fisher’s exact test for qualitative independent variables was applied. Bonfferoni correction of the $\alpha$-value for multiple comparisons was carried out. Correlations between two variables were assessed using Spearman’s rho test. All statistical tests were two-side. A $P$-value less than 0.05 was considered to be significant.

Results

LMVD assessment

D2-40 is a commercially available mouse monoclonal antibody directed against human podoplanin, which is a mucin-type transmembrane protein in the lymphatic endothelial cells [21]. It is a highly specific marker of lymphatic endothelium and has been proven valuable in distinguishing lymph vessels from blood vessels and detecting lymphatic invasion in various malignant neoplasms [22-24]. Despite the basal epithelial cell layers of the epidermis and the myoepithelial cells of human breast, prostate and salivary gland can be stained by D2-40,
morphology of these cells are different from the characteristic of lymphatic endothelium [25, 26].

In the present study, D2-40 staining was mainly located in the cytoplasm or membrane of lymphatic endothelial cells, while tumor cells and blood vessel endothelium had no staining. The ductal cancer in situ (DCIS) foci displayed weaker residual discontinuous myoepithelial staining. Tumor lymphatic vessel invasion (LVI) was established when at least one tumor cell cluster (“tumor emboli”) was clearly visible inside a D2-40 positive lymph vessel according to Hasebe et al. [27]. Representative examples of the staining are shown in Figure 1.

For LMVD assessment, the mean number of visual microvessels was 12.95±1.73 (range 1.04-42.10) lymphatic microvessels per 200× field (LMV per 200× field) in the 41 breast cancer specimens. For the same subjects, the mean LMVD in normal breast tissues and lymph nodes were 2.24±0.18 (range 1.00-5.03) and 5.49±0.52 (range 0-32.01) LMV per 200× field, respectively. The difference of LMVD among the three types of tissues was statistically significant (Friedman test, \( P < 0.01 \)). Dunnett’s post-test showed the mean LMVD in tumor tissues was higher than that in the normal breast tissues and lymph nodes, respectively (\( P < 0.01 \)). However, the difference in LMVD between the normal tissues and the lymph nodes was not statistically significant (\( P > 0.05 \)). The mean LMVD in the lymph nodes of the metastasis group was higher than that in the non-metastasis group (Mann Whitney test, \( P = 0.003 \)). Between the two groups, the difference of LMVD was not
statistically significant in the cancer tissues, neither in the normal breast epitheliums ($P = 0.409$ and $P = 0.377$, respectively) (Table 1).

These data showed LMVD in the breast cancer tissues was significantly higher than that in the normal tissues; LMVD in the lymph nodes with metastasis was higher than that without metastasis. Lymphatic vessels are well-known to have a discontinuous basement membrane and lack tight interendothelial junctions. It is therefore believed that the lymphatic vessels would be easier for tumor cells to enter than the blood vessels [2], and the LMVD enhancement significantly increases a potential for tumor cells to invade lymphatic vessel surface[28].

**ECM1/VEGF-C mRNA and protein expression**

The mean relative expressions of ECM1 and VEGF-C mRNA by real-time RT-PCR in the breast cancer specimens, the normal epitheliums and lymph nodes were respectively shown in Table 2 and Table 3. The difference of ECM1 mRNA expression among these tissues was statistically significant (one-way ANOVA, $P < 0.01$). Multiple comparison (Tukey’s test) showed that ECM1 mRNA expression in the breast cancer samples was overall significantly higher, in comparison to that in the normal tissues or in the lymph nodes ($P < 0.05$, respectively); while there was no difference between the normal tissues and the lymph nodes ($P > 0.05$). The results of VEGF-C mRNA expression among the three types of tissues were in idem.
ECM1 was mainly located in the cytoplasm, with scant staining noted on the cell membrane or the stroma, but no staining in the nucleus. Within the draining lymph nodes, the ECM1 staining was specific to the metastatic cancer cells and was primarily in their cytoplasm. Notably, in the normal breast epithelium, there was little or no staining at all. Representative examples of the staining are shown in Figure 2. The difference in ECM1 staining positive rate among tumor tissues (31, 75.6%), normal breast tissues (4, 9.8%) and lymph nodes (13, 31.7%) was significantly different \( (\chi^2 = 39.08, P < 0.01) \). Multiple comparison \( (\chi^2 \text{ division}, \alpha = 0.0125) \) showed ECM1 staining positive rate in the cancer tissues was higher than that in the normal tissues and that in the lymph nodes, respectively \( (P < 0.001) \). Difference in the ECM1 staining positive rate between the normal tissues and lymph nodes was not significant, however \( (P > 0.0125, \text{Table 2}) \).

In breast cancer cells, VEGF-C staining was observed in the cytoplasm, which was often more intense in the invasive edge and/or intraductal component (Figure 3). In contrast, very little or no staining was observed in normal ductal epithelium occasionally. According to the criteria for immunostaining evaluation, VEGF-C expression in the cancer specimens \( (33/41, 80.5\%) \) was higher than that in the normal tissues and lymph nodes \( (19/41, 46.3\% \text{ and } 15/41, 36.6\%, \text{ respectively}; P < 0.001) \). But difference between normal epithelium and the matched lymph nodes was not significant \( (P > 0.0125, \text{Table 3}) \).
**Difference in ECM1/VEGF-C expression between metastatic and non-metastatic groups**

Difference in the expression of ECM1 mRNA in tumor tissues between the metastasis group and the non-metastasis group was not significant (Mann-Whitney test, \( P = 0.314 \)); neither that in the normal breast epithelium tissues, nor that in the lymph nodes (\( P = 0.754 \) 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 (Table 5).

Difference in the ECM1 staining positive rate between metastatic (15/19, 78.9%) and non-metastatic (16/22, 72.7%) tumors was not significant (\( P > 0.05 \)); similarly in the normal tissues between the two groups (3/19, 15.8% and 1/22, 4.55%, respectively) (Fisher’s exact test, \( P > 0.05 \), Table 4). ECM1 positive rate in the lymph node metastases was 68.4% (13/19). Likewise, there was no difference of VEGF-C expression in the above three tissues between metastasis and non-metastasis groups (Fisher’s exact test, \( P > 0.05 \), Table 5).

In the metastatic group, the difference of ECM1 positive staining rate between the primary tumor (15/19, 78.9%) and the metastatic focus (13/19, 68.4%) was not statistically significant (\( P > 0.05 \), Table 4). Of two cases, we found ECM1 was negative in the primary tumor, while ECM1 was expressed in the corresponding lymph node metastases. In the same way, VEGF-C staining rate in the two tissues were both 68.4% (13/19), although the staining positive cases did not all coincide (Table 5).
**ECM1/VEGF-C Expression and Clinical Characteristics**

We evaluated the correlation between ECM1/VEGF-C expressions and the clinicopathological characteristics, including age, histological type, histological grade, tumor size, lymph node metastasis or non-metastasis and the status of ER, PR and Her-2/neu. The analysis was summarized in Table 6 and Table 7, respectively. The ECM1 mRNA expression was not associated with the clinical variables (Unpaired t test with Welch’s correction, $P > 0.05$, respectively). The expression positive rate of ECM1 protein seemed to be associated with the status of ER (Chi-square, $P = 0.045$). The estrogen receptor status of patients with ECM1-positive tumors were more likely to be positive than those without ECM1 staining. However, ECM1 staining was not correlated with tumor size, lymph node status, PR status and the status of Her-2/neu (Fisher’s exact test, $P > 0.05$). Besides, the mRNA and protein expressions of VEGF-C were neither associated with any of the clinicopathological characteristics ($P > 0.05$, respectively; Table 7).

**ECM1/VEGF-C protein expression and LMVD**

We first found these sections which were ECM1-positive had higher LMVD (Figure 4). As shown in Table 8, between the ECM1-positive cases and ECM1-negative cases, differences in LMVD was statistical significant in the tumor tissues (Mann Whitney test, $P = 0.045$), so as to that in the lymph nodes (Mann Whitney test, $P < 0.001$). A positive correlation was further established between ECM1 staining intensity and LMVD in the breast cancer sections,
so as to the lymph node sections (Spearman’s correlation coefficient were 0.347 and 0.604, respectively; \( P < 0.05 \), Table 9). However, the correlation was not linear.

LMVD in the VEGF-C-positive lymph nodes was higher than that in the VEGF-C-negative ones (Mann Whitney test, \( P < 0.001 \)); LMVD in the lymph nodes was correlated with VEGF-C staining (Spearman’s correlation coefficient was 0.566, \( P < 0.001 \)). But LMVD in the breast cancer specimens was not associated with VEGF-C staining (\( P > 0.05 \), Table 10).

We further analysed LMVD in the breast cancer tissues and the lymph nodes in terms of both ECM1 and VEGF-C staining (Table 11). LMVD in both ECM1- and VEGF-C-positive (E+V+) tumor specimens was higher than that in both ECM1- and VEGF-C-negative (E-V-) ones (Mann Whitney test, \( P = 0.029 \); Figure 5A). Additionally, both LMVD in E+V- and E-V+ tumor specimens were higher than that in the E-V- ones; LMVD in E+V+ tumor specimens was higher than that in E-V+ and E+V- ones, although the differences did not reach statistically significance (Mann Whitney test, \( P > 0.05 \), respectively). However, LMVD in the lymph nodes of different assemblies in terms of both ECM1 and VEGF-C staining was statistically different (one way anova, \( P = 0.025 \)), but did not show a significant tendency (Figure 5B).
Discussion

Previous study showed the lymphangiogenic property of ECM1 [6]. However, whether this protein contributes to the formation of new lymphatic vessels in tumor progression is still poorly understood. The present study revealed a positive correlation between ECM1 protein expression and LMVD, both in the tumor specimens and the lymph nodes. Besides, in the lymph nodes the VEGF-C expression was correlated with LMVD. These results indicated that ECM1 had a closer relationship with LMVD than VEGF-C. LMVD reflects the status of lymphangiogenesis and the incidence of lymphatic metastasis. Therefore, like VEGF-C, ECM1 was a potent enhancer of tumor lymphangiogenesis, and may contribute to increased metastatic spread of breast cancer cells to lymph nodes. Furthermore, LMVD in the both ECM1- and VEGF-C-positive tumor specimens was statistically higher than that in the both ECM1- and VEGF-C-negative ones and the either ECM1- or VEGF-C-positive ones, suggesting that ECM1 and VEGF-C might have a synergistic effect on lymphangiogenesis of breast cancer..

In the present study, cytoplasmic ECM1 was significantly elevated in breast cancer specimens, compared with the peritumoral normal counterparts from the same patients. Han et al. [29] and Wang et al. [10] also reported that ECM1 was overexpressed in breast cancer tissues. Moreover, the mRNA and protein levels of ECM1 detected by real-time RT-PCR and by IHC were consistent, suggested that the elevated ECM1 protein expression may derive from increased transcription of the gene.
Previous study demonstrated breast cancer with lymph node metastasis was more likely to be ECM1-positive (10/13, 76.9%) than those without metastasis (3/9, 33.3%) [10]. However, we failed to find significant differences in ECM1-staining from different patients with regard to lymph node metastasis, in accordance with an analysis of a single hospital-based cohort of patients, in which ECM1 expression was not statistically associated with the status of lymph node metastasis [8]. The difference among our findings and those reported by Wang et al. [10] and Lal et al. [8] may be due to the different number of the cases and the use of antibodies from different producers, as well as different compositions of histological types. The objects of Lal et al. [8] and our study were mainly infiltrating breast cancer, while Wang et al. [10] did not illustrate. The difference in the relative expression of ECM1 mRNA between the metastasis and non-metastasis cases was not statistically significant, either. Additionally, we found the expression of VEGF-C had no statistically difference between the metastasis and non-metastasis cases, in concert with the report by Kinoshita et al. [30]. Whereas other studies demonstrated there existed association between the VEGF-C expression and lymph node metastasis [31, 32]. Addition to methodological variability, particularly the antibody and specimens used in the different studies, may be the cause, at least in part, of disagreement in these results. Recent studies have demonstrated that the expansion of lymphatic networks within the lymph nodes was prior to the onset of metastasis [3]. So, the status of lymph node metastasis cannot be early prediction of lymphatic invasion. These findings, as well as the absence of a correlation between ECM1/VEGF-C expression in the tumor cells and the
lymph node metastasis, may be explained by the fact that metastatic establishment in lymph nodes is a complex process in which multiple growth factors are involved. Lymphangiogenesis initiated by lymphangiogenic factors secreted from tumor cells is just the onset of metastasis. So the result of the ECM1/VEGF-C expression was not associated with the lymph node metastasis is understandable. One possible explanation is that ECM1/VEGF-C might be responsible for early events of lymphatic spread prior to the lymph node metastasis [30].

In order to explore whether the ECM1 expression was associated with the metastasis character of breast cancer cells, we analyzed the ECM1 expression between the primary tumors and metastatic focus in the individuals with metastasis. Theoretically, the metastatic focus in nodes and the primary nests were consanguine. Additionally, we found that there were two cases of which ECM1 was negative in the primary tumor, but expressed in the corresponding lymph node metastases. Han et al. [29] and Wang et al. [10] also found the case which was ECM1-negative in the primary tumor had ECM1-positive staining in its corresponding metastatic focus. This suggested that ECM1 was associated with the breast cancer cells which had a potential capability to metastasis, although the exact mechanism is still unclear. Recently study reported that ECM1 was selectively expressed in the Type 2 helper T cells (T\textsubscript{H2} lymphocytes), and regulated T\textsubscript{H2} cell migration through controlling the expression of KLF2 and S1P\textsubscript{1} [33]. Whether ECM1 regulates metastasis associated genes or
interacts with other extracellular matrix or both to involve in tumor cells migration is still unknown.

A recently published work suggested that overexpression of TFAP2α or TFAP2γ induced \textit{ECM1} expression in human mammary epithelial cells and thus modified ER responsiveness [34]. In the present study, ER positive tumors seemed to be more likely to be ECM1-positive. This finding indicated that ECM1 was associated with estrogen responsiveness of breast cancer. Further investigations are needed to determine this correlation and the role of ECM1 in the breast estrogen-receptor-axle, and whether ECM1 can be a new target to breast cancer hormonotherapy.

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 molecular that for tumor lymphangiogenesis and we found it may synergy with ECM1 to facilitate lymphatic metastasis. We only demonstrated the correlation, and these findings supported a role of ECM1 in the lymphatic progression of breast cancer, an area that will require further study to explore the mechanisms.
Conclusions

Our data demonstrated that both 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 was positively correlated with estrogen responsiveness and LMVD, but was not correlated the status of lymph node metastasis. ECM1 and VEGF-C may have a synergistic effect on lymphangiogenesis to facilitate lymphatic metastasis of breast cancer.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

QWW and ZMZ designed and participated in all steps of the study. HQS performed most of the experiments. HQS and JL participated in the analysis of IHC data, in statistical analysis and discussion of the results. YFH proceeded surgeries and follow up of patients. QMY and QLY participated in the experiments of IHC staining. All authors read and approved the final manuscript.

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Figures

Figure 1 - LMVD labelled by D2-40 with IHC (200×)

A-C Invasive ductal breast cancer: A. Stained lymphatic microvessels peripheral tumor (long arrow) were 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, clutter and dense array; C. a cluster of tumor cells (arrow) in a stained vessel, as “tumor emboli”. D. The luminal epithelial cells were negative for D2-40 staining. Myoepithelial cells of normal ducts and lobules revealed positive D2-40 immunostaining (short arrow), but with a granular, branching membranous staining pattern, different from the characteristic staining of lymphatic endothelium (long arrow). E-F Lymph nodes with metastasis: E. stained lymphatic microvessels in the metastasis, with irregular lumens and cracked tube walls; F. dilated lymphatic microvessels subcapsular metastasis, no-stained tumor cells in it, as “LVI” (arrow).

Figure 2 - Representative IHC staining of ECM1

A-D: ECM1 was mainly expressed in the cytoplasm of breast cancer cells (200×): A. negative for ECM1; B. + for ECM1 staining; C. ++ for ECM1 staining; D. +++ for ECM1 staining. E-F normal breast epithelium (200×): E. negative for ECM1; F. breast ductal epithelial cells with + for cytoplasmic ECM1 staining. G-H lymph node metastases: G. metastatic cells with + for ECM1 staining (200×); H. cytoplasm of metastatic cells with ++ for ECM1 staining (100×).

Figure 3 – Representative IHC staining of VEGF-C (200×)
A-D: VEGF-C staining was mainly located in the cytoplasm of breast cancer cells: A. negative for VEGF-C; B. + for VEGF-C staining; C. ++ for VEGF-C staining; D. +++ for VEGF-C staining. E-F normal breast epithelium: E. negative for VEGF-C; F. breast ductal epithelial cells with ++ for cytoplasmic VEGF-C staining. G-H lymph node metastases: G. metastatic cells with negative for VEGF-C staining; H. cytoplasm of metastatic cells with + for VEGF-C staining.

**Figure 4 - Representative examples of D2-40 and ECM1 staining in the matched sections (100×)**

These sections which were ECM1-positive staining had higher LMVD.

A-D: Lymphatic microvessels labelled by D2-40.

E-H: ECM1 IHC staining: E&G: negative for ECM1; F&H: ++ for ECM1 staining.

A-B, E-F: invasive ductal breast cancer; C-D, G-H: lymph nodes with metastasis.

A&E, B&F and C&G, D&H: the matched sections from the same specimen, respectively.

**Figure 5 – LVMD in breast cancer specimens and lymph nodes in terms of ECM1/VEGF-C staining**

A: LMVD in both ECM1- and VEGF-C-positive (E+V+) tumor specimens was higher than that in both ECM1- and VEGF-C-negative (E-V-) ones ($P < 0.05$).

B: LMVD in the 41 lymph nodes of different assemblies in terms of both ECM1 and VEGF-C staining was statistically different ($P < 0.05$), but did not show a significant tendency between these assemblies.

Abbreviations: E-V-: negative for both ECM1 and VEGF-C staining; E-V+: negative for ECM1 staining but positive for VEGF-C staining; E+V-: positive for ECM1 staining but negative for VEGF-C staining; E+V+: positive for both ECM1 and VEGF-C staining.
**Tables**

Table 1 – Comparison of LMVD between metastasis group and non-metastasis group

| Tissue type   | Metastasis group | Non-metastasis group | U    | P   |
|---------------|------------------|----------------------|------|-----|
| Cancer tissues | 15.16±2.94       | 11.05±1.97           | 177.0 | 0.409 |
| Normal tissues | 2.42±0.27        | 2.09±0.24            | 176.0 | 0.377 |
| Lymph nodes   | 9.32±2.16        | 2.18±0.32            | 97.5  | 0.003* |

* P < 0.05 is considered statistically significant.
Table 2 - The ECM1 expression in breast cancer specimens, normal epithelium and lymph nodes

| Tissue type     | Case | ECM1 mRNA expression | Positive staining rate (%) | Staining grades of ECM1 | \( \chi^2 \) |
|-----------------|------|-----------------------|---------------------------|-------------------------|-------------|
| Cancer tissues  | 41   | 1.25±0.33^A           | 75.6^D                    | 10 22 7 2               |             |
| Normal tissues  | 41   | 0.46±0.10^B           | 9.8^E                     | 37 3 1 0                |             |
| Lymph nodes     | 41   | 0.38±0.18^C           | 4.86**                    | 28 9 3 1 39.08**        |             |

** \( P < 0.01, P < 0.05 \) is considered statistically significant.

“+”, “++” and “+++” for ECM1 immunochemistry staining were all grouped together as “+”.

A vs B and A vs C: \( P < 0.05 \), respectively; B vs C: \( P > 0.05 \).

\( \chi^2 \) division: \( \alpha = 0.05/4 = 0.0125 \); D vs E and D vs F: \( P < 0.001 \), respectively; E vs F: \( P > 0.0125 \).
**Table 3 - The VEGF-C expression in breast cancer specimens, normal epithelium and lymph nodes**

| Tissue type       | Case | ECM1 mRNA expression | F  | Positive staining grades of VEGF-C | \( \chi^2 \) |
|-------------------|------|-----------------------|----|-----------------------------------|--------------|
| Cancer tissues    | 41   | 2.63±0.32⁴ᵃ             | 80.5ᵈ | - 8 20 9 4                       |              |
| Normal tissues    | 41   | 1.08±0.17ᵇ              | 46.3ᵉ | + 22 17 2 0                      |              |
| Lymph nodes       | 41   | 1.33±0.17ᶜ 15.05***     | 36.6ᶠ | ++ 26 10 3 2                     | 23.08***     |

*** P < 0.001, P < 0.05 is considered statistically significant.

“+”, “++” and “+++” for VEGF-C immunochemistry staining was all grouped together as “+”.

A vs B and A vs C: \( P < 0.01 \), respectively; B vs C: \( P > 0.05 \).

\( \chi^2 \) division: \( \alpha = 0.05/4 = 0.0125 \); D vs E and D vs F: \( P < 0.01 \), respectively; E vs F: \( P > 0.0125 \).
Table 4 - Comparison of the *ECM1* expression between metastasis group and non-metastasis group

| Tissue type     | *ECM1* mRNA expression | Positive rate of ECM1 (%) |  
|-----------------|------------------------|---------------------------|
|                 | Metastasis             | Non-metastasis            | *P* | Metastasis | Non-metastasis | *P* |
| Cancer tissues  | 1.11±0.39              | 1.48±0.54                 | 0.314 | 78.9(15/19) | 72.7(16/22)   | > 0.05 |
| Normal tissues  | 0.53±0.18              | 0.40±0.09                 | 0.754 | 15.8(3/19)  | 4.55(1/22)    | > 0.05 |
| Lymph nodes     | 0.62±0.36              | 0.20±0.13                 | 0.178 | 68.4(13/19) | (0/22)        | < 0.01* |

* *P* < 0.05 is considered statistically significant.

Within parenthesis: numbers of eligible cases.
Table 5 - Comparison of VEGF-C expression between metastasis group and non-metastasis group

| Tissue type   | VEGF-C mRNA level | Positive rate of VEGF-C (%) |
|---------------|-------------------|-----------------------------|
|               | Metastasis        | Non-metastasis              | Metastasis | Non-metastasis | P     |
| Cancer tissues| 2.27±0.39         | 2.92±0.48                   | 0.455      | 68.4 (13/19)   | 90.9 (20/22) | > 0.05 |
| Normal tissues| 0.95±0.18         | 1.18±0.27                   | 0.948      | 47.4 (9/19)    | 45.5 (10/22) | > 0.05 |
| Lymph nodes   | 1.21±0.23         | 1.46±0.24                   | 0.314      | 68.4 (13/19)   | 9.1 (2/22)   | <0.001* |

* P < 0.05 is considered statistically significant.

Within parenthesis: numbers of eligible cases.
Table 6 - The correlation of ECM1 expression with clinicopathological characteristics

| Clinicopathological characteristics | ECM1 positive |   |   |   |
|-------------------------------------|---------------|---|---|---|
|                                     | Case | case | rate (%) | P | ECM1 mRNA | P |
| Age (years)                         |      |      |          |   |            |   |
| < 60                                | 30   | 22   | 73.3     | 1.54±0.43 |
| ≥ 60                                | 11   | 9    | 81.8     | 0.700 | 0.68±0.46 | 0.183 |
| Histological grade (invasive ductal)|      |      |          |   |            |   |
| I+II                                | 16   | 12   | 75.0     | 1.76±0.71 |
| III+IV                              | 20   | 16   | 80.0     | 0.720 | 1.18±0.39 | 0.483 |
| Tumor size                          |      |      |          |   |            |   |
| T1                                  | 15   | 10   | 66.7     | 1.49±0.60 |
| T2                                  | 23   | 19   | 82.6     | 1.34±0.46 |
| T3                                  | 3    | 2    | 66.7     | 0.512 | 0.11±0.08 | 0.275 |
| Lymph nodal status                  |      |      |          |   |            |   |
| N0                                  | 22   | 16   | 72.7     | 1.48±0.54 |
| N1                                  | 12   | 10   | 83.3     | 1.32±0.58 |
| N2                                  | 5    | 3    | 60.0     | 0.81±0.60 |
| N3                                  | 2    | 2    | 100      | 0.121 | 0.54±0.50 | 0.424 |
| Estrogen receptor status            |      |      |          |   |            |   |
| -                                   | 10   | 5    | 50.0     | 1.02±0.62 |
| +++++                               | 31   | 26   | 83.9     | 0.045* | 1.40±0.41 | 0.611 |
| Progesterone receptor status        |      |      |          |   |            |   |
| -                                   | 8    | 4    | 50.0     | 1.32±0.90 |
| +++++                               | 33   | 27   | 81.8     | 0.082 | 1.30±0.37 | 0.987 |
| HER2/neu score                      |      |      |          |   |            |   |
|   | (0-1) | (2-3) |   |   |   |
|---|-------|-------|---|---|---|
| - | 13    | 28    | 11| 20| 84.6| 1.48±0.69 |
| + | 11    | 20    | 71.4| 0.458| 1.23±0.39 | 0.757 |

“+”, “++” and “+++” for ECM1 immunochemistry staining was all grouped together as “+”.

* $P < 0.05$ is considered statistically significant.
Table 7 - The correlation of VEGF-C expression with clinicopathological characteristics

| Clinicopathological characteristics | VEGF-C positive | VEGF-C mRNA |
|-------------------------------------|-----------------|-------------|
|                                     | Case | rate (%) | P | expression level | P |
| Age (years)                         |      |          |   |                   |   |
| <60                                 | 30   | 76.7     | 2.41±0.33 |
| ≥60                                 | 11   | 90.9     | 0.412 | 3.20±0.76 | 0.505 |
| Histological grade (invasive ductal) |      |          |   |                   |   |
| I+II                                | 16   | 87.5     | 2.72±0.58 |
| III+IV                              | 20   | 70.0     | 0.257 | 2.65±0.45 | 0.908 |
| Tumor size                          |      |          |   |                   |   |
| T1                                  | 15   | 80.0     | 3.00±0.63 |
| T2                                  | 23   | 78.3     | 2.14±0.31 |
| T3                                  | 3    | 100      | 0.670 | 4.58±1.68 | 0.237 |
| Lymph nodal status                  |      |          |   |                   |   |
| N0                                  | 22   | 90.9     | 2.92±0.48 |
| N1                                  | 12   | 66.7     | 2.10±0.53 |
| N2                                  | 5    | 60.0     | 2.51±0.86 |
| N3                                  | 2    | 100      | 0.606 | 2.65±0.07 | 0.710 |
| Estrogen receptor status            |      |          |   |                   |   |
| -                                   | 10   | 90.0     | 2.42±0.63 |
| ++++                                | 31   | 77.4     | 0.653 | 2.69±0.38 | 0.606 |
| Progesterone receptor status        |      |          |   |                   |   |
| -                                   | 8    | 75.0     | 2.14±0.75 |
| ++++                                | 33   | 81.8     | 0.642 | 2.75±0.35 | 0.217 |
| HER2/neu score                      |      |          |   |                   |   |
|   | (0-1) | 13  | 10  | 76.9 | 2.70±0.66 |
|---|------|-----|-----|------|-----------|
| + | (2-3) | 28  | 23  | 82.1 | 0.693     |
|   |       |     |     | 2.60±0.36 | 0.918     |

“+”, “++” and “+++” for VEGF-C immunochemistry staining was all grouped together as “+”. 
| Tissue type       | ECM1 staining | U   | P      |
|-------------------|---------------|-----|--------|
| Cancer tissues    | 7.50±1.63(10) | 15.17±2.14(31) | 85.50 | 0.045* |
| Lymph nodes       | 2.71±0.62(28) | 11.46±2.77(13) | 48.00 | 0.000*** |

“+”, “++” and “+++” for ECM1 immunochemistry staining was all grouped together as “+”.

* $P < 0.05$ and *** $P < 0.001$ is considered statistically significant.

Within parenthesis: numbers of eligible cases.
**Table 9 - Correlation between ECM1 stained intensity and LMVD (LMV per 200× field of vision)**

| Tissue type       | -             | +       | ++        | +++       | r      | P      |
|-------------------|---------------|---------|-----------|-----------|--------|--------|
| Cancer tissues    | 7.50±1.63(10) | 14.73±2.81(22) | 15.57±3.80(7) | 1.50±2.50(2) | 0.347  | 0.026* |
| Lymph nodes       | 2.71±0.62(28) | 10.44±3.06(9) | 14.00±9.08(3) | 13(1)     | 0.604  | <0.001*|

* P < 0.05 is considered statistically significant.

Within parenthesis: numbers of eligible cases.
Table 10 - Correlation between VEGF-C staining and LMVD (LMV per 200× field of vision)

| Tissue type     | VEGF-C staining |   |   |   |
|-----------------|-----------------|---|---|---|
|                 | -               | + | U | P |
| Cancer tissues  | 8.88±2.57(8)    | 13.94±2.04(33) | 93.50 | 0.211 |
| Lymph nodes     | 3.15±1.19(26)   | 11.88±2.32(25) | 75.00 | <0.001*** |

“+”, “++” and “+++” for VEGF-C immunochemistry staining was all grouped together as “+”.

*** $P < 0.05$ is considered statistically significant.

Within parenthesis: numbers of eligible cases.
Table 11 - LMVD in terms of ECM1/VEGF-C staining (LMV per 200× field of vision)

| ECM1 staining | VEGF-C staining (Cancer tissues) | ECM1 staining | VEGF-C staining (Lymph nodes) |
|---------------|----------------------------------|---------------|-------------------------------|
| -             | 3.67±0.88(3) 7.43±1.59(7)        | -             | 2.20±1.24(5) 17.0(1)          |
| +             | 12.0±3.44(5) 15.7±2.45(26) 0.054 | +             | 32.0(1) 9.75±2.37(12) 0.025* |

“+”, “++” and “+++” for ECM1 or VEGF-C immunochemistry staining was all grouped together as “+”.

* P < 0.05 is considered statistically significant.

Within parenthesis: numbers of eligible cases.
Figure 5