Increased Angiogenesis and Lymphangiogenesis in Inflammatory versus Noninflammatory Breast Cancer by Real-Time Reverse Transcriptase-PCR Gene Expression Quantification

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ABSTRACT

Purpose: Inflammatory breast cancer is a distinct and aggressive form of locally advanced breast cancer with unique clinical and pathological features. Recently, histologic evidence of intense angiogenesis was found in inflammatory breast cancer specimens. The aim of this study was to confirm the angiogenic phenotype of inflammatory breast cancer and to investigate its potential to induce lymphangiogenesis.

Experimental Design: Real-time quantitative reverse transcriptase-PCR was used to measure levels of mRNA of tumor angiogenesis and lymphangiogenesis-related factors [vascular endothelial growth factor (VEGF)-A, VEGF-C, VEGF-D, Flt-1, KDR, Flt-4, Ang-1, Ang-2, Tie-1, Tie-2, cyclooxygenase-2, fibroblast growth factor-2 (FGF-2), Egr-1, Prox-1, and LYVE-1] in tumor specimens of 16 inflammatory breast cancer and 20 noninflammatory breast cancer patients. Tissue microarray technology and immunohistochemistry were used to study differential protein expression of some of the angiogenic factors in inflammatory breast cancer and noninflammatory breast cancer. Active lymphangiogenesis was further assessed by measuring lymphatic endothelial cell proliferation.

Results: Inflammatory breast cancer specimens had significantly higher mRNA expression levels than noninflammatory breast cancer specimens of the following genes: KDR (P = 0.033), Ang-1, (P = 0.0001), Tie-1 (P = 0.001), Tie-2 (P = 0.001), FGF-2 (P = 0.002), VEGF-C (P = 0.001), VEGF-D (P = 0.012), Flt-4 (P = 0.001), Prox-1 (P = 0.005), and LYVE-1 (P = 0.013). High mRNA levels of FGF-2 and cyclooxygenase-2 corresponded to increased protein expression by immunohistochemistry. Inflammatory breast cancer specimens contained significantly higher fractions of proliferating lymphatic endothelial cells than noninflammatory breast cancer specimens (P = 0.033).

Conclusions: Using real-time quantitative reverse transcriptase-PCR and immunohistochemistry, we confirmed the intense angiogenic activity in inflammatory breast cancer and demonstrated the presence of active lymphangiogenesis in inflammatory breast cancer. This may help explain the high metastatic potential of inflammatory breast cancer by lymphatic and hematogenous route. Both pathways are potential targets for the treatment of inflammatory breast cancer.

INTRODUCTION

Inflammatory breast cancer is a distinct and aggressive form of locally advanced breast carcinoma with unique clinical and pathological features (1). Inflammatory breast cancer is characterized by enlargement of the breast and by skin changes, such as erythema and edema. These symptoms are typically rapidly progressive, distinguishing inflammatory breast cancer from other forms of locally advanced breast cancer. Pathologically, there is often extensive lymphovascular invasion with large tumor emboli. Inflammatory breast cancer represents 5% of all breast cancers. Because of its tendency to invade locally and its high metastatic potential, prognosis remains poor. Novel insights into the molecular mechanisms responsible for the phenotype of this disease might contribute to the development of new treatment strategies.

The growth and metastatic spread of breast cancer are related to new blood vessel formation or angiogenesis (2, 3). Few studies have investigated the angiogenic phenotype of inflammatory breast cancer. Most of these studies used human inflammatory breast cancer xenografts as experimental models for inflammatory breast cancer (4, 5). Using semiquantitative reverse transcriptase-PCR, Shirakawa et al. (5) found that angiogenic factors were overexpressed in human inflammatory breast cancer in comparison with noninflammatory breast cancer. Recently, a quantitative morphologic study of angiogenesis in human inflammatory breast cancer was performed by Colpaert et al. (6). Relative microvessel area, determined with the
MATERIALS AND METHODS

Frozen Tissue Samples. Tumor specimens of 16 consecutive inflammatory breast cancer patients and 20 control non-stage-matched breast cancer patients were collected and snap-frozen in liquid nitrogen. All inflammatory breast cancer patients presented with a recently developed diffuse enlargement of the breast with redness and edema of more than one third of the skin of the breast. The average age of the inflammatory breast cancer patients was 60 years (range, 41 to 80 years). The noninflammatory breast cancer control population consisted of 6 T1, 4 T2, 7 T3, and 3 T4a breast tumors. The average age of the noninflammatory breast cancer patients was 61 years (range, 42 to 78 years). Informed consent was obtained from all patients before their participation in this study. All protocols were reviewed and approved by the ethical committee of the General Hospital Sint-Augustinus.

Real-Time Quantitative Reverse Transcriptase-PCR. Total RNA was extracted with the RNeasy Mini RNA isolation kit (Qiagen, Valencia, CA) following manufacturer’s instructions. The Agilent 2100 Bioanalyzer was used to assess the quality of the RNA. One microgram of total RNA was then reverse transcribed into cDNA with random primers with the High-Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA). PCR primers and TaqMan probes targeting VEGF-A, VEGF-C, VEGF-D, Flt-1, KDR, Flt-4, Ang-1, Ang-2, Tie-1, Tie-2, cyclooxygenase-2 (COX-2), fibroblast growth factor-2 (FGF-2), Egr-1, Prox-1, LYVE-1, 18S rRNA, and β-actin were purchased as Assays-on-demand Products for Gene Expression (Applied Biosystems). Real-time quantitative reverse transcriptase-PCR was performed with the ABI Prism 7700 Sequence Detector (Applied Biosystems) following manufacturer’s instructions. 18S rRNA and β-actin were selected as internal controls for RNA input and reverse transcription efficiency with the TaqMan Human Endogenous Control Plate (Applied Biosystems). All PCR reactions were done in duplicate for both target gene and internal control. After control for equal PCR efficiency of target genes and internal controls, relative gene expressions were presented with the 2−ΔΔCt method (10). For LYVE-1 mRNA, a standard curve was generated because of unequal PCR efficiency between target gene and internal controls.

Tumor Histology. The fractions of tumor epithelium and tumor-associated stroma in the tissue used for real-time quantitative reverse transcriptase-PCR were estimated. The tissue adjacent to the part of the tumor specimen used for RNA isolation was embedded in paraffin. H&E-stained sections were evaluated by light microscopy. The relative surface area of tumor epithelium in the total tissue section was determined by a surgical pathologist (P. Vermeulen). A score of 1 to 6 was given for the relative area (percentage) of tumor epithelium versus stroma (1, 0 to 1%; 2, 1 to 10%; 3, 10 to 30%; 4, 30 to 50%; 5, 50 to 75%; 6, >75%).

Immunohistochemistry

Immunohistochemical Staining for VEGF-A, FGF-2, and COX-2. A tissue microarray containing 39 inflammatory breast cancer specimens (five cylinders per tumor) and 46 non-stage-matched breast tumor specimens (three cylinders per tumor) was constructed. Four-micrometer sections were cut, deparaffinized, and rehydrated through graded alcohols. For VEGF-A immunostaining, slides were heated in Tris EDTA (pH 9.0) for 3 minutes with a pressure cooker. For FGF-2 immunostaining, antigen retrieval was performed by heating the slides for 30 minutes in a warm water bath at 98°C. Endogenous peroxidase was quenched for 10 minutes with peroxidase blocking reagent (DakoCytomation Glostrup, Denmark). Primary antibodies, anti-VEGF-A (VG1; ref. 11; 1:2), anti-FGF-2 (1:200; Santa Cruz Biotechnology, Santa Cruz, CA), and anti-COX-2 (1:200; Cayman Chemical, Ann Arbor, MI) were incubated for 60 minutes at room temperature. Antibody staining was visualized with the ChemMate Envision detection system (DakoCytomation). Sections were counterstained with hematoxylin and mounted for microscopy. Immunostaining intensity of the tumor cells (0, no staining; 1, weak; 2, moderate; 3, strong) was evaluated by light microscopy at two different times. Staining was uniform in all cylinders of a single tumor in most cases.
When this was not so, the predominant score was taken for further analysis.

**Double Immunostaining for LYVE-1/Ki67.** For the quantification of proliferating lymphatic endothelial cells, a LYVE-1/Ki67 immunohistochemical double stain of 15 inflammatory breast cancer and 31 noninflammatory breast cancer tissue sections was performed on a Dako Autostainer (DakoCytomation). A monoclonal antibody directed against Ki-67 (MIB-1, DakoCytomation) was applied for 30 minutes to the rehydrated paraffin sections after antigen retrieval at 97°C in Tris buffer (pH 9). EnVision + Dual Link System, Peroxidase (DakoCytomation) and 3,3’-diaminobenzidine were used to visualize binding of the first antibody. The sections were then incubated for 120 minutes with an antibody against LYVE-1 (1:20; RELIAttech). EnVision System Alkaline Phosphatase and Fuchsion + Substrate-Chromogen (DakoCytomation) were used to visualize binding of this second antibody. The fractions of proliferating lymphatic endothelial cells were assessed in the whole tissue section by two observers (I. Auwera and C. Colpaert) and were calculated as the number of lymphatic endothelial cells with Ki-67–stained nuclei per 100 lymphatic endothelial cells.

**Statistical Analysis.** Differences in target gene expression levels between inflammatory breast cancer and noninflammatory breast cancer specimens were evaluated with the Mann-Whitney U test. Further analysis was done with the Spearman correlation coefficient. Immunostaining intensity scores and the percentage of proliferating lymphatic endothelial cells were compared between inflammatory breast cancer and noninflammatory breast cancer with the Mann-Whitney U test and the χ² test. The Kappa test was used to analyze concordance between two measurements. *P* ≤ 0.05 was considered to be significant. All analyses were performed using the SPSS 11 software (SPSS, Inc., Chicago, IL).

**RESULTS**

**Real-Time Reverse Transcriptase-PCR**

**Differential Expression of Genes Encoding for Angiogenesis-related Factors in Inflammatory Breast Cancer and Noninflammatory Breast Cancer.** The results of real-time quantitative reverse transcriptase-PCR for the angiogenesis-related factors are shown in Table 1. Inflammatory breast cancer specimens contained significantly higher expression levels of the following mRNAs than noninflammatory breast cancer specimens: KDR (P = 0.033), Ang-1 (P = 0.0001), Tie-1 (P = 0.001), Tie-2 (P = 0.001), and FGF-2 (P = 0.002). Moreover, Flt-1 (P = 0.080) and COX-2 mRNA levels (P = 0.063) showed a statistical trend toward higher expression in inflammatory breast cancer. There were no significant differences in the mRNA levels of the other angiogenesis-related factors between inflammatory breast cancer and noninflammatory breast cancer specimens. Spearman correlation analysis revealed significant positive correlations between those markers that are associated with the vascular endothelium (Flt-1, KDR, Ang-1, Ang-2, Tie-1, and Tie-2; *P* < 0.01). The relative gene expression of VEGF-A was positively correlated with the fractions of tumor epithelium versus tumor-associated stroma present in the inflammatory breast cancer and noninflammatory breast cancer samples (τ = 0.360, *P* = 0.037). In contrast, FGF-2 mRNA (τ = -0.463, *P* = 0.006), Ang-1 mRNA (τ = -0.505, *P* = 0.002), and Tie-2 mRNA (τ = -0.360, *P* = 0.036) expressions were negatively correlated with the fractions of tumor epithelium versus tumor-associated stroma present in the samples.

**Differential Expression of Genes Encoding for Lymphangiogenesis-related Factors in Inflammatory Breast Cancer and Noninflammatory Breast Cancer.** Relative mRNA expression levels of the lymphangiogenesis-related factors are shown in Table 2. Inflammatory breast cancer expressed significantly higher levels of all five lymphangiogenesis-associated mRNAs (*P* < 0.05). There was a significant correlation between VEGF-C, VEGF-D, and Flt-4 mRNA expression (*P* < 0.01). We also found a significant negative correlation between the tumor/stroma ratios and VEGF-D mRNA (τ = -0.460, *P* = 0.006) or Flt-4 mRNA (τ = -0.427, *P* = 0.012).

**Tumor Histology.** We compared the fractions of tumor epithelial cells versus stromal cells between the inflammatory breast cancer and noninflammatory breast cancer samples used.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Results of real-time reverse transcriptase-PCR quantification of angiogenesis-related genes in inflammatory breast cancer and noninflammatory breast cancer specimens</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>VEGF</td>
</tr>
<tr>
<td><strong>Noninflammatory breast cancer</strong></td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>20</td>
</tr>
<tr>
<td>Min</td>
<td>0.098</td>
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<tr>
<td>Median</td>
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<tr>
<td>Max</td>
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<tr>
<td>Mean</td>
<td>1.433</td>
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<tr>
<td>SD</td>
<td>4.168</td>
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<tr>
<td><strong>Inflammatory breast cancer</strong></td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>16</td>
</tr>
<tr>
<td>Min</td>
<td>0.085</td>
</tr>
<tr>
<td>Median</td>
<td>0.350</td>
</tr>
<tr>
<td>Mean</td>
<td>0.380</td>
</tr>
<tr>
<td>SD</td>
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<tr>
<td><em>P</em> value</td>
<td>0.176</td>
</tr>
</tbody>
</table>

**Abbreviations:** N, number.
for analysis and found significantly higher fractions of tumor epithelial cells in the samples of noninflammatory breast cancer patients ($P = 0.005$; Table 3).

**Immunohistochemistry**

**Immunostaining of Tissue Microarray for VEGF-A, FGF-2, and COX-2.** Immunoreactivity of VEGF-A in the breast cancer specimens was mainly restricted to the cytoplasm of the tumor cells. There was no difference in VEGF-A immunostaining intensity of the tumor cells of inflammatory breast cancer and noninflammatory breast cancer specimens ($P = 0.768$). According to the criteria of Landis (12), there was a good concordance ($\kappa = 0.77$) between the two sets of score values of VEGF-A expression. FGF-2 staining was intensely positive in the epithelium of the normal glands and in the macrophages of the tumor-associated stroma. There was also FGF-2 immunostaining in the nuclei of the endothelial cells. There was an excellent concordance ($\kappa = 0.89$) between the two independent score value sets of FGF-2 expression. No statistically significant difference in FGF-2 protein expression was found in inflammatory breast cancer versus noninflammatory breast cancer ($P = 0.216$). In the cylinders belonging to the tumors with highest mRNA expression levels (above 95th percentile of inflammatory breast cancer values), protein expression was abundant and clearly different from all other tumors (Fig. 1). This observation could also be made for COX-2 when results of reverse transcriptase-PCR and immunohistochemistry were compared (Fig. 1).

**Active Lymphangiogenesis in Inflammatory Breast Cancer and Noninflammatory Breast Cancer.** Lymphatic endothelial cell proliferation was significantly higher in inflammatory breast cancer than in noninflammatory breast cancer (Fig. 2): median percentage of proliferating lymphatic endothelial cells was 2.32% (range, 0 to 8.0%) in inflammatory breast cancer and 0% (range, 0 to 11.4%) in noninflammatory breast cancer ($P = 0.033$). About half of the noninflammatory breast cancer specimens (16 of 31) contained no dividing lymph vessels (in comparison to only 3 of 15 for the inflammatory breast cancer specimens; $\chi^2$, $P = 0.041$). The fractions of proliferating lymphatic endothelial cells determined by the two observers were correlated ($r_s = 0.636$; $P < 0.001$), if analyzed as a continuous variable. A moderate concordance ($\kappa = 0.552$) was found when proliferating lymphatic endothelial cells were considered as being present or absent.

**DISCUSSION**

The occurrence of metastasis in breast cancer is correlated with the extent of new blood vessel growth (2), which is now recognized both as a promising prognostic indicator (13) and a target for therapy (14, 15). Multiple angiogenic growth factors and their receptors are known to be key regulators of the angiogenic process. Primary breast cancers express many angiogenic polypeptides, of which, VEGF is the most abundant (16). In this study, we aimed not only to confirm the presence of increased tumor angiogenesis in inflammatory breast cancer but also to identify the main

**Table 2** Results of real-time reverse transcriptase-PCR quantification of lymphangiogenesis-related genes in inflammatory breast cancer and noninflammatory breast cancer specimens

<table>
<thead>
<tr>
<th>Gene</th>
<th>Noninflammatory breast cancer</th>
<th>Inflammatory breast cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Min</td>
<td>Median</td>
</tr>
<tr>
<td>VEGF-C</td>
<td>0.139</td>
<td>0.286</td>
</tr>
<tr>
<td>VEGF-D</td>
<td>0.051</td>
<td>0.133</td>
</tr>
<tr>
<td>Fln-4</td>
<td>0.303</td>
<td>0.920</td>
</tr>
<tr>
<td>Prox-1</td>
<td>0.195</td>
<td>0.855</td>
</tr>
<tr>
<td>Lyve-1</td>
<td>0.096</td>
<td>2.922</td>
</tr>
</tbody>
</table>

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**Table 3** Relative fractions of tumor epithelial cells versus tumor-associated stromal cells in inflammatory breast cancer and noninflammatory breast cancer samples used for reverse transcriptase-PCR analysis (1, 0–1%; 2, 1–10%; 3, 10–30%; 4, 30–50%; 5, 50–75%; 6, >75%)

<table>
<thead>
<tr>
<th>Tumor Type</th>
<th>Score 1</th>
<th>Score 2</th>
<th>Score 3</th>
<th>Score 4</th>
<th>Score 5</th>
<th>Score 6</th>
<th>Total</th>
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</thead>
<tbody>
<tr>
<td>Inflammatory breast cancer</td>
<td>5</td>
<td>3</td>
<td>1</td>
<td>4</td>
<td>2</td>
<td>0</td>
<td>15</td>
</tr>
<tr>
<td>Noninflammatory breast cancer</td>
<td>0</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>6</td>
<td>4</td>
<td>19</td>
</tr>
<tr>
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<td>5</td>
<td>6</td>
<td>4</td>
<td>7</td>
<td>8</td>
<td>4</td>
<td>34</td>
</tr>
</tbody>
</table>

NOTE. Inflammatory breast cancer samples contained significantly less tumor epithelial cells than noninflammatory breast cancer samples ($P < 0.05$).
factors that drive the angiogenic process in this disease with real-time quantitative reverse transcriptase-PCR and immunohistochemistry. To this purpose, we used a non–stage-matched control population as opposed to a stage matched. Because inflammatory breast cancer is a fast growing tumor, detected early after sudden onset, its kinetics are more likely to be comparable with those of early-stage tumors rather than locally advanced breast cancer.

Real-time quantitative reverse transcriptase-PCR represents a sensitive and powerful tool for analyzing transcription (17). It offers the potential to rapidly and quantitatively analyze a number of gene products with limited material. However, a complication of analyzing mRNA levels in tumor specimens with quantitative reverse transcriptase-PCR is the diversity of cellular populations that are present, which makes it difficult to identify cell-specific gene expression patterns. We microscopically scored the fractions of tumor epithelium and tumor-associated stroma in the inflammatory breast cancer and noninflammatory breast cancer samples used for reverse transcriptase-PCR analysis to get an idea of the main sites of mRNA production in inflammatory breast cancer and noninflammatory breast cancer. The lower fractions of tumor epithelium in inflammatory breast cancer samples correspond to the pathologic features of inflammatory breast cancer, which often grows diffusely without forming a discrete mass.

With real-time quantitative reverse transcriptase-PCR we found an up-regulation of the Ang1/Tie2-signaling pathway, which has been shown to function in the maintenance and survival of endothelial cells, as well as in the expansion of the vasculature (18). Among the angiogenic factors, the VEGF (VEGF-A) and its receptors have been studied most extensively and are believed to be most essential for the activation and maintenance of the vascular system (19). We did not find an altered mRNA expression level of VEGF-A in inflammatory breast cancer when compared with noninflammatory breast cancer, although its receptor KDR was up-regulated in inflammatory breast cancer versus noninflammatory breast cancer. KDR is predominantly expressed in endothelial cells and its activation results in a mitogenic and migratory response (20). The positive correlation between VEGF-A mRNA expression and the fractions of tumor epithelium versus tumor-associated stroma in the inflammatory breast cancer and noninflammatory breast cancer samples corresponds to the immunohistochemical localization of VEGF-A protein expression in the tumor cells rather than in the stromal cells.

Our results provide evidence for a possible role of FGF-2 in the activation of neovascularization in inflammatory breast cancer. FGF-2 mRNA expression levels were significantly increased in inflammatory breast cancer speci-
Lymphangiogenesis in Inflammatory Breast Cancer

A secreted FGF-binding protein that mobilizes and activates stored extracellular FGF-2 can serve as an angiogenic switch for different tumor cell lines (21). The negative correlation between FGF-2 mRNA expression levels and the tumor/stroma ratios of inflammatory breast cancer and noninflammatory breast cancer samples in this study identifies the tumor-associated stroma as the main source of FGF-2, corresponding to FGF-2 immunoreactivity in inflammatory breast cancer and noninflammatory breast cancer. Taken together, these data confirm that the angiogenic process is increased in inflammatory breast cancer when compared with noninflammatory breast cancer. This could be attributable to the overexpression of Rhoc GTPase in inflammatory breast cancer, which is implicated in the control of the production of angiogenic factors by inflammatory breast cancer cells (22).

Despite of the results of real-time reverse transcriptase-PCR gene expression quantification in inflammatory breast cancer and noninflammatory breast cancer, we did not find statistically significant differences in the protein expression levels between inflammatory breast cancer and noninflammatory breast cancer for the factors that were studied. mRNA and protein expression levels correlated only in the cases in which the mRNA expression was highly abundant. However, because tissue microarray technology was used, we cannot rule out that the equal protein expression is attributable to sampling bias because of tumor heterogeneity, especially in inflammatory breast cancer (23).

The lymphatic system constitutes also an important pathway of tumor dissemination and early metastasis to lymph nodes is a frequent complication in human breast cancer. Inflammatory breast cancer shows extensive lymphatic spread in the breast and particularly in cutaneous and subcutaneous lymphatics. Nearly all inflammatory breast cancer patients have lymph node involvement at time of diagnosis (1). In this study, we aimed to investigate the presence of lymphangiogenesis in inflammatory breast cancer and its potential role in inflammatory breast cancer metastasis.

In contrast to the study of Shirakawa et al. (5), we did find evidence for the occurrence of lymphangiogenesis in inflammatory breast cancer with real-time quantitative reverse transcriptase-PCR. The results show that the Flt-4 signal transduction pathway is increased in inflammatory breast cancer specimens when compared with noninflammatory breast cancer specimens. Stimulation of the Flt-4 signal transduction pathway by a Flt-4–specific mutant of VEGF-C or VEGF-D is sufficient to induce lymphangiogenesis in transgenic mice (24). The overexpression of VEGF-C in breast cancer cells increases intratumoral lymphangiogenesis and results in enhanced metastasis to regional lymph nodes and to lungs (25). VEGF-D can induce both tumor angiogenesis and lymphangiogenesis and promotes the lymphatic spread of tumors (26). We also found higher mRNA expression levels in inflammatory breast cancer for LYVE-1 and Prox-1, both considered as specific markers for the lymphatic endothelium. This difference in expression level of lymphangiogenic factors was corroborated by the significantly higher fraction of proliferating lymphatic endothelial cells, as a measure of lymphangiogenesis, in inflammatory breast cancer versus noninflammatory breast cancer. To the best of our knowledge, this is the first study to show this.

In conclusion, we demonstrated that inflammatory breast cancer is a highly angiogenic and lymphangiogenic tumor, which may help explain its potential to metastasize through the hematogenous and the lymphatic route. Our findings suggest that both processes are novel targets for future interventions in the treatment of patients with Inflammatory breast cancer.

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